The PINK1 p.I368N Mutation Affects Protein Stability and Kinase Activity with Its Structural Change

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Background: Mutations in the recessive genes PINK1 and PARKIN are the most common causes of early-onset Parkinson’s disease (PD). The mitochondrial ubiquitin (Ub) kinase PINK1 mediates, together with the cytosolic E3 Ub ligase PARKIN, mitochondrial quality control. Thereby, damaged mitochondria are identified to prevent their accumulation and eventually cell death. A detailed understanding of PINK1 mutations will help to further our understanding of PD.

Objective: The aim of this study was to examine the exact molecular pathogenic mechanisms of PINK1 p.I368N.

Methods: We investigated molecular mechanisms on the structural and functional level in patients’ fibroblasts and in cell-based, biochemical assays.

Results: Under endogenous conditions, PINK1 p.I368N is expressed, imported in healthy mitochondria similar to PINK1 wild type. Upon mitochondrial damage, however, full-length PINK1 p.I368N is unstable on the outer mitochondrial membrane and consequently mitochondrial quality control declines. We found that stress-induced interaction between PINK1 p.I368N and TOM40 of the mitochondrial protein import machinery is abolished. Analysis of a structural PINK1 p.I368N model additionally suggested impairments of Ub kinase activity. We further confirmed experimentally that the kinase activity of the PINK1 p.I368N mutant is abolished.

Conclusions: We revealed two mechanisms that lead to loss of function of PINK1 upon mutation.

Key words: Parkinson’s disease, PINK1, ubiquitin, mitochondria, mitophagy
Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease, characterized by the selective dopaminergic neuronal loss in the substantia nigra. PD has a big impact on aging society and therefore an elucidation of its cause is top priority subjects. Most cases of PD are sporadic, however some causative genes which were identified from PD pedigrees have been provided clues for mechanism of PD.

PTEN-induced putative kinase 1 (PINK1) and Parkin are causative autosomal recessive PD genes. Mitochondrial kinase PINK1 carries pivotal role for mitochondrial quality control with E3 ligase PARKIN.

In healthy mitochondria, newly translated full-length PINK1 (~63 kDa) is constitutively imported through the TOM; the translocase of the outer mitochondrial membrane (OMM) complex and then through the TIM; the translocase of the inner mitochondrial membrane (IMM) complex. Upon import, the N-terminal mitochondrial targeting sequence (MTS) of PINK1 is cleaved off by the matrix processing peptidase (MPP) and the intermediate isoform (~60 kDa) is further processed by the Presenilin-associated rhomboid-like protein (PARL) in the inner mitochondrial membrane (IMM) to generate a 52 kDa PINK1 fragment. This cleaved form of PINK1 is exported back to the cytosol by an unknown mechanism and degraded by the Ub/proteasome system (UPS).

Upon mitochondrial depolarization, full-length PINK1 is no longer imported into mitochondria, but accumulates, with the kinase domain facing the cytosol, on the OMM and forms a dimeric structure associated with the TOM complex. This allows phosphorylation of its substrates, the small modifier protein Ub and its E3 ligase PARKIN, at a conserved Ser65 residue. Both PINK1–mediated phosphorylation events fully activate the auto-inhibited enzymatic functions of PARKIN and further facilitate its recruitment from the cytosol (Figure-1). Then, PINK1 and PARKIN together cooperatively label damaged mitochondria in a feed forward mechanism with phosphorylated poly-Ub chains that serve as a “tag” for their removal via the autophagy/lysosome system.

Missense mutations in PINK1 can interfere with mitochondrial quality control through different molecular mechanisms. For instance PINK1 p.Q456X results in instability of its transcript through non-sense mediated decay, leading to a complete loss-of-function on the protein level. In contrast, the mutant p.G411S is expressed and upon damage forms a dimer on the OMM similar to PINK1 wild type (WT). However, in addition to partial loss of its kinase activity, in a heterodimer with PINK1 WT, p.G411S also exerts a partial, dominant-negative effect. This lowers overall protection through mitochondrial quality control and emphasizes risk for PD even in heterozygous

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carriers.

Towards a complete dissection of PINK1 regulation and a rationalized drug design in the future, we focused on the novel p.I368N mutation and investigated the association of PINK1 kinase activity and its stability using newly synthesized pS65-Ubiqitin (Ub) antibody and patient fibroblast. In patients’ fibroblasts, we found that stabilization of PINK1 p.I368N on the OMM is greatly decreased under mitochondrial stressed condition. Moreover, p.I368N structural analysis revealed changes in the active site of the kinase domain that should affect ATP binding. Indeed, in cells we observed loss of kinase activity even under conditions of forced expression. Taken together, our study identified two molecular mechanisms that can drive pathogenicity of PINK1 missense mutations and might help highlighting future drug design.

Material and Methods

1. Genetic examination

Material for analysis of genomic DNA was extracted from blood and fibroblasts using a standard protocol. To confirm the genotype (c.T1103A/c.T1103A; p.I368N/p.I368N) and exclude other mutations in PINK1, all coding exons were directly sequenced by Sanger method and analyzed against the reference sequence NM_032409.2 (NCBI36/hg18). Gene rearrangements were analyzed by multiple ligation-dependent probe amplification (MLPA) technique using available tests SALSA P051 PD probes (MRC Holland) – probes for all PINK1 exons.

2. Modeling PINK1 structures and refinement

The modeling of the full-length human PINK1 protein, NP_115785.1 (581 amino acid residues), has been described recently. In brief, each individual domain was modeled as a separate unit and built into a composite full-length structure. The hybrid model is derived from consensus between the programs PRIME (Prime v3.0, Schrödinger, LLC, New York, NY) , YASARA SSP/Homology/PSSM Method , DISTILL (Porter) and TASSER and combines homology, threading, ab initio, and compositing techniques.

3. Modification and mutation modeling

Mutations of amino acids were completed using the Maestro within the Schrödinger suite and parameterized using OPLS2.0 and Amber FF. Additionally, Maestro was used for placing mutated residues (or extending) automatically within an existing peptide chain. Also, MacroModel features within Maestro allow for the quick minimization of the structure for local geometry fixes to correct stereochemistry and packing of the amino acids. Further minimization was completed on the post-Schrödinger model using OPLS2005 within YASARA2, which has an AMBER set and can be used to further parameterize modifications to import into existing molecular dynamics integration engines, as the parameters for the modification are well documented for YASARA and Schrödinger.

4. Molecular dynamics simulation and methods

Molecular dynamics simulation (MDS) was completed on each model for conformational sampling, using methods previously described. Each PINK1 system was minimized with relaxed restraints using Steepest Descent and Conjugate Gradient PR, and equilibrated in solvent with physiological salt conditions, as described in the literature. After equilibration was established, each system was allowed to run an additional molecular dynamics (MD) production length of > 250 nanoseconds. The primary purpose of MD for this study was conformational stability, refinement, and interaction calculations that may occur at the active site or dimer interface. The protocol for refinement include the following steps: (1) Minimization with explicit water molecules and ions, (2) Energy minimization of the entire system, and (3) MDS for >2 ns to relax to the force field.

5. Cell Culture

Primary human dermal fibroblasts were grown in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, BioWest), 1% PenStrep and 1% non-essential amino acids (both Invitrogen). Control fibroblasts were received from cell Applications. Cells with PINK1 p.I368N mutations have been deposited at Coriell Biorepository (ND40077 and ND40068).
HeLa cells (ATCC) were cultured in DMEM supplemented with 10% FBS. All cells were maintained at 37°C 5% CO₂ in a humidified atmosphere.

6. Chemical treatments, siRNA and DNA transfection of cells

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), epoxomicin and cycloheximide were purchased from Sigma-Aldrich, valinomycin from Axxora. siRNA transfections were performed with 20 nM control (all stars negative control) or PINK1-specific siRNA (5’ GACGCTGTTCCTCGTTATGAA-3’) using HiPerfect (all from Qiagen) or Lipofectamine2000 (Invitrogen). siRNA resistant PINK1-V5 constructs have been described before. The mutations p. L347P and p. I368N were introduced by site-directed mutagenesis and verified by Sanger sequencing. The standard transfection protocol for DNA was as follows: for one well of a 12–well plate 1 μg of DNA and 2.5 μl of Lipofectamine2000 (Invitrogen) were each mixed with 100 μl of Opti-MEM media (Invitrogen), incubated for 5 min at room temperature, mixed together and incubated 20 min before adding to the cells.

7. Antibodies

The following primary antibodies were used for Western blot (WB) and/or immunofluorescence (IF): anti–CDC37 (WB, 1:1,000, #4793, Cell Signaling), anti–HSP90 (WB, 1:2,000, 610419, BD), mouse anti–GAPDH (WB, 1:150,000, H86504M, Meridian Life Science), mouse anti–Mitofusin 1 (WB, 1:5,000, ab57602, Abcam), rabbit anti–PINK1 (WB, 1:2,000, BC100–494, Novus Biologicals), rabbit anti–PINK1 (WB, 1:2,000, IF, 1:100, #6946, Cell Signaling), mouse anti–TOM20 (IF, 1:250, sc–17764, Santa Cruz Biotechnology), anti–TOM40 (WB, 1:1,000, 18409–1-AP, ProteinTech Group), mouse anti–Ub (WB, 1:1,000, MAB1510, ubi–1, Millipore), rabbit anti–pS65–Ub (WB, 1:5,000–1:15,000, IF, 1:250, in house), mouse anti–V5 (WB, 1:5,000, R960–25, Invitrogen), rabbit anti–V5 (WB, 1:5,000, ab9115, Abcam), Streptavidin HRP (WB, 1:150, 0000, Pierce).

8. Protein extraction, SDS–PAGE and Western blot (WB)

Cells were collected and lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP–40, 0.5% deoxycholate, 0.1% SDS) plus Complete protease and PhosSTOP phosphatase inhibitors (Roche Applied Science) on ice for 30 min. Proteins were collected as supernatants after centrifugation at 20,000 g for 15 min at 4°C. SDS–PAGE was performed using 8–16% or 16% Tris Glycine gels (Invitrogen). Proteins were transferred onto PVDF membranes and detected using standard immunoblotting procedures. To detect phosphorylated PINK1, 8% Tris Glycine gels containing 50 mM Phos–tag acrylamide (Wako chemicals) and 100 mM ZnCl₂ were used. After electrophoresis, Phos–tag acrylamide gels were washed using transfer buffer with 0.01% SDS and 1 mM EDTA for 20 min and then with transfer buffer containing 0.01% SDS without EDTA for 10 min to remove excess Zn²⁺ before transfer.

9. Immunofluorescence (IF)

Fibroblasts were seeded onto PDL (P0899, Sigma–Aldrich) coated glass coverslips treated with valinomycin, CCCP or left untreated. Cells were then fixed with 4% (w/v) paraformaldehyde, permeabilized with 1% Triton–X–100, blocked with 10% goat serum and incubated with primary antibodies followed by secondary antibodies (anti–rabbit Alexa Fluor–488, anti–mouse IgG Alexa Fluor–568, 1:1000, Invitrogen). Nuclei were stained with Hoechst 33342 (1:5000, Invitrogen). For staining of PINK1 tyramide signal amplification (T20922, Invitrogen) was used. Coverslips were mounted onto microscope slides using fluorescence mounting medium (Dako). High resolution confocal images were taken with an AxioObserver microscope equipped with an ApoTome Imaging System (Zeiss).

10. (Co–)Immunoprecipitation

HeLa cells were transiently transfected with V5–6xHis–tagged PINK1 WT, p.I368N or p.L347P for 48 h and were then treated with 15 μM CCCP for 2 h or left untreated. Cells were lysed in co–IP buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol and 0.02% NP–40) supplemented with protease and phosphatase inhibitors (Roche Applied Science) and briefly sonicated. For PINK1–V5 immunoprecipitation (IP), total cell
lysate was incubated overnight at 4°C with mouse anti-V5-agarose beads (A7345, Sigma-Aldrich). Formed immuno-complexes were spun down, washed 3x with co-IP buffer and eluted from beads with 50 μl 2x SDS sample buffer and boiled at 95°C. 10 μg of input cell lysates and 10 μl of immunoprecipitates were analyzed by Western blot.

11. In vitro kinase assay

Immunoprecipitated WT and p.I368N PINK1-V5 from CCCP treated and untreated HeLa cells were washed twice with co-IP buffer and once with in vitro phosphorylation buffer (20 mM HEPES pH 7.4, 50 mM NaCl, 5 mM MgCl2, 0.1 mM EGTA, 0.01% Triton X-100). After complete removal of the buffer 100 μl of the in vitro reaction mixture was added. The reaction contained 1 μg of N-terminally biotinylated Ub (Boston Biochem), 1 mM TCEP (Gold Bio) and 2 mM ATP trisodium salt (Sigma-Aldrich) in phosphorylation buffer. Reactions were carried out at 37°C under constant agitation for 24 h, stopped by addition of 6x SDS sample buffer, heated to 56°C for 15 min and analyzed by immunoblotting using pS65-Ub antibody. Detection of biotinylated Ub with streptavidin-HRP (Pierce) served as loading control.

12. Densitometry and statistical methods

Densitometric analysis of Western blots was performed with the Image Studio software (LiCor). All quantitative results are expressed as mean ± SEM from at least three independent experiments. Statistical comparisons were performed using one-way and two-way ANOVA, respectively, with Tukey’s post-hoc test (*, p<0.05; **, p<0.005; ***, p < 0.0005). All analyses were performed using GraphPad Prism version 6.

Results

1. Clinical features of PINK1 p.I368N are similar to sporadic late-onset PD

Here we examined two patients that are homozygous carriers of a recently identified PINK1 c.T1103A (p.I368N) mutation (45). Though the analyzed siblings both carry a homozygous mutation, there was no consanguineous marriage of their parents. Both patients have early-onset and slowly progressive PD that started at relatively young age (28 and 33 years, respectively), consistent with the typical clinical presentation of homozygous PINK1 loss-of-function (46). Some clinical features (e.g. laterality of parkinsonism, resting tremor, bradykinesia and good response to levodopa) are indistinguishable from sporadic late-onset PD. Both
patients show no depression or anxiety, which are occasionally recognized as a non-motor symptom of PINK1-associated PD.

2. Structural changes of the p.I368N mutation distort the ATP-binding site of PINK1

The residue Ile368 is located in the C-lobe of PINK1’s kinase domain (Figure-2A) that is highly conserved among different species. Using homology modeling, we have recently generated a structural model of full-length PINK1 at an all-atom resolution. To examine how mutation of I368 affects PINK1 structure, we performed MDS. We compared the appearance of PINK1 WT with I368N using superposition overlay (data not shown). The orientation of I368N in the structure made the ATP-binding pocket deformed (Figure-2B). This suggests that a distortion but not a compaction occurs in the ATP-binding pocket of PINK1 I368N and this change might damage a necessary catalytic arrangement within the kinase domain of PINK1.

3. PINK1 p.I368N mutant protein is not properly stabilized upon dissipation of the mitochondrial membrane potential

To study the effects of the PINK1 I368N mutation, we used fibroblasts that were donated by PD patients carrying the PINK1 I368N mutation. First, we examined the PINK1 protein level upon mitochondrial stress. Under basal conditions, PINK1 is constitutively cleaved by PARL and degraded by the proteasome. Upon dissipation of the mitochondrial membrane potential, PINK1 cleavage is suppressed and PINK1 accumulates on the OMM. While PINK1 protein robustly accumulated upon treatment with the K+ ionophore valinomycin, PINK1 p.I368N protein accumulation was significantly decreased (Figure-3A). To confirm these findings, fibroblasts were immunostained with antibodies against PINK1 and the mitochondrial marker TOM20 showed that PINK1 p.I368N is localized to damaged mitochondria after the mitochondrial uncoupler CCCP treatment. Consistent with WB analysis, PINK1 signal was weaker in PINK1 I368N cells compared to control (Figure-3B). Quantitative RT-PCR of PINK1 mRNA levels showed no significant differences between control and I368N mutant fibroblasts with or without mitochondrial depolarization. Although PINK1 mRNA level slightly increased with valinomycin treatment (data not shown). This suggests that the transcription level of PINK1 p.I368N mutant is maintained and that observed differences in PINK1 levels reflect changes on protein level rather than mRNA levels.

4. The PINK1 p.I368N mutation affects kinase activity

Recently, we have generated and characterized new antibodies that specifically detect PINK1 phosphorylated Ubiquitin (phospho-Serine 65 Ubiquitin) pS65-Ub. Consistent with very low PINK1 protein levels in PINK1 I368N mutant cells, pS65-Ub was almost undetectable upon mitochondrial depolarization. In contrast to p.I368N, pS65-Ub signal was time-dependently increased in controls (Figure-3A).

To further confirm this by an independent method, fibroblasts were treated with valinomycin and analyzed by IF (Figure-3C). Consistent with the lack of pS65-Ub signal in patients’ cells, Mitofusin1 ubiquitylation were not observed (see Figure-3A). Overall, these results suggest that the PINK1 p.I368N mutation reduces protein stabilization under mitochondrial stress condition resulting in decrease of mitochondrial quality control.

5. Cleaved forms of PINK1 p.I368N protein are stabilized upon proteasome inhibition

Since PINK1 I368N mutant cells have significantly less PINK1 protein than control cells and gene expression differences on mRNA levels could not be detected, we performed further experiments to detect the cause of decreased PINK1 I368N levels. In order to reveal whether differences in PINK1 protein levels are caused by aberrant processing or protein instability, we co-treated cells with a mitochondrial uncoupler and a proteasome inhibitor to stabilize the cleaved forms of PINK1. WB analysis showed that although full-length PINK1 protein (~63 kDa) was markedly reduced in p.I368N mutant cells upon mitochondrial depolarization, the PARL-cleaved form (~52 kDa) was stabilized upon epoxomicin treatment to the same extent as PINK1 WT in the control cells (Figure-4A, B). Full-length PINK1 and in particularly the PARL-cleaved form has been reported before as an insoluble protein. Thus, we separately

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analyzed soluble and insoluble fractions of control and p.I368N mutant fibroblasts that had been treated with a combination of valinomycin and epoxomicin. Full-length WT PINK1 was mostly found in the soluble fraction, while its cleaved form strongly accumulated in the insoluble fraction upon proteasome inhibition (data not shown). While the PARL-cleaved form of p.I368N accumulated in the insoluble fraction similar to PINK1 WT, full-length mutant PINK1 remained barely detectable in either

**Figure-3** Full-length PINK1 protein and kinase activity are decreased in p.I368N mutant fibroblasts

A: WT control and two PINK1 p.I368N fibroblasts were treated with 1 μM valinomycin for 0, 2, 4 or 8 hours and total lysates were analyzed by WB with the indicated antibodies. GAPDH served as a loading control. Slower migrating PINK1 and Mitofusin1 protein species indicate activated and/or modified forms and were only observed in control cells. pS65-Ub signal increased in control cells over time but was undetectable in PINK1 p.I368N mutant cells.

B: Representative images of control and PINK1 p.I368N fibroblasts with or without 6 days of 10 μM CCCP treatment stained with antibodies against PINK1 (green) and TOM20 (red). Nuclei are shown in blue. Scale bars indicate 10 μm. PINK1 signal increase at mitochondria upon stress was largely diminished in PINK1 p.I368N cells.

C: Representative images of fibroblasts upon treatment with 1 μM valinomycin. Cells were stained with anti pS65-Ub (green) and TOM20 (red). Nuclei are shown in blue (Hoechst). Scale bars indicate 10 μm. Upon treatment, pS65-Ub is induced only in control, but not in PINK1 p.I368N fibroblasts.
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**Figure-4** PARL-cleaved forms of PINK1 WT and p.I368N are similarly stabilized upon proteasome inhibition
A: Control and PINK1 p.I368N fibroblasts were pretreated with 500 nM epoxomicin for 1 h followed by 10 µM CCCP treatment for additional 24 h, as indicated. Only PARL-cleaved PINK1 p.I368N (∼52 kDa, black arrowhead), but not the full-length form (∼63 kDa, white arrowhead) accumulated with 500 nM epoxomicin ± CCCP in mutant fibroblasts. Anti-pS65-Ub and total Ub antibodies were used as controls for CCCP and epoxomicin treatment, respectively. GAPDH served as a loading control.
B: WB quantification of full-length and PARL-cleaved PINK1 species from control and p.I368N mutant fibroblasts as performed in (A). Values of the full-length PINK1/GAPDH ratio were normalized to control cells treated only with CCCP. Values of the cleaved PINK1/GAPDH ratios were normalized to control cells treated only with epoxomicin. Error bars indicate mean ± SEM from three independent experiments (two-way ANOVA with Tukey’s post hoc; *, p<0.05; ***, p<0.0005).

**Figure-5** The decreased stability of PINK1 p.I368N can be masked by high expression levels
HeLa cells were simultaneously transfected with PINK1 siRNA and V5 empty vector, PINK1 WT or p.I368N mutant. Cells were left untreated or incubated with 10 µM CCCP for 4 h. Cell lysates were analyzed by WB, probed with anti-V5, PINK1 and pS65-Ub antibodies. GAPDH was used as a loading control. Auto-phosphorylated PINK1 (anti-V5) (asterisk) was detected on phos-tag gels only in lysates from PINK1-V5 transfected, CCCP treated cells. In PINK1-V5 p.I368N transfected cells, CCCP--induced phosphorylation of Ub was largely abolished.
fraction. Yet, we could not identify any distinct, additional cleavage product for p.I368N that would indicate aberrant processing of mutant PINK1.

6. Protein instability of PINK1 p.I368N is masked upon overexpression

To examine whether full length PINK1 I368N is an inherently unstable protein, we compared PINK1 levels upon overexpression in HeLa cells. In contrast to fibroblasts, the expression level of full length PINK1 I368N was similar to WT upon standard transfection (Figure-5). This indicates that the decreased stability of PINK1 I368N can be masked by high expression levels and stability deficits can only be observed under endogenous or low level exogenous PINK1 expression. Our data show that high-level PINK1 overexpression can override important regulatory mechanisms.

Altogether these results show that while generation, processing and stability of PINK1 p.I368N are not impaired under basal conditions, specifically the mutant full-length form does not properly accumulate on the OMM upon mitochondrial stress.

7. Full-length PINK1 p.I368N is not stabilized on the OMM by the HSP90/CDC37 co-chaperone complex

To elucidate the reasons for the destabilization of the p.I368N mutation, we investigated key protein interactions of PINK1 by co-immunoprecipitation (Figure-6A, B). We focused on the HSP90/CDC37 chaperone complex that supports folding of several kinases in the cytosol including PINK1. The PD-associated PINK1 mutation p.L347P had been reported before as unstable due to its lower binding affinity for HSP90/CDC37 and thus was included as a control. Although HSP90 which is known as a PINK1 associated chaperone was co-immunoprecipitated with WT, p.I368N, p.L347P, the signal obtained with both mutants were less than WT. In addition, we examined the interaction of PINK1 with the integral OMM protein TOM40.
that forms the channel of the protein import complex\(^{32}\). It is known that full-length PINK1 accumulates into a higher molecular weight protein complex with the import machinery\(^{10, 33}\), which regulates its insertion into the OMM, stability and enzymatic function towards PARKIN activation. In line with this, PINK1–V5 WT was found to interact with TOM40 specifically upon CCCP treatment, while the PINK1 mutations p.I368N or p.L347P failed, despite comparable amounts of immunoprecipitated PINK1–V5. Together, these data suggest that the stability of the PINK1 p.I368N mutation is altered through reduced interactions with stabilizing chaperone and the mitochondrial protein import complex.

8. The PINK1 p.I368N mutant affects kinase activity

At equal expression levels, only PINK1 WT was auto-phosphorylated upon mitochondrial depolarization as shown by separation and detection on phos-tag gel (see Figure-5).

To investigate kinase activity of the PINK1 mutation independent of protein instability, HeLa cells were transfected with WT or p.I368N mutant. Accordingly, pS65–Ub levels were only increased in HeLa cells transfected with PINK1 WT, but not in cells expressing the p.I368N mutant or the empty vector. To directly confirm lack of kinase activity of PINK1 p.I368N, we performed in vitro kinase assays with recombinant Ub. PINK1–V5 WT and p.I368N were immunoprecipitated from overexpressing cells that had been treated with or without CCCP and samples were incubated with biotinylated mono-Ub as a substrate. Phosphorylated Ub was detected exclusively in presence of PINK1 WT obtained from CCCP–treated cells (Figure-7).

Altogether, these data confirm the lack of p.I368N kinase activity even at protein levels comparable to PINK1 WT and the protective mitochondrial quality control.

Discussion

While missense mutations in PINK1 and PARKIN typically result in biological loss-of-function, their particular pathomechanisms may vary. Several papers have indicated specific molecular defects of individual missense variants along a sequential and complex-regulated mitochondrial quality control pathway\(^{5, 22}\). This is of particular interest not only towards a complete understanding of the PINK1/PARKIN pathway, but also towards a rationalized drug design in the future.

Although numerous missense mutations have been identified in PINK1 from both patients and controls so far\(^{34}\), the molecular pathomechanisms of PINK1 mutations have not been well elucidated, in particular not on the endogenous level in patients’ cells. In this study, we revealed two additional molecular mechanisms that can lead to loss of PINK1 function and to increased disease risk. PINK1 p.I368N was normally expressed, imported into and processed in mitochondria under basal conditions, in contrast to PINK1 WT, however, stabilization of the full-length PINK1 p.I368N
protein on the OMM was strongly impaired. WB analysis showed that although the full-length form of PINK1 was decreased, the PARL-cleaved form (~52KDa) accumulated in I368N mutants similar to controls after treatment with UPS inhibitor. Yet, proteasome inhibition blocks progression of mitochondrial quality control and N-terminally processed PINK1 itself, which tends to aggregate in the insoluble fraction\(^7\)\(^8\)\(^9\), and has been suggested to block PARKIN translocation from the cytosol\(^10\). This could indicate an overall enhanced and potentially continuous import of PINK1 p.I368N that is coupled to PARL cleavage and degradation by the UPS in the cytosol (Figure-8). It has previously been suggested that the N-terminus of PINK1 is still imported into the inter-membrane space even after depolarization\(^56\). Furthermore, one possibility could not be excluded that PINK1 I368N might be degraded faster by UPS inhibitor resisted unknown protease.

In addition to this, we found remarkably less interactions of PINK1 p.I368N with the cytosolic chaperones HSP90 and CDC37. This chaperone system promotes folding of many kinases and has been shown to regulate stability and subcellular distribution of PINK1\(^50\)\(^57\)\(^59\). Mutations such as p.L347P that showed less binding to HSP90/CDC37\(^51\)\(^60\) were suggested before to be subject to misfolding and increased turnover through either the Ub/proteasome system or another protease\(^48\). While we cannot formally exclude aberrant cleavage of the PINK1 p.I368N mutant in either compartment, a particular instability of the protein in the cytosolic vs. mitochondrial environment was obvious.

It has been suggested that import of PINK1 into mitochondria is directly regulated by binding to HSP90 and CDC37. The chaperone complex not only assists in folding, stability, and thus kinase activity of PINK1\(^46\)\(^50\), but also supports to retention and accumulation of PINK1 on the OMM. As such, a reduced interaction would increase PINK1 inside mitochondria\(^60\), which in turn would give more substrate to MPP in the matrix and PARL in the IMM. Of note, loss of those proteases resulted in OMM accumulation of endogenous, full-length PINK1, which induced mitophagy even in the absence of membrane depolarization\(^8\)\(^9\)\(^56\).

In this regard, it is important to mention that PINK1 WT interacted with the mitochondrial import central channel TOM40 specifically upon CCCP treatment, but both of the mutations that had reduced HSP90/CDC37 binding, failed. It is known that upon mitochondrial depolarization PINK1 auto-phosphorylates\(^53\)\(^61\) and dimerizes into a higher molecular weight protein complex together with the TOM machinery in the OMM\(^10\)\(^12\)\(^62\). However, how PINK1 keep stabilization on OMM remained unsolved. One interpretation is PINK1 stays in TOM complex with resistance to high pH or translocates laterally from TOM complex and integrates into lipid phase of OMM by disrupting membrane potential. It is unclear whether PINK1 then remains in the TOM complex or integrates laterally into the lipid phase of OMM\(^9\)\(^10\)\(^56\). TOM complex is thought that it assists in orientation of dimerized PINK1 to promote intermolecular phosphorylation and consequently activating Ub and

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**Figure-8** Kinase dead p.I368N on the damaged mitochondria

PINK1 p.I368N is imported to the damaged mitochondria, processed then returns to the cytosol to be degraded by Ub/proteasome system (UPS).
Parkin as substrates of PINK1. Further analysis would be needed for this kinase inactive I368N which also might have disability with dimerization, autophosphorylation and formation with TOM complex.

In the structure of PINK1, Ile368 is positioned next to the catalytic loop residues 360–367 of the kinase domain. The catalytic loop is supposed to provide conserved catalytic base Asp362 which is essential for phosphotransferring to substrate. We surmise p.I368N structural change might hinder phosphor–binding or phosphotransfer reaction as similar as other PD causative mutatnt nearby I368N: N367S or L369P.

Importantly, a recent study had identified the ATP–analog kinetin to rescue a kinase-defective mutant PINK1 p.G309D. Future rationalized drug design that is based on a detailed understanding of the pathomechanisms should give more such small molecules. In addition to active site binders, the complex and as of yet poorly understood life cycle of PINK1 that includes its import, cleavage and re-routing between both mitochondrial membranes might reveal further allosteric sites or strategies to enhance activity and/or retention/stabilization on the OMM.

When PINK1 was expressed exogenously in cells, full length PINK1 existed beyond the import amount to the mitochondria and subsequently Parkin recruited to even healthy mitochondria in a quantitative manner. In line, our data indicate that subtle defects seen with physiological levels of PINK1 can be masked or overruled by overexpression. Here, we showed that full-length PINK1 p.I368N is unstable on the OMM under endogenous conditions, but this effect was not seen in the overexpression condition.

In summary, we have shown that the PINK1 p.I368N mutation affects protein stabilization specifically on the OMM and results in complete loss of kinase activity towards Ub likely caused by distortion of the ATP–binding site. Together with previous studies, this highlights the presence of several distinct pathomechanisms that inactivate PINK1 and emphasizes at novel therapeutic avenues that could be pursued in a structure–function based drug design.

Conclusions

Mutations in PINK1 and PARKIN are the most common forms of early onset PD. Both proteins play significant roles in stress-induced mitochondrial quality control that protects cell death. In this study, we examined the PINK1 p.I368N mutation on the clinical and genetic as well as the structural and functional level. We identified two distinct molecular mechanisms that can lead to inactivation of PINK1 function and thus result in loss of neuroprotection. The PINK1 kinase activity as post translational modification would highlight future avenues for drug design and further elucidate a pathogenesis of PD.

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