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Differential submitochondrial localization of PINK1 as a molecular switch for mediating distinct mitochondrial signalling pathways

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Abstract

Mutations in mitochondrial kinase PINK1 cause Parkinson disease (PD), but the submitochondrial site(s) of PINK1 action remains unclear. Here, we report that three-dimensional structured illumination microscopy (3D-SIM) enables super-resolution imaging of protein submitochondrial localization. Dual-color 3D-SIM imaging analysis revealed that PINK1 resides in the cristae membrane and intracristae space but not on the outer mitochondrial membrane (OMM) of healthy mitochondria. Under normal physiological conditions, PINK1 colocalizes with its substrate TRAP1 in the cristae membrane and intracristae space. In response to mitochondrial depolarization, PINK1, but not TRAP1, translocates to the OMM. The PINK1 translocation to the OMM of depolarized mitochondria is independent of new protein synthesis and requires combined action of PINK1 transmembrane domain and C-terminal region. We found that mitochondrial depolarization-induced PINK1 OMM translocation is required for recruitment of parkin to the OMM of damaged mitochondria. Our findings suggest that differential submitochondrial localization of PINK1 serves as a molecular switch for mediating two distinct mitochondrial signalling pathways in maintenance of mitochondrial homeostasis. Furthermore, our study provides evidence for the involvement of deregulated PINK1 submitochondrial localization in PD pathogenesis.

Keywords

Parkinson disease; PINK1; parkin; TRAP1; three-dimensional structured illumination microscopy

1. Introduction

Parkinson disease (PD) is the most common neurodegenerative movement disorder [1-3]. While PD is best known for the loss of dopaminergic neurons in the substantia nigra, increasing evidence indicates that the disease also involves neurodegeneration in other brain regions, including cerebral cortex [4-7]. The pathogenic mechanisms that cause neurodegeneration in PD remain unclear, but mitochondrial dysfunction has been strongly implicated in PD pathogenesis [8-10]. Human genetic studies have identified a number of

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homozygous mutations in mitochondrial serine/threonine kinase PINK1 that cause autosomal recessive, early-onset PD [11-14]. In addition, heterozygous mutations in PINK1 has been implicated as a significant risk factor in the development of late-onset PD [15-17]. These findings underscore the importance of determining the sites and mechanisms of PINK1 action.

Mitochondria contain two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which separate two aqueous compartments: the intermembrane space (IMS) and the matrix. Compartmentalization provided by the double membrane structure is fundamental to many aspects of mitochondrial function, including redox control, energy production, metabolism, and programmed cell death [18,19]. Because the resolution of confocal microscopy is not sufficient to resolve submitochondrial compartments, most published studies of PINK1 submitochondrial localization [20-24] relied on the use of subcellular/submitochondrial fractionation, a method which is prone to artifacts resulting from impurities in the preparation of fractions. A few PINK1 localization studies [25-27] used immunogold labeling electron microscopy, which suffers from potential artifacts resulting from the harsh chemical fixation, embedding, and sectioning procedures. These methodological limitations may contribute to the conflicting conclusions on PINK1 submitochondrial localization: several studies, including ours, showed that PINK1 is localized in the IMM and IMS with its kinase domain facing the IMS [24,28,25,23,26], whereas others reported that PINK1 resides on the OMM with its kinase domain facing the cytoplasm [29,27,21]. The submitochondrial localization of PINK1 remains contentious and unresolved.

Three-dimensional structured illumination microscopy (3D-SIM) is a recently developed, super-resolution fluorescence imaging technique which improves resolution through reconstruction of multiple images produced with periodic illumination patterns in different orientations [30,31]. Here, we show that 3D-SIM is a useful tool for studying protein submitochondrial localization. Dual-color 3D-SIM super-resolution imaging analysis reveals that PINK1 resides in the IMM and IMS but not in the OMM under normal physiological conditions and that PINK1 colocalizes with mitochondrial molecular chaperone TRAP1, a PINK1 substrate identified in our previous study [24]. When mitochondria are depolarized, PINK1, but not TRAP1, translocates to the OMM and colocalizes with E3 ubiquitin-protein ligase parkin, a PINK1 substrate with a key role in mitophagy. Furthermore, our study has identified the structural determinants of PINK1 translocation to the OMM and obtained evidence linking the impairment of PINK1 translocation to PD pathogenesis.

2. Materials and Methods

2.1 Expression constructs and antibodies

The expression constructs encoding C-terminal GFP-tagged human PINK1 WT, PD-linked PINK1 L347P and W437X mutants were generated by using standard molecular biological techniques. Plasmids containing PINK1 C92F and Δ TM mutant cDNAs were provided by R. Youle (National Institutes of Health, Bethesda, MD) and the cDNAs were subcloned to generate constructs for expression of C-terminal GFP-tagged PINK1 C92F and Δ TM mutants. The validity of all constructs was confirmed by DNA sequencing. The vector for

mitochondrial matrix-targeted mito-dsRed (pDsRed2-Mito) was obtained from Clontech (Mountain View, CA) and the plasmid mCherry-parkin from Addgene (Cambridge, MA). The following antibodies were used in this study: anti-TOM20 (FL-145) and anti-TRAP1 (TR1 or HSP75; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome *c* (Clone 6H2.B4) and anti-TIM23 (BD biosciences, San Jose, CA). All secondary antibodies (FITC and TRITC) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.2 Cell transfection and treatment

HeLa cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. HeLa were transfected with the indicated plasmids using Lipofectamine 2000 (Life Technologies, Grand Island, NY) by following the manufacturer's instructions. For analysis of the effects of various treatments on PINK1 submitochondrial localization, cells were either untreated or treated for two hours with 10 µM CCCP, 10 µM valinomycin, 10 µM oligomycin, 15 µM nocodazole, 80 µM antimycin A, or 10 µM nigericin. All chemicals were purchased from Sigma-Aldrich (St Louis, MO). For analysis of protein synthesis inhibition on mitochondrial depolarization-induced PINK1 OMM translocation, cells were pretreated with either 10 µM emetine or 100 µM cyclohexamide (Sigma-Aldrich, St Louis, MO) for one hour followed by addition of CCCP and co-incubation of emetine or cyclohexamide with 10 µM CCCP for 2 hours.

2.3 PINK1 knockout mice and primary neuronal culture

A breeding colony of PINK1 knockout mice on 129/SvEv background was established from breeding pairs provided by G. Auburger (University Medical School, Frankfurt am Main, Germany) as described [32]. Primary cortical cultures were prepared from wild type or PINK1 knockout mouse embryos as described previously [33,34]. Briefly, cortices were dissected from embryonic day 18 (E18) mouse embryos, and cortical neurons were dissociated and plated on poly-L-lysine coated coverslips or MatTek glass bottom dishes. Neurons were cultured in Neurobasal Media supplemented with 0.5 mM L-glutamine, B27, and 100 IU/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Grand Island, NY). Neurons were transfected with the indicated constructs on day *in vitro* 4 using Lipofectamine 2000 (Life Technologies, Grand Island, NY). Culture media were changed to the supplemented Neurobasal media lacking B27 (Life Technologies, Grand Island, NY) at 24 hours after transfection, and neurons were treated with 10 µM CCCP for three hours.

2.4 3D-SIM super-resolution microscopy

Cells were fixed with 4% paraformaldehyde in culture media for 10 min, permeabilized with a solution containing 0.1% saponin (Sigma-Aldrich, St Louis, MO) and 2% horse serum in 1× PBS, stained with the indicated primary antibodies followed by secondary antibodies conjugated to FITC or TRITC, and then mounted in ProLong Gold antifade reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Images were captured at room temperature using a CFI Apochromat TIRF 100×/1.49 NA oil immersion objective lens on an N-SIM microscope (Nikon Instruments, Melville, NY) equipped with

CCD camera (DU-897), Perfect Focus System, and SIM Illuminator using 488 nm and 561 nm lasers. All images were acquired and reconstructed using NIS-Elements software (Nikon Instruments, Melville, NY). Image stacks were acquired in 100 nm intervals for 8-15 Z planes. In each plane, 15 images were acquired with a rotating illumination pattern (5 phases, 3 angles) in two color channels (488 nm and 561 nm) independently. The series of images were reconstructed using NIS-Elements software to yield three-dimensional images with resolution beyond the limit of diffraction. Brightness was adjusted using NIS-Elements software and figures were compiled using Photoshop CS4 software (Adobe Systems, San Jose, CA).

2.5 Image analysis

All image analysis was performed using the ImageJ software (National Institutes of Health). For line scans, a straight line was drawn through the mitochondria between the two arrowheads indicated on the images, and the fluorescence intensity profiles of each color channel was determined by using the Plot Profile function in ImageJ. The value of fluorescence intensity was normalized to that of the single brightest point in each channel and is expressed as arbitrary unit. Graphs were made in SigmaPlot 11.0 (Systat Software, San Jose, CA), and images were annotated to indicate where the line was drawn using Photoshop CS4 software.

2.6 Quantification of colocalization

Quantification of the colocalization of PINK1 with TRAP1 or parkin was performed on unprocessed images. The background was subtracted from the images by setting the threshold of each channel to the background value, and the percentage of PINK1 overlapping with TRAP1 or parkin was determined by Mander's coefficients using the JACOP plugin for ImageJ [35,36]. The percentages of overlap were averaged from 30 randomly selected mitochondria for each experiment, and the experiment was repeated three times. Graphs were generated with SigmaPlot 11.0 software and figures made using Photoshop CS4 software.

2.7 Statistical analysis

Data were subjected to statistical analyses by one- or two-way analysis of variance with a Tukey's *post hoc* test using the SigmaPlot software (Systat Software, San Jose, CA). Results are expressed as mean $\pm$ s.e.m. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Dual-color 3D-SIM super-resolution imaging analysis enables visualization of protein submitochondrial localization

Mitochondria have a complex architecture with four distinct compartments: the OMM, IMM, IMS, and matrix (Fig. 1A). Due to the presence of the cristae junction, the IMM is divided into two structurally distinct subdomains: the inner boundary membrane, which parallels the OMM, and the cristae membrane, which invaginates into the matrix [37]. Likewise, the IMS is divided into two subcompartments: the peripheral IMS, which lies

between the OMM and the inner boundary membrane, and the intracristae space, which is bordered by the cristae membrane (Fig. 1A). To determine if 3D-SIM super-resolution microscopy is capable of resolving submitochondrial compartments, we performed dual-color 3D-SIM fluorescence imaging analysis of HeLa cells with mitochondria labeled by the mitochondrial matrix marker mito-dsRed and antibodies against the OMM marker TOM20, the IMM marker TIM23, or the IMS marker cytochrome *c* (Fig. 1 B-G). Dual-color 3D-SIM images showed that the TOM20-positive OMM surrounds the mitochondrial matrix labeled by mito-dsRed, and there is no overlap between these two submitochondrial compartments (Fig. 1B,C). We observed that the TIM23-positive IMM wraps along and protrudes into the mito-dsRed-labeled matrix, often in a striated pattern which is indicative of the cristae membrane (Fig. 1D). The TIM23-positive, cristae membrane is enclosed inside the TOM20-positive OMM (Fig. 1E). The TIM23-positive, inner boundary membrane appears in close proximity or colocalizes with the TOM20-positive OMM at a number of sites (arrows in Fig. 1E), which likely represent the so-called mitochondrial contact sites seen in electron microscopic images [38,39]. We found that cytochrome *c*-positive IMS also wraps along and protrudes into the mito-dsRed-labeled matrix in a striated pattern which is indicative of the intracristae space (Fig. 1F). The cytochrome *c*-positive, intracristae space exhibits substantial overlap with the TIM23-positive, cristae membrane (Fig. 1G). Together, these results demonstrate the capability of 3D-SIM to resolve submitochondrial compartments and support its use as a tool for studying protein submitochondrial localization.

3.2 PINK1 resides in the cristae membrane/intracristae space of healthy mitochondria and translocates to the OMM upon mitochondrial depolarization

Next, we performed dual-color 3D-SIM super-resolution fluorescence imaging analyses to examine the submitochondrial localization of PINK1. Because of lack of a reliable anti-PINK1 antibody for immunostaining of endogenous PINK1, we imaged HeLa cells expressing C-terminal GFP-tagged PINK1 (PINK1-GFP) with various markers of submitochondrial compartments (Fig. 2A). We found that mitochondria displayed a tubular morphology under basal conditions and PINK1-GFP fluorescence was confined inside the boundary defined by the TOM20 immunofluorescence, indicating that PINK1 is not localized to the OMM of healthy mitochondria. The PINK1-GFP fluorescence had a striated pattern along the mito-dsRed-labeled matrix and showed substantial overlap with the TIM23 and cytochrome *c* immunofluorescence (Fig. 2A), indicating that PINK1 is localized to the cristae membrane and intracristae space of healthy mitochondria.

Our dual-color 3D-SIM imaging analysis revealed that treatment of cells with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) caused fragmentation of mitochondria into spherical organelles and redistribution of PINK1-GFP fluorescence from the cristae membrane and intracristae space to the OMM (Fig. 2B), indicating PINK1 translocation to the OMM of damaged mitochondria. Because CCCP is a protonophore that dissipates mitochondrial membrane potential (Ψ) and pH gradient (pH) leading to impaired ATP production, we examined the effects of additional ionophores and other drugs to determine the cause of PINK1 redistribution (Fig. 3). We found that the K^+ ionophore valinomycin, which dissipates Ψ but not pH , was capable of inducing PINK1 redistribution to the OMM (Fig. 3B,G), whereas the H^+/K^+ antiporter, nigericin, which

reduces pH but not Ψ , was unable to cause PINK1 redistribution (Fig. 3C,G). In addition, the mitochondrial complex III inhibitor antimycin A, which causes increased ROS generation and mitochondrial depolarization, could also trigger PINK1 redistribution (Fig. 3D,G). In contrast, the ATP synthase inhibitor, oligomycin, or the microtubule-depolymerizing drug, nocodazole, could not induce PINK1 redistribution (Fig. 3E,F,G). These results indicate that PINK1 translocates to the OMM following loss of mitochondrial membrane potential but not on loss of pH gradient, ATP, or microtubule integrity.

3.3 PINK1 colocalizes with TRAP1 in the IMM/IMS of healthy mitochondria and colocalizes with parkin on the OMM of dysfunctional mitochondria

Our finding of PINK1 localization in the cristae membrane and intracristae space of healthy mitochondria (Fig. 2A) suggests a signalling role of PINK1-mediated phosphorylation in these compartments. We have previously identified the mitochondrial molecular chaperone, TRAP1, as a substrate of PINK1-mediated phosphorylation [24], but the submitochondrial localization of TRAP1, like PINK1, has also been controversial. While our subcellular fractionation analysis showed that TRAP1 is localized to the IMM and IMS [24], others reported that TRAP1 resides in the matrix [40]. We therefore performed dual-color 3D-SIM imaging analyses to determine TRAP1 submitochondrial localization. We found that TRAP1 resides in the cristae membrane and intracristae space (Fig. 4A,B) and that TRAP1 colocalizes with PINK1 in healthy mitochondria under basal conditions (Fig. 4C,K). However, unlike PINK1, TRAP1 does not undergo mitochondrial depolarization-induced translocation to the OMM (Fig. 4E-G,M). These data support a physiological role of the PINK1-TRAP1 signalling pathway in regulating the activities of healthy mitochondria.

PINK1 has been proposed to recruit parkin from the cytosol to mitochondria for initiating mitophagy [27,41–43], but the specifics of PINK1-mediated parkin recruitment remain unclear. In contrast to previous reports that overexpression of PINK1 caused parkin translocation to healthy mitochondria [27,44], our dual-color 3D-SIM super-resolution imaging analyses revealed that parkin failed to translocate to mitochondria under basal conditions even with high levels of PINK1 overexpression (Fig. 4D,M) because PINK1 is restricted to the IMM/IMS of healthy mitochondria (Fig. 2A) and cannot interact with cytosolic parkin. When mitochondria were depolarized by CCCP, we observed PINK1 translocation to the OMM of damaged mitochondria concurrently with parkin recruitment to the OMM (Fig. 4H-J,M), where these two proteins showed extensive colocalization (Fig. 4J,L). Taken together, these results demonstrate that increased PINK1 protein expression levels alone are insufficient to trigger parkin recruitment to mitochondria, and they indicate that mitochondrial depolarization-induced PINK1 translocation from the IMM/IMS to the OMM is required for parkin recruitment to mitochondria.

Our 3D-SIM imaging analyses revealed that ectopic parkin expression in HeLa cells, which lack endogenous parkin, induced perinuclear clustering of CCCP-depolarized mitochondria (Fig. 4H-J), consistent with published studies [44,27]. However, different from the previous report that parkin was localized to the outside boundaries of clustered mitochondria (Vives-Bauza et al., 2010), we found that parkin was enriched in the mitochondrion - mitochondrion contacts (Fig. 4H). Our results demonstrate the power of dual-color 3D-SIM super-

resolution imaging in visualization of protein spatial distributions and support a direct role of parkin in promoting the clustering of dysfunctional mitochondria.

3.4 Mitochondrial depolarization-induced PINK1 translocation to the OMM is independent of new PINK1 protein synthesis

To further characterize mitochondrial depolarization-induced PINK1 translocation, we investigated the spatiotemporal dynamics of PINK1 redistribution following CCCP treatment. Due to rapid movement of mitochondria and the time required to acquire a dual-color 3D-SIM image, we were unable to resolve submitochondrial compartments in live cells. Our time-course analysis using fixed cells showed that PINK1 began to appear in the OMM of fragmented mitochondria around 15-30 min of CCCP treatment and reached the maximum by 60 min and then sustained to at least 2 h (Fig. 5). The increase in the OMM-localized PINK1 was accompanied by a gradual disappearance of the IMM/IMS-localized PINK1 with continued CCCP treatment (Fig. 5). By 60 min, PINK1 was almost solely localized on the OMM and there was very little PINK1 inside the mitochondria (Fig. 5).

To determine whether mitochondrial depolarization-induced PINK1 translocation to the OMM is due to the recruitment of newly synthesized PINK1, we pretreated cells with the protein synthesis inhibitor, emetine, for 1 hour followed by co-incubation with emetine and CCCP for 2 hours. 3D-SIM imaging analysis revealed that 3-hour emetine treatment failed to block PINK1 redistribution to the OMM of depolarized mitochondria (supplementary material Fig. S1). Similar result was also obtained by repeating the experiments using another protein synthesis inhibitor, cycloheximide (supplementary material Fig. S1). These results indicate that mitochondrial depolarization-induced PINK1 translocation to the OMM is independent of new PINK1 protein synthesis, suggesting that OMM-localized PINK1 could come from pre-existing pools of cytosolic PINK1 or IMM/IMS-localized PINK1.

3.5 The ability of PINK1 to translocate to the OMM is impaired by PD-linked PINK1 C92F and W437X mutations

PINK1 contains a hydrophobic region (residues 94-110; Fig. 6A) that has been proposed to act as a transmembrane domain or a stop-transfer signal to prevent full translocation of PINK1 into the mitochondrial matrix (Zhou et al., 2008; Becker et al., 2012). Our 3D-SIM imaging analysis of transfected HeLa cells revealed that, different from previous reports (Zhou et al., 2008; Becker et al., 2012), deletion of PINK1 hydrophobic transmembrane domain (PINK1TM) did not cause PINK1 to mislocalize to the mitochondrial matrix under either the basal or CCCP-treated conditions (Fig. 6F,G). We found that the PINK1TM mutant remained in the IMM/IMS and was incapable of translocating to the OMM following CCCP treatment (Fig. 6D,E,N), indicating that the PINK1 transmembrane domain is required for mitochondrial depolarization-induced PINK1 OMM translocation.

Our 3D-SIM imaging analysis showed that, although PD-linked PINK1 C92F mutation did not change the IMM/IMS localization of PINK1 under basal conditions, the mutation significantly reduced the mitochondrial depolarization-induced PINK1 translocation to the OMM (Fig. 6H,I,N). In contrast, PD-linked PINK1 L347P mutation, which is known to disrupt PINK1 kinase function (Pridgeon et al., 2007), had no effect on PINK1

submitochondrial localization in either healthy or CCCP-depolarized mitochondria (Fig 6J,K,N), indicating that the PINK1 kinase activity is not required for mitochondrial depolarization-induced PINK1 OMM translocation. We found that PD-linked PINK1 W437X (X is the stop codon), a truncation mutation which deletes the C-terminal region, abolished the ability of PINK1 to translocate to the OMM following CCCP treatment without affecting the IMM/IMS localization of PINK1 under basal conditions (Fig. 6L-N), indicating an essential role of the PINK1 C-terminal region in PINK1 translocation to the OMM. Our finding that mitochondrial depolarization-induced PINK1 translocation to the OMM is disrupted by PD-linked PINK1 C92F and W437X mutations provides evidence linking impairment of PINK1 OMM translocation to PD pathogenesis.

3.6 PINK1 translocates to the OMM to recruit parkin upon mitochondrial depolarization in neurons and the translocation requires combined action of PINK1 transmembrane and C-terminal domains

Next, we performed dual-color 3D-SIM imaging analyses of mitochondria in primary mouse cortical neurons and found that the resolution provided by 3D-SIM is able to differentiate the OMM from mitochondrial interior structures in neurons (Fig 7A,B). We found that, under normal culture conditions, C-terminal GFP-tagged PINK1 WT resided inside but not on the OMM of healthy, tubular mitochondria in cortical neurons (Fig 7C). In response to CCCP treatment, PINK1 WT translocated to the OMM of fragmented mitochondria with spherical morphology (Fig. 7D,G) and this translocation was impaired by PINK1 Δ TM or PINK1 W437X mutation (Fig. 7E-G).

We then performed 3D-SIM imaging analysis to determine the relationship between PINK1 OMM translocation and parkin recruitment to mitochondria using primary cortical neurons from wild-type (*PINK1*^{+/+}) and PINK1 knockout (*PINK1*^{-/-}) mice [32]. We transfected *PINK1*^{+/+} and *PINK1*^{-/-} neurons with mCherry-parkin to visualize parkin subcellular localization. Our 3D-SIM imaging analysis revealed that in response to CCCP treatment, parkin was recruited from the cytosol to the OMM of damaged mitochondria in *PINK1*^{+/+} neurons (Fig. 8A). In contrast, parkin remained in the cytosol in GFP-transfected *PINK1*^{-/-} neurons following CCCP treatment (Fig. 8B), indicating that PINK1 is required for parkin recruitment to the OMM of CCCP-depolarized mitochondria. We then took advantage of the *PINK1*^{-/-} phenotype and performed rescue experiments by expressing C-terminal GFP-tagged PINK1 WT or PINK1 mutants in *PINK1*^{-/-} neurons. We found that the defective parkin OMM recruitment phenotype of *PINK1*^{-/-} neurons was rescued by PINK1 WT expression (Fig. 8C,G). There was a clear association between mitochondrial depolarization-induced PINK1 OMM translocation and parkin recruitment to the OMM, as shown by the extensive colocalization between these two proteins (Fig. 8C, F). By contrast, the OMM translocation-defective PINK1 Δ TM and PINK1 W437X mutants were unable to rescue the defective parkin OMM recruitment phenotype of *PINK1*^{-/-} neurons (Fig. 8D, E, G) and showed significantly reduced colocalization with parkin (Fig. 8D-F), indicating an impaired capability of these mutant to facilitate parkin recruitment to the OMM of CCCP-depolarized mitochondria. Together, these results provide strong evidence that mitochondrial depolarization-induced PINK1 OMM translocation is required for parkin recruitment to the OMM of depolarized mitochondria.

4. Discussion

Mitochondria are multi-functional organelles with crucial roles in health and disease. Because mitochondrial functions are highly compartmentalized, it is important to determine the spatial localization of proteins in submitochondrial compartments. A major barrier to visualizing protein submitochondrial localization has been the resolution limit of conventional fluorescence microscopy. In this work, we have shown that dual-color 3D-SIM fluorescence microscopy enables super-resolution imaging of submitochondrial compartments and provides a useful tool for studying protein submitochondrial localization and its regulation.

The importance of defining the submitochondrial localization of PINK1 protein is underscored by the identification of PINK1 mutations as a major cause of early-onset PD [11-14]. Previous studies using biochemical fractionation and electron microscopy have generated conflicting results regarding PINK1 protein levels and submitochondrial localization. Some studies reported that the levels of endogenous and exogenous PINK1 protein under normal physiological conditions are undetectable or extremely low due to rapid degradation of PINK1, thus arguing against a role of PINK1 in healthy mitochondria [20,44,45]. By contrast, other studies reported significant levels of endogenous and exogenous PINK1 protein under normal physiological conditions, but localized PINK1 to either the IMM/IMS [23,24,28,26,25] or to the OMM [29,27,21]. This work is the first study to use dual-color 3D-SIM super-resolution imaging for visualization of PINK1 submitochondrial localization. The super-resolution imaging clearly showed that, under normal physiological conditions, PINK1 is not present on the OMM of healthy mitochondria, but rather PINK1 resides in the IMM/IMS where it is mainly localized in the cristae membrane and intracristae space. Given the critical involvement of the cristae membrane and intracristae space in the control of many mitochondrial activities such as respiration, metabolism, and redox control [19], our findings support an intramitochondrial role of PINK1 in these submitochondrial compartments to regulate the activities of healthy mitochondria under normal physiological conditions.

We have previously identified a mitochondrial signalling pathway by which PINK1 phosphorylates mitochondrial molecular chaperone TRAP1 to protect cells against oxidative stress [24]. Increasing evidence indicates that TRAP1 functions as a key modulator of mitochondrial respiration and mitochondrial stress response, but the submitochondrial localization of TRAP1 remains controversial and poorly defined [40,24,46-49]. Our 3D-SIM super-resolution imaging analysis revealed that, like PINK1, TRAP1 resides in the cristae membrane and intracristae space of healthy mitochondria. Furthermore, TRAP1 colocalizes with PINK1 in the cristae membrane/intracristae space under normal physiological conditions. These results support that the PINK1-TRAP1 signalling pathway functions in the cristae membrane/intracristae space of polarized mitochondria to regulate mitochondrial activities.

Our 3D-SIM super-resolution imaging analysis showed that, in response to mitochondrial depolarization, PINK1 changes its submitochondrial localization from the cristae membrane/intracristae space to the OMM of depolarized mitochondria. By contrast, the

submitochondrial localization of TRAP1 is unchanged following mitochondrial depolarization and TRAP1 remains in the cristae membrane/intracristae space of depolarized mitochondria. We found that the OMM-localized PINK1 recruits and colocalizes with parkin on the OMM of depolarized mitochondria, supporting the proposed role of PINK1 in activating parkin for mitophagy [44,41,42,50]. Together, these findings suggest a model in which differentially localized PINK1 serves as a critical molecular switch to trigger two separate signalling cascades in mitochondrial stress responses for maintaining mitochondrial homeostasis. During low levels of mitochondrial stress, PINK1 phosphorylates TRAP1 in the cristae membrane/intracristae space to activate adaptive responses for maintaining the homeostasis of polarized mitochondria. Under high levels of mitochondrial stress that cause mitochondrial depolarization, PINK1 translocates to the OMM and phosphorylates parkin to activate the selective clearance of damaged mitochondria via mitophagy.

The ability to visualize PINK1 submitochondrial distribution using 3D-SIM super-resolution imaging allowed us to study cellular events that control PINK1 localization to different submitochondrial compartments. We found that the change of PINK1 submitochondrial localization from the IMM/IMS to the OMM is dependent on the loss of mitochondrial membrane potential but not on the loss of pH gradient, ATP production, or microtubule integrity. These results argue against the model which localizes PINK1 to the OMM in the presence of mitochondrial membrane potential [29,27,21]. How does loss of mitochondrial membrane potential cause PINK1 to localize on the OMM? According to a current model, PINK1 is imported into polarized mitochondria, cleaved by the IMM-localized protease PARL and then retrotranslocated to the cytosol for rapid degradation by the proteasome. Loss of mitochondrial membrane potential blocks mitochondrial import of newly synthesized PINK1 and thereby causes PINK1 to accumulate on the OMM of depolarized mitochondria [20,44,45]. Different from this model, our 3D-SIM analysis revealed that mitochondrial depolarization-induced PINK1 localization to the OMM is independent of new protein synthesis, suggesting that OMM-localized PINK1 could originate from a pre-existing pool of cytosolic PINK1 when its mitochondrial import is blocked by loss of mitochondrial membrane potential. Interestingly, our time-course analysis revealed an increase in the OMM-localized PINK1 with a concomitant decrease in the IMM/IMS-localized PINK1. These results, together with the previous report of rapid inhibition of PARL-mediated PINK1 cleavage by mitochondrial depolarization [51], raise the possibility that loss of mitochondrial membrane potential may cause retrotranslocation of PINK1 from the IMM/IMS to the OMM of depolarized mitochondria.

Our study revealed that PINK1 N-terminal, putative transmembrane domain is required for PINK1 translocation to the OMM of depolarized mitochondria but is dispensable for PINK1 localization in the IMM/IMS. Our finding that deletion of this domain did not cause PINK1 mislocalization in the mitochondrial matrix argues against its proposed role as a stop-transfer sequence for preventing complete translocation of PINK1 across the IMM into the mitochondrial matrix (Zhou et al., 2008; Becker et al., 2012). The involvement of PINK1 transmembrane domain in mitochondrial depolarization-induced PINK1 translocation to the OMM is further supported by our results showing that PD-linked PINK1 C92F mutation flanking the N-terminus of the transmembrane domain impaired the ability of PINK1 to translocate to the OMM of depolarized mitochondria without affecting its IMM/IMS

localization. Furthermore, our analysis of PD-linked PINK1 W437X mutant revealed that PINK1 C-terminal domain is also required for PINK1 translocation to the OMM of depolarized mitochondria but not for PINK1 localization in the IMM/IMS. Our finding that both the transmembrane and C-terminal domains of PINK1 are necessary but neither one is sufficient for PINK1 localization in the OMM of depolarized mitochondria indicates that combined action of these two domains are required for mitochondrial depolarization-induced PINK1 translocation to the OMM in neurons as well as in non-neuronal cells.

5. Conclusions

In summary, our study demonstrates the capability of 3D-SIM fluorescence microscopy for super-resolution imaging of protein distribution in submitochondrial compartments. The findings obtained from this study provide novel insights into PINK1 submitochondrial localization and its regulation. Our data support differential submitochondrial localization of PINK1 as a key molecular switch for mediating two distinct mitochondrial signalling pathways in the maintenance of mitochondrial homeostasis. Moreover, our results reveal the deregulation of PINK1 submitochondrial localization as a novel mechanism for PD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Super-resolution imaging of submitochondrial compartments by dual-color 3D-SIM fluorescence microscopy. (A) Diagram depicting the compartments and subdomain structures of the mitochondria. (B-G) Dual-color 3D-SIM fluorescence imaging analysis of HeLa cells labeled with the mitochondrial matrix (MTX) marker mito-dsRed and antibodies against the OMM marker TOM20, the IMM marker TIM23, or the IMS marker Cytochrome *c* (Cyt. *c*). Enlarged view (C) of the boxed region in (B) shows that the TOM20-positive OMM and the mito-dsRed-labeled MTX can clearly be distinguished from each other. The arrows in (E) indicate the sites where the TIM23-labeled IMM appears in close proximity to the TOM20-positive OMM. Scale bars: 5 μm (B) or 1 μm (C-G). Each panel shows representative images from three independent experiments with at least 100 mitochondria examined for each experiment. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads indicated in (C-G).

Fig. 2. PINK1 resides in the cristae membrane/intracristae space under normal conditions and translocates to the OMM following mitochondrial depolarization. (A and B) HeLa cells expressing PINK1-GFP were either untreated (A) or treated with 10 μ M CCCP for 2 h (B) followed by staining with the indicated antibodies and 3D-SIM imaging. The MTX was visualized using mito-dsRed, and the OMM, IMM, and IMS using antibodies against TOM20, TIM23, and Cyt. *c*, respectively. Scale bars: 1 μ m. Each panel shows representative images from three independent experiments with at least 100 mitochondria examined for each experiment. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads.

Fig. 3.

PINK1 translocates to the OMM following loss of Ψ but not loss of pH, ATP, or microtubule integrity. (A-F) HeLa cells expressing PINK1-GFP were either untreated (A) or treated with valinomycin (B), nigericin (C), antimycin A (D), oligomycin (E), or nocodazole (F) for 2 h followed by staining with anti-TOM20 antibodies and 3D-SIM imaging. Scale bars: 1 μ m. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads. (G) Quantification of the percentage of mitochondria with PINK1 localized to the OMM following the indicated treatment of HeLa cells. For each experiment, 30 mitochondria were randomly selected for quantification, and the experiment was repeated three times. Data represent mean $\pm$ s.e.m. ($n = 3$ independent experiments). *, $P < 0.05$ versus the untreated control, one-way analysis of variance with a Tukey's *post hoc* test.

Fig. 4. PINK1 colocalizes with TRAP1 in the IMM/IMS of healthy mitochondria and colocalizes with parkin on the OMM following mitochondrial depolarization. (A-J) HeLa cells were either untransfected (B,F) or transfected with mito-dsRed (A, E,H), PINK1-GFP (C,G), or GFP-parkin (H,I) or co-transfected with PINK1-GFP and mCherry-parkin (D,J). Cells were either untreated (A-D) or treated with 10 μM CCCP for 2 h (E-J) followed by staining with anti-TRAP (A-C and E-G) and/or anti-TOM20 (B,F,I) antibodies and 3D-SIM imaging. Scale bars: 1 μm. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads. (K-M) Quantification shows the percentage of PINK1 colocalized with TRAP1 (K), the percentage of PINK1 colocalized with parkin (L), and the percentage of mitochondria with OMM-localized PINK1, TRAP1 or parkin under the basal or CCCP-treated conditions. For each experiment, 30 mitochondria were randomly selected for quantification, and the experiment was repeated three times. Data represent mean ± s.e.m. ($n = 3$ independent experiments). *, $P < 0.05$ versus the corresponding untreated control, one-way analysis of variance with a Tukey's *post hoc* test.

Fig. 5.

Time course of mitochondrial depolarization-induced PINK1 translocation to the OMM. HeLa cells expressing PINK1-GFP were treated with 10 μ M CCCP for the indicated lengths of time followed by staining with anti-TOM20 antibodies and 3D-SIM imaging. Scale bars: 1 μ m. Each panel shows representative images from three independent experiments with at least 100 mitochondria examined for each experiment. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads.

Fig. 6. Mitochondrial depolarization-induced PINK1 OMM translocation is impaired by PINK1 TM, C92F and W437X mutations. (A) Domain structure of PINK1. The locations of mitochondrial targeting sequence (MTS), transmembrane domain (TM), Ser/Thr kinase domain, and PD-linked PINK1 point mutations are indicated. (B-M) HeLa cells expressing C-terminal GFP-tagged PINK1 WT (B, C) or indicated PINK1 mutants (D-M) were either untreated or treated with 10 μ M CCCP for 2 h as indicated, followed by staining with anti-TOM20 antibodies and 3D-SIM imaging. The MTS was visualized using mito-dsRed. Scale bars: 1 μ m. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads. (N) Quantification shows the percentage of mitochondria with OMM-localized PINK1 under the basal or CCCP-treated conditions. For each experiment, 30 mitochondria were randomly selected for quantification, and the experiment was repeated three times. Data represent mean $\pm$ s.e.m. ($n = 3$ independent experiments). *, $P < 0.05$ versus the corresponding untreated control; #, $P < 0.05$ versus the CCCP-treated PINK1 WT, two-way analysis of variance with a Tukey's *post hoc* test.

Fig. 7.

The transmembrane domain and C-terminal region of PINK1 are required for mitochondrial depolarization-induced PINK1 OMM translocation in neurons. (A,B) Dual-color 3D-SIM imaging analysis of primary mouse cortical neurons labeled with mito-dsRed and anti-TOM20 antibodies. Enlarged view (B) of the boxed region in (A) shows a clear separation of TOM20-positive OMM from mito-dsRed-labeled MTX in neurons. (C-F) Primary mouse cortical neurons expressing C-terminal GFP-tagged PINK1 WT (C, D) or indicated PINK1 mutants (E,F) were either untreated (C) or treated with 10 μM CCCP for 3 h (D-F) followed by staining with anti-TOM20 antibodies and 3D-SIM imaging. Scale bars: 1 μm. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads. (G) Quantification of the percentage of mitochondria with PINK1 localized to the OMM following CCCP treatment. For each experiment, 30 mitochondria were randomly selected for quantification, and the experiment was repeated three times. Data represent mean ± s.e.m. ($n = 3$ independent experiments). *, $P < 0.05$ versus the CCCP-treated PINK1 WT, one-way analysis of variance with a Tukey's *post hoc* test

Fig. 8.

PINK1 OMM translocation-defective mutants are incapable of recruiting parkin to damaged mitochondria in neurons. (A-E) Dual-color 3D-SIM imaging analysis of PINK1^{+/+} mouse cortical neurons (A) or PINK1^{-/-} mouse cortical neurons (B-E) expressing mCherry-parkin (A-E) and GFP (B), C-terminal GFP-tagged PINK1 WT (C) or indicated PINK1 mutants (D,E) following treatment with 10 μ M CCCP for 3 h. The OMM in (A) was visualized by staining with anti-TOM20 antibodies. Scale bars: 1 μ m. Line scans show the fluorescence intensity profiles of each fluorescence signal along a line drawn through the mitochondria between the two arrowheads. (F) Quantification of the percentage of PINK1 colocalized with parkin. For each experiment, 30 mitochondria were randomly selected for quantification, and the experiment was repeated three times. Data represent mean $\pm$ s.e.m. ($n = 3$ independent experiments). *, $P < 0.05$ versus the PINK1 WT-transfected PINK1^{-/-} neurons, one-way analysis of variance with a Tukey's *post hoc* test. (G) Quantification of the percentage of mitochondria with parkin recruited to mitochondria. For each experiment, 30 mitochondria were randomly selected for quantification, and the experiment was repeated three times. Data represent mean $\pm$ s.e.m. ($n = 3$ independent experiments). *, $P < 0.05$ versus the GFP vector-transfected PINK1^{-/-} neurons; #, $P < 0.05$ versus the PINK1 WT-transfected PINK1^{-/-} neurons, two-way analysis of variance with a Tukey's *post hoc* test.