The membrane scaffold SLP2 anchors a proteolytic hub in mitochondria containing PARL and the *i*-AAA protease YME1L

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Abstract

The SPFH (stomatin, prohibitin, flotillin, HflC/K) superfamily is composed of scaffold proteins that form ring-like structures and locally specify the protein–lipid composition in a variety of cellular membranes. Stomatin-like protein 2 (SLP2) is a member of this superfamily that localizes to the mitochondrial inner membrane (IM) where it acts as a membrane organizer. Here, we report that SLP2 anchors a large protease complex composed of the rhomboid protease PARL and the *i*-AAA protease YME1L, which we term the SPY complex (for SLP2–PARL–YME1L). Association with SLP2 in the SPY complex regulates PARL-mediated processing of PTEN-induced kinase PINK1 and the phosphatase PGAM5 in mitochondria. Moreover, SLP2 inhibits the stress-activated peptidase OMA1, which can bind to SLP2 and cleaves PGAM5 in depolarized mitochondria. SLP2 restricts OMA1-mediated processing of the dynamin-like GTPase OPA1 allowing stress-induced mitochondrial hyperfusion under starvation conditions. Together, our results reveal an important role of SLP2 membrane scaffolds for the spatial organization of IM proteases regulating mitochondrial dynamics, quality control, and cell survival.

Keywords membrane scaffold; mitochondria; OMA1; rhomboid; SLP2; YME1L

Subject Categories Membrane & Intracellular Transport

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Introduction

Mitochondria are emerging as cellular signaling platforms deeply integrated into various cell survival and cell death cascades.

Proteolytic events at the inner membrane (IM) represent central regulatory steps in these processes, emphasizing the importance of mitochondrial IM proteases beyond their roles as gatekeepers of protein quality. The IM proteases YME1L and OMA1 mediate the processing of the dynamin-like GTPase OPA1, balancing fusion and fission of mitochondrial membranes, and regulating cristae morphogenesis [1–3]. YME1L also catalyzes the regulatory turnover of PRELID1, required at an early stage in the synthesis of the mitochondrial phospholipid cardiolipin (CL) [4]. OMA1 is a stress-activated peptidase, which ensures protein quality control, fine-tunes mitochondrial bioenergetic function, and controls cellular apoptotic resistance [5–9]. The rhomboid protease PARL cleaves the PTEN-induced kinase PINK1 in the IM, which regulates the activity of respiratory complex I and the clearance of damaged mitochondria by mitophagy [10–13]. Moreover, PARL processes phosphoglycerate mutase family member 5 (PGAM5) under stress conditions [14]. PGAM5 is a mitochondrial phosphatase that is cleaved into two forms and regulates mitochondrial dynamics, respiration, and cell survival [15–18]. IM proteases thus have a pivotal role in determining the form and function of mitochondria as well as the regulation of cell signaling and survival.

Increasing evidence points to a functional compartmentalization of the IM, which is considered to be the most protein-rich membrane in the cell [19]. Punctuating the landscape of the IM are large, multiprotein complexes such as ATP synthase and respiratory chain supercomplexes [20,21], the mitochondrial contact site and cristae organizing system (MICOS) [22], and membrane scaffold proteins of the SPFH (stomatin, prohibitin, flotillin, HflC/K) family [23–26]. These scaffold proteins form ring-like structures that specify the local protein–lipid composition in a variety of cellular membranes. The first mitochondrial SPFH proteins to be identified were prohibitins, which are composed of alternating subunits of

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PHB1 and PHB2 [25,27]. Prohibitin ring complexes assemble with *m*-AAA proteases into so-called PMA complexes modulating proteolysis [28,29]. Moreover, they stabilize fusion-active forms of OPA1 and ensure mitochondrial translation and the remodeling of CL [28,30–32], thus maintaining mitochondrial architecture, respiratory function, and apoptotic resistance. SLP2 (STOML2) represents another mitochondrial SPFH family member [33]. SLP2 defines CL-rich membrane domains important for the stability of respiratory complexes and the bioenergetic function of T cells [34,35]. While SLP2 has been shown to physically interact with CL [36] and prohibitins [37], other endogenous binding partner proteins of this membrane scaffold have not been identified.

Here, we used an unbiased proteomic approach to characterize interaction partners of the rhomboid protease PARL in the IM. We identified a large proteolytic hub composed of SLP2, PARL, and the *i*-AAA protease YME1L, which we termed the SPY complex. SLP2 is shown to regulate the proteolytic activities of PARL and the peripherally associated OMA1 peptidase. Our experiments thus reveal the importance of a defined spatial membrane organization for the coordination of proteolytic functions in the IM.

Results

PARL is part of large protein complexes in the mitochondrial inner membrane

To gain insight into the function of the rhomboid protease PARL [38], we used an unbiased proteomic approach to identify its interaction partners. We generated FITR293T *PARL* knockout cells by CRISPR/Cas9-mediated genome editing and functionally complemented them with PARL-FLAG. Mitochondrial extracts from these cells were subjected to immunoprecipitation in order to identify PARL-binding proteins (Fig EV1A). Mass spectrometric analysis revealed co-purification of a selective group of mitochondrial proteins, including the *i*-AAA protease YME1L, the *m*-AAA protease subunit AFG3L2, and the membrane scaffold SLP2 (Fig 1A and B, Dataset EV1). Subunits of prohibitin complexes were not significantly enriched (Dataset EV1). Similarly, known substrates of PARL, the mitochondrial kinase PINK1 [10] and the phosphatase PGAM5 [14], did not efficiently interact with PARL, neither under normal conditions nor in depolarized mitochondria, likely due to their transient binding to the proteolytically active protease (Fig EV1B, Dataset EV1). These proteomic data thus suggest that PARL is part of larger complexes in the IM of mitochondria.

To characterize these complexes further, we determined the complexome profile of proteins co-purifying with PARL in MEFs, combining BN-PAGE with quantitative mass spectrometry [39]. Analysis of the electrophoretic migration profiles of proteins that significantly interacted with PARL-FLAG revealed a striking comigration of PARL, SLP2, AFG3L2, and YME1L as part of complexes migrating at ~2 MDa (Fig 1C). These results were corroborated by BN-PAGE of mitochondria isolated from MEFs lacking SLP2, PARL, or YME1L (Fig 1D). In the absence of SLP2, YME1L formed significantly smaller complexes of ~200 kDa, while PARL was not detected by immunoblotting (Fig 1D), indicating that the membrane scaffold SLP2 promotes the assembly of PARL and YME1L into large protein

complexes in the IM. In contrast, deletion of *Slp2* did not impair the formation of large AFG3L2-containing complexes indicating that AFG3L2 is part of independent large assemblies in the IM [28] (Fig EV1C). Genetic ablation of *Yme1l* or *Parl* did not disrupt the formation of large complexes to the same extent as *Slp2* deletion (Fig 1D). SLP2 was detected as part of large complexes in YME1L- and PARL-deficient cells, whereas PARL accumulated in slightly smaller complexes in the absence of YME1L (Fig 1D). Complexome analysis using high-resolution BN-PAGE revealed that only a fraction of SLP2 assembles with YME1L and PARL into large complexes (Fig 1E). Immunoprecipitation experiments in wild-type cells confirmed the physical interaction of endogenous SLP2, PARL, and YME1L: We successfully co-purified SLP2 and YME1L with PARL-specific antibodies as well as PARL and YME1L with SLP2-specific antibodies (Fig 2A). Notably, we observed interactions between PARL and SLP2 in *Yme1l*^{-/-} cells and between PARL and YME1L in *Slp2*^{-/-} cells (Fig 2B). Similarly, SLP2 and YME1L maintained their interaction in *Parl*^{-/-} cells (Fig 2C), indicating that SLP2, PARL, and YME1L can interact independently.

Therefore, to unambiguously demonstrate that SLP2 forms one complex with both IM proteases, we performed immunoprecipitations using cells expressing SLP2-FLAG, eluted native complexes with FLAG peptides, and analyzed them by BN-PAGE. We observed a large macromolecular complex of ~2 MDa that was immunoreactive for SLP2, PARL, and YME1L but not for PHB2 (Fig 2D). High-resolution BN-PAGE revealed a significant size shift of SLP2-containing complexes upon deletion of either *Parl* or *Yme1l* (Fig 2E). These results demonstrate that the membrane scaffold SLP2 promotes the assembly of PARL and YME1L into one large protein complex in the IM.

SLP2 has been localized predominantly to mitochondria [34,36,37], but seemingly confounding reports of interactions with OM and IM proteins prompted us to define its submitochondrial localization [33,37]. We performed proteinase K protection assays that revealed that SLP2 resides in the mitochondrial matrix (Fig 2F). SLP2 behaved like the integral IM protein PHB2 upon alkaline extraction of mitochondrial membranes at pH 11.5, indicating a tight association with the IM (Figs 2G and EV2). However, SLP2 lacks a putative transmembrane domain and, consistently, was recovered in the supernatant fraction at high pH (Figs 2G and EV2).

Together, we conclude that SLP2 is a matrix protein associated with the IM, where it assembles with both YME1L and PARL into large complexes. We termed these proteolytic hubs SPY complexes (SLP2–PARL–YME1L, Fig 2H).

SLP2 regulates PARL activity within the SPY complex

We assessed in further experiments how the assembly into SPY complexes affects the activity of the associated proteases. We first measured the steady state levels of known proteolytic substrates of the *i*-AAA protease YME1L in MEFs lacking SPY complex subunits. These substrates included the lipid transfer protein PRELID1 [4], the IM translocase subunit TIMM23 [40], and the dynamin-like GTPase OPA1 [1] (Fig 3A). While PRELID1 and TIMM23 accumulated when the protease is absent (Fig 3A) or inactivated (Fig EV3A), their levels in *Slp2*^{-/-} or *Parl*^{-/-} cells did not differ from wild-type levels (Figs 3A and EV3B). Similarly,

Figure 1. PARL is part of a large assembly in the IM.

- A Volcano plot representation of PARL interaction partners. Mitochondria isolated from *PARL*^{-/-} FITR293T cells expressing PARL-FLAG were subjected to co-immunoprecipitation (IP). Co-purifying proteins were separated by SDS-PAGE and identified by quantitative MS ($n = 3$) (Dataset EV1).
- B Heat map of log_2 -transformed LFQ intensities for three independent experiments. All significant ($\text{FDR} < 0.05$) proteins showing a positive ratio between PARL IP and control in (A) are shown. Gray color shows missing quantitative information. Clustering was performed using the complete method with Euclidean distance.
- C Complexome analysis of MEF mitochondria ($n = 3$). Heat maps of the relative abundances of proteins significantly enriched in the eluate of PARL immunoprecipitates at a FDR level of 0.05 (A) and identified by mass spectrometry after BN-PAGE analysis are shown after hierarchical clustering.
- D BN-PAGE analysis of mitochondria lacking SLP2, YME1L, or PARL. Mitochondria isolated from corresponding MEFs were solubilized using 1.5% (w/v) digitonin at a protein concentration of 2.5 mg/ml. Solubilized proteins were separated by a 3–13% BN-PAGE and analyzed using SLP2-, YME1L-, and PARL-specific antibodies.
- E High-resolution complexome analysis of SPY complex members SLP2, YME1L, and PARL using MEF mitochondria ($n = 3$). Heat maps and migration profiles are shown after separation using a 3–9% BN-PAGE. SLP2, YME1L, and PARL migrate in a high molecular weight complex (~2 MDa), whereas SLP2 is additionally present in complexes of ~1.6 MDa).

Figure 2.

the cleaved form d of OPA1 generated by YME1L cleavage was present in these cells (Figs 3A and EV3B). We observed the accumulation of the cleaved S-OPA1 forms c and e in *Slp2*^{-/-} cells that we could attribute to increased cleavage by OMA1 (see

Fig 5D). Regardless, proteolysis by YME1L can occur independently from its assembly into SPY complexes.

PARL activity was examined in *Slp2*^{-/-} cells by monitoring the proteolysis of its substrate PGAM5 [14]. In light of

Figure 2. SLP2 forms a proteolytic hub in the IM.

- A Co-immunoprecipitation of endogenous SLP2, PARL, and YME1L in mitochondria isolated from human FTR293T cells using either SLP2- or PARL-specific antibodies. IgG was used as a negative control. In, input (10%).
- B, C Co-immunoprecipitation of endogenous SLP2 and YME1L with PARL-specific antibodies in mitochondria isolated from wild-type MEFs (WT) and MEFs lacking YME1L, SLP2, or PARL. In, input (10%).
- D Assembly of SLP2, PARL, and YME1L into a high molecular weight complex. Mitochondria isolated from FTR293T cells (Con) and cells inducibly expressing SLP2-FLAG (FLAG) were solubilized in digitonin and subjected to immunoprecipitation using FLAG-specific antibodies. Native eluates of the precipitate were analyzed by BN-PAGE and immunoblotting using SLP2-, PARL-, YME1L-, and PHB2-specific antibodies. In, input (8%); E, eluate (100%).
- E High-resolution BN-PAGE analysis of mitochondria lacking PARL, YME1L, or SLP2. Mitochondria isolated from corresponding MEFs were solubilized using 1.5% (w/v) digitonin at a protein concentration of 2.5 mg/ml. Solubilized proteins were separated by a 3–9% gradient gel containing 0–10% glycerol and analyzed using SLP2-specific antibodies.
- F, G Submitochondrial localization of SLP2. (F) Mitochondria were isolated from MEFs and fractionated in the presence or absence of proteinase K as indicated. (G) Mitochondrial membranes were extracted with sodium carbonate at the indicated pH and separated into pellet (P) and supernatant (S) fractions by centrifugation. Fractions were analyzed by SDS-PAGE and immunoblotting. MFN2 served as OM marker, TIMM23, SMAC/DIABLO, and YME1L as IMS markers, and AFG3L1 and AFG3L2 as matrix marker proteins.
- H Topology of the SLP2–PARL–YME1L (SPY) complex in the IM. IM, inner membrane; IMS, intermembrane space.

Source data are available online for this figure.

discrepant reports on the localization of PGAM5 [14,15,41], we first confirmed the localization of both mature, full-length PGAM5 (L-PGAM5) and its proteolytically processed, short form (S-PGAM5) at the IM (Fig EV4). Only unprocessed L-PGAM5 accumulated in *PARL*^{−/−} cells, whereas about equimolar amounts of L-PGAM5 and S-PGAM5 accumulated in wild-type cells (Fig 3B). Overexpression of PARL-FLAG resulted in the almost complete conversion of L-PGAM5 to S-PGAM5 in both wild-type and *PARL*^{−/−} cells (Fig 3B). In contrast, expression of proteolytic inactive PARL^{S277A}-FLAG did not restore PGAM5 processing in PARL-deficient cells (Fig 3B). We conclude that PARL cleaves PGAM5 not only under conditions of mitochondrial stress as previously reported [14], but mediates PGAM5 processing under basal conditions as well.

We next examined whether assembly into the SPY complex affected cleavage of PGAM5 by PARL and monitored the rate of conversion of L-PGAM5 to S-PGAM5 in *Slp2*^{−/−} and *Yme1l*^{−/−} cells subjected to a cycloheximide (CHX) chase. While loss of YME1L did not affect PGAM5 processing, we observed accelerated production of S-PGAM5 in *Slp2*^{−/−} cells (Fig 3C), which could be blocked by RNAi-mediated depletion of PARL (Fig 3D). These observations indicate that SLP2 negatively regulates PGAM5 processing by PARL.

To determine whether PGAM5 was a substrate of PARL within the SPY complex, we performed BN-PAGE in cells expressing PARL-FLAG or the proteolytically inactive variant PARL^{S277A}-FLAG

(Fig 3E). We found PGAM5 to comigrate with catalytically inactive but not wild-type PARL in the SPY complex (Fig 3E). We therefore conclude that SLP2 modulates PARL-mediated cleavage of PGAM5 within the SPY complex.

The SPY complex facilitates PINK1 cleavage by PARL

To corroborate these findings, we turned our attention to another PARL substrate, PINK1. PARL cleavage generates a ~52 kDa form of PINK1, which is released from mitochondria and degraded by 26S proteasomes, while mitochondrial depolarization causes the accumulation of a ~63 kDa form of PINK1 at the mitochondrial surface and the induction of mitophagy [12]. We therefore examined the PARL-dependent formation of the ~52 kDa form of PINK1 in cells lacking SLP2, PARL, or YME1L that transiently expressed an HA-tagged variant of PINK1 and were treated with the proteasomal inhibitor MG132 (Fig 4A). Loss of SLP2 or YME1L significantly impaired the PARL-dependent accumulation of the ~52 kDa form of PINK1 (Fig 4A and B). BN-PAGE analysis of *Parl*^{−/−} cells expressing PARL-FLAG or PARL^{S277A}-FLAG revealed that transiently expressed PINK1-HA comigrated with SPY complexes containing proteolytically inactive PARL, indicating that PINK1 is cleaved in association with SPY complexes (Fig 4C). Similarly, at least a fraction of endogenous PINK1 was part of a high molecular weight complex comigrating with SLP2 (Fig 4D). Interestingly, depletion of SLP2 reduced the association of PINK1 with this complex, demonstrating

Figure 3. SLP2 modulates PGAM5 processing by PARL in the SPY complex.

- A Steady state levels of proteolytic substrates of YME1L. Whole-cell extracts of *Yme1l*^{−/−}, *Parl*^{−/−}, and *Slp2*^{−/−} MEFs were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. a–b, L-OPA1 forms. c–e, S-OPA1 forms.
- B PGAM5 processing depends on proteolytically active PARL. PARL and catalytic inactive PARL^{S277A} harboring C-terminal FLAG epitopes were expressed under the control of a tetracycline (tet)-inducible promoter in wild-type (WT) and *PARL*^{−/−} FTR293T cells as indicated. Processing of L-PGAM5 to S-PGAM5 was monitored by immunoblotting.
- C Accelerated processing of L-PGAM5 in *Slp2*^{−/−} cells. Processing of L-PGAM5 was analyzed by immunoblotting in wild-type (WT), *Yme1l*^{−/−}, *Parl*^{−/−}, and *Slp2*^{−/−} MEFs after inhibition of cytosolic protein synthesis by cycloheximide (CHX). A quantification of L-PGAM5 levels at different time points is shown in the lower panel. Two-way ANOVA analysis ($n = 3$; **** $P < 0.0001$). Error bars indicate SEM.
- D The accelerated processing of L-PGAM5 in *Slp2*^{−/−} cells is mediated by PARL. PARL was depleted from *Slp2*^{−/−} cells by RNAi prior to CHX treatment. A quantification of L-PGAM5 levels at different time points is shown. Two-way ANOVA analysis ($n = 3$; ** $P < 0.01$, **** $P < 0.0001$). Error bars indicate SEM.
- E PGAM5 associates with the SPY complex harboring proteolytically inactive PARL. Mitochondria isolated from FTR293T cells (WT) or *PARL*^{−/−} FTR293T cells expressing PARL-FLAG (PARL-WT) or PARL^{S277A}-FLAG (PARL-S277A) were solubilized in digitonin and were analyzed by BN-PAGE and immunoblotting using FLAG- and PGAM5-specific antibodies.

Figure 3.

the specificity of this interaction and suggesting that SLP2 supports the recruitment of PINK1 to PARL within the SPY complex (Fig 4D). Together, we conclude that the SPY complex facilitates the cleavage of PINK1 by PARL.

OMA1 cleaves PGAM5 under stress and is regulated by SLP2

Dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) has been reported to trigger the activation of PARL [14]. Thus, we sought to determine whether the SPY complex also mediates the regulation of PARL activity under such stress conditions.

Mitochondrial depolarization by the protonophore carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) resulted in the rapid proteolysis of L-PGAM5 (Fig 5A), which occurred more rapidly than under basal conditions (Fig 3C). Deletion of *Slp2* or *Yme1l* did not further accelerate the formation of S-PGAM5 (Fig 5A), indicating that SLP2 does not impair PGAM5 proteolysis after CCCP treatment. This was not due to dissociation of the SPY complex, as SLP2 and YME1L were efficiently immunoprecipitated with PARL in depolarized mitochondria (Fig EV1B). Intriguingly, we still observed proteolysis of L-PGAM5 in CCCP-treated *Parl*-deficient cells (Fig 5A), pointing to the involvement of another protease.

Figure 4. The SPY complex facilitates PINK1 processing by PARL.

- A** PARL-dependent cleavage of PINK1-HA is reduced in *Slp2*^{-/-} and *Yme1l*^{-/-} MEFs. Whole-cell extracts of *Slp2*^{-/-}, *Parl*^{-/-}, and *Yme1l*^{-/-} MEFs expressing PINK1-HA were analyzed by SDS-PAGE and immunoblotting using the indicated antibodies. Cells were treated with 20 μ M MG132 or 20 μ M CCCP for 4 h.
- B** Quantification of the protein ratio (log₂) PINK1-HA 52 kDa/63 kDa in the presence of MG132 ($n = 3$; * $P < 0.05$; one-way ANOVA). n.s., not significant. Error bars indicate SEM.
- C** PINK1-HA associates with the SPY complex harboring proteolytically inactive PARL. Mitochondria isolated from FTR293T cells (WT) or *PARL*^{-/-} FTR293T cells expressing PARL-FLAG (PARL-WT) or PARL^{S277A}-FLAG (PARL-S277A) were solubilized in digitonin and analyzed by BN-PAGE and immunoblotting using FLAG-, HA-, and SLP2-specific antibodies.
- D** PINK1 associates with SLP2. Mitochondria isolated from FTR293T cells (WT) or *PARL*^{-/-} FTR293T cells, depleted of SLP2 by siRNA as indicated, were solubilized in digitonin and analyzed by BN-PAGE and immunoblotting using SLP2- and PINK1-specific antibodies.

Figure 5.

Figure 5. OMA1 cleaves PGAM5 under stress and is regulated by SLP2.

- A Processing of L-PGAM5 to S-PGAM5 in depolarized mitochondria of wild-type (WT), *Slp2*^{-/-}, *Yme1*^{-/-}, and *Parl*^{-/-} MEFs. PGAM5 processing was monitored by immunoblotting at the indicated time points after inhibition of cytosolic protein synthesis with cycloheximide (CHX). A quantification of L-PGAM5 levels at different time points is shown. L-PGAM5 levels at *t* = 0 were set to 100%. Two-way ANOVA analysis (*n* = 3; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001). Arrowheads denote intermediate PGAM5 cleavage products. Error bars indicate SEM.
- B OMA1-mediated processing of PGAM5 in depolarized *Parl*^{-/-} mitochondria. Processing of L-PGAM5 to S-PGAM5 was assessed as in (A) in the presence or absence of CCCP (20 μM) in *Parl*^{-/-} mitochondria which were depleted of OMA1 by siRNA as indicated. The arrowhead denotes an intermediate PGAM5 cleavage product. SCR, scrambled.
- C Impaired PGAM5 processing in depolarized *Oma1*^{-/-} mitochondria lacking PARL. Processing of L-PGAM5 to S-PGAM5 was examined as in (A) after 2 h in wild-type (WT) and *Oma1*^{-/-} mitochondria in the presence or absence of CCCP (20 μM, 2 h), which were depleted of PARL by siRNA as indicated. *Unspecific cross-reaction.
- D OMA1 mediates accelerated processing of L-OPA1 in *Slp2*^{-/-} MEFs. Processing of OPA1 and PGAM5 was monitored in *Slp2*^{-/-}, *Oma1*^{-/-}, and *Slp2*^{-/-}*Oma1*^{-/-} cells by immunoblotting after inhibition of cytosolic protein synthesis with cycloheximide (CHX). a–b, L-OPA1 forms. c–e, S-OPA1 forms.
- E OMA1 interacts with SLP2. *OMA1*^{-/-} FITR293T cells ectopically expressing OMA1-myc were transfected with pcDNA5 (Control) or pcDNA5-SLP2-FLAG (FLAG). Isolated mitochondria were solubilized in digitonin and subjected to immunoprecipitation using FLAG-specific antibodies. Native eluates of the precipitate were analyzed by BN-PAGE and immunoblotting using SLP2-, YME1L-, PARL-, and myc-specific antibodies. In, input (8%); E, eluate (100%). Samples were analyzed by SDS-PAGE in parallel (lower panel).
- F Quantification of immunoprecipitation efficiencies in (E).
- G Model for the regulation of IM proteases by the membrane scaffold SLP2. SLP2 inhibits processing of L-OPA1 by OMA1 and of PGAM5 by PARL, while facilitating PINK1 cleavage by PARL, thus modulating mitochondrial morphology, quality control, and cell survival.

We turned our attention to the stress-activated peptidase OMA1 [5] and tested whether it was able to cleave PGAM5 in depolarized mitochondria. L-PGAM5 cleavage was abolished in the presence of CCCP when OMA1 was depleted from *PARL*^{-/-} cells or PARL from *OMA1*^{-/-} cells (Fig 5B and C) or in *PARL*^{-/-}*OMA1*^{-/-} cells (Fig EV5A), demonstrating that the stress-induced proteolysis of L-PGAM5 observed in *PARL*^{-/-} cells depended on OMA1. On the other hand, dissipation of $\Delta\Psi_m$ accelerated PGAM5 processing in *OMA1*^{-/-} cells, confirming the previously reported stress-activated PGAM5 processing by PARL [14]. Together, these data demonstrate accelerated PGAM5 processing by OMA1 and PARL under stress.

The activation of OMA1 in depolarized mitochondria and its ability to cleave PGAM5 raises the possibility that the accelerated processing of PGAM5 in *Slp2*^{-/-} cells is due to OMA1. However, depletion of PARL from *Slp2*^{-/-} cells completely impaired PGAM5 processing (Fig 3D). The OMA1 substrate L-OPA1 was previously reported to be destabilized in SLP2-deficient cells leading to mitochondrial fragmentation [42]. We observed the accumulation of L-OPA1 in *Slp2*^{-/-}*Oma1*^{-/-} cells (Fig 5D). Consistently, depletion of OMA1 preserved tubular mitochondria in *Slp2*^{-/-} cells under normal and stress conditions (Fig EV5B and C). These results demonstrate that SLP2 affects the proteolysis of both L-PGAM5 by PARL and L-OPA1 by OMA1.

OMA1 was not identified as subunit of the SPY complex in our proteomic analysis. We reasoned that the low expression level might have hampered the identification of the membrane-embedded peptidase by mass spectrometry. We therefore performed immunoprecipitation experiments in cells expressing SLP2-FLAG and a myc-tagged variant of OMA1, eluted native complexes with FLAG peptides, and analyzed them by BN-PAGE (Fig 5E and F). Indeed, a fraction of OMA1 was detected as part of large assemblies comigrating with SLP2, PARL, and YME1L, demonstrating that OMA1 can interact with SPY complexes when overexpressed.

Discussion

Our results reveal assembly of the membrane scaffold SLP2 with at least two IM proteases, PARL and YME1L, into a large proteolytic

hub in the IM that we term the SPY complex. We demonstrate that SLP2 regulates the activity of PARL toward PGAM5 and PINK1 and restricts OPA1 cleavage by OMA1 (Fig 5G). As SLP2-associated peptidases have been functionally linked to the regulation of mitochondrial dynamics, quality control, and cell survival, our findings suggest that the regulation of these processes is spatially organized and occurs at defined sites at the IM. Interestingly, α - and γ -secretases were recently identified as part of large multiprotease complexes at the plasma membrane, suggesting that spatially coordinated proteolysis of membrane proteins is of broad relevance [43].

The association of SLP2 with the *i*-AAA protease YME1L is reminiscent of PMA complexes containing the structurally related prohibitin scaffolds and *m*-AAA proteases, homologous IM proteases that are active at the matrix side [28,29]. While prohibitins are membrane-anchored and localized to the IMS, SLP2 binds to the inner surface of the IM. Therefore, membrane scaffolds at both membrane sides assemble with AAA proteases in the IM, which expose their catalytic sites to the opposite membrane surface. Loss of SLP2 did not impair the accumulation of known YME1L substrates, indicating normal proteolysis by YME1L. This may reflect substrate-specific effects and is reminiscent of prohibitins that negatively modulate the turnover of certain *m*-AAA protease substrates only [29]. Moreover, altered proteolysis by YME1L in the absence of SLP2 might only become apparent if the protease is limiting, that is, under conditions of high substrate load. Nevertheless, it appears likely that SLP2 may contribute to YME1L-mediated proteolysis ensuring its spatial organization in the IM. For instance, assembly into SPY complexes may facilitate the coordinated proteolysis of common substrates of both YME1L and PARL.

While the functional relationship of YME1L with SLP2 remains to be defined, the association of PARL with SLP2 modulates PARL activity. PARL cleavage of both PGAM5 and PINK1 occurs in association with the SPY complex. Whereas the association with SPY complexes appears to facilitate PINK1 processing, PARL assembly into these complexes limits its activity toward PGAM5. We observed accelerated processing of PGAM5 by PARL in mitochondria lacking SLP2. It is an attractive possibility that SLP2, acting as a membrane scaffold, defines the lipid environment for

proteolysis and modulates substrate accessibility, thereby putting the breaks on or facilitating PARL-mediated proteolysis in a substrate-specific manner. In agreement with previous findings [14], we observed accelerated PGAM5 processing by PARL upon mitochondrial depolarization. This may reflect PARL activation and an increased specific activity of the protease that can be mimicked by PARL overexpression in energized mitochondria. Notably, we demonstrate that the stress-activated peptidase OMA1 can partially substitute for PARL in PGAM5 processing upon mitochondrial $\Delta\Psi_m$ dissipation, identifying PGAM5 as a novel OMA1 substrate. The protease–substrate relationship between both proteins may underscore the markedly divergent metabolic phenotypes observed in OMA1- and PGAM5-deficient mice under stress conditions [14,17,44].

The loss of SLP2 affects the activities of both PARL and OMA1, the latter resulting in the degradation of L-OPA1 forms and mitochondrial fragmentation. Thus, SLP2 limits OMA1 activity toward L-OPA1 under conditions of stress-induced mitochondrial hyperfusion (SIMH) [42], which ensures respiration and protects against mitophagy under starvation conditions [45,46]. OMA1 can associate with SLP2 at least when overexpressed suggesting that SPY complexes defined by SLP2 membrane scaffolds determine the spatial organization of additional regulatory processes at the level of the IM.

Materials and Methods

Cell lines and culture

Immortalized embryonic fibroblasts (MEFs) were maintained in DMEM (Gibco) supplemented with nonessential amino acids (PAA) and 10% (v/v) FBS (Gibco) at 37°C, 5% (v/v) CO₂ in a humidified incubator. *Yme1l*^{-/-}, *Oma1*^{-/-}, *Part*^{-/-}, and *Slp2*^{-/-} MEFs were previously described [1,35,38]. Embryonic fibroblasts were obtained from *Slp2*^{-/-}*Oma1*^{-/-} knockout embryos (E13.5) and immortalized to generate *Slp2*^{-/-}*Oma1*^{-/-} MEFs as previously described [1]. Inducible cell lines carrying epitope-tagged variants of PARL and SLP2 were generated from Flp-In™ T-REX™ HEK293 cells (FTR293T, Invitrogen), which were cultured with 7.5% (v/v) tetracycline-reduced FBS (Biochrom AG) prior to induction with tetracycline.

Generating knockout and stable cell lines

CRISPR/Cas9 genome editing was performed to delete *PARL* in FTR293T cells [47]. For gene targeting, a DNA fragment specific for human *PARL* DNA (5'-TGCTTTGATTCCTCCTG-3') and (5'-CTTCACCACTTGTCCTG-3') were synthesized, subcloned into the pX335 (Addgene), and expressed in FTR293T cells. Single cells were isolated by limited dilution and analyzed using the surveyor assay to examine gene editing. Positive clones were verified by immunoblotted using PARL-specific antibodies and sequenced to confirm the disruption of the *PARL* gene.

Complementary DNA (cDNA) encoding human PARL or SLP2 was amplified by PCR using cDNA of HeLa cells as template. Complementary DNA (carrying 3' sequence of the FLAG epitope when indicated) was subcloned into the pcDNA5/FRT/TO (Invitrogen). Mutant variants of PARL were generated by site-directed mutagenesis.

In-gel digestion

The eluate from immunoprecipitation was digested following the protocol published previously [48]. In brief, each gel lane was divided into seven parts and after several washing steps proteins were reduced (10 mM DTT at 56°C for 30 min) and alkylated (IAA, 30 min, room temperature in the dark). After trypsin digestion (12 ng/μl, 40 μl) overnight at 37°C, generated peptides were extracted using an increasing content of acetonitrile and concentrated in a speed vac. Prior to LC-MS/MS analysis, samples were primed using the STAGE tip technique [49].

Liquid chromatography and mass spectrometry

Peptides were eluted from C18 tips with 30 μl of 0.1% formic acid in 80% acetonitrile (ACN), concentrated in a speed vac, and re-suspended in 10 μl buffer A (0.1% formic acid). The liquid chromatography–tandem mass spectrometry (LC-MS/MS) is equipped with an EASY nLC 1000 coupled to the quadrupole-based QExactive instrument (Thermo Scientific) via a nano-spray electroionization source. Peptides were separated on an in-house packed 50 cm column (1.9 μm C18 beads, Dr. Maisch) using a binary buffer system: (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile. The content of buffer B was raised from 7 to 23% within 40 min and followed by an increase to 45% within 5 min. Eluting peptides were ionized by an applied voltage of 2.4 kV. The capillary temperature was 275°C and the S-lens RF level was set to 50. MS1 spectra were acquired using a resolution of 70,000 (at 200 m/z), an automatic gain control (AGC) target of 3e6, and a maximum injection time of 20 ms in a scan range of 300–1,750 Th. In a data-dependent mode, the 10 most intense peaks were selected for isolation and fragmentation in the HCD cell using normalized collision energy of 25. Dynamic exclusion was enabled and set to 20 s. The MS/MS scan properties were 35,000 resolution at 200 m/z, an AGC target of 5e5, an isolation window of 1.8 Th, and a maximum injection time of 120 ms [50].

MaxQuant analysis and bioinformatics

Raw data were processed using MaxQuant 1.5.1.2 [51] and the implemented Andromeda search engine [52]. MS/MS spectra were correlated against the human Uniprot database (downloaded November 2014) including a list of common contaminants. We used 7 and 4.5 ppm MS/MS tolerances for first and main search, respectively. The FDR at the peptide-spectrum match and the protein level was controlled by the implemented decoy algorithm using the revert database. Match-between runs re-quantify and LFQ quantification [53] algorithms were enabled and used by default settings. N-term acetylation and oxidation at methionine residues were defined as variable modifications, whereas carbamidomethylation at cysteine residues was defined as a fixed modification. LFQ intensities were log2-transformed and a two-tailed *t*-test was applied to identify significant differently pulled down proteins between the control and PARL-FLAG. To correct for multiple testing, we used a permutation-based FDR calculation with a fudge-factor *s*₀ of 0.1 and a FDR cutoff of 5% (# of permutations: 500). For visualization of selected identified interaction partners, we utilized the heatmap.2 function in the *gplots* package

in R. The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE [54] partner repository with the dataset identifier PXD004914.

Submitochondrial localization

Mitochondria were treated with 0.5 µg/ml proteinase K (PK) for 10 min with or without prior swelling in 10 mM HEPES-KOH pH 7.4 containing 1 mM EDTA. PK digestion of mitochondrial proteins was stopped with 1 mM PMSF. When indicated, 0.5% (v/v) Triton X-100 was added prior to PK treatment. Samples were precipitated by trichloric acid and analyzed by SDS-PAGE and immunoblotting.

Protein cross-linking and co-immunoprecipitation

Protein A-sepharose was washed in buffer B (0.1 M Tris-HCl pH 8, 0.5 M NaCl) and then incubated with 80 µg of the respective antibody in buffer A (0.1 M Tris-HCl pH 8) for 2 h at room temperature with gentle agitation. Dimethyl pimelimidate (0.2 M) cross-linking was performed in sodium borate buffer (0.2 M, pH 9.2) for 30 min and stopped by the addition of 0.2 M ethanolamine pH 8 for 2 h. After three final washing steps in PBS, the beads were used for co-immunoprecipitation [28].

Primary antibodies

Rabbit polyclonal antibodies against PARL were generated in response to the peptide 366-EIRTNGPKKGGGSK-379 of human PARL. The following commercially available antibodies were used: SLP2 (Protein Tech Group, 1:1,000), MYC (Cell signaling, 1:5,000), OPA1 (BD Biosciences, 1:500), penta-HIS (Qiagen, 1:1,000), YME1L (Protein Tech Group, 1:1,000), SDHA (Invitrogen, 1:10,000), PHB2 (1:1,000) [30], AGF3L2 (1:1,000) [55], SMAC/DIABLO (MoBiTec, 1:1,000), TIMM23 (Abcam, 1:2,000), MFN2 (Sigma, 1:1,000), PRELID1 (PX19 Abnova, 1:1,000), PGAM5 (Sigma-Aldrich, 1:1,000), PINK1 (Novus, 1:2,000), HA (Roche, 1:1,000), and FLAG (Sigma-Aldrich, 1:1,000).

Statistical analysis

For each statistical analysis, three independent biological replicates were performed. Calculations of means, standard deviation of the mean, and parametric *t*-test (unpaired, two-tailed) were performed. *P*-values were categorized as follows: **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, and *****P* ≤ 0.0001. One-way ANOVA was used to evaluate the statistical significance of alterations in PINK1 cleavage; two-way ANOVA was used to compare turnover and conversion rates of proteolytic substrates.

Miscellaneous

The following procedures were performed as previously described: complexome profiling [39], BN-PAGE and co-immunoprecipitation using FLAG-specific antibodies [28], and isolation of mitochondria [1].

Expanded View for this article is available online.

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Author contributions

TW and SM characterized SPY complexes biochemically; SS performed the purification of PARL-containing complexes; HN and MK performed the mass spectrometric analysis; TK and RRD performed BN-PAGE analysis; JM and RRD generated MEFs harboring floxed *Slp2* and *Oma1*; MM, UB, and TK performed the complexome analysis; HGS performed submitochondrial localization analysis; and TW and TL drafted the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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