Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL

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PINK1 is a mitochondrial kinase mutated in some familial cases of Parkinson's disease. It has been found to work in the same pathway as the E3 ligase Parkin in the maintenance of flight muscles and dopaminergic neurons in *Drosophila melanogaster* and to recruit cytosolic Parkin to mitochondria to mediate mitophagy in mammalian cells. Although PINK1 has a predicted mitochondrial import sequence, its cellular and submitochondrial localization remains unclear in part because it is rapidly degraded. In this study, we report that the mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid-like protein (PARL) mediates cleavage of PINK1 dependent on mitochondrial membrane potential. In the absence of PARL, the constitutive degradation of PINK1 is inhibited, stabilizing a 60-kD form inside mitochondria. When mitochondrial membrane potential is dissipated, PINK1 accumulates as a 63-kD full-length form on the outer mitochondrial membrane, where it can recruit Parkin to impaired mitochondria. Thus, differential localization to the inner and outer mitochondrial membranes appears to regulate PINK1 stability and function.

Introduction

Two genes mutated in autosomal recessive cases of Parkinson's disease, PINK1 (Valente et al., 2004) and Parkin (Kitada et al., 1998), have been found to work in the same pathway (Clark et al., 2006; Park et al., 2006; Yang et al., 2006) linked to mitochondrial maintenance (Greene et al., 2003; Palacino et al., 2004; Gautier et al., 2008). PINK1 is a kinase localized to mitochondria (Valente et al., 2004), whereas Parkin is an E3 ubiquitin ligase located in the cytosol (Shimura et al., 1999; Shimura et al., 2000). Upon mitochondrial damage or uncoupling, Parkin relocalizes to mitochondria and can mediate the autophagic elimination of damaged mitochondria (Narendra et al., 2008), which is dependent on PINK1 kinase activity (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). These results suggest that PINK1 and Parkin may normally participate in a mitochondrial quality control pathway that is defective in certain familial forms of Parkinson's disease.

How PINK1 recruits Parkin to damaged mitochondria is unclear. Although endogenous PINK1 protein expression is constitutively low owing to rapid turnover, PINK1 proteolysis is inhibited by mitochondrial uncoupling, allowing a robust increase in expression when a mitochondrion is damaged and loses membrane potential (Lin and Kang, 2008; Matsuda et al., 2010; Narendra et al., 2010). The protease that mediates this constitutive PINK1 turnover is unknown. Rhomboid-7 was reported to be necessary for PINK1 cleavage and activity in *Drosophila melanogaster* cells (Whitworth et al., 2008). However, Rhomboid-7 is localized to the inner mitochondrial membrane (Hill and Pellegrini, 2010), whereas ectopic and endogenous PINK1 have been reported to span the outer mitochondrial membrane with the C-terminal kinase region facing the cytosol poised to induce Parkin translocation (Zhou et al., 2008; Narendra et al., 2010). However, PINK1 has an apparent N-terminal mitochondrial import sequence predicted to mediate import through the TIM23 complex either to the matrix or spanning the inner membrane. Consistent with a predicted interaction between PINK1's N terminus and the TIM23 import machinery, PINK1's N terminus

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Abbreviations used in this paper: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; Cyt c, cytochrome *c*; ΔMTS, MTS deleted; FL, full length; KO, knockout; MEF, mouse embryonic fibroblast; MPP, mitochondrial processing protease; MTS, mitochondrial targeting sequence; PARL, presenilin-associated rhomboid-like protein; PK, proteinase K; WT, wild type.

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is sufficient to target ECFP to the matrix (Silvestri et al., 2005), and deletion of PINK1's putative transmembrane domain results in targeting of its kinase domain to the matrix (Zhou et al., 2008). Finally, in some studies, PINK1 has been localized to the inner membrane with its kinase domain facing the intermembrane space (Silvestri et al., 2005; Plun-Favreau et al., 2007; Pridgeon et al., 2007). As these previous studies were performed in the absence of uncouplers that were recently found to influence PINK1 stability, it remains unclear how and where endogenous PINK1 is degraded.

Results and discussion

Presenilin-associated rhomboid-like protein (PARL) is required for proteolytic cleavage of PINK1

To identify the protease mediating PINK1 turnover on mitochondria, we knocked down known mitochondrial proteases by siRNA and examined the PINK1 expression pattern. We found that in contrast to siRNAs against Afg3L2, ClpP, Oma1, HtrA2/Omi, Paraplegin, and Yme1 (unpublished data), siRNA for PARL led to increased expression of endogenous PINK1 in the absence of the depolarizing agent carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP; Fig. 1 a). In addition, the molecular mass of the PINK1 band (Fig. 1 a, orange arrowhead) in the absence of CCCP was slightly lower than that of endogenous PINK1 stabilized by CCCP, predicted to represent full-length (FL) PINK1 (63 kD; Fig. 1 a, blue arrowhead) based on molecular mass. The ∼60-kD band would be consistent with the molecular mass of PINK1 with a mitochondrial targeting sequence (MTS) deleted (ΔMTS) after mitochondrial processing protease (MPP) cleavage (Fig. 1 a). When mitochondria were uncoupled with CCCP, both scrambled siRNA– and PARL siRNA–transfected cells showed the same level of FL PINK1 accumulation, indicating that knockdown of PARL did not affect PINK1 expression in uncoupled mitochondria. This contrasts with our previous work using short hairpin RNAi targeting a different site in PARL mRNA that was interpreted to exclude PARL as a protease for PINK1 cleavage, likely owing to insufficient knockdown of endogenous PARL in HeLa cells and to insufficient expression of ectopic PINK1 in PARL knockout (KO) mouse embryonic fibroblasts (MEFs; Narendra et al., 2010). To confirm the effect of PARL knockdown on endogenous PINK1 cleavage, we analyzed MEFs derived from wild-type (WT) or PARL KO mice. Given that we could not detect endogenous mouse PINK1 in MEF cells, cells were transfected with a human PINK1-V5/His construct. Consistent with the results observed in HeLa cells for endogenous PINK1, PARL KO MEFs displayed a 60-kD form of PINK1, likely to be ΔMTS-PINK1, in the absence of CCCP (Fig. 1 b, orange arrowhead).

Treatment of cells with a proteosome inhibitor stabilizes a 52-kD PINK1 fragment (Lin and Kang, 2008; Zhou et al., 2008), suggesting that proteosome-independent proteolysis yields a 52-kD form of PINK1 that is subsequently degraded by the proteosome (Narendra et al., 2010). When we treated PARL KO MEFs transiently overexpressing PINK1-V5/His with MG132, the 52-kD fragment of PINK1 (Fig. 1 c, red arrowhead) was absent, and a 60-kD predicted ΔMTS-PINK1 (Fig. 1 c, orange arrowhead) was stabilized, suggesting that PARL-mediated proteolysis normally generates the 52-kD fragment. Rhomboid proteases such as PARL cleave proteins in and around membrane-spanning domains (Urban et al., 2001; Sík et al., 2004; Strisovsky et al., 2009). PARL-mediated cleavage of PINK1 in the predicted membrane-spanning domain between residues 94 and 110 would yield a protein fragment of 52 kD, which is consistent with the molecular mass of the fragment stabilized by MG132 and absent in the PARL KO MEFs.

To further analyze the effect of PARL on PINK1 cleavage, we performed in vitro mitochondrial import assays. Mitochondria freshly isolated from WT and PARL KO MEFs were incubated with ^{35}S-labeled PINK1 in the presence or absence of CCCP. After import, each sample was split in two and treated with or without 5 µg/ml proteinase K (PK) to degrade non-imported protein (Fig. 1 d). The 52-kD species of PINK1 was found to accumulate in WT MEF mitochondria (Fig. 1 d, red arrowheads) but was absent in PARL KO samples, corroborating that PARL-mediated proteolysis generates the 52-kD form. As in PARL KO cells (Fig. 1 c), mitochondria isolated from PARL KO MEFs did not generate the 52-kD fragment but yielded a new 60-kD species of PINK1 predicted to be ΔMTS-PINK1 (Fig. 1 d, orange arrowheads). Generation of both the 52-kD form of PINK1 in WT mitochondria and the 60-kD form of PINK1 in PARL KO mitochondria was prevented by CCCP, indicating that they required inner mitochondrial membrane import (Fig. 1 d). When we incubated the mitochondria containing imported ^{35}S-labeled PINK1 with PK, the 60-kD ΔMTS-PINK1 in PARL KO mitochondria and the 52-kD PINK1 in WT mitochondria were more stable than FL PINK1 (Fig. 1 d, bottom). These results suggest that in contrast to FL, the CCCP-stabilized form of PINK1 that localizes to the outer mitochondrial membrane facing the cytosol (Narendra et al., 2010), the 60-kD ΔMTS-PINK1, which appears in the absence of PARL, and the 52-kD PINK1 are protease-protected within polarized mitochondria (Fig. 1 d).

To confirm that ΔMTS-PINK1 reflects a form imported into the mitochondria to allow MPP cleavage, we compared PK sensitivity after import of radiolabeled PINK1 relative to control proteins in the outer mitochondrial membrane and intermembrane space (Fig. 1 e). FL, radiolabeled PINK1 (Fig. 1 e, blue arrowhead) and Tom20 were rapidly degraded by 100 µg/ml PK treatment, whereas ΔMTS-PINK1 ((Fig. 1 e, orange arrowhead), intermembrane space protein Htra2/Omi, and matrix protein Hsp70 were more stable.

We have previously shown that experimentally stabilized PINK1 on the mitochondrial outer membrane recruits Parkin even in the absence of mitochondrial uncoupling (Narendra et al., 2010). Therefore, if ΔMTS-PINK1 is sequestered within the mitochondria, as indicated in Fig. 1 (d and e), it would not be positioned to recruit Parkin. Indeed, when we transfected HeLa cells stably overexpressing YFP-Parkin with scrambled control siRNA or siRNA for PARL as in Fig. 1 a, no mitochondrial translocation of Parkin was observed (Fig. 1 f). Supporting this conclusion, the 60-kD form of ΔMTS-PINK1 stabilized in coupled mitochondria in the absence of CCCP in PARL KO MEFs fails to recruit mCherry-Parkin to mitochondria (Fig. 1 g).

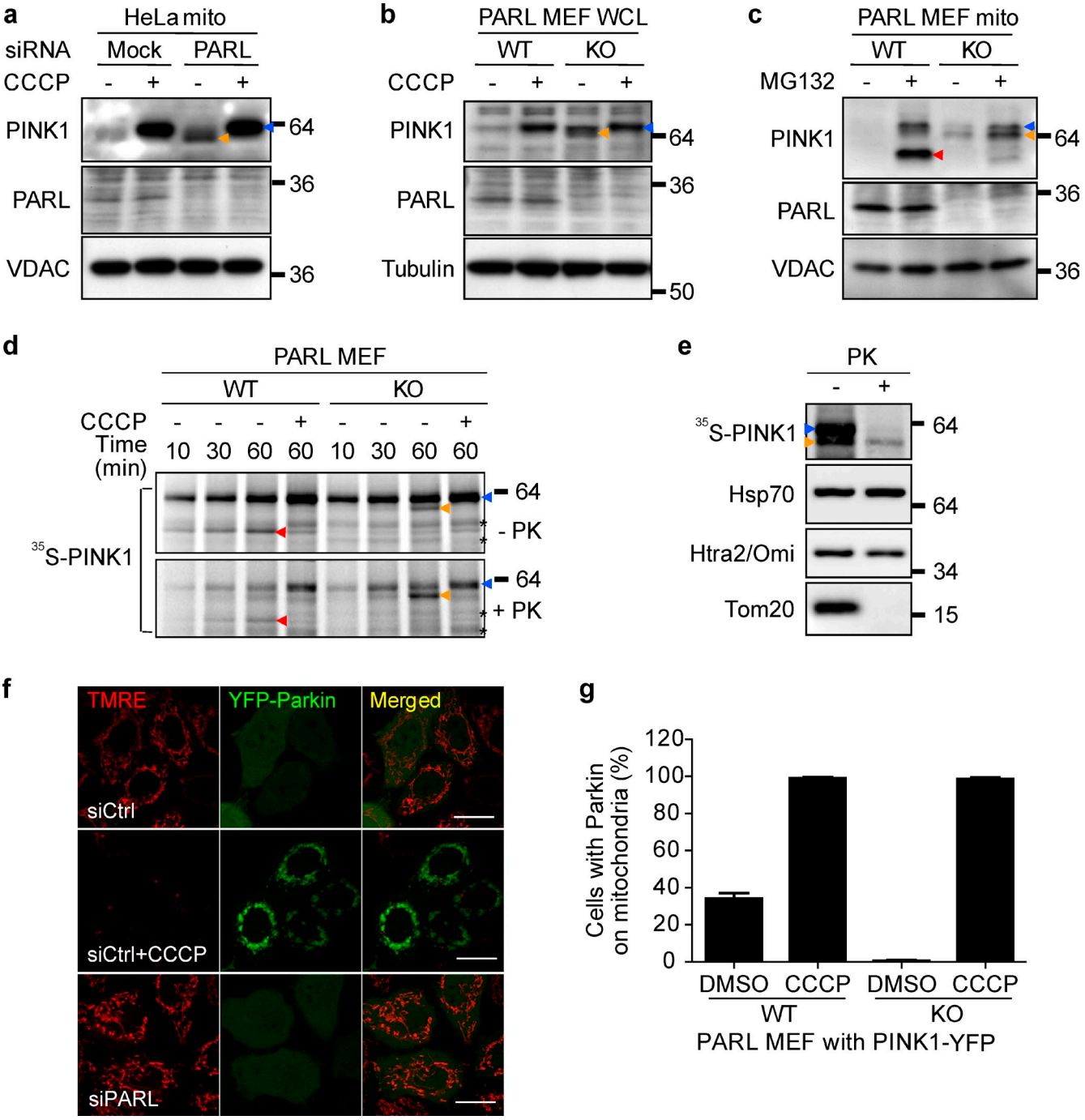

Figure 1. **Constitutive cleavage of PINK1 is mediated by PARL.** (a) HeLa cells were transfected with scrambled control siRNA or PARL siRNA. After 4 h incubation with or without 10 µM CCCP, mitochondria were isolated, and mitochondrial protein extracts were assayed for endogenous levels of PINK1 and PARL by immunoblotting. VDAC1 is a mitochondrial marker. (b) MEFs from PARL WT and KO mice were transfected with PINK1-V5/His for 2 d and treated with DMSO or 10 µM CCCP for 4 h. Exogenous PINK1 levels were assayed by immunoblotting. Tubulin is a loading control. (c) PARL WT and KO MEFs were transfected with PINK1-V5/His as in b and treated with DMSO or 10 µM MG132. After 4 h of treatment, cells were fractionated, and exogenous PINK1 levels in mitochondrial fraction were measured with immunoblotting. VDAC1, mitochondrial loading control. (d) [^{35}S]methionine-labeled PINK1 was incubated for different times with mitochondria isolated from PARL WT or KO MEFs in the presence or absence of 1 µM CCCP. After import, samples were treated with or without 5 µg/ml PK. Radiolabeled PINK1 was detected using digital autoradiography. Asterisks, nonspecific bands. (e) [^{35}S]-PINK1 was imported into PARL KO mitochondria for 60 min as in d, and these mitochondria were incubated in the presence or absence of high PK (100 µg/ml) for 10 min. Hsp70, Htra2/Omi, and Tom20 were identified by immunoblotting as markers for mitochondrial matrix, inter membrane space, and outer membrane, respectively. (f) HeLa cells stably expressing YFP-Parkin were transfected with control (siCtrl) or PARL siRNA (siPARL) for 192 h. After transfection, cells were treated with either DMSO or 10 µM CCCP for 1 h, stained with TMRE, and analyzed by live cell imaging. Bars, 20 µm. (g) PARL WT and KO MEFs transfected with PINK1-YFP and mCherry-Parkin were treated with 10 µM DMSO or CCCP for 3 h. Cells (≥50/treatment) were counted for mitochondrial translocation of Parkin. Counting results were represented as mean ± SEM from four replicates. Blue arrowheads, FL PINK1; orange arrowheads, ΔMTS-PINK1; red arrowheads, 52-kD PINK1.

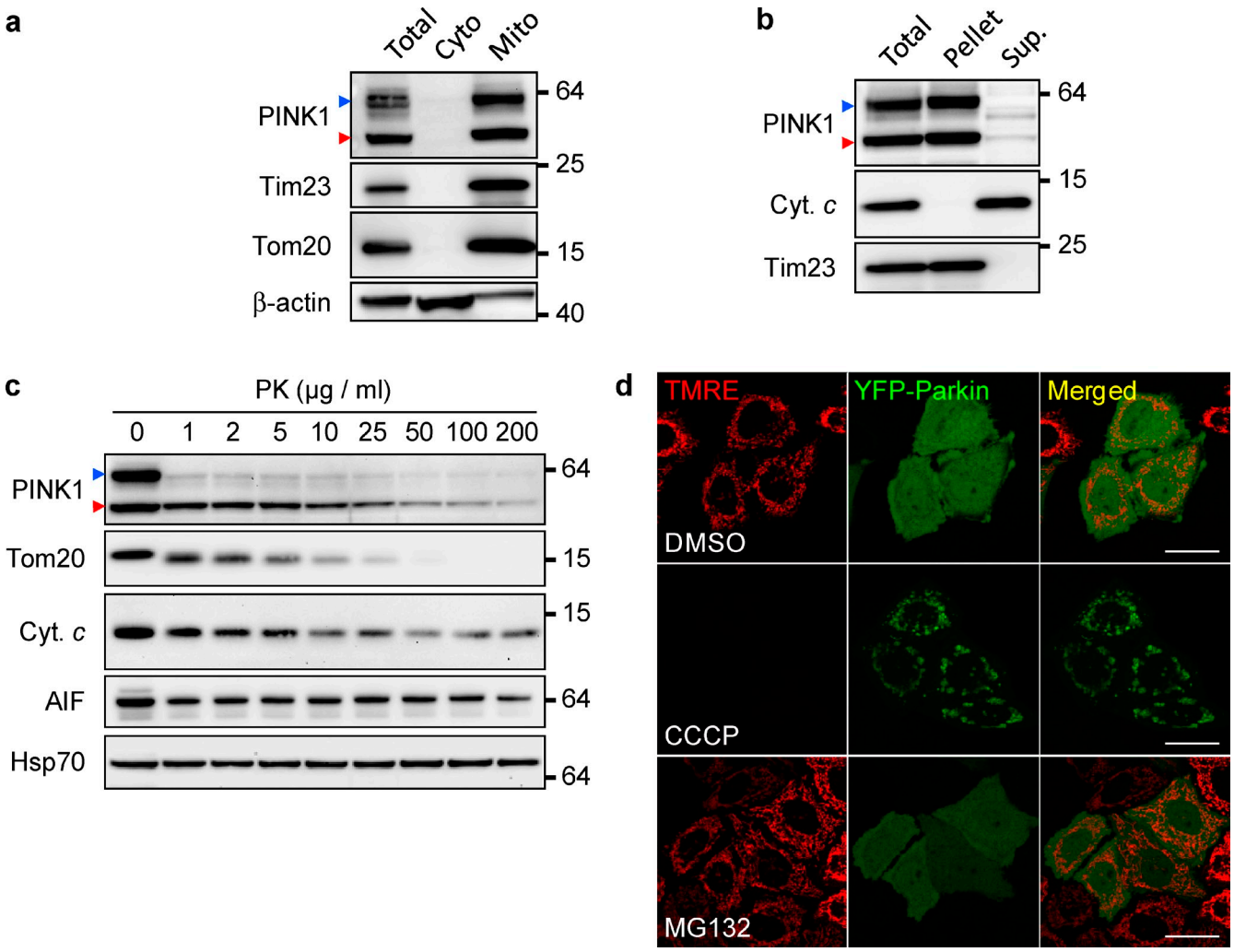

Figure 2. **The 52-kD form of endogenous PINK1 is found inside mitochondria and does not recruit Parkin.** (a) HeLa cells were initially treated with 50 μM MG132 for 10 h and then together with 10 μM CCCP for a final 3 h. Cells were fractionated and analyzed by immunoblotting using antibodies against the indicated proteins. (b) The mitochondrial fraction from panel a was subjected to alkaline extraction using sodium carbonate and immunoblotted for PINK1, Cyt *c*, and Tim23. (c) Mitochondria from panel a were incubated for 30 min on ice with various concentrations of PK followed by immunoblotting using antibodies against PINK1 and the indicated mitochondrial markers. (d) HeLa cells stably expressing YFP-Parkin were treated with DMSO, 10 μM CCCP, or 10 μM MG132 for 3 h followed by staining with TMRE and confocal imaging. Bars, 20 μm. Blue arrowheads, FL PINK1; red arrowheads, 52-kD PINK1.

A mutant form of PINK1 lacking the transmembrane region in PINK1 between amino acids 91 and 117 does not accumulate in cells upon CCCP treatment (Fig. S1, a and b) and does not function to recruit Parkin after CCCP treatment in marked contrast to WT PINK1 (Fig. S1 c). Thus, the transmembrane domain appears to be essential for proper positioning of WT PINK1 in the outer mitochondrial membrane to recruit Parkin.

Although overexpressed PINK1 accumulates as a 52-kD form in the cytosol after proteosome inhibition (Muqit et al., 2006; Tang et al., 2006; Lin and Kang, 2008; Takatori et al., 2008; Weihofen et al., 2008), the location and topology of the endogenous 52-kD PINK1 produced by PARL-mediated proteolysis has not been conclusively elucidated. To address this, we treated cells first with MG132 to accumulate the 52-kD PINK1 followed by treatment with CCCP to accumulate FL 63-kD PINK1 to compare the localization of the two proteins in the same samples. Endogenous FL PINK1 (Fig. 2 a, blue arrowhead) and 52-kD PINK1 (Fig. 2 a, red arrowhead) were detected in the mitochondrial fraction but not in the cytosolic fraction. Mitochondrial fractions subjected to alkaline (Na_2CO_3) extraction yielded FL (Fig. 2 b, blue arrowhead) and 52-kD (Fig. 2 b, red arrowhead) PINK1 in the pellet fraction, suggesting that both forms of PINK1 are integrated within mitochondrial membranes. To determine whether FL and 52-kD PINK1 exist in the same submitochondrial compartment, we conducted a PK protection assay. Although FL PINK1 (Fig. 2 c, blue arrowhead) was rapidly degraded by low concentrations of PK (1 μg/ml), the 52-kD PINK1 (Fig. 2 c, red arrowhead) was very stable and could be detected even after incubation with 100 μg/ml PK (Fig. 2 c), indicating that endogenous FL PINK1 and 52-kD PINK1 are in different compartments, corroborating the in vitro import results shown in Fig. 1 d. We addressed the activity of endogenous 52-kD PINK1 to recruit Parkin. When we treated HeLa cells stably expressing YFP-Parkin with MG132 for 8 h to accumulate the 52-kD PINK1 (Fig. 1 c; Narendra et al., 2010), we detected no mitochondrial translocation of Parkin (Fig. 2 d),

which is consistent with import and PARL-mediated cleavage of PINK1 in the inner mitochondrial membrane in the absence of uncoupling. Although the 52-kD form of PINK1 was not functional for Parkin translocation because of its different submitochondrial localization compared with CCCP-stabilized FL PINK1, this fragment may have other functions within the mitochondria. Thus, it will be interesting to determine how this fragment is degraded.

Mutagenesis screen of amino acid residues affecting PARL-mediated cleavage of PINK1

Rhomboid proteases prefer to cleave specific sequences near to transmembrane domains and within membrane-spanning helices that are partially destabilized by helix-breaking amino acids such as glycine and proline (Urban and Freeman, 2003; Strisovsky et al., 2009). Interestingly, the predicted membrane-spanning region of PINK1 between Ala^{93} and Ile^{111} contains more than one third glycine and proline residues (Fig. 3 a), which is consistent with the identified high susceptibility to PARL cleavage. Moreover, the PINK1 transmembrane domain is highly conserved from zebrafish to human (Fig. S2). *Drosophila* PINK1 has a predicted transmembrane domain that is less homologous to man and contains fewer helix-breaking residues, suggesting that it may have a different sensitivity to the fly PARL orthologue Rhomboid 7 (Fig. S2). To explore PINK1 cleavage mediated by PARL, we mutated each amino acid in this domain to residues with a bulky side chain that we predicted may interfere with substrate recognition by PARL. N-terminal amino acids 91–98 of the WT PINK1-YFP construct were mutated to phenylalanine, and amino acids 99–110 were mutated to tryptophan (Fig. 3 a).

HeLa cells transfected with mutant PINK1-YFP constructs were either untreated or treated with CCCP or MG132 and analyzed by immunoblotting (Fig. 3 b). In the absence of CCCP or MG132, PINK1-YFP mutants G97F and R98F displayed dramatically increased levels of FL and/or ΔMTS-PINK1 (Fig. 3 b, green arrowheads; compare control yellow rectangle with red rectangles) in contrast to WT and all of the other mutant forms of PINK1 (Fig. 3 c). However, in the presence of MG132, G97F and R98F PINK1 mutants also displayed partial cleavage to the 52-kD form (Fig. 3 b, red arrowheads), suggesting that PARL-mediated PINK1 cleavage was not completely prevented by these mutations.

Although WT PINK1-YFP expression is below the level of detection, the PINK1-YFP R98F mutant is found localized to mitochondria in the absence of CCCP based on subcellular fractionation (Fig. S3 a) and confocal imaging (Fig. 3 d). To determine the submitochondrial location of PINK1 R98F, a mitochondrial PK protection assay was performed. Mitochondria isolated from HeLa cells transfected with PINK1-YFP R98F were treated with increasing amounts of PK, and the degradation pattern of PINK1 R98F was compared with the degradation patterns of mitochondrial proteins representing each compartment of mitochondria (Fig. 4 a). PINK1-YFP R98F (Fig. 4 a, green arrowhead) exhibited increased protease protection relative to WT PINK1 (Fig. 2 c), suggesting that the mutant is imported but incompletely processed by PARL-mediated proteolytic activity. To further investigate the submitochondrial location of PINK1-YFP R98F, we performed immunostaining of fixed cells that were either untreated or permeabilized with 0.005% digitonin or 0.25% Triton X-100. Control experiments showed that this assay could distinguish between mitochondrial proteins localized inside (cytochrome *c* [Cyt *c*]) or outside (Tom20) of the mitochondrial outer membrane (Fig. 4 b). HeLa cells transfected with WT PINK1-YFP followed by treatment with CCCP for 3 h showed positive immunostaining in all cells expressing PINK1-YFP after permeabilization with 0.005% digitonin. However, anti-GFP immunoreactivity was absent in most HeLa cells transfected with R98F PINK1-YFP using the same permeabilization conditions, indicating that the C terminus of PINK1-YFP R98F is protected by the mitochondrial outer membrane in the absence of CCCP treatment (Fig. 4, c and d).

Consistent with localization within mitochondria, expression of R98F PINK1-YFP did not induce mitochondrial translocation of mCherry-Parkin in HeLa cells (Figs. 4 e and S3 b) or PINK1 KO cells (Fig. 4 f). CCCP treatment of PINK1 KO cells expressing R98F PINK1-YFP induced Parkin translocation, indicating that this mutant is functional for inducing Parkin translocation when located on the outer mitochondrial membrane.

Interestingly, in the screen for PINK1 mutants displaying incomplete PARL-mediated cleavage, we also identified A103W and G105W mutations that instead stabilize the 52-kD fragment of PINK1 (Fig. 3 b, red arrowheads and blue rectangles) in the absence of MG132 treatment. Using differential permeabilization conditions described in Fig. 4 b, both A103W and G105W PINK1 mutants were protected by the mitochondrial outer membrane and did not recruit Parkin to mitochondria in HeLa cells or PINK1 KO MEFs (not depicted).

In coupled mitochondria, PINK1 appears to be guided to mitochondria by the N-terminal targeting sequence after translation and imported into the inner mitochondrial membrane via the general mitochondrial import machinery, TOM and TIM23 complexes (Fig. 5). In this study, it would encounter MPP, which cleaves the MTS for most MTS-containing mitochondrial proteins to generate a 60-kD ΔMTS-PINK1. PINK1 appears to be cleaved to a 52-kD form within the inner mitochondrial membrane by PARL-mediated proteolytic activity. The 52-kD PINK1 is then degraded by an MG132-sensitive protease. Thus, a prominent function of PARL in the PINK1–Parkin pathway appears to be facilitating the rapid degradation of PINK1 by mediating the cleavage of PINK1 in the mitochondrial inner membrane. The PARL KO mouse displays defects in postnatal growth and lifespan (Cipolat et al., 2006) that may in part be caused by accumulation of ΔMTS-PINK1 in the mitochondrial inner membrane space.

Our results indicate that a protein with an MTS can be differentially targeted to inner and outer mitochondrial membranes depending on the presence of mitochondrial membrane potential and may explain the discrepancies regarding PINK1 location in the literature. In yeast, a bipartite targeting sequence of NADH cytochrome B5 reductase (Mcr1p) drives it to the mitochondrial inner membrane, where it is cleaved and released into the intermembrane space. Interestingly, in the absence of membrane

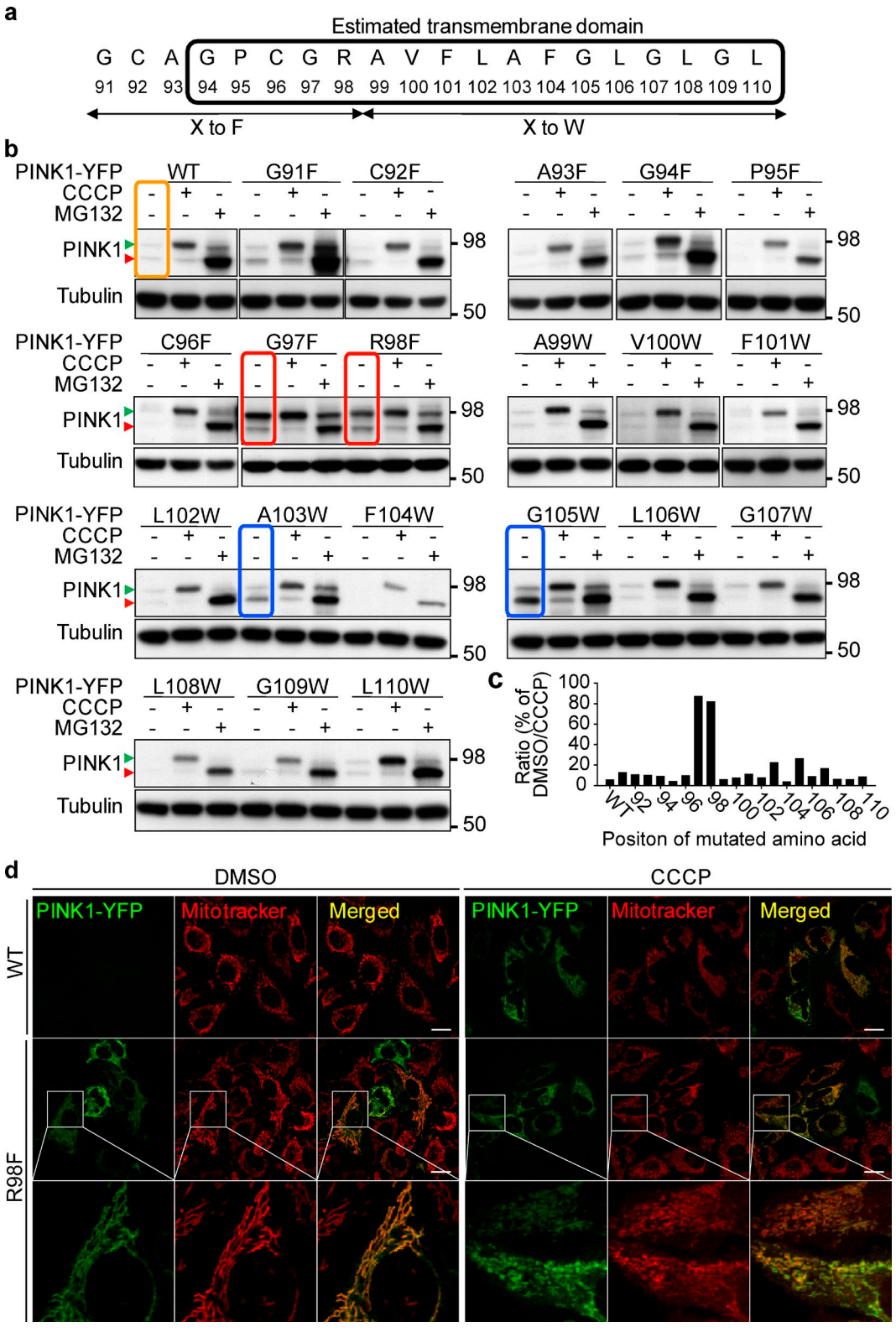

Figure 3. **Point mutations in the transmembrane domain of PINK1 partially inhibit its proteolytic cleavage.** (a) Amino acids throughout the predicted transmembrane domain of PINK1 were mutated to phenylalanine (amino acids 91–98) or tryptophan (amino acids 99–110). (b) HeLa cells transfected with the indicated PINK1-YFP mutants were treated with either DMSO, 10 µM CCCP, or 10 µM MG132 for 3 h. 20 µg cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies against PINK1 and tubulin. (c) The band intensity of FL PINK1 in DMSO- or CCCP-treated lanes from b was densitometrically measured using Multi Gauge (Fujifilm). After corrections for background and loading, the band intensity ratio of DMSO/CCCP-treated samples for each PINK1 mutant was measured. (d) YFP-tagged WT PINK1 and PINK1 R98F mutants were transfected into HeLa cells. Cells were stained with MitoTracker red before treatment with 10 µM CCCP for 3 h and analyzed by confocal microscopy. (bottom) Enlarged views of the boxed areas are shown. Bars, 20 µm. Green arrowheads, FL and ΔMTS-PINK1; red arrowheads, 52-kD PINK1.

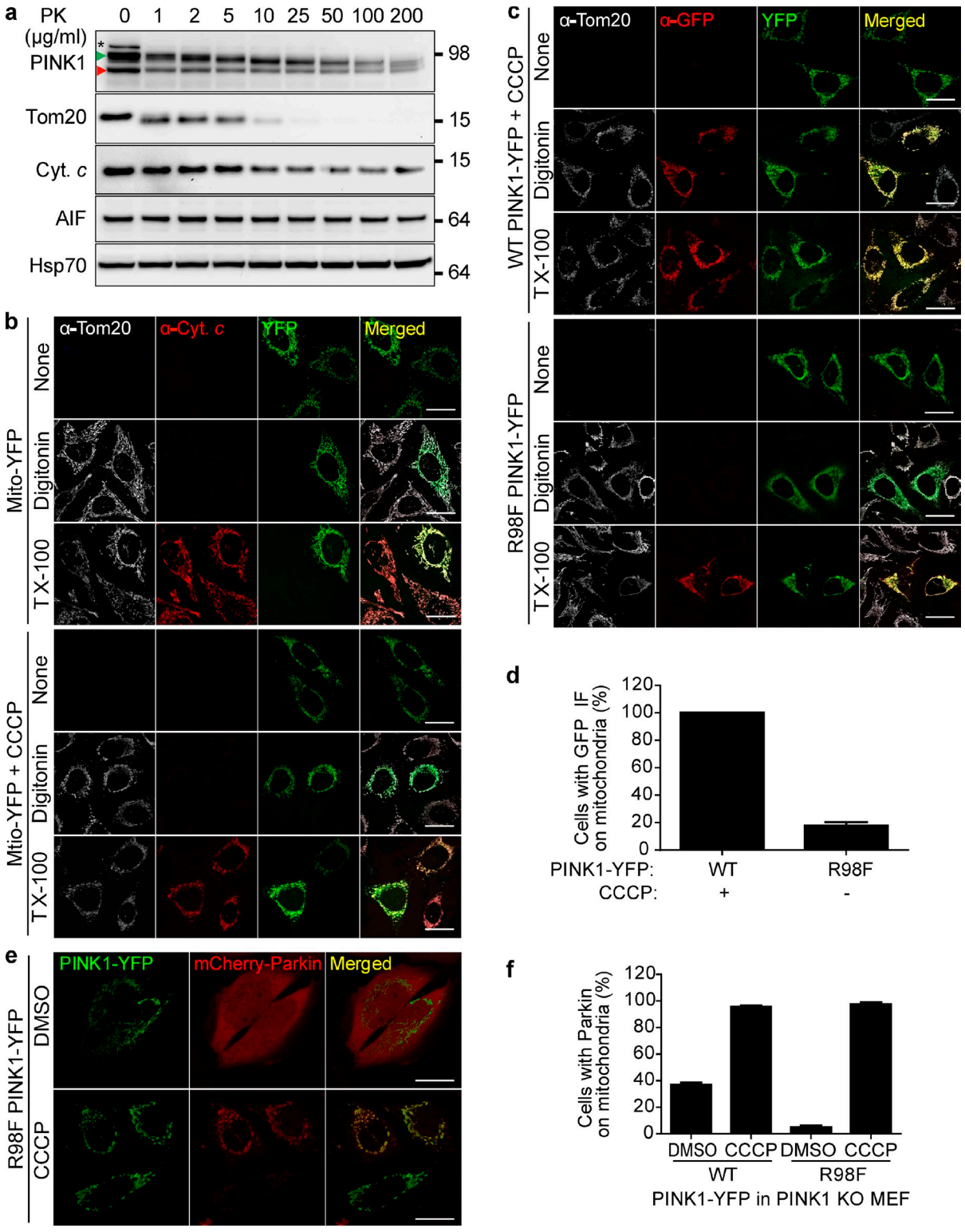

Figure 4. **The PINK1 R98F mutant resistant to PARL-mediated cleavage is located inside mitochondria.** (a) Mitochondria isolated from HeLa cells transfected with YFP-tagged PINK1 R98F were incubated with various concentrations of PK for 30 min on ice and immunoblotted for PINK1, Tom20, Cyt *c*, AIF, and Hsp70. Green arrowhead, FL and ΔMTS-PINK1; red arrowhead, 52-kD PINK1. (b) HeLa cells transfected with mito-YFP were treated with DMSO or 10 μM CCCP for 3 h followed by incubation in either PBS alone or PBS containing 0.005% digitonin or 0.25% Triton X-100 (TX-100). Cells were immunostained using antibodies against Tom20 and Cyt *c* and analyzed by confocal microscopy. (c) HeLa cells were transfected with YFP-tagged WT PINK1 or PINK1 R98F mutant for 18 h. Cells were then treated with DMSO or CCCP for 3 h, permeabilized, and immunostained with the indicated antibodies. Images were taken by confocal microscopy. (d) HeLa cells (≥150/condition) stained in c were counted for GFP immunofluorescence. Counting results were represented as mean ± SEM from four replicates. (e) HeLa cells cotransfected with YFP-tagged PINK1 R98F mutant and mCherry-Parkin were incubated with either DMSO or 10 μM CCCP for 1 h followed by confocal imaging. (f) PINK1 KO MEFs transfected with YFP-tagged WT PINK1 or PINK1 R98F mutant together with mCherry-Pax were treated with DMSO or 10 μM CCCP for 3 h, and cells were counted for mitochondrial translocation of Parkin (≥50 cell counts for each sample). Counting results were represented as mean ± SEM from four replicates. Bars, 20 μm.

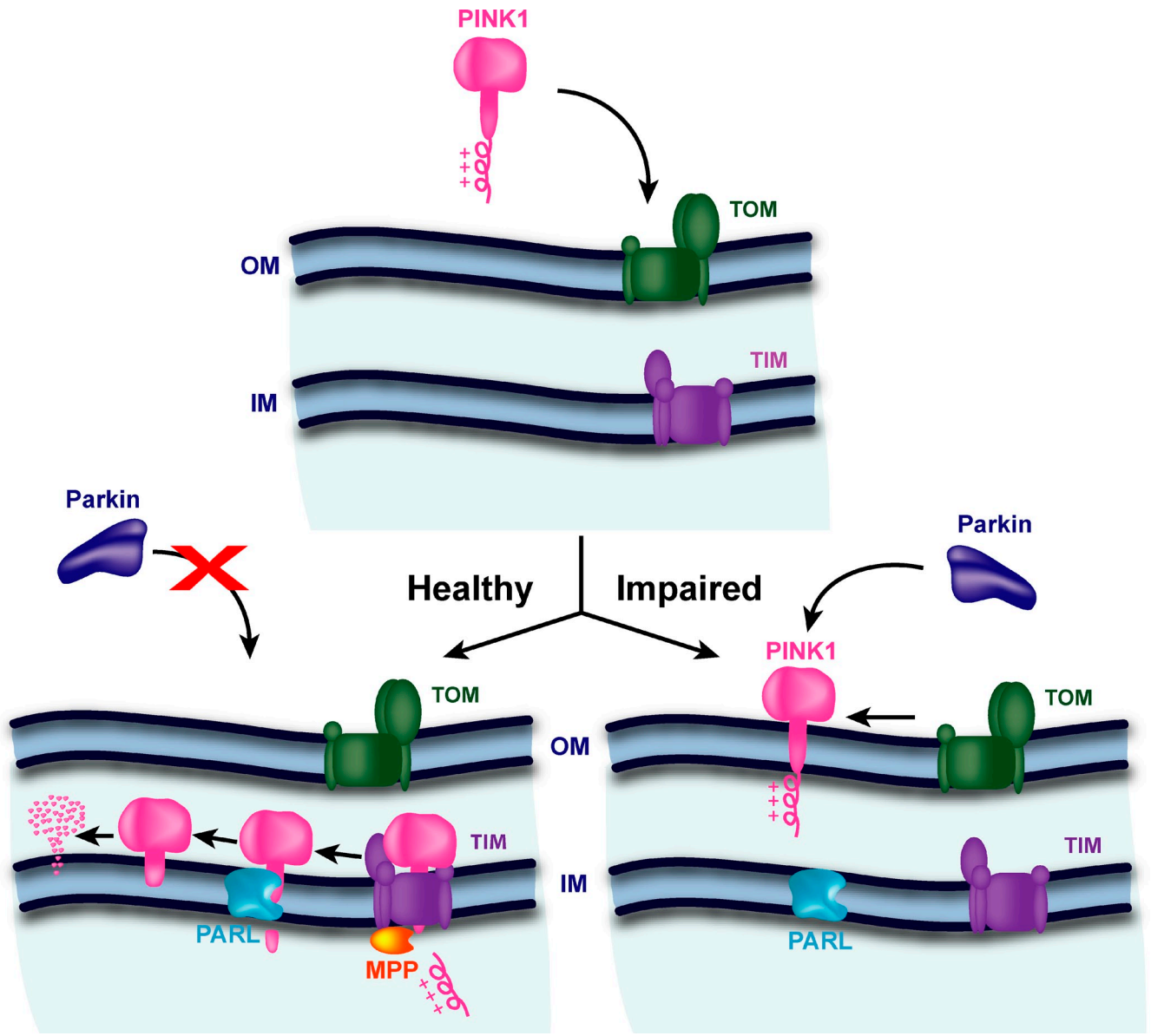

Figure 5. **Model for PINK1 import and processing.** OM, outer membrane; IM, inner membrane.

potential, Mcr1p is found predominantly on the outer membrane, reflecting our findings with PINK1 (Haucke et al., 1997). It seems plausible that additional proteins will be found that are regulated by differential mitochondrial membrane targeting. Interestingly, expression of FL Oma1 was recently indicated to increase on mitochondrial membrane potential collapse, allowing it to accumulate and cleave the mitochondrial fusion protein Opa1 (Head at el., 2009). Opa1 degradation by Oma1 may prevent fusion of damaged mitochondria with healthy mitochondria and be coupled to Pink1-mediated recruitment of Parkin to facilitate mitophagy.

Materials and methods

Cell culture and chemicals

HeLa cells and MEFs were maintained in DME supplemented with 10% fetal calf serum, 20 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM nonessential amino acids, and penicillin/streptomycin. HeLa cells stably expressing YFP-Parkin were grown under selection in 300 µg/ml hygromycin (Sigma-Aldrich). All chemicals for cell culture were purchased from Invitrogen. PARL KO and WT MEFs were provided by L. Pellegrini (University of Cambridge, Cambridge, England, UK), and PINK1 KO MEFs by Z. Zhang (Burnham Institute for Medical Research, La Jolla, CA).

Constructs and mutagenesis

PARL siRNA (5′-AAATCCAGGGTCCAGAGTTAT-3′) were synthesized by QIAGEN. To achieve efficient knockdown of PARL, cells were transfected twice over a period of 96 h. PINK1-V5/His was provided by M. Cookson (National Institutes of Health, Bethesda, MD). For site-directed mutagenesis, primers were designed using Primer X, a web-based mutagenic primer design program (http://www.bioinformatics.org/primerx/), and produced by Operon. 15 rounds of PCR reactions were performed using Phusion DNA polymerase (Finnzymes) and WT PINK1-YFP constructs as a template. Introduction of point mutations was confirmed by sequencing.

Transfection

Cells were cultured in borosilicated chamber slides for imaging, 6-well plates for whole cell lysates, and a 100- or 150-mm culture dish for subcellular fractionation. 1 d after seeding, cells were transfected with the

indicated constructs using Fugene HD (Roche) or Lipofectamine 2000 (Invitrogen) according to the manufacturers' guidelines.

Western blotting

For whole cell lysates, cells were washed twice with cold PBS and directly lysed with 1× sample buffer. For mitochondrial fraction, cells were fractionated as described previously (Narendra et al., 2008), and mitochondrial pellets were lysed with 1× sample buffer. 20 µg proteins was separated on 4–12% Tris-glycine or Bis-Tris SDS-PAGE. The following antibodies were used in this study: anti-PINK1 (Novus Biologicals), anti-PARL N-terminal (provided by L. Pellegrini), anti-Tom20 (Santa Cruz Biotechnology, Inc.), anti-AIF (Sigma-Aldrich), and anti-Htra2/Omi (R&D Systems). The following polyclonal antibodies were used in this study: anti-VDAC (EMD), anti-tubulin, anti–β-actin (Sigma-Aldrich), anti-Hsp70 (Cell Signaling Technology), anti-Tom20 (BD), anti-Tim23, and anti–Cyt *c* (BD) monoclonal antibodies.

Subcellular fractionation, PK treatment, and alkaline extraction of isolated mitochondria

Before fractionation, HeLa cells were treated with 50 µM MG132 for 10 h followed by 10 µM CCCP for 3 h to accumulate both FL and 52-kD forms of PINK1. Harvested cells were homogenized using a Teflon pestle (Thomas Scientific) in 20 mM Hepes-KOH, pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF, and 2 mg/ml BSA and centrifuged at 800 *g* at 4°C for 10 min to obtain a postnuclear supernatant. Mitochondria were pelleted by centrifugation at 10,000 *g* at 4°C for 20 min. The supernatant fraction was centrifuged further for 30 min at 100,000 *g* to obtain a cytosolic protein fraction. Cytosolic fractions were concentrated using TCA precipitation. Mitochondrial samples treated by alkaline extraction were resuspended in freshly prepared 0.1 M Na_2CO_3, pH 11.5, and incubated on ice for 30 min with occasional vortexing. Membranes were isolated by centrifugation at 100,000 *g* for 30 min at 4°C and solubilized in SDS-PAGE loading dye.

For PK protection assays, mitochondria freshly isolated from HeLa cells were resuspended in 20 mM Hepes-KOH, pH 7.4, 250 mM sucrose, 80 mM KOAc, and 5 mM MgOAc and incubated with various concentrations of PK (Sigma-Aldrich) for 30 min on ice. Digestion was stopped with 1 mM PMSF followed by TCA precipitation of samples, separation by SDS-PAGE, and Western blotting.

In vitro import of PINK1 into isolated mitochondria

Generation of radiolabeled PINK1 precursor was performed by in vitro transcription followed by translation using rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine/cysteine protein-labeling mix (PerkinElmer) as previously described (Lazarou et al., 2007). PINK1 translation products were incubated with freshly isolated mitochondria in import buffer (20 mM Hepes-KOH, pH 7.4, 250 mM sucrose, 80 mM KOAc, 5 mM MgOAc, 5 mM methionine, 1 mM DTT, and 5 mM ATP) at 24°C for various times as indicated in Fig. 1 d. Dissipation of membrane potential was performed using 1 µM CCCP. Samples subjected to protease treatment were incubated on ice for 10 min in 5 µg/ml PK before protease inactivation with 1 mM PMSF. 50 µg of mitochondrial pellets was precipitated using TCA and subjected to SDS-PAGE. Radiolabeled PINK1 was detected by digital autoradiography.

Immunocytochemistry and live cell imaging

For immunocytochemistry, cultured cells in borosilicated chamber slides were fixed with 4% paraformaldehyde in PBS (USB; Affymetrix) and permeabilized with the indicated detergent. After 30 min of blocking with 10% BSA in PBS, cells were stained with primary antibodies anti-Tom20 polyclonal antibody (Santa Cruz Biotechnology, Inc.) and anti–Cyt *c* monoclonal antibody (BD) or anti-Tom20 monoclonal antibody (BD) and anti-GFP polyclonal antibody (Invitrogen), and were then stained with secondary antibodies goat anti–mouse or rabbit IgG conjugated with Alexa Fluor 594 or 647, respectively. For live cell imaging, cells were pulsed with 600 nM TMRE for 5 min to evaluate mitochondrial membrane potential or 10 nM MitoTracker red for 30 min to see the mitochondrial morphology. Cells were imaged using an inverted microscope (LSM510 Meta; Carl Zeiss, Inc.) with a 63× 1.4 NA oil differential interference contrast Plan Apo objective. Image contrast and brightness were adjusted in the accompanying image browser (LSM; Carl Zeiss, Inc.).

Online supplemental material

Fig. S1 shows that the transmembrane domain of PINK1 is required for accumulation in CCCP and for Parkin recruitment. Fig. S2 shows the sequence conservation for predicted PINK1 transmembrane domains from various species. Fig. S3 shows the accumulation of FL and ΔMTS forms of R98F mutant PINK1 on mitochondria that do not induce Parkin translocation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201008084/DC1.

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