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Structure of PINK1 in complex with its substrate ubiquitin

Alexander F. Schubert¹, Christina Gladkova¹, Els Pardon^{2,3}, Jane L. Wagstaff¹, Stefan M.V. Freund¹, Jan Steyaert^{2,3}, Sarah L. Maslen¹, and David Komander^{1,*}

¹Medical Research Council Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, CB2 0QH, UK

²VIB-VUB Center for Structural Biology, VIB, 1050 Brussels, Belgium

³Structural Biology Brussels, Vrije Universiteit Brussel, 1050 Brussels, Belgium

Abstract

Autosomal recessive juvenile Parkinsonism (AR-JP) is caused by mutations in a number of PARK genes, in particular in the E3 ubiquitin ligase Parkin (PARK2), and in its upstream protein kinase PINK1 (PARK6). PINK1 phosphorylates ubiquitin and the Parkin ubiquitin-like domain on structurally protected Ser65 to trigger mitophagy. We here report a crystal structure of a nanobody stabilised complex between *Pediculus humanus corporis* (Ph)PINK1 bound to ubiquitin in the ‘C-terminally retracted’ (Ub-CR) conformation. The structure reveals many peculiarities of PINK1, including the architecture of the C-terminal region, and reveals how the PINK1 N-lobe binds ubiquitin via a unique insertion. The flexible Ser65-loop in the Ub-CR conformation reaches the activation segment, facilitating placement of Ser65 in a phosphate accepting position. The structure also explains how autophosphorylation in the N-lobe stabilises structurally and functionally important insertions, and reveals the molecular basis for AR-JP causing mutations, some of which disrupt ubiquitin binding.

Parkinson’s disease (PD) is a neurodegenerative condition caused by the loss of dopaminergic neurons in the substantia nigra, which manifests clinically in the form of characteristic motor defects¹. Most PD cases are sporadic and found in people above the age of 60, but roughly 10% of cases are hereditary forms with an earlier onset. Autosomal recessive juvenile Parkinsonism (AR-JP) is caused by mutations in around 15 PARK genes that have been annotated to date². The Ser/Thr kinase PINK1 (PARK6) was one of the first PARK genes to be identified³, and genetic mutation in flies reconciled Parkinsonism-like symptoms, and linked PINK1 with the E3 ubiquitin ligase Parkin (PARK2)^{4–8}. Detailed

*All reagents and materials are available upon request from the corresponding author (dk@mrc-lmb.cam.ac.uk).

Data availability statement

Coordinates and structure factors have been deposited with the protein data bank under accession codes 6EQI. Uncropped versions of all gels are displayed in Supplementary Figure 1. All data and reagents are available upon request from the corresponding author.

Author contribution

A.S. and D.K. designed the study, and A.S. performed all experiments. C.G. S.M.V.F. and J.L.W. designed and characterised Ub-CR reagents, S.L.M. performed mass-spectrometry, and E.P. and J.S. generated nanobodies. D.K. wrote the manuscript.

Author information

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functional reconstruction have since linked PINK1 and Parkin to mitophagy, an important mitochondrial quality control process^{9–11}.

Mitochondrial damage and subsequent loss of mitochondrial membrane potential leads to the stabilisation of PINK1 on the mitochondrial outer membrane (MOM)¹², and subsequent PINK1 dimerisation, autophosphorylation and activation^{13–16}. PINK1 phosphorylates ubiquitin (hereafter phospho-ubiquitin)^{17–21} and the Parkin Ubl domain on Ser65^{16,21–25}. Together, phosphorylation and phospho-ubiquitin binding lead to localised Parkin activation on mitochondria, where Parkin ubiquitinates MOM proteins²⁶ triggering a feed-forward mechanism²¹. Mitochondrial ubiquitination recruits mitophagy adaptors and initiates clearance of the damaged mitochondria^{9,10,27,28}.

PINK1 is one of the most divergent human protein kinases²⁹. It contains an N-terminal mitochondrial targeting and a transmembrane segment, an unconserved region, a kinase domain with three insertions in the N-lobe, and a conserved C-terminal region (CTR) of unknown function and structure (Fig. 1a, Extended Data Fig. 1). Whilst distinct from other kinases, PINK1 is highly conserved across species (Extended Data Fig. 1). This had led to identification of well-behaved insect PINK1 homologues including *Pediculus humanus corporis* (*Ph*)PINK1 that can be expressed in large quantities in *E.coli*³⁰.

Enabling structural studies of PINK1

It was striking that PINK1 phosphorylates Ser65 in the ubiquitin fold, since its side chain is protected and hence an unlikely phosphorylation site. Building on previous work on phospho-ubiquitin²⁰ (Extended Data Fig. 2), we have recently shown that wild-type ubiquitin adopts an equilibrium between the ‘common’ ubiquitin conformation and a distinct second conformation, in which β 5-strand slippage retracts the C terminus and extends the Ser65-containing loop by two residues³¹ (Extended Data Fig. 2). This ‘ubiquitin C-terminally retracted’ (Ub-CR) conformation is lowly populated, but can be stabilised by point mutations including T66V/L67N (hereafter Ub TVLN, ³¹, Extended Data Fig. 2). Consequently, PINK1 prefers to phosphorylate Ub-TVLN compared to the common ubiquitin conformation ³¹.

We exploited this insight, and raised llama nanobodies against a chemically cross-linked *Ph*PINK1-Ub TVLN complex (Fig. 1b). Nanobody-696 (Nb696) did not bind Ub TVLN and only interacted weakly with *Ph*PINK1 (Extended Data Fig. 3a), but formed a gel filtration-stable, non-covalent trimeric complex when both were present (Extended Data Fig. 3a-c). Also, neither Ub TVLN nor Nb696 alone impacted *Ph*PINK1 stability, but Ub TVLN and Nb696 together increased the melting temperature of *Ph*PINK1 by $\sim 7^\circ\text{C}$ (Extended Data Fig. 3d).

Structure of a *Ph*PINK1 / Ub TVLN / nanobody complex

Further optimisation generated a homogeneous complex of *Ph*PINK1 (aa 143-575) bound to Nb696 and Ub TVLN (Fig. 1c, Extended Data Fig. 3e,f). Subsequent λ -phosphatase treatment of the complex generated *Ph*PINK1 homogeneously phosphorylated at three sites (Extended Data Fig. 3g). The complex was crystallised, resulting in a 3.1 Å structure with

well-defined electron density (Fig. 1d-e, 2, Extended Data Table 1, Extended Data Fig. 4a). Nb696 and Ub TVLN closely resemble previously published structures^{31,32} (Extended Data Fig. 4b-c). The nanobody binds Ub TVLN and both lobes of the kinase domain (Fig. 1d, Extended Data Fig. 4d-e), explaining how it stabilises the complex. Most importantly, the structure reveals how *Ph*PINK1 binds Ub TVLN via the N-lobe, in particular through insertion-3, and also via the kinase C-lobe where Ub TVLN Ser65 is placed in a phospho-accepting position (Fig. 1d-e, see below). This suggests that we have captured a precatalytic state of the PINK1-ubiquitin phosphorylation event.

Unique structural features of PINK1

*Ph*PINK1 adopts the well-known bilobal kinase fold³³ (Fig. 1e, 2a, Extended Data Fig. 5a). Comparison with an active protein kinase structure, e.g. phosphorylase kinase (pdb id 2phk, 34, Extended Data Fig. 5a, b) reveals highly similar conformations of the catalytically important DFG ('DFG-in', Asp357, Phe358, Gly359) and HRD (His332, Arg333, Asp334) motifs^{35,36}, formation of an essential Glu-Lys contact in the ATP binding site (Glu214-Lys193 in *Ph*PINK1), and highly similar R- and C-spines³⁶ confirm an active conformation of *Ph*PINK1 (Extended Data Fig. 5c).

The *Ph*PINK1 structure illuminates unique features of PINK1 such as the architecture of the CTR (Fig. 2a, b, Extended Data Fig. 1). Four additional helices (α J- α M) pack tightly against the C-lobe such that the last two helices (α L and α M) are embedded by CTR α J and α K on one side, and C-lobe helices (α E, α H, α I) on the other side (Fig. 2a, b), forming an extended hydrophobic core (Extended Data Fig. 5d). This architecture explains why loss of the CTR affects PINK1 activity and stability^{30,37}. Exposed surface residues of the CTR do not show a high degree of evolutionary conservation (Extended Data Fig. 5e), and it is unclear whether and how the CTR contributes to PINK1 function beyond stabilisation.

The most striking differences between our *Ph*PINK1-Ub TVLN complex and other kinase structures are located in the kinase N-lobe, which comprises the three unique insertions, but also features an α B and an unusually short, single turn α C helix³⁶ that harbours Glu214 (Fig. 2c-e, Extended Data Fig. 5c).

Of the three insertions, insertion-1 (5 aa in *Ph*PINK1, 34 aa in hPINK1, Extended Data Fig. 1) connecting strands β 2 and β 3, is disordered (Fig. 2c). Insertion-2 (19 aa in *Ph*PINK1, 21 aa in hPINK1) connects the α C helix to the β 4 strand, and forms a short β -strand (β i, parallel to β 4) and an α i-helix atop the α B- α C surface (Fig. 2d).

Insertion-3 (27 aa in *Ph*PINK1 and hPINK1) is located between β 4 and β 5 and forms an ordered subdomain that lacks secondary structure yet binds ubiquitin (Fig. 1e, 2a-e, 3). This element is the best-conserved insertion in PINK1 (Extended Data Fig. 1), underscoring its functional importance. Finally, the N-lobe harbours two conserved phosphorylated residues, Ser202 and Ser204 (Ser228 and Ser230 in hPINK1)(Fig. 2e) that are discussed below.

Ub TVLN binding to *PhPINK1*

Our complex structure enabled analysis of a rare kinase-substrate complex. *PhPINK1* binds Ub TVLN via a 751 Å² interface, and forms bipartite interactions with the N-lobe and the activation segment in the C-lobe (Fig. 3a). The N-lobe part of the interface includes a pocket generated by the Gly-rich loop (aa 164-174), insertion-3 and Phe196-Tyr198, which engulfs the ubiquitin Ala46-Gly47 hairpin (Fig. 3b, Extended Data Fig. 6a). Tyr198 is a key residue that forms hydrophobic contacts with ubiquitin Ile44 and especially Val70, and a hydrogen bond with Gly47 (Fig. 3b). Several additional hydrogen bonds are formed between insertion-3 and ubiquitin (Fig. 3b).

The C-lobe part of the interface involves the *PhPINK1* activation segment (aa 360-385) that contacts the extended Ser65-containing loop of Ub TVLN (Fig. 3c). The latter is held in place by polar contacts (ubiquitin Glu64-*PhPINK1* Asn383/Ala385, ubiquitin Val(Thr)66-*PhPINK1* Asn383, ubiquitin His68-*PhPINK1* Asp379) that resemble those reported in kinase – substrate peptide complexes (Fig. 3c, d) and place Ser65 near a phospho-accepting position. Flexibility in the Ser65-loop and kinase N-to-C-lobe dynamics would enable catalysis in presence of nucleotide.

Mutational analysis confirmed the ubiquitin interface. *PhPINK1* Y198E did not affect autophosphorylation (Extended Data Fig. 7a), but abrogated Ub TVLN and ubiquitin phosphorylation (Fig. 3f). Similarly, a G47E mutant in ubiquitin or Ub TVLN was no longer phosphorylated (Fig. 3f). Structural AR-JP mutations in insertion-3 (*PhPINK1* G281D or P268L, Fig. 3b, see below) reduced or abrogated kinase activity towards ubiquitin or Ub TVLN, and in the case of G281D also reduced autophosphorylation (Fig. 3f, Extended Data Fig. 7a). *PhPINK1* D379A that would prevent the Ub TVLN His68 contact (Fig. 3c), reduced Ub TVLN and ubiquitin phosphorylation, but not *PhPINK1* autophosphorylation, while AR-JP mutation G382V abrogated kinase activity completely (Fig. 3f, Extended Data Fig. 7a). This confirms the ubiquitin binding mode, explaining previous biochemical data^{19,22,38} (Extended Data Fig. 6b).

Strikingly, ubiquitin binding seems to be optimised towards the Ub-CR conformation. The β5-strand shift in Ub TVLN (Extended Data Fig. 2) juxtaposes Val70 and Tyr198 (Fig. 3b), and allows His68 to contact the activation segment via Asp379 (Fig. 3c, Extended Data Fig. 6c). Superposition of wild-type ubiquitin onto Ub TVLN suggests that substrate-positioning interactions with the activation segment can only be established with an extended Ser65-loop (Fig. 3e, Extended Data Fig. 6d). This is consistent with NMR data that the Ser65-loop in wild-type ubiquitin does not partake in *PhPINK1* contacts³¹, and was also confirmed by hydrogen deuterium exchange mass spectrometry (HDX-MS) that revealed that binding of Ub TVLN involves the kinase activation segment, while wild-type ubiquitin does not contact the kinase C-lobe (Extended Data Fig. 6e-f). Nonetheless, wild-type ubiquitin binds to *PhPINK1* and is phosphorylated, albeit at a ~50-fold reduced rate³¹, and NMR data showed interactions via Ala46-Gly47, Ile44 and Arg423¹, consistent with HDX-MS data (Extended Data Fig. 6f). It is a possibility that the ‘common’ ubiquitin conformer binds the N-lobe and transforms to the Ub-CR conformer whilst bound. This would stabilise the interface and enable activation segment interactions and Ser65 phosphorylation.

Phosphorylation organises N-lobe insertions

PINK1 activity is regulated by (auto)phosphorylation^{13,15,39}. Three phosphorylation sites resisted λ -phosphatase treatment and were resolved in the crystal structure of active *Ph*PINK1: Thr305, Ser202 and Ser204. Thr305 is not conserved, and is on an exposed position in the C-lobe (Extended Data Fig. 7b). In contrast, Ser202 and Ser204 are highly conserved (Ser228 and Ser230 in hPINK1), and autophosphorylation of hPINK1 Ser228 is important for kinase activity^{13,15}. The structure explains the important role of phosphoSer202 and phosphoSer204 in coordinating insertion-3 and insertion-2, respectively (Fig. 4a). PhosphoSer204 provides an anchor point for insertion-2 to sit atop the α B- α C helix. Similarly, insertion-3 residues Arg282 and Asn283 wrap around phosphoSer202, locking the end of insertion-3 in place (Fig. 4a).

Hence, while Ub TVLN does not contact phosphoSer202 directly, the role of phosphorylation in structuring insertion-3 may impact ubiquitin binding. Consistently, *Ph*PINK1 S202A/S204A but also *Ph*PINK1 R282A/N283A have lost the ability to phosphorylate Ub TVLN or ubiquitin (Fig. 4b). Moreover, these mutants are less autophosphorylated, confirming severe kinase impairment¹⁵ (Fig. 4b, Extended Data Fig. 7a). Indeed, the AR-JP mutation G281D would also be predicted to disrupt insertion-3, and has similar effects (Fig. 3f).

To corroborate more directly whether phosphorylation is important for ubiquitin binding, we performed pull-down and thermal stability assays with Nb696, which binds on the opposite surface of the kinase domain and does not contact the phosphorylation sites (Fig. 1d, Extended Data Fig. 4d). A *Ph*PINK1/Nb696/Ub TVLN trimeric complex is no longer formed when dephosphorylated *Ph*PINK1 is used (Fig. 4c). Interestingly, re-phosphorylation of *Ph*PINK1 by adding MgATP reinstates complex formation (Fig. 4c). Also, *Ph*PINK1 S202A/S204A and *Ph*PINK1 R282A/N283A display reduced thermal stability (by ~3-4 °C), and are no longer stabilised by Ub TVLN/Nb696 (Extended Data Fig. 7c).

Together, this suggests that phosphorylation in the N-lobe, and in particular organisation of insertion-3 by phosphoSer202, is crucial for kinase integrity and activity, as well as for ubiquitin binding to the N-lobe. The location of Ser202 and Ser204 would require autophosphorylation *in trans*, and would be an early event in PINK1 activation (see further discussion of autophosphorylation in Extended Data Fig. 7d).

Molecular basis for AR-JP

Lastly, the structure of *Ph*PINK1-Ub TVLN provides molecular explanations for numerous mutations identified in AR-JP patients. We collated mutation information from previous reviews^{2,9} and PDMutDB (<http://www.molgen.vib-ua.be/PDMutDB/>) and mapped mutations on the *Ph*PINK1 structure (Fig. 5, Extended Data Fig. 8, Supplementary Table 1). All reported homozygous hPINK1 mutations affect residues conserved in *Ph*PINK1 (Supplementary Table 1). Also, several heterozygous mutations in hPINK1 map to conserved residues in *Ph*PINK1 and likely affect PINK1 structure or function (Fig. 5). Other heterozygous mutations however are not conserved in *Ph*PINK1 (e.g. in the extended

hPINK1 insertion-1) or are in regions where a functional impact is unclear (Extended Data Fig. 8a).

PINK1 mutations can be categorised into structural mutations (Fig. 5a, Extended Data Fig. 8b-c) and mutations that impact on activity or substrate binding (Fig. 5b, Extended Data Fig. 8d). A hot-spot for structural mutations is the activation segment (Extended Data Fig. 8c), highlighting the importance of its integrity. A well-studied N-lobe mutation is T313M, a disputed phosphorylation site^{15,40}. The Thr313 equivalent Ser285 in *PhPINK1* is inaccessible for phosphorylation (Extended Data Fig. 8d) but a larger Met residue would disrupt the N-lobe and insertion-3, impeding substrate- and autophosphorylation^{15,40}. Mutations around the ATP-binding pocket (Fig. 5), and mutation of the DFG and HRD motifs, and α C-helix Glu residue (hPINK1 E240K) would affect kinase activity (Fig. 5, Supplementary Table 1). Finally, several mutations are located in the ubiquitin binding interfaces (Fig. 5b) and include insertion-3 G309D (Gly281 in *PhPINK1*) and P296L (Pro268 in *PhPINK1*) (see Fig. 3b), and in the activation loop G409V (Gly382 in *PhPINK1*) (Fig. 3c). As shown above, these patient mutations introduced into *PhPINK1* reduce or abrogate ubiquitin phosphorylation (Fig. 3f).

Discussion

Our structure of *PhPINK1* bound to one of its substrates, ubiquitin, provides molecular explanations for a cohort of patients with early-onset Parkinson's disease, and provides detailed insight into how ubiquitin, a well-folded protein and unlikely kinase target, is phosphorylated on protected Ser65. We show that *PhPINK1* utilises an only recently identified Ub-CR conformer of ubiquitin³¹ which is able to bind the kinase N-lobe, in part via a unique binding element, insertion-3. Crucially, the Ub-CR conformer, but not the common ubiquitin conformation, is able to form substrate-like interactions with the activation segment of *PhPINK1*. The structural and biochemical data provided here and in ³¹ suggest that the lowly populated Ub-CR conformer of ubiquitin is the PINK1 kinase substrate, indicating its physiological importance. Moreover, rather than 'selecting' or 'inducing' the Ub-CR conformation, *PhPINK1* may bind to the common conformer of the substrate and allow it to transition to a phosphorylatable Ub-CR substrate conformation in situ. Similar mechanisms of local transitions upon substrate binding could be in place for the Parkin Ubl (for which a Ub-CR conformer has not been reported, see Extended Data Fig. 6), or indeed for other kinases that phosphorylate protected residues in folded substrates and e.g. PKR-mediated phosphorylation of eIF2 α seems conceptually similar⁴¹. However, complex structures of kinases bound to their protein substrates are scarce as the mechanism of phosphorylation enables high turnover and does not maintain stable precatalytic states. We trapped such a state by exploiting a nanobody that stabilised the *PhPINK1*-Ub TVLN complex. The resulting structure explains a large body of biochemical data, and will help reconcile controversies in the literature. For example, the structure explains the architecture of the PINK1 CTR, and the unusual features of the N-lobe with its insertions, regulatory phosphorylation sites, and variations of common kinase elements. An apo structure of *Tribolium castaneum* (*Tc*)PINK1 lacking insertion-3 and hence unable to bind and phosphorylate ubiquitin was reported recently⁴². Together, the structures finally explain why PINK1 is one of most divergent protein kinases in the human kinome²⁹. They also

represent a first key step towards identifying and optimizing activators of PINK1-mediated mitophagy events that may benefit patients with Parkinson disease⁴³. A new concept emerging from our studies³¹ is that ubiquitin conformational modulators may affect ubiquitin phosphorylation levels that may lead to new pharmacological strategies.

Online Methods

Molecular Biology

Ubiquitin constructs were cloned into pET17b or into pOPIN-E, *PhPINK1* constructs were cloned into pOPIN-K or pOPIN-S 44, the Nb696 construct was cloned into a pMESy4 vector. Site directed mutagenesis and construct optimisation was carried out using the QuikChange and the InFusion protocol using Phusion polymerase (NEB).

Protein purification

PhPINK1 constructs were expressed in Rosetta 2 (DE3) pLacI cells in 2xTY media. Protein expression was induced at OD₆₀₀ of 0.6 - 0.8 with 200 μ M IPTG and cells were harvested after 12 h at 20 °C. GST-tagged *PhPINK1* constructs (aa 115-575) were purified as described previously 20. The GST-tag was removed using PreScission protease. SUMO-tagged *PhPINK1* constructs (aa 115-575) were purified using HisPur™ Cobalt Resin (Thermo Fisher Scientific). The His-SUMO tag was cleaved using SENP1 during dialysis in cleavage buffer (25 mM Tris (pH 8.5)), 200 mM NaCl, 2 mM β -mercaptoethanol) overnight at 4 °C. *PhPINK1* constructs (aa 115-575) were purified by SEC (Superdex 75 or Superdex 200, GE Life Science) in buffer A (25 mM Tris (pH 8.5)), 150 mM NaCl, 5 % glycerol, 10 mM DTT). Ubiquitin constructs were purified as described previously 20 with SEC (Superdex 75, GE Life Science) in buffer B (25 mM Tris (pH 7.4) 150 mM NaCl) as final step. *PhPINK1* was dephosphorylated using a 1:5 ratio of λ -phosphatase in buffer A supplemented with 0.5 mM MnCl₂. λ -phosphatase was removed by SEC in buffer A. For purification of *PhPINK1* (aa 143-575) for crystallography see 'Protein preparation for crystallization'.

PhPINK1 – Ub TVLN crosslinking for nanobody selection

130 μ l of 1 mM C-terminally His-tagged Ub TVLN was added to 1.8 ml of 24 μ M *PhPINK1* in 25 mM Tris (pH 8.5), 200 mM NaCl, 2 mM β -mercaptoethanol. The reaction was started by adding 100 μ l of 2 mg/ml crosslinker mix from the CovalX stabilization K200 kit (CovalX). The reaction was incubated for 1 h at 22 °C. The formed complex was further purified via the C-terminal His-tag of Ub TVLN using HisPur™ Cobalt Resin (Thermo Fisher Scientific) and eluted with buffer A supplemented with 250 mM imidazole. The complex was subjected to SEC (Superdex 200, GE Life Science) in buffer A as a final purification step. Complex containing fractions were pooled, concentrated to 1.1 ~mg/ml, vitrified in liquid nitrogen, and stored at -80 °C for immunisation.

Generation and selection of *PhPINK1*-Ub TVLN specific nanobodies by phage display

A llama was immunised with a total of 820 μ g of crosslinked *PhPINK1* – Ub TVLN over a period of 6 weeks using previously established protocols 45. From the anti-coagulated blood of the immunised llama, lymphocytes were used to prepare cDNA which served as a

template to amplify the open reading frames coding for the variable domains of the heavy-chain only antibodies. The PCR fragments were ligated into the pMESy4 phage display vector and transformed in *E. coli* TG1 cells. A nanobody library of 10^9 transformants was obtained with an insert rate of 100 % as determined by PCR. After super infection with helper phage, the nanobody repertoire of this library was expressed on the tip of the phage and a selection of phage particles expressing nanobodies that specifically bind to target protein was performed⁴⁵. Two different selection methods were used to select for *PhPINK1*-Ub TVLN specific binders. First, the crosslinked material was solid phase coated on MaxiSorp ELISA plates, and phage displaying nanobodies were added directly to the cross-linked material. In the second step, phage were incubated first with 1 μ M of free *PhPINK1*, and then added to the crosslinked complex. Selections were done in 25 mM Tris (pH 8.5), 150 mM NaCl, 1 mM DTT.

After selection, phage were eluted by incubating the complex-coated wells with 100 μ l of trypsin (250 μ g ml⁻¹) for 30 min. After infection of freshly grown TG1 cells, 48 colonies were randomly picked. Nanobodies were prepared from each clone and binding to the respective antigens was analysed by ELISA. The open reading frames of the positive clones were sequenced. This revealed 23 different nanobody families. Nb696 was present only when phage were counter-selected with *PhPINK1*.

Nb696 was expressed in the periplasm of *E. coli* as described ⁴⁵, in WK6Su- cells in 2xTY media. Protein expression was induced at OD₆₀₀ of 0.6 - 0.8 with 200 μ M IPTG and cells were harvested after 12 h at 30 °C. The cell pellet was resuspended in TES buffer (200 mM Tris, 0.5 mM EDTA, 0.5 M Sucrose (pH 8.0)) for 1 h at 4 °C and centrifuged at 15,000 x g at 4 °C. The nanobody containing supernatant was further purified using HisPur™ Cobalt Resin (Thermo Fisher Scientific) without removing the His-tag. As a final step, Nb696 was purified by SEC (Superdex 75, GE Life Science) in buffer B.

Protein preparation for crystallisation

The *PhPINK1* crystallisation construct (aa 143-575) was expressed as a SUMO-fusion construct as described above and purified using a HiTrap™ Nickel column (GE Life Science). The SUMO-tag was removed during dialysis in cleavage buffer (25 mM Tris (pH 7.2)), 150 mM NaCl, 5 % glycerol, 2 mM β -mercaptoethanol) overnight at 4 °C and captured on the nickel column. The flow-through was further purified using a RESOURCE™ Q column (GE Life Science). Pure *PhPINK1* containing fractions were pooled and mixed with Nb696 and Ub TVLN in a 1:2:2 ratio. The complex was further purified by SEC (Superdex 200, GE Life Science) in (25 mM HEPES (pH 7.0)), 300 mM NaCl, 5 % glycerol, 10 mM DTT). Complex containing fractions were pooled, concentrated and dephosphorylated using a 1:5 ratio of λ -phosphatase in 25 mM HEPES (pH 7.0), 300 mM NaCl, 5 % glycerol, 0.5 mM MnCl₂ 10 mM DTT for 1 h at 22 °C. As a final purification step and to remove the λ -phosphatase, the complex was subjected to SEC (Superdex 200, GE Life Science) in crystallisation buffer (25 mM HEPES (pH 7.0)), 300 mM NaCl, 5 % glycerol, 10 mM DTT) and concentrated to 7-9 mg/ml. The *PhPINK1*-Nb696-Ub TVLN complex was crystallised at 7 mg/ml using sitting-drop vapour diffusion against 1.3 M Ammonium Tartrate Dibasic, 0.1 M BIS-TRIS Propane pH 7.0 from a 1:1

protein to mother liquor ratio, in 100 nl drops at 22 °C. Crystals were optimised using streak seeding at 8.9 mg/ml complex concentration in 1.4 M Ammonium Tartrate, 0.1 M BIS-TRIS Propane pH 6.3 at 22 °C. Crystals were collected after 3-4 days, cryoprotected in 35 % glycerol, 1.0 M Ammonium Citrate, 0.1 M BIS-TRIS Propane pH 7.0, and vitrified in liquid nitrogen.

Data collection, phasing and refinement

Diffraction data was collected at the Diamond Light Source, beam line I-04 (0.98 Å, 100 K), and processed using Xia2 46 (version 0.5.270-ge1c2909e-dials-1.5). The crystal structure was determined by molecular replacement in Phaser 47 (as implemented in Phenix version 1.11.1-2575) using a polyalanine model of CDPK1 from *Plasmodium bergheii* (pdb 3Q5I, unpublished) for *PhPINK1*, the nanobody of the LacY/Nanobody complex (pdb 5GXB, 48) for Nb696, and the phosphoUb TVLN structure (pdb 5OXH, 31) for Ub TVLN. The original solution was rebuilt at 3.1 Å from the models, in multiple rounds of model building in Coot 49 (version 0.7.2) and refinement in PHENIX 50 (version 1.11.1-2575) and Refmac (CCP4 version 6.4.0). Final Ramachandran statistics: 96.7 % favoured, 3.1 % allowed, and 0.2 % outliers. Structural figures were generated using PyMol (version 1.8.6.2 Enhanced for Mac OS X, www.pymol.org). The following residues lacked electron density and were not modelled: *PhPINK1* aa 143-146 (N-terminus), 181-186 (insertion-1), 227-228 (insertion-2), 514-538 (CTR), and 575 (C-terminus); Nb696 127-134 (C terminus); Ub-TVLN 74-76 (C terminus). Data collection and refinement statistics can be found in Extended Data Table 1.

Phos-tag assays

Ubiquitin constructs were phosphorylated by incubating 15 µM substrate with *PhPINK1* (aa 115-575, concentrations as indicated) in 25 mM Tris (pH 7.4), 150 mM NaCl, 10 mM MgCl₂, 10 mM ATP, 1 mM DTT at 22 °C. Reactions were quenched at the indicated time points in EDTA-free LDS sample buffer. Samples were separated with Mn²⁺ Phos-tag SDS-PAGE, using 17.5% (w/v) acrylamide gels supplemented with 50 µM Phos-tag AAL solution (Wako Chemicals) and 50 µM MnCl₂ using an EDTA-free Tris-Glycine running buffer. Gels were stained with Instant Blue SafeStain (Expedeon).

Pull-down assays

Pull-downs were performed by mixing 10 µM Nb696 with 10 µM *PhPINK1* (aa 115-575) and 20 µM Ub TVLN in pull-down buffer (25 mM Tris (pH 8.5), 150 mM NaCl, 1 mM β-mercaptoethanol) at 22 °C. Dephosphorylated *PhPINK1* was pre-incubated with 10 mM ATP/MgCl₂ for 1 h at 22 °C prior the pull-down experiment (deP *PhPINK1* + ATP/MgCl₂). The complex was captured via the His-tagged Nb696 using HisPur™ Ni-NTA Resin (Thermo Fisher Scientific) on Proteus Clarification Mini Spin Columns (Generon). Unbound protein was removed by several centrifugation rounds at 14,000 x g in pull-down buffer at 22 °C. The samples were eluted with pull-down buffer supplemented with 250 mM imidazole and LDS sample buffer was added. Loading (L) and bait (B) samples were diluted 1:5 with LDS sample loading buffer and samples were resolved on 4-12% SDS PAGE gradient gels (NuPage) and stained with Instant Blue SafeStain (Expedeon).

Thermal denaturation assays

Assays were performed by mixing 4 μ M *Ph*PINK1 (aa 115-575) or indicated *Ph*PINK1 mutants with 8 μ M Nb696, and/or 20 μ M Ub TVLN in 25 mM Tris (pH 8.5), 150 mM NaCl. SYPROTM Orange (Thermo Fisher Scientific) was added to the samples and melting curves were recorded using a PCR Rotorgene 6000 (Corbett Research) and analysed with the Rotor-Gene 6000 Series Software (version 1.7, build 75, Corbett Research). Representative melting curves and the derived melting temperatures were plotted with GraphPad Prism (version 7.0b). Nine technical replicates were measured per sample.

Mass spectrometric analysis of phosphorylation states

Intact proteins were subjected to LC-MS analysis. Briefly, a modified NanoAcquity (Waters, UK) delivered a flow of approximately 50 μ L/min. A C8 UPLC BEH 1.7 μ m, 2.1 x 5 mm pre-column (Waters, UK), trapped the proteins prior to separation on a C8 BEH 1.7 μ m, 1.0 x 100 mm UPLC column. Proteins were eluted with a 60 min gradient of acetonitrile (2% to 80% (v/v)). The analytical column outlet was directly interfaced via an electrospray ionisation source, with a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Xevo G2, Waters, UK). Data was acquired over a m/z range of 350–2000, in positive ion mode with lock mass calibration using [Glu1]-fibrino peptide B (50 fmol/ μ L). Scans were summed together manually and deconvoluted using MaxEnt1 (MassLynx version 4.1, Waters, UK).

Hydrogen Deuterium Exchange Mass Spectrometry

Deuterium exchange reactions of *Ph*PINK1 alone and in presence of Nb696 / Ub wt or Nb696 / Ub TVLN were initiated by diluting the protein in D₂O (99.8% (v/v) D₂O ACROS, Sigma, UK) in HDX buffer (25 mM Tris (pH 8.5), 150 mM NaCl, 1 mM TCEP) to give a final D₂O percentage of ~93%. For all experiments, deuterium labelling was carried out at 23°C at 3 s, 30 s and 300 s, in triplicate. The labelling reaction was quenched by the addition of chilled 2.4% (v/v) formic acid in 2 M guanidinium hydrochloride and immediately frozen in liquid nitrogen. Samples were stored at -80°C prior to analysis.

The quenched protein samples were rapidly thawed and subjected to proteolytic cleavage by pepsin followed by reversed phase HPLC separation. Briefly, the protein was passed through an Enzymate BEH immobilised pepsin column, 2.1 x 30 mm, 5 μ m (Waters, UK) at 200 μ L/min for 2 min, the peptic peptides were trapped and desalted on a 2.1 x 5 mm C18 trap column (Acquity BEH C18 Van-guard pre-column, 1.7 μ m, Waters, UK). Trapped peptides were subsequently eluted over 11 min using a 3–43% (v/v) gradient of acetonitrile in 0.1% (v/v) formic acid at 40 μ L/min. Peptides were separated on a reverse phase column (Acquity UPLC BEH C18 column 1.7 μ m, 100 mm x 1 mm (Waters, UK) and detected on a SYNAPT G2-Si HDMS mass spectrometer (Waters, UK) over a m/z of 300 to 2000, with the standard electrospray ionization (ESI) source with lock mass calibration using [Glu1]-fibrino peptide B (50 fmol/ μ L). The mass spectrometer was operated at a source temperature of 80°C and a spray voltage of 2.6 kV. Spectra were collected in positive ion mode. Data was acquired using MassLynx (version 4.1 SCN924, Waters, UK).

Peptide identification was performed by MS^E 51 using an identical gradient of increasing acetonitrile in 0.1% (v/v) formic acid over 11 min. The resulting MS^E data were analysed using Protein Lynx Global Server software (version 3.0.1, Waters, UK) with an MS tolerance of 5 ppm.

Mass analysis of the peptide centroids was performed using DynamX software (version 3.0, Waters, UK). Only peptides with a score >6.4 were considered. The first round of analysis and identification was performed automatically by the DynamX software, however, all peptides (deuterated and non-deuterated) were manually verified at every time point for the correct charge state, presence of overlapping peptides, and correct retention time. Deuterium incorporation was not corrected for back-exchange and represents relative, rather than absolute changes in deuterium levels. Changes in H/D amide exchange in any peptide may be due to a single amide or a number of amides within that peptide.

Three data sets were generated in total: *PhPINK1* alone, *PhPINK1*-Nb696-Ub-TVLN, and *PhPINK1*-Nb696-Ub wt. The peptide masses of *PhPINK1* alone were subtracted from the respective peptide masses of *PhPINK1*-Nb696-Ub-TVLN or *PhPINK1*-Nb696-Ub wt, respectively. The mass differences were plotted with the middle amino acid of each peptide with Graph Pad Prism (v.7.0b)

Extended Data

Extended Data Figure 1. Similarity of *PhPINK1* with other PINK1 orthologues.

PINK1 orthologues from different species were aligned using MUSCLE52. *PhPINK1* secondary structure elements are shown and coloured as in Fig. 1. The β6 and the β9 sheets often observed in kinases form highly analogous interactions but are not annotated as β-strand secondary structure elements in *PhPINK1*. The amino acid sequences of regions with no structural information are coloured grey. Catalytic residues are highlighted in red and

phosphorylation sites in orange. The position of insertion-1, -2, and -3 are indicated as yellow boxes. Stars indicate residues mutated in AR-JP patients and their colour is based on the here provided structural information (see Fig. 5 and Supplementary Table 1).

Extended Data Figure 2. Background to the Ub-CR conformation in Ub TVLN.

(a) We previously published that Ser65-phosphorylated ubiquitin adopts an equilibrium between the ‘common’ ubiquitin conformation and a second ‘C-terminally retracted’ Ub-CR conformation, in which the β 5-strand slides in its binding groove by two residues^{20,53}. Illustrated are the shape of ubiquitin and the relative position of the last β -strand in the two conformations; a side-view (boxed) shows how the hydrophobic Leu side chains in ubiquitin slide in their respective pockets. (b) Using Chemical Exchange Saturation Transfer (CEST) NMR experiments⁵⁴ we found that unphosphorylated wild-type ubiquitin also adopts a very small (‘invisible’) population of a similar Ub-CR conformation³¹. (c) We were able to stabilise the Ub-CR conformation in unphosphorylated ubiquitin by mutating Thr66 to Val, and Leu67 to Asn. The resulting Ub TVLN mutant adopts the Ub-CR conformation in the

unphosphorylated state, binds *Ph*PINK1 with higher affinity via its exposed Ser65-containing loop, and is consequently a ~50-fold better substrate for *Ph*PINK131. Phosphorylated Ub TVLN adopts the Ub-CR conformation as well31. **(d)** There are numerous important structural differences between ubiquitin in the ‘common’ wild-type conformation and in the Ub-CR conformation in Ub TVLN. The latter extends the Ser65 loop, which becomes flexible and solvent exposed, while the C-terminus is shortened. Moreover, the important Ile44 hydrophobic patch becomes disjointed, as Val70 and His68 move on the ubiquitin surface. The new conformation hence has numerous unique features that can be exploited by binding partners. Indeed, the properties of Ub-CR as a superior substrate and the knowledge that wild-type ubiquitin exists in the Ub-CR conformation is consistent with the idea that PINK1 utilises this conformation during the phosphorylation process31.

Extended Data Figure 3. Nb696 stabilises the *PhPINK1*-Ub TVLN complex.

(a) Nb696-mediated (B, bait) pull-down experiments of Ub TVLN, *PhPINK1* (aa 115-575), and *PhPINK1* + Ub TVLN (L, loading; E, elution). Nb696 does not pull down Ub TVLN alone, interacts with *PhPINK1* only weakly, but stably binds *PhPINK1* and Ub TVLN when both are present. Experiments were performed in duplicate. (b) Gel filtration profile of *PhPINK1* (aa 115-575) alone (dashed line) and *PhPINK1* (aa 115-575) with Nb696 and Ub TVLN. (c) Gel filtration fractions showing that *PhPINK1* co-elutes with Nb696 and Ub TVLN. A protein contamination (*), potentially a truncation product of *PhPINK1*, co-elutes

with the complex. **b** and **c** correspond to a representative example from an experiment performed in duplicate. **(d)** Melting temperatures of *PhPINK1* (aa 115-575) alone, *PhPINK1*/Ub TVLN, *PhPINK1*/Nb696, or *PhPINK1*/Nb696/Ub TVLN. When both Nb696 and Ub TVLN are added, the melting temperature of *PhPINK1* increases by $\sim 7^\circ\text{C}$, consistent with complex stabilisation. The left panel shows representative melting curves for each sample. The right panel plots the derived values. Experiments have been done in nine technical replicates. The average melting temperatures are indicated. **(e-f)** Further construct optimisation by removing flexible N-terminal residues resulted in *PhPINK1* (aa 143-575), which also formed a stable trimeric complex with Nb696 and Ub TVLN on gel filtration. Gel filtration runs with identical compositions have been performed eleven times, and a representative experiment is shown. **(f)** Coomassie stained SDS PAGE of protein containing fractions of the representative experiment shown in **e**. The fractions of three gel filtration runs have been analysed with identical results. **(g)** *PhPINK1* (residues 143-575) was expressed in *E.coli* and is autophosphorylated in the *PhPINK1*-Nb696-Ub-TVLN complex at six to nine sites. (*top left*). Dephosphorylation of the complex with λ -phosphatase (λ -PP) results in a species with three phosphorylation sites on *PhPINK1* (*top right*). λ -phosphatase treatment does not affect Nb696 or Ub TVLN intact mass in the MgATP-free conditions (*bottom*). A second lower abundant, presumably acetylated, *PhPINK1* species was also detected and shows the same phosphorylation pattern (*, *top left*). The dephosphorylated complex was used for crystallisation. Experiments have been done in technical duplicates.

Extended Data Figure 4. Electron density and structural details.

(a) Electron density of the *PhPINK1*-Nb696-Ub TVLN complex. The structure is shown as in Fig. 1d and covered by a $2|F_o|-|F_c|$ electron density map contoured at 1σ . Detailed views (*boxed*) show the same electron density map for each component and for selected interfaces (the latter at 1.5σ). **(b)** Nb696 from the *PhPINK1*-Nb696-Ub TVLN complex structure is compared to two most similar structures in the PDB according to DALI analysis 55 (http://ekhidna.biocenter.helsinki.fi/dali_server): Nanobody TP377 (pdb 5E0Q, 32, beige, RMSD 0.624 Å) and a Nanobody targeting *mVsig4* (pdb 5IMK, 56, light blue, RMSD 0.460 Å).

The right panel shows the superposition of structures. As expected, the stable nanobody scaffold is highly similar apart from divergent variable regions (red). **(c)** Ub TVLN from the *Ph*PINK1-Nb696-Ub TVLN complex (red) is structurally highly similar to recent Ub-CR structures (e.g. Ub L67S (pdb 5OXI, 31, green, RMSD 0.766 Å). The structure is also highly similar to wild-type ubiquitin (pdb 1UBQ, 57, grey, RMSD 0.669 Å) despite slippage of the last β -strand. The right panel shows the superposition of the structures. **(d)** Structure as in Fig. 1e, with the nanobody included. The nanobody interacts with the ATP binding groove, N-lobe, C-lobe and Ub TVLN. The nanobody does not contact the N-lobe phosphorylation sites but binds on the opposing kinase surface (see Fig. 2). **(e)** Structural detail of nanobody interfaces. Hydrogen bonds are shown as dotted lines.

Extended Data Figure 5. *PhPINK1* structure comparison and structure-based sequence alignment.

(a) Structural similarity searches using DALI 55 with the obtained *PhPINK1* structure reveals highest similarity with LIM domain kinase (LIMK1, pdb 5HVK 58, DALI score 23.6), and death-associated protein kinase 1 (DAPK1, pdb 5AV3 59, DALI score 23.5), but also shows that >100 kinase structures have DALI scores >23. We then used the isolated, divergent N-lobe of *PhPINK1* in a second search, revealing high similarity to Polo-like kinase 4 (PLK4, pdb 4YUR 60, DALI score 9.5). We chose to compare *PhPINK1* to

phosphorylase kinase (pdb 2PHK 34, DALI score 8.3), since this enzyme had been characterised in various states including bound to a substrate peptide (see Fig. 3). *Left*, *PhPINK1* structure (*left*, coloured as in Fig. 2); *middle*, phosphorylase kinase; *right*, superposition. **(b)** Structure-based sequence alignment of *PhPINK1* kinase domain and phosphorylase kinase from **(a)**, using PROMALS3D61. The *PhPINK1* secondary structure elements are displayed as in Extended Data Fig. 1. **(c)** Direct comparison with phosphorylase kinase reveals hallmarks of an active kinase conformation in *PhPINK1*. *Left*, Spine analysis showing aligned C- and R-spines within the kinase core. *Middle*, two insets show the corresponding residues under a transparent surface. *Right*, zoom-in into the ATP binding site. In *PhPINK1*, ATP is modelled from the phosphorylase kinase superposition. Key structural elements include a crucial Glu-Lys salt bridge involving the α C helix, the DFG motif in the 'in' position, and the HRD motif in an active conformation. Striking similarities in the conformation of all elements support the notion that *PhPINK1* is in an active conformation. **(d)** The CTR extends the kinase domain and forms a contiguous hydrophobic core with the kinase C-lobe. CTR helices are shown in dark purple and the kinase C-lobe in pink, and hydrophobic Leu, Ile, Phe and Trp residues are shown in stick representation. **(e)** ConSurf analysis (<https://consurf.tau.ac.il/2016/>) of *PhPINK1* (*left*) and a close-up of the C-terminal region (CTR, *right*) coloured according to level of conservation. The CTR shows no apparent conserved patches except a hydrophobic patch between α K and α L that originates from Leu-zipper interactions between helices.

Extended Data Figure 6. Ub TVLN and Ser65-loop binding to *PhPINK1*.

(a) View focussing on the Ub TVLN Ala46/Gly47 hairpin, which occupies a pocket on the *PhPINK1* N-lobe between the Gly-rich loop, Tyr198, and insertion-3. *PhPINK1* is shown under a surface to highlight shape complementarity. (b) Ub TVLN bound to *PhPINK1*, showing the position of Lys residues. Several Lys residues display disordered side chains and were modelled in their most favourable rotamers. Lys6 and Lys48 are close to the interface with *PhPINK1*, which may disallow binding to ubiquitin moieties ubiquitinated at these sites (i.e. internal ubiquitin molecules in a ubiquitin polymer). This is consistent with

recent data that *PhPINK1* phosphorylates Lys6 and Lys48 chains with strong preference at the distal ubiquitin 38.

(c-d) Direct comparison of Ub TVLN and wild-type ubiquitin binding to *PhPINK1*. **(c)** In Ub TVLN, the Ub-CR conformation enables optimised contacts with Val 70, and allows His68 to contact the activation segment. **(d)** In wild-type ubiquitin, the Gly47 hairpin is identical and can form contacts with the N-lobe, but Tyr198 in *PhPINK1* is juxtaposed to the larger and more hydrophilic His68, and Val70 is not involved in the interaction. Moreover, the Ser65 loop is unable to contact the activation segment. The difference in the ability of the Ser65-loops to partake in *PhPINK1* interaction was seen in NMR studies 31. **(e-f)** Hydrogen deuterium exchange mass spectrometry (HDX-MS) studies of *PhPINK1* bound to Nb696 and Ub TVLN or wild-type ubiquitin (see Methods). Experiments were performed as technical triplicate. Differences in *PhPINK1* deuterium uptake upon complex formation with either Nb696+Ub-TVLN or Nb696+Ub WT are shown for 3 s, 30 s, and 300 s timepoints. Mass differences are plotted on the middle amino acids of the respective peptides. The data revealed identical binding sites for ubiquitin and Ub TVLN in the N-lobe (pink, yellow boxes) consistent with the complex structure. Importantly, Ub TVLN also binds to the C-lobe activation segment (orange box), whereas this interaction is not seen with wild-type ubiquitin. It is also apparent that Ub TVLN protects *PhPINK1* binding sites more efficiently suggesting that the complex is more stable (compare 300 s datasets, red). **(g-h)** Structures of the Parkin Ubl domain resemble wild-type ubiquitin (shown here in green Parkin Ubl R33Q crystal structure, pdb 3B1L, unpublished). **(g)** Conservation of residues at the *PhPINK1* interface (including Ala46/Gly47) would suggest a similar *PhPINK1* binding mode as compared to wild-type ubiquitin. A Ub-CR conformer for the Parkin Ubl has however not been reported, and a superposition **(h)** shows that like wild-type ubiquitin, the Parkin Ubl would unlikely be able to bind the activation segment. NMR analysis however suggests that the Parkin Ubl Ser65 loop displays higher structural flexibility 62.

Extended Data Figure 7. *PhPINK1* autophosphorylation.

(a) Autophosphorylation states of indicated bacterially expressed *PhPINK1* (aa 115-575) proteins, all of which were purified identically. The top row, middle panels shows that these states can be collapsed by λ -phosphatase treatment to a singly-phosphorylated species in the absence of Nb696 (compare Extended Data Fig. 3) The identified phosphorylation state of the *PhPINK1* variants is depicted in Fig. 3f and 4b. Some mutations abrogate kinase autophosphorylation, while others (Y198E) are fully phosphorylated. Experiments have been done in technical duplicates. (b) Structure of *PhPINK1* from the complex, showing that

phospho-Thr305 resides in an exposed area of the C lobe, far from the ATP- or ubiquitin-binding sites. It is unclear why this site has been spared by λ -phosphatase treatment; one explanation is that the neighbouring Pro306 may impede λ -phosphatase binding **(c)** Thermal denaturation analysis of *PhPINK1* with mutated N-lobe autophosphorylation sites (S202A/S204A) or lacking the ability to coordinate pSer202 with insertion-3 (R282A/N283A). Both mutants are already impaired in stability (by 3-4 °C as compared to wild-type *PhPINK1*) already suggesting that phosphorylation stabilises the N-lobe. Moreover, addition of Nb696 and Ub TVLN, which increases stability of wild-type *PhPINK1* (see Extended Data Fig. 3), has no effect on either mutant. This is further evidence that phosphorylation is required for ubiquitin binding. The left panel shows representative melting curves for each sample. The right panel plots the derived values. Experiments have been done in nine technical replicates. The average melting temperatures are indicated. **(d)** One autophosphorylation site that may have been lost with λ -phosphatase treatment, is on Ser375 in the activation segment (corresponding to Ser402 in hPINK1). Indeed, while the exposed position of Ser375 explains how it may be targeted by λ -phosphatase, the conformation of the activation segment in *PhPINK1* does not reveal how the reportedly crucial phosphorylation event on this site^{13,15} impacts kinase activity. Traditionally, activation segment phosphorylation is coordinated by the HRD motif (aa 332-334 in *PhPINK1*, 360-362 in hPINK1), yet in *PhPINK1*, the shortest side chain distance between Arg333 and Ser375 is >13Å; instead, Arg333 is in close proximity to the backbone of Lys380 and to the side-chain of Asp379 (which also binds ubiquitin His68) (see Fig. 3c, Extended Data Fig. 6c). The activation segment conformation is supported by the α EF- α F-loop below, and by intra-loop interactions, e.g. Arg374-Gln378 and Lys380-Tyr404. On the other hand, the activation segment may be flexible enough for phosphoSer375 to fold back and bind Arg333. It is also possible that phosphorylation of Ser375 (Ser402) is transiently important for kinase activation, and then no longer essential for ubiquitin phosphorylation. A structure of phosphorylated *PhPINK1* at this site will be required to understand the reported importance of activation segment phosphorylation for kinase activity.

Extended Data Figure 8. Additional insight into PINK1 AR-JP mutations.

(a) Detailed view showing PINK1 mutations that are not conserved, or in positions where a direct impact on PINK1 structure is not obvious. Notably, these mutations are all annotated as heterozygous, and may exist in conjunction with other mutations. (b) PINK1 fold mutations shown in Fig. 5a are coloured blue, and other residues in white. Displaying the surface of *PhPINK1* shows that the mutated residues affect the core of the protein and hardly affect *PhPINK1* surface properties. (c) Detailed view of the activation segment, depicting the cluster of mutations shown in Fig. 5, as well as exposed autophosphorylation site Ser375 (Ser402 in hPINK1). (d) Detailed view of mutations in insertion-3, including well-studied T313M (Ser285 in *PhPINK1*).

Extended Data Table 1
Data collection and refinement statistics.

Values in parentheses are for highest-resolution shell.

<i>PhPINK1/Nb696/Ub-TVLN</i>	
Data collection	
Space group	$P3_1 2 1$
Cell dimensions	
a, b, c (Å)	105.71, 105.71, 149.45
α, β, γ (°)	90, 90, 120
Resolution (Å)	49.83 – 3.10 (3.31 – 3.10)
R_{merge}	0.14 (1.18)
$I / \sigma I$	11.67 (2.10)
Completeness (%)	99.50 (99.98)
Redundancy	7.1 (7.1)
Refinement	
Resolution (Å)	49.83 – 3.10
No. reflections / test set	17963 / 1775
$R_{\text{work}} / R_{\text{free}}$	0.203 / 0.234
No. atoms	
Protein	4426 (592 aa)
Ligand/ion	18
Water	9
B -factors	
Protein	85.60
Ligand/ion	92.07
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.67

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *PhPINK1* constructs, nanobody design, and crystal structure of the trimeric *PhPINK1*/Nb696/Ub TVLN complex.

(a) Human PINK1 (hPINK1) and *Pediculus humanus corporis* PINK1 (*PhPINK1*) constructs used in this study. The mitochondrial targeting sequence (MTS), transmembrane domain (TM), insertions-1 (i1), -2 (i2), and -3 (i3), and the C-terminal region (CTR) are indicated. (b) Nanobodies were raised against the chemically crosslinked *PhPINK1* - Ub T66V, L67N (Ub TVLN) complex. (c) Nanobody 696 (Nb696) forms a stable, non-covalent trimeric complex with *PhPINK1* and Ub TVLN on gel filtration. A single fraction from a gel filtration run is shown. See also Extended Data Fig. 3f for full gel and Supplementary Figure 1 for uncropped gels. (d) Crystal structure of the *PhPINK1*/Nb696/Ub TVLN complex, with *PhPINK1* under a surface, Nb696 in grey, and Ub TVLN in red. The ubiquitin-binding Insertion-3 is shown in yellow. Ubiquitin Ser65 is displayed as spheres. See also Extended Data Table 1 and Extended Data Fig. 4. (e) As in d, with 90° rotation and showing only *PhPINK1* and Ub TVLN.

Figure 2. Unique features of PINK1.

(a) *PhPINK1* structure overview. Kinase domain (KD) (pink), C-terminal region (CTR, dark pink), insertions-1, -2, and -3 (yellow), and N-lobe phosphorylation sites (spheres) are indicated. Dashed lines indicate disordered loops. (b) The CTR circles back around the kinase C-lobe (transparent grey surface). See Extended Data Fig. 5d-e. (c) Close-up of the N-lobe showing the position of the insertions. The kinase C-lobe is displayed as a white surface. See Extended Data Fig. 5a-b for a comparison with phosphorylase kinase. (d) As in c, but focussing on the unusual α B and α C helices of *PhPINK1*. (e) N-lobe autophosphorylation sites of *PhPINK1*, pSer202 (Ser228 in hPINK1) and pSer204 (Ser230 in hPINK1). Also see Fig. 4.

Figure 3. The ubiquitin binding site.

(a) A bipartite interaction between *PhPINK1* (as in Fig. 2) and Ub TVLN (red) is formed via the N-lobe (insertion-3, $\beta 3$ - αB loop, Gly-rich loop) and the activation segment. ATP was modelled from a superposition with phosphorylase kinase (pdb 2PHK 34). (b) Detail of the *PhPINK1* N-lobe - Ub TVLN interaction. Dotted lines indicate hydrogen bonds. Red residue labels indicate AR-JP mutations (see Fig. 5). Also see Extended Data Fig. 6. (c) Detail of the Ub TVLN Ser65 loop interacting with the activation segment. The Ub-CR conformation enables activation loop contacts of the Ser65 loop and His68 (see Extended Data Fig. 2d, 6).

(**d**) Substrate peptide interaction in phosphorylase kinase bound to a peptide substrate (pdb 2PHK 34). (**e**) Model of *PhPINK1* with wild-type ubiquitin, which is unable to engage the activation segment, also see Extended Data Fig. 6c-f and 31. (**f**) Phos-tag-based time course analysis of Ub TVLN (*top*) and ubiquitin (*bottom*) phosphorylation with indicated *PhPINK1* (aa 115-575) and Ub mutants. The autophosphorylation status of *PhPINK1* based on intact protein mass spectrometry (Extended Data Fig. 7a) is indicated. Experiments were performed in duplicate. See Supplementary Figure 1 for uncropped gels.

Figure 4. Phosphorylation organises the N-lobe.

(a) Detailed view on the N-lobe showing how phosphoSer202 and phosphoSer204 upstream of the αB helix contribute to integrity of the N-lobe. PhosphoSer202 coordinates the ubiquitin-binding insertion-3 (Arg282, Asn283) but does not directly contact ubiquitin. (b) Phos-tag-based time course analysis of Ub TVLN (*top*) and ubiquitin (*bottom*) phosphorylation by *PhPINK1* (aa 115-575) mutants. The *PhPINK1* autophosphorylation status based on intact protein mass spectrometry (Extended Data Fig. 7a) is indicated. Experiments were performed in duplicate. (c) Nb696-mediated (B, bait) pulldown of the

*Ph*PINK1 - Ub TVLN complex (L, loading; E, elutate). Dephosphorylated (deP) *Ph*PINK1 weakly interacts with Nb696 but no longer with Ub TVLN, and addition of MgATP reinstates phosphorylation and complex formation. Shown is a representative example from duplicate experiments. Also see Extended Data Fig. 7. See Supplementary Figure 1 for uncropped gels.

Figure 5. *PhPINK1* explains human AR-JP mutations.

(a) Structure of *PhPINK1* with AR-JP mutations highlighted that would affect kinase structure. Shown are residues from homozygous (**bold**) and heterozygous patients according to 2,9 and PDMutDB (<http://www.molgen.vib-ua.be/PDMutDB/>) which are conserved between *PhPINK1* and hPINK1 (see Extended Data Fig. 1, Supplementary Table 1). Most fold-mutations do not affect surface properties of *PhPINK1* (Extended Data Fig. 8b). (b) As in a, but focussing on residues affecting the ATP-binding pocket and catalytic residues of

PINK1, as well as the ubiquitin-binding site. See Fig. 3f for biochemical effects of *Ph*PINK1 P268L, G281D and G382V. Also see Extended Data Fig. 8.