PAQR9 modulates BAG6-mediated protein quality control of mislocalized membrane proteins

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Abstract

Protein quality control is crucial for maintaining cellular homeostasis and its dysfunction is closely linked to human diseases. The post-translational protein quality control machinery mainly composed of BCL-2-associated athanogene 6 (BAG6) is responsible for triage of mislocalized membrane proteins (MLPs). However, it is unknown how the BAG6-mediated degradation of MLPs is regulated. We report here that PAQR9, a member of the Progesterone and AdipoQ receptor (PAQR) family, is able to modulate BAG6-mediated triage of MLPs. Analysis with mass spectrometry identified that BAG6 is one of the major proteins interacting with PAQR9 and such interaction is confirmed by co-immunoprecipitation and co-localization assays. The protein degradation rate of representative MLPs is accelerated by PAQR9 knockdown. Consistently, polyubiquitination of MLPs is enhanced by PAQR9 knockdown. PAQR9 binds to the DUF3538 domain within the proline-rich stretch of BAG6. PAQR9 reduces the binding of MLPs to BAG6 in a DUF3538 domain-dependent manner. Taken together, our results indicate that PAQR9 plays a role in the regulation of protein quality control of MLPs via affecting the interaction of BAG6 with membrane proteins.

Keywords: Protein quality control; mislocalized membrane proteins; BAG6; PAQR9; ubiquitination; protein degradation
Protein quality control is crucial for maintaining cellular homeostasis, and its dysfunction is closely linked to numerous human diseases such as neurodegenerative disorders [1]. Different types of protein quality control exist to ensure aberrant proteins are recognized and either corrected or degraded [2]. The cells have evolved two pathways, co-translational and post-translational ones, to ensure secretory and membrane proteins to successfully target to endoplasmic reticulum (ER) upon synthesis from cytosolic ribosomes; otherwise the hydrophobic domains of these proteins would make them form aggregates causing damage to the cell [3]. A co-translational pathway couples the synthesis of nascent polypeptide from ribosomes to ER targeting. For many proteins destined to the ER, their N-terminal signal sequence is quickly bound by signal recognition particle (SRP) upon emergence of the polypeptide from the ribosomes [4]. SRP shields the hydrophobic domain of the polypeptides from contacting aqueous cytosol and targets the ribosome-bound polypeptide to the ER via interacting with the SRP receptors on the ER [5].

The post-translational pathway is another route taken by membrane proteins; their transmembrane domain(s) are not located in the N-terminal end, unlike the tail-anchored proteins that contain ER-targeting sequences at the C-terminal half of the protein [6]. These proteins are delivered to the ER via small glutamine rich tetra-tricopeptide repeat containing protein α (SGTA) and BCL2 associated athanogene 6 (BAG6) [3]. The polypeptides of these membrane protein precursors are first recognized and bound by SGTA upon synthesis from ribosomes, and the SGTA binding protects the hydrophobic domain from contacting aqueous cytosol [7, 8]. Following SGTA binding, these polypeptides take two destinations dependent on the nature of the polypeptide and the capacity of the machineries downstream of SGTA. For most tail-anchored proteins, SGTA hands them over to TRC40 (Get3 in yeast) via a bridging complex containing UBL4A (Get5 in yeast) and TRC35 (Get4 in yeast) with both binding the
C-terminal end of BAG6, the scaffold domain of the protein [9, 10]. TRC40 then delivers these polypeptide to the ER membrane via the TRC40 receptors located on the ER [11-15]. In the cell, some fractions of ER-targeted precursor proteins can be inefficiently delivered to the ER and therefore mislocalize to the cytosol. For these mislocalized hydrophobic proteins (MLPs), a unique protein quality control machinery composed of BAG6 and its associated proteins takes center stage to maintain cellular proteostasis [16-19]. BAG6 contains two major domains in its N-terminal and middle region, i.e., an ubiquitin-like domain (UBL) and a proline-rich domain [19]. The proline-rich domain promiscuously binds the hydrophobic region of MLPs [20, 21], while the UBL domain binds an E3 ubiquitin ligase RNF126 [22]. RNF126 causes polyubiquitination of MLPs, leading to proteasome-mediated degradation of these proteins. However, it is currently unknown how the BAG6-mediated degradation of MLPs is modulated by other molecules. We address this question in this study. We report here that PAQR9, a member of the Progesterone and AdipoQ receptor (PAQR) [23], plays a key role in controlling BAG6-mediated binding and degradation of MLPs. PAQR9 was also previously characterized as an integral membrane progesterone receptor named as mPRa [24]. However, it is unclear whether PAQR9 possesses other biological activities.
Materials and Methods

Antibodies and reagents

Cycloheximide (CHX) and polybrene were from Sigma-Aldrich (St. Louis, MO, USA), MG132 was from BD Biosciences (Franklin Lakes, New Jersey, USA); Antibodies against Flag tag, α-tubulin and PAQR9 were from Sigma-Aldrich; Antibodies against GFP tag, Myc tag and mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Antibody against HA was from ABclonal (Boston, MA, USA); Antibodies against calnexin, BAG6, Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG were from Abcam (Cambridge, UK).

Cell culture and transfection

The human embryonic kidney cells (HEK293T) and human cervix adenocarcinoma HeLa cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% (v/v) FBS (fetal bovine serum). Cells were maintained at 37°C under 5% CO2 in a humidified incubator. Transient transfection was performed with the polyetherimide (PEI) Transfection Reagent (Sigma-Aldrich) for HEK293T and Polyjet (Signagen Laboratories, Rockville, MD, USA) for Hela cells.

Lentivirus packaging and infection

Knockdown of PAQR9 was performed by lentivirus-based method. HEK293T cells were used for packaging virus, and the virus was harvested from the supernatant after transfection for 48 and 72 h. After the filtered supernatants were centrifuged at 20,000 rpm, 4°C for 2 h, then adding appropriate amounts of virus into DMEM of Hela cells. The infected stable cell lines were examined the infection efficiency of lentivirus by real-time PCR after incubation for 72 h.

RNA isolation and real-time PCR
Total RNA of cells was lysed by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified according to manufacturer’s instructions. RNA was reverse-transcribed with FastQuant RT Kit (with gDNase) (Tiangen, Shanghai, China) to obtain cDNA. Real-time PCR was performed with the SYBR Green PCR system (Applied Biosystems, Foster City, CA) and conducted with an ABI Prism 7900 sequence detection system (Applied Biosystems). The primers used in Real-Time PCR were as follows: 5′-CTGGAACGGTGAGGTGACA-3′ and 5′-AAGGGACTTCTGTGAACATGCA-3′ for human β-actin; 5′-CACGTGGTCAAGCTGCTGTC-3′ and 5′-GCCGAAGCCGTAGTAGCTGA-3′ for human PAQR9.

Plasmids construction

PAQR9 and BAG6 cDNAs were cloned by reverse transcription (RT)-PCR from total RNA isolated from human cell lines and confirmed by sequencing. The mutants of BAG6 were generated by a PCR based method and confirmed by DNA sequencing. BAG6 was also cloned into pLVXm-N-flag-IRES-Puro and pEGFP-C1 vector. PAQR9, OP90 and mutants of BAG6 were cloned into pEGFP-C1 vector. TCRα was cloned into p3XFLAG-CMV-10 expression vector. APP-C99 was cloned into pEGFP-N1 vector.

Protein isolation, co-immunoprecipitation and Western blotting

We lysed tissues in RIPA buffer (Yeasen, Shanghai, China) with fresh protease inhibitors (Sigma-Aldrich), and the supernatant was centrifuged at 4°C for 20 min at 13,000rpm. The ubiquitination of protein needs to be treated with or without 10 μM MG132 for an additional 6 hours to block proteasomal degradation of protein after transfection for 24h. Then the co-immunoprecipitation and western blotting assays were performed as previously reported [25].

Immunofluorescence studies
Hela cells were cultured on glass coverslips and transfected with different plasmids. At 24h after transfection, the cells were fixed with paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X100 in PBS for 10 min. The cells were blocked in 3% bovine serum albumin in PBS for 1h, then incubated with primary antibodies and secondary antibodies for 1h, respectively. The nuclei were stained with Hoechst 33342 (Molecular Probes) for 5 min. Fluorescence images were acquired with ZEISS (LSM880).

**CRISPR/Cas9 technology**

BAG6 gene deletion was described as previously reported [26]. The sgRNA sequence was designed according to the website http://crispr.mit.edu and inserted into lentiCRISPRv2 (from Addgene), then confirmed by DNA sequencing. Immunoblotting with anti-BAG6 was used to detect the efficiency after lentivirus infection in Hela cells.

**Statistical analysis**

The data were shown as mean ± s.e.m. The results were analyzed with Student’s t-test, and values of P < 0.05 were considered statistically significant.
**Results and Discussion**

**PAQR9 interacts with BAG6**

PAQR9 is a member of the PAQR family and its function has not been fully characterized [23]. In order to explore the potential biological activity of PAQR9, we used a mass spectrometry-based method to identify potential PAQR9-interacting proteins. Flag-tagged full length PAQR9 was overexpressed in HEK293T cells, and the cell lysate was used in immunoprecipitation with an anti-Flag antibody. The immunoprecipitated proteins were analyzed by mass spectrometry. Such analysis revealed that BAG6 was one of the most abundant proteins pulled down by the anti-Flag antibody (Figure 1A).

We next employed co-immunoprecipitation assays to validate the interaction between PAQR9 and BAG6. When Flag-tagged BAG6 and GFP-fused PAQR9 were co-expressed in HEK293T cells, an anti-Flag antibody pulled down PAQR9 in a co-immunoprecipitation assay (Figure 1B). Inversely, a Flag-tagged PAQR9 pulled down Myc-tagged BAG6 in a co-immunoprecipitation assay (Figure 1C). Furthermore, endogenous PAQR9 interacted with endogenous BAG6 in the co-immunoprecipitation assay (Figure 1D). When expressed in HeLa cells, the GFP-fused PAQR9 was clearly co-localized with calnexin, a marker for the ER (Figure 1E, upper panel), indicating that PAQR9 is mainly localized in the ER. When both PAQR9 and BAG6 were co-expressed, they were partially co-localized (Figure 1E, middle panel). BAG6 was also localized in the nucleus (Figure 1E, lower panel), consistent with a previous report demonstrating that BAG6 is mainly a nuclear protein [27]. We also analyzed the localization of HA-tagged and Flag-tagged PAQR9 proteins and found that they were co-localized with calnexin (Figure S1). Collectively, these data indicated that PAQR9 interacts with BAG6 and is co-localized with BAG6 in the ER.

**PAQR9 affects degradation and ubiquitination of mislocalized membrane proteins**

It has been discovered that BAG6 plays a significant role in the protein quality control by...
mediating degradation of mislocalized membrane proteins (MLPs) [3]. As our analysis indicated PAQR9 interacts with BAG6, we explored whether or not PAQR9 could affect the degradation of MLPs. We analyzed three representative membrane proteins that have been investigated previously to be regulated by BAG6-mediated degradation, i.e., human TCRα [28], a truncated human APP protein named as APP-C99 [18, 29], and a truncated mouse rhodopsin (OP90) [30] (Figure 2A). In HeLa cells, we successfully silenced the expression of PAQR9 by using two PAQR9-specific shRNAs as judged by real-time quantitative RT-PCR (Figure 2B). We next analyzed the protein half-lives of the three membrane proteins. Knockdown of PAQR9 significantly accelerated the protein degradation rate of TCRα, APP-C99 and OP90 proteins (Figure 2C to 2E), indicating that PAQR9 can inhibit the degradation of MLPs. As PAQR9 itself is a membrane protein [24], we also analyzed whether or not the degradation of PAQR9 is modulated by BAG6. As shown in Figure 2F, the protein degradation rate of PAQR9 was not affected by BAG6 overexpression.

To further investigate whether PAQR9 could alter the ubiquitin-mediated proteasomal degradation of the MLPs, we analyzed the ubiquitination level of the MLPs in the presence or absence of MG132, a proteasome inhibitor. Firstly, we found treatment with MG132 could increase the accumulation of TCRα, APP-C99, and OP90 (Figure 3A), indicating that the degradation of these membrane proteins are mediated by protein ubiquitination. We next analyzed the polyubiquitination levels of the membrane proteins. As shown in Figure 3B to 3D, the polyubiquitination levels of the three membrane proteins were enhanced by PAQR9 knockdown in HEK293T cells in the presence of MG132. These results, therefore, indicated that PAQR9 could suppress the polyubiquitination of MLPs.

**Identification of DUF3538 domain of BAG6 as the PAQR9-interacting motif**

We further analyzed the domain(s) of BAG6 involved in its interaction with PAQR9. BAG6
contains five functional domains (Figure 4A), i.e., an ubiquitin like domain (UBL, amino acids 13-87), a long proline-rich stretch (amino acids 195–681), a domain of unknown function (DUF3538, amino acids 273–394) within the proline-rich stretch, nuclear localization signal (NLS, amino acids 1030-1055), and a BCL2-associated athanogene domain (BAG, amino acids 1050-1111) [3, 31]. It is noteworthy that the “BAG” domain is not a canonical BAG domain as indicated by structural analyses [10, 32]. With a co-immunoprecipitation assay, we found that PAQR9 mainly interacted with the long proline-rich stretch and the DUF3538 domain (Figure 4B). We also analyzed the 1-194 aa fragment of BAG6 was this region was previously reported to have preference to hydrophobic substrate [33]. We found that this region could not interact with PAQR9 by a co-immunoprecipitation assay (Figure S2).

As the DUF3538 domain is embedded inside the proline-rich domain, we assumed that the DUF3538 domain is critical for the interaction of BAG6 with PAQR9. In order to validate our assumption, we analyzed a BAG6 mutant that lacked the DUF3538 domain. Deletion of the DUF3538 domain of BAG6 led to a loss of its interaction with PAQR9 (Figure 4C, lane 3 compared to lane 4). We found that when the mutant BAG6 was expressed alone (Figure 4C, lane 2), its expression level was higher than that of co-expression with PAQR9, likely caused by reduced degradation of BAG6 when its DUF3538 domain was deleted.

Furthermore, we analyzed whether or not overexpression of the DUF3538 domain was able to compete off the interaction of BAG6 with PAQR9. Using a co-immunoprecipitation assay, we found that overexpression of the DUF3538 domain alone was able to dose-dependently reduce the interaction of BAG6 with PAQR9 (Figure 4D). These results, therefore, verified that the DUF3538 domain of BAG6 is the major motif that interacts with PAQR9.

PAQR9 suppresses the interaction of BAG6 with membrane proteins
Based on the finding that PAQR9 could bind the DUF3538 domain, we made another hypothesis that PAQR9 was able to compete off the interaction of MLPs with BAG6 as the proline-rich stretch of BAG6 is involved in the interaction of MLPs with BAG6 [3]. Consistent with our hypothesis, overexpression of PAQR9 dose-dependently reduced the interaction of BAG6 with TCRα, APP-C99 and OP90 respectively (Figure 5A to 5C).

Furthermore, we analyzed whether PAQR9 could affect the interaction of BAG6 with RNF126, the E3 ubiquitin ligase recruited by BAG6 to execute polyubiquitination of MLPs [22]. Intriguingly, PAQR9 dose-dependently reduced the interaction between BAG6 and RNF126 (Figure 5D), consistent with our observations that the polyubiquitination levels of MLPs were reduced by PAQR9. These data, therefore, indicate that one of the biochemical activities of PAQR9 is to reduce the interaction of MLPs with BAG6 via its interaction with BAG6.

The DUF3538 domain of BAG6 is crucial for the degradation of MLPs

We next explored that functional consequence of PAQR9 interaction with the DUF3538 domain of BAG6. As PAQR9 could lessen the interaction of MLPs with BAG6, which is involved in the degradation of these membrane proteins via recruitment of an E3 ubiquitin ligase RNF126 [3], we assumed that the DUF3538 domain played a critical role in modulating the degradation of MLPs. We established BAG6 knockout HeLa cells, in which endogenous BAG6 was silenced by a CRISPR-Cas9 strategy (Figure 6A). As expected, reconstitution with wild-type BAG6 in these cells increased the degradation rate of overexpressed TCRα, APP-C99 and OP90 proteins respectively (Figure 6B to 6D, middle 4 lanes compared to the first 4 lanes). Intriguingly, overexpression of the DUF3538 domain alone further accelerated the degradation rate of these membrane proteins (Figure 6B to 6D, the last 4 lanes compared to the middle 4 lanes). We hypothesized this phenomenon was caused by the reduction of PAQR9-BAG6 interaction due to the overexpressed DUF3538 domain that competed for PAQR9 interaction, thus...
leading to accelerated degradation of MLPs. To further support our hypothesis, we analyzed the behavior of BAG6 with deletion of the DUF3538 domain. Reconstitution of the BAG6-deleted cells with BAG6(ΔDUF3538) slowed down the degradation rate of the overexpressed TCRα, APP-C99 and OP90 (Figure 6E to 6G, the last 4 lanes compared to the middle 4 lanes, and Figure S3). Based on these results, we propose a model to illustrate the function of PAQR9 (Figure 6H). We speculate that one of the biological functions of PAQR9 is to modulate protein quality control by altering the degradation of MLPs via interaction with the DUF3538 domain of BAG6, whereby blocking RNF126-mediated polyubiquitination and proteasome-mediated degradation of these membrane proteins (Figure 6H).

In summary, our study indicates that PAQR9 is a new player in the regulation of BAG6-mediated protein quality control of MLPs. PAQR9 binds to the DUF3835 domain of BAG6 embedded within the proline-rich stretch, thus preventing the interaction of the hydrophobic domain of MLPs with BAG6 and inhibiting polyubiquitination and proteasomal degradation of MLPs. Furthermore, the interaction of PAQR9 with the DUF3538 domain appears to hinder the interaction of RNF126 with the ubiquitin-like domain of BAG6, further blocking ubiquitination of MLPs by RNF126. Consequently, PAQR9 functions as a negative regulator on BAG6 mediated degradation of MLPs. Such functioning of PAQR9 may change the balance of the functionalities of BAG6. BAG6 serves two mutually exclusive functions in the cell. The C-terminal end of BAG6 functions as a scaffold to facilitate handover of membrane proteins from SGTA to TRC40. Conversely, the N-terminal and middle region of BAG6 are involved in triage of MLPs via facilitating ubiquitination of MLPs by RNF126 associated with the N-terminal end of BAG6. It was known that overexpression of SGTA antagonizes BAG6-mediated degradation of MLPs [18], while likely favoring maturation and insertion into the ER. In this way, PAQR9 may have a similar function as SGTA by blocking triage of MLPs and consequently favoring ER insertion of membrane proteins.
In addition to BAG6 mediated quality control of mislocalized membrane proteins, it was recently found that proteins of the ubiquilin family can also bind transmembrane proteins in the cytosol and prevent their aggregation [34, 35]. Ubiquilins recruit E3 ligase to cause ubiquitination of membrane proteins, subsequently leading to protein degradation [35]. Interestingly, UBQLN4, a member of ubiquilin family, can bind BAG6 and mediate ubiquitination of misassembled proteins [34][36]. It will be interesting to investigate in the future whether the function of ubiquilins is also modulated by PAQR9.

It is noteworthy that PAQR9 was previously characterized as a membrane progesterone receptor named as mPRH [24]. Progesterone is an important hormone that regulates a wide spectrum of cognitive, neuroendocrine, neuroimmune and neuroprotective functions [36]. Two groups of nonclassical progesterone receptors have been identified recently including the class II PAQR family members and the b5-like haeme/steroid-binding protein family members. The PAQR family members involved in progesterone signaling include PAQR7 (mPRα), PAQR8 (mPRβ), PAQR5 (mPRγ), PAQR6 (mPRδ), and PAQR9 (mPRε). It was reported that PAQR9 was localized in the plasma membrane in MDA-MB-231 breast cancer cells [24]. However, our study revealed that PAQR9 is mainly localized in the ER in HeLa cells (Figure 1E), consistent with a previous report showing that membrane progestin receptors were mainly localized in the ER in HEK293 and MDA-MB-231 cells [37]. Such discrepancy might be explained by the possibility that the cellular localization of PAQR9 varies dependent on the cell type. In addition, it cannot be ruled out that PAQR9 has a dual functionalities to serve as a receptor for progesterone and meanwhile modulate BAG6-mediated protein quality control. This will be an intriguing question to be addressed in the future.
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Conflict of Interest declaration

We declare no conflict of interest.

Author contribution

Y.C. conceptualized the study. X.Y. and Y.C. designed the study and wrote the paper. X.Y., Y.L. and Y.H. performed the experiments. L.X. and Q.C. provided technical assistance.
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Figure Legends

Figure 1. Interaction and co-localization of PAQR9 with BAG6
(A) A short list of proteins that potentially interact with PAQR9 as identified by mass spectrometry
(B, C) Interaction between overexpressed PAQR9 and BAG6. HEK293T cells were transfected with the plasmids as indicated, and the cell lysate was used in immunoprecipitation (IP) and immunoblotting (IB) with the antibodies as indicated after transfection for 24 h.
(D) Interaction between endogenous PAQR9 and BAG6. The cell lysate of HEK293T cells was used in IP and IB with the antibodies as indicated.
(E) Subcellular distribution of PAQR9 and BAG6. Hela cells were transiently transfected with GFP-fused PAQR9 and BAG6 as indicated. The cells were used in immunofluorescent staining. The ER was stained with an antibody against calnexin. The nucleus was stained with Hoechst 33342. Intensity plots of signal intensity (y axis) against distance in μm (x axis) are used to indicate occurrence of overlaps between the red and green channels (shown on the right panels).

Figure 2. PAQR9 affects degradation of mislocalized membrane proteins
(A) The structure of the three membrane proteins used in the study. The transmembrane domains of these proteins are all localized in the C-terminal half.
(B) The efficiency of knocking-down of PAQR9. Hela cells were infected with the control lentivirus (mock) or the lentivirus containing PAQR9-specific shRNAs (shPAQR9). The mRNA level was determined by real time RT-PCR. These data are shown as mean ± SEM with *** for p < 0.001 between the indicated groups.
(C-E) The protein stability of the three membrane proteins is affected by PAQR9. Hela cells as described in B were transiently transfected with Flag-tagged TCRα (C), GFP-fused APP-C99 (D), or GFP-fused OP90 (E). The cells were then treated with...
cycloheximide (100 µg/ml) for various times, followed by immunoblotting with the antibodies as indicated. Quantitation of the immunoblotting results from three independent experiments is shown in the right panel. These data are shown as mean ± SEM with * for p < 0.05 and ** for p < 0.01 as compared to the mock group.

(F) The protein stability of PAQR9 was not affected by BAG6. Hela cells were transiently transfected with His-tagged PAQR9 and Myc-tagged BAG6 as indicated and treated with cycloheximide (100 µg/ml) for various times, followed by immunoblotting. Quantitation of the immunoblotting results from three independent experiments is shown in the right panel.

Figure 3. PAQR9 regulates polyubiquitination of mislocalized membrane proteins

(A) MG132 treatment elevates the protein levels of MLPs. HEK293T cells were transiently transfected with the plasmids as indicated, and then treated with or without 10 µM MG132 for 6 h before harvest, followed by immunoblotting (IB) with the antibodies as indicated.

(B-E) PAQR9 affects polyubiquitination of membrane proteins. HEK293T cells were transiently transfected with the plasmids as indicated, and then treated with or without 10 µM of MG132 for 6 h before harvest, followed by immunoprecipitation (IP) and immunoblotting (IB) with the antibodies as indicated. Quantitation of the IP results from three independent experiments for B, C and D is shown in E. These data are shown as mean ± SEM with * for p < 0.05 between the groups as indicated.

Figure 4. PAQR9 interacts with the DUF3538 domain of BAG6

(A) Schematic diagram showing amino acid sequences and functional domains of BAG6.

(B) The DUF3538 domain of BAG6 interacts with PAQR9. PAQR9 and different domains of BAG6 were transiently transfected in HEK293T cells and the cell lysate was used in immunoprecipitation (IP) and immunoblotting (IB) using the antibodies as indicated.
(C) Deletion of the DUF3538 domain loses the interaction of BAG6 with PAQR9. HEK293T cells were transiently transfected with the plasmids as indicated, followed by IP and IB.

(D) Overexpression of the DUF3538 domain reduces BAG6-PAQR9 interaction. HEK293T cells were transiently transfected with the plasmids as indicated, followed by IP and IB.

Figure 5. PAQR9 disrupts the interaction of BAG6 with membrane proteins.

(A-C) PAQR9 dose-dependently reduces the interaction of BAG6 with MLPs. HEK293T cells were transiently transfected the plasmids as indicated, followed by immunoprecipitation (IP) and immunoblotting (IB) with the antibodies as indicated.

(D) PAQR9 disrupt the interaction of BAG6 with RNF126. HEK293T cells were transiently transfected the plasmids as indicated, followed by IP and IB.

Figure 6. The DUF3538 domain of BAG6 is indispensable for the activity of PAQR9

(A) The efficiency of disrupting Bag6 gene by CRISPR/Cas9 technology. Hela cells were infected with lentivirus that contained CRISPR/Cas9 to target Bag6 gene.

(B-D) The DUF3538 domain of BAG6 accelerates the degradation of membrane proteins. The cells in A were transiently transfected with the plasmids as indicated, treated with cycloheximide (100 μg/ml) for various times, followed by immunoblotting. The arrows indicate the protein containing only the DUF3538 domain.

(E-G) Deletion of DUF3538 domain of BAG6 delays the degradation of membrane proteins. The cells in A were transiently transfected the plasmids as indicated, and then treated with cycloheximide (100 μg/ml) for various times, followed by immunoblotting.

Quantitation of the immunoblotting results from three independent experiments for E, F and G is shown in Figure S3.

(H) A proposed model to illustrate the function of PAQR9. PAQR9 interacts with the DUF3538 domain of BAG6 to suppress the degradation of MLPs, thereby blocking RNF126-mediated polyubiquitination and proteasomal degradation of the membrane proteins.
proteins.
