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Cleaved PGAM5 is released from mitochondria depending on proteasome-mediated rupture of the outer mitochondrial membrane during mitophagy

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Running title: Release of cleaved PGAM5 from mitochondria during mitophagy

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Summary

PGAM5 is a unique type of protein phosphatase that exists in mitochondria. It has been shown to exist in the inner mitochondrial membrane through its transmembrane domain and to be cleaved within the transmembrane domain upon mitochondrial dysfunction. However, its submitochondrial localization remains controversial; many researchers claim that PGAM5 localizes to the outer mitochondrial membrane based on the findings that PGAM5 associates with many cytoplasmic proteins. Here, we found that cleaved PGAM5 was released from mitochondria during mitophagy, a selective form of autophagy specific for mitochondria, and that the release was inhibited by proteasome inhibitors in HeLa cells stably expressing the E3 ubiquitin ligase Parkin. However, treatment of parental HeLa cells lacking Parkin with mitophagy-inducing agents caused PGAM5 cleavage but did not cause its release from mitochondria. Thus, cleaved PGAM5 appears to be released from mitochondria depending on proteasome-mediated rupture of the outer membrane during mitophagy, which has been previously shown to precede autophagy-mediated degradation of whole mitochondria. This study suggests that PGAM5 senses mitochondrial dysfunction in the inner mitochondrial membrane and serves as a signaling intermediate that regulates the cellular response to mitochondrial stress upon its cleavage and release from mitochondria.

Keywords: mitochondria; mitophagy; Parkin; PGAM5.

Abbreviations:

OA, oligomycin A and antimycin A; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta\psi_m$, membrane potential; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PGAM5, phosphoglycerate mutase family member 5; TM, transmembrane.

Introduction

Mitophagy is the autophagy-based degradation machinery selective for mitochondria and constitutes a major part of the mitochondrial quality control system (*1, 2*). A large body of evidence has revealed that the E3 ubiquitin ligase Parkin and mitochondrial serine/threonine kinase PINK1 play crucial roles in mitophagy induction. In damaged mitochondria with loss of membrane potential ($\Delta\psi_m$), PINK1 accumulates on the outer mitochondrial membrane (OMM) and recruits Parkin to mitochondria where Parkin is activated. Parkin then ubiquitinates and degrades various OMM proteins, triggering proteasome-mediated OMM rupture prior to complete degradation of mitochondria through autophagy machinery (*3, 4*).

Phosphoglycerate mutase family member 5 (PGAM5) is a unique type of protein phosphatase that exists in mitochondria and is proposed to be involved in various biological processes such as apoptosis, necroptosis, lipid metabolism, inflammation, and immune responses (*5-10*). We have previously shown that PGAM5 exists in the inner mitochondrial membrane (IMM) through its N-terminal transmembrane (TM) domain (*11*) . However, its submitochondrial localization remains controversial; several reports have shown that PGAM5 functions through binding to cytoplasmic proteins, such as Drp1, FUNDC1, nucleoside diphosphate kinase B, KEAP1, Nrf2, and RIPK1, suggesting that PGAM5 exists in the OMM (*6, 12-15*).

A strong piece of evidence of the existence of PGAM5 in the IMM at least in unstressed cells is that PGAM5 is cleaved within the TM domain in response to loss of mitochondrial $\Delta\psi_m$ and that the responsible proteases that cleave PGAM5 are the IMM-resident proteases, presenilin-associated rhomboid-like protein (PARL) and OMA1 (overlapping activity with *m*-AAA protease) (*11, 16*). These findings strongly suggest that PGAM5 is integrated into the IMM, rather than the OMM, through its N-terminal TM

domain, and intramembrane cleavage of PGAM5 may be an important component of the sensing system of loss of mitochondrial $\Delta\psi_m$.

PGAM5 has recently been proposed to be involved in regulation of mitophagy (*12, 15, 17*). Because recent reports have shown that the cleaved form of PGAM5 is released from mitochondria under certain conditions by a mechanism that is not fully understood (*8, 18, 19*), we examined whether PGAM5 is cleaved and released from mitochondria during mitophagy.

Materials and Methods

Reagents

Antimycin A, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and oligomycin A were purchased from Wako Chemical (Osaka, Japan). Oligomycin A was also purchased from Cayman Chemical (Ann Arbor, MI). MG132 and epoxomicin were purchased from Enzo Life Sciences (Farmingdale, NY) and Peptide Institute (Osaka, Japan), respectively.

Cell culture

HeLa cells stably expressing HA-tagged Parkin (Parkin-HeLa cells) were established previously (*20*) and cultured in Dulbecco's modified Eagle's medium (DMEM) (high glucose; Wako Chemical) containing 8% fetal bovine serum (FBS), 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 1× MEM non-essential amino acids (Thermo Fisher Scientific, Waltham, MA), and 5 μg/ml puromycin under a 5% CO_2 atmosphere at 37°C. HeLa cells were cultured in DMEM (high glucose) containing 8% FBS, 100 U/ml penicillin G and 0.1 mg/ml streptomycin under a 5% CO_2 atmosphere at 37°C.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 3.7% paraformaldehyde and permeabilized with 0.25% Triton X-100. After blocking with 2.5% BSA, the cells were stained with a 1:100- or 1:200-diluted anti-PGAM5 rat monoclonal antibody (clone K1B6) (*11*) and 1:200-diluted anti-Tom20 antibody (FL-145; Santa Cruz Biotechnology, Dallas, TX). Immune complexes were detected with 1:200-diluted anti-rat IgG Alexa Fluor 488 and 1:200-diluted anti-rabbit IgG Alexa Fluor 546 (Thermo Fisher Scientific). Nuclei were

counterstained with 20 μM Hoechst 33342 (bisbenzimide; Sigma-Aldrich, St. Louis, MO). Images were acquired by a confocal microscopy (LSM710; Zeiss, Jena, Germany).

Subcellular fractionation

Parkin-HeLa cells were collected in ice-old PBS and centrifuged at 2,400 *g* for 2 min. The cells were suspended in 800 μl homogenization buffer [20 mM HEPES buffer (pH 7.9) containing 0.22 M mannitol, 0.08 M sucrose, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. After centrifugation at 2,400 *g* for 2 min, the cells were resuspended in 800 μl homogenization buffer. After centrifugation at 2,400 *g* for 2 min, the cells were resuspended in 400 μl homogenization buffer and homogenized using a syringe with a 27-G needle. The homogenate was centrifuged at 310 *g* for 5 min, and the resulting supernatant was transferred to a new tube as the post-nuclear supernatant (PNS). After a sample (22 μl) of the PNS was set aside for immunoblot analysis, the residual PNS was centrifuged at 5,000 *g* for 5 min. Approximately 300 μl supernatant was obtained as the cytosolic fraction. The mitochondria-enriched pellet was suspended in 300 μl homogenization buffer. An equal volume of cytosol and mitochondrial fractions together with the PNS were subjected to immunoblot analysis.

Immunoblot analysis

Cells were lysed in buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 1% Triton X-100, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 21,500 *g* for 15 min, the supernatants were collected as cell lysates. Cell lysates were then fractionated by SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. The membranes were probed with primary

antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein bands were visualized using the enhanced chemiluminescence system and analyzed with an ImageQuant LAS4000 (GE Healthcare, Piscataway, NJ) or ChemiDoc Touch (Bio-Rad, Hercules, CA). The following primary antibodies were used in this study: anti-PGAM5 antibody (RTL) (5), anti-Tom20 antibody (F-10 or FL-145; Santa Cruz Biotechnology), anti-ubiquinol-cytochrome c reductase core protein 1 (UQCRC1) (clone 16D10AD9AH5; Thermo Fisher Scientific), and anti-α-tubulin antibody (clone 11H10; Cell Signaling, Danvers, MA). HRP-conjugated anti-mouse IgG (GE Healthcare) and HRP-conjugated anti-rabbit IgG (Cell Signaling) were used as secondary antibodies.

Results and Discussion

As shown in many studies, PGAM5 was confined to mitochondria that were stained with an antibody against the OMM protein Tom20 in unstimulated HeLa cells stably expressing HA-tagged Parkin (Parkin-HeLa cells) (**Fig. 1A and B**). When the cells were treated with CCCP, an uncoupler (protonophore) that effectively induces mitophagy, for 12 h, mitochondria accumulated in perinuclear regions. However, in approximately 30% of cells, PGAM5 was diffusely distributed in the cytosol and no longer co-localized with Tom20 **(Fig. 1A-C)**. In immunoblotting, a doublet band of PGAM5 observed in unstimulated cells converged with a lower band that corresponded to the cleaved molecule in response to CCCP (**Fig. 1D**). Subcellular fractionation data showed that the proportion of cleaved PGAM5 in the cytosol to that in mitochondria was much higher in CCCP-treated cells than in untreated cells (**Fig. 1E**). However, CCCP induced cleavage of PGAM5, but did not induce the release of PGAM5 from mitochondria in parental HeLa cells that are known not to express endogenous Parkin (**Fig. 2**). These results suggest that the cleaved form of PGAM5 is released from mitochondria during Parkin-dependent mitophagy.

We next examined whether the release of PGAM5 from mitochondria depended on Parkin-induced OMM rupture through the ubiquitin-proteasome system. We found that the CCCP-induced dynamic changes in the location of PGAM5 were clearly inhibited by treatment of the cells with proteasome inhibitors MG132 or epoxomicin (**Fig. 1A and C**), although PGAM5 cleavage was not inhibited (**Fig. 1D**). Subcellular fractionation data also showed that the proportion of cleaved PGAM5 in the cytosol to that in mitochondria decreased in response to the proteasome inhibitors (**Fig. 1F**). However, the effects of the inhibitors on the release of PGAM5 from mitochondria was much weaker in subcellular fractionation than in immunofluorescence. The proteasome inhibitors may prevent

proteasome-dependent OMM rupture but may not be enough to maintain the integrity of the OMM in CCCP-treated cells. Thus, a substantial amount of cleaved PGAM5 might be artificially released from mitochondria during homogenization of cells in subcellular fractionation. These results suggest that cleaved PGAM5 is released from mitochondria upon proteasome-dependent OMM rupture triggered by Parkin.

Although CCCP effectively induces mitophagy, it affects various biological processes other than those in mitochondria as a protonophore. Thus, we employed simultaneous treatment of cells with oligomycin A and antimycin A (OA), which inhibit mitochondrial electron transport chain complex III and ATP synthase, respectively, and have been previously shown to induce mitophagy (*21*). We initially tested various doses of OA and found that the combination of 500 nM oligomycin A and 50 nM antimycin A induced a time-dependent decrease of Tom20 in a manner similar to that induced by 10 μM CCCP (**Fig. 3A**). Subcellular fractionation demonstrated that OA treatment also induced the release of cleaved PGAM5 from mitochondria to the cytosol (**Fig. 3B**). However, it was difficult to determine the proportion of cells with cytosolic PGAM5 in OA-treated cells using immunofluorescence because the immunofluorescence signal of cytosolic PGAM5 in OA-treated cells was generally low and difficult to distinguish from a background signal. This appeared to be caused by the lower efficiency of PGAM5 cleavage in OA-treated cells than in CCCP-treated cells (**Fig. 3A**). The OA-induced release of cleaved PGAM5 from mitochondria was also inhibited by treatment of the cells with the proteasome inhibitors (**Fig. 3C**), although cleavage of PGAM5 was not inhibited (**Fig. 3D**). Taken together, PGAM5 appears to be cleaved in the initial step of mitophagy and released from mitochondria depending on proteasome-dependent rupture of the OMM during mitophagy **(Fig. 4)**.

In this study, we employed CCCP or OA as stimuli to induce robust mitophagy in

which most mitochondria within a cell lose their function in a synchronized manner. However, under more physiological settings, PGAM5 may be released from a fraction of mitochondria that reduce their $\Delta\psi_m$ and therefore lose their intact OMM structure even under mildly stressed conditions. Thus, there is the possibility that cytoplasmic proteins, which have been reported as binding proteins of PGAM5, function together with PGAM5 that is cleaved and released from the IMM upon mitochondrial dysfunction. It has been recently reported that cleaved PGAM5 dephosphorylates and thus stabilizes β-catenin in the cytosol, leading to cell-intrinsic activation of Wnt signaling (*19*). In this report, an interesting outcome of this activation was proposed to be biogenesis of mitochondria. Thus, PGAM5 might regulate the balance between degradation and biogenesis of mitochondria during mitophagy. Further elucidation of the role of PGAM5 outside mitochondria will shed light on its importance as a signaling intermediate that coordinates cellular response to mitochondrial stress.

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Supplementary Data

Supplementary Data are available at *JB* online.

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Conflict of Interest

None declared.

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Figure legends

Figure 1. CCCP induces the release of PGAM5 from mitochondria depending on proteasome activity in Parkin-HeLa cells.

(A) Parkin-HeLa cells were pretreated with 1 μM MG132 or epoxomicin for 30 min and then treated with 10 μM CCCP for 12 h. The cells were stained with antibodies against PGAM5 and Tom20 (OMM marker). *Yellow arrowheads* indicate cells with PGAM5 released from mitochondria. *Scale bar*, 20 μm. The experiments were done three times with similar results. **(B)** Magnified cells in the highlighted boxes in **(A)**. *Scale bar*, 10 μm. **(C)** The proportion of cells with cytosolic PGAM5. At least 120 cells were determined in each condition in **(A)**. Data are shown as the mean ± SD (n = 3). *Epox*, epoxomicin. **(D)** Parkin-HeLa cells were pretreated with 1 μM MG132 or epoxomicin for 30 min and then treated with 10 μM CCCP for the indicated times. The cells were subjected to immunoblot analysis using antibodies against PGAM5, Tom20, and α-tubulin (cytosol marker). FL and CL indicate full length and cleaved PGAM5, respectively. The experiments were done three times with similar results. **(E, F)** Parkin-HeLa cells were treated with 10 μM CCCP for 10 h and then subjected to subcellular fractionation. In **(F)**, cells were pretreated with 5 μM MG132 or epoxomicin (Epox) for 1 h prior to CCCP treatment. The post-nuclear supernatant (P), mitochondrial fraction (Mt), and cytosolic fraction (Cy) were subjected to immunoblot analysis using antibodies against PGAM5, Tom20, UQCRC1 (matrix marker), and α-tubulin. The experiments were done three times with similar results.

Figure 2. CCCP does not induce the release of PGAM5 from mitochondria in HeLa cells.

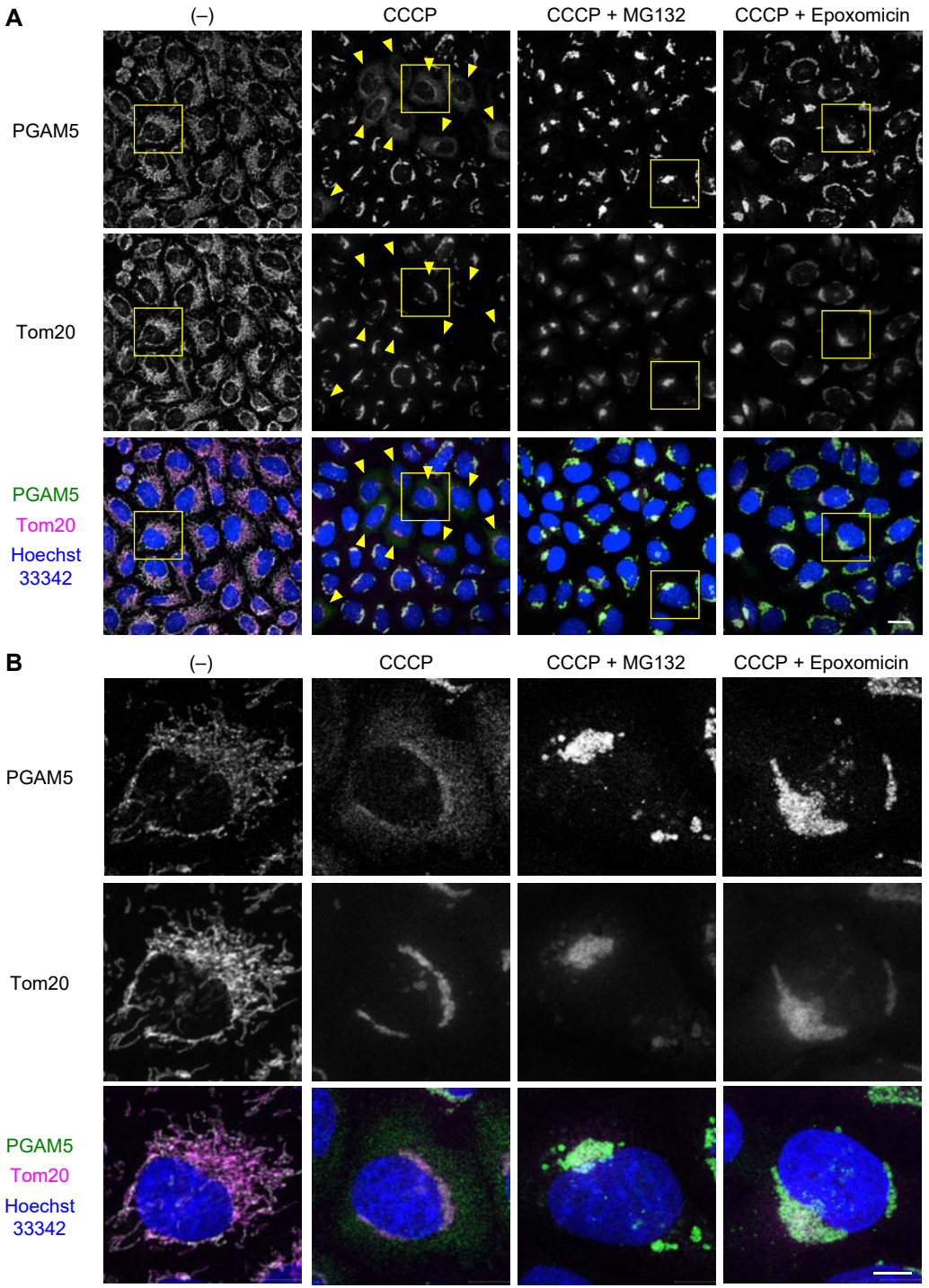
(A) HeLa cells were treated with 10 μM CCCP for 12 h. The cells were stained with antibodies against PGAM5 and Tom20. *Scale bar*, 20 μm. The experiments were done three times with similar results. **(B)** HeLa cells were treated with 10 μM CCCP for the indicated times. The cells were subjected to immunoblot analysis using the indicated antibodies. FL and CL indicate full length and cleaved PGAM5, respectively. The experiments were done three times with similar results.

Figure 3. Simultaneous treatment of Parkin-HeLa cells with oligomycin A and antimycin A induces the release of PGAM5 from mitochondria depending on proteasome activity.

(A) Parkin-HeLa cells were treated with the indicated doses of oligomycin A, antimycin A, and CCCP for the indicated times. The cells were subjected to immunoblot analysis using the indicated antibodies. FL and CL indicate full length and cleaved PGAM5, respectively. The experiments were done two times with similar results. **(B, C)** Parkin-HeLa cells were treated with 500 nM oligomycin A and 50 nM antimycin A (OA) for 10 h and then subjected to subcellular fractionation. In **(C)**, cells were pretreated with 5 μM MG132 or epoxomicin (Epox) for 1 h prior to OA treatment. The post-nuclear supernatant (P), mitochondrial fraction (Mt), and cytosolic fraction (Cy) were subjected to immunoblot analysis using the indicated antibodies. The experiment was repeated three times with similar results. **(D)** Parkin-HeLa cells were pretreated with 1 μM MG132 or epoxomicin for 30 min and then treated with 500 nM oligomycin A and 50 nM antimycin A for the indicated times. The cells were subjected to immunoblot analysis using the indicated antibodies. The experiment was repeated three times with similar results.

Figure 4. A proposed model of release of cleaved PGAM5 from mitochondria during mitophagy

PGAM5 is cleaved within the TM domain in response to loss of mitochondrial membrane potential ($\Delta\psi_m$) in the initial step of PINK1-Parkin-dependent mitophagy and released from mitochondria depending on proteasome-mediated rupture of the OMM.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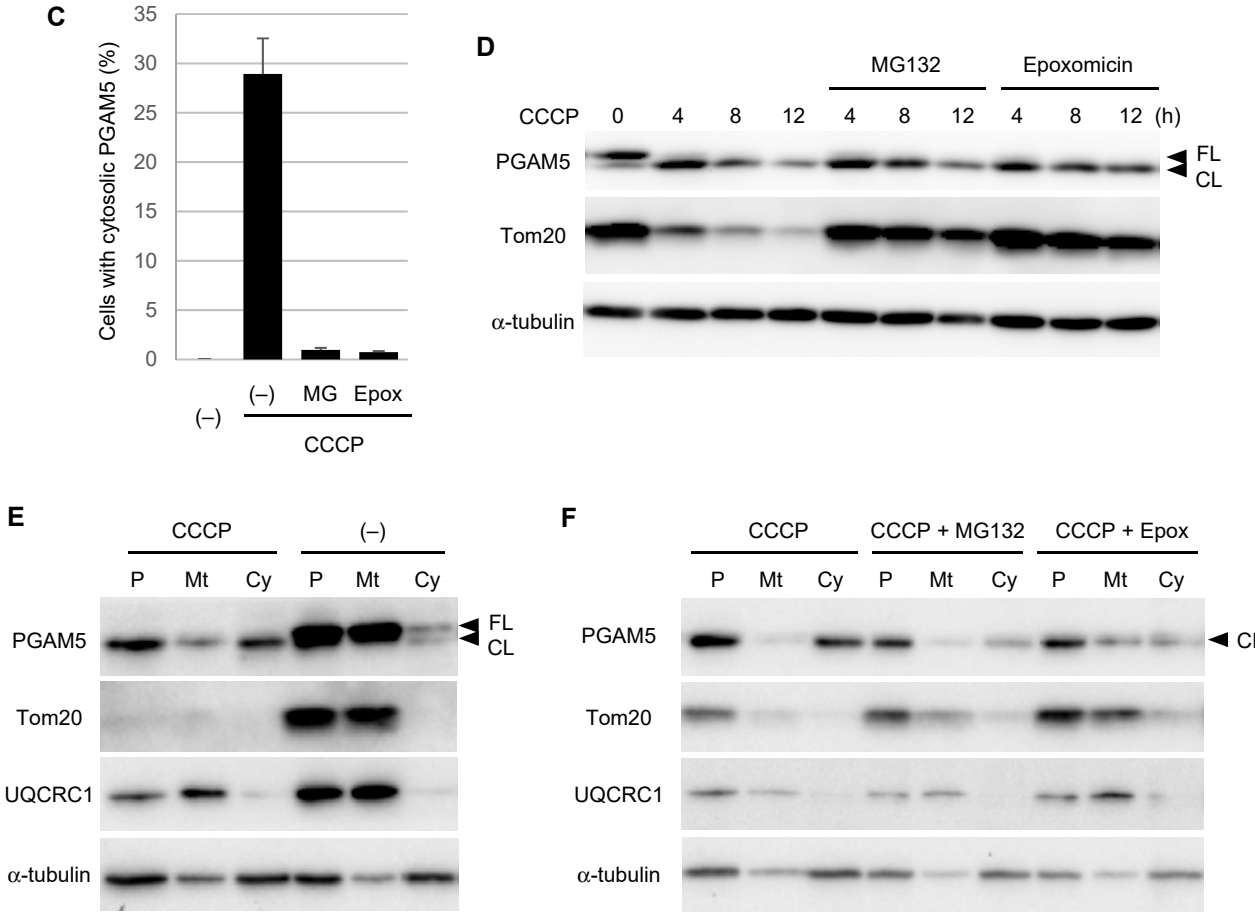
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Fig. 2

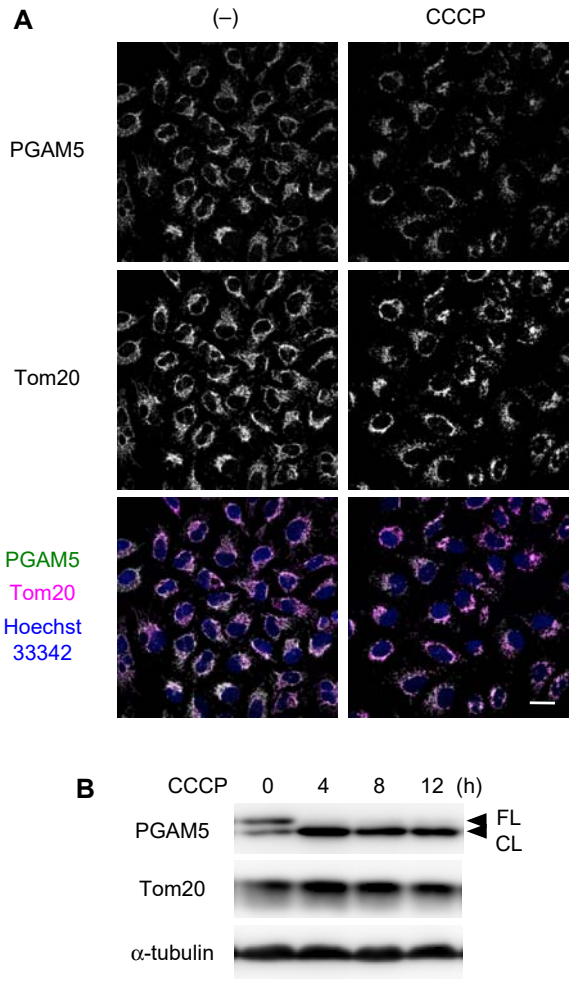
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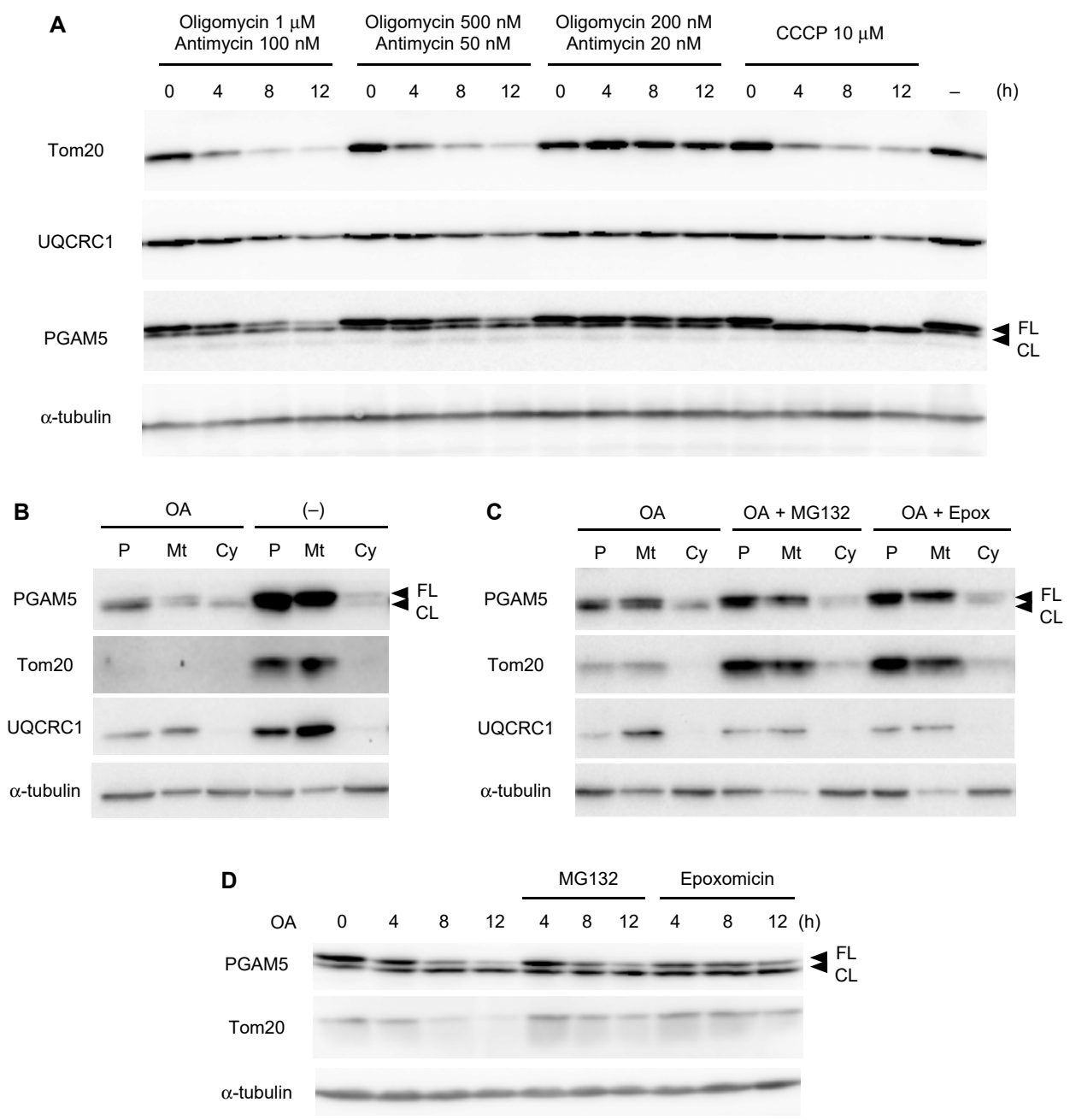
Fig. 3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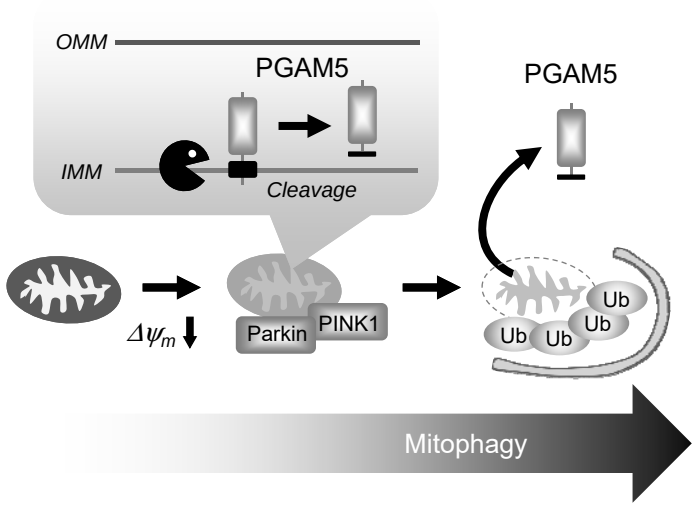
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Ub
Ub
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Mitophagy