Secretion of misfolded cytosolic proteins from mammalian cells is independent of chaperone-mediated autophagy

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In eukaryotic cells, elimination of misfolded proteins is essential for maintaining protein homeostasis and cell viability. Misfolding-associated protein secretion (MAPS) is a protein quality-control mechanism that exports misfolded cytosolic proteins via a compartment characteristic of late endosomes, but how cytosolic proteins enter this compartment is unclear. Because chaperone-mediated autophagy (CMA) is a known mechanism that imports cytosolic proteins bearing a specific CMA motif to lysosomes for degradation and because late endosomes and lysosomes overlap significantly in mammalian cells, we determined here whether CMA is involved in targeting protein cargoes to the lumen of late endosomes in MAPS. Using HEK293T and COS-7 cells and immunoblotting and -staining and coimmunoprecipitation methods, we show that, unlike CMA, the secretion of misfolded proteins in MAPS does not require cargo unfolding, is inhibited by serum starvation, and is not dependent on the CMA motif in cargo. Intriguingly, knockdown of lysosome-associated membrane protein 2 (LAMP2), which consists of three isoforms, including a variant proposed to form a protein channel, can use a unique unconventional protein secretion mechanism termed MAPS2 to export misfolded cytosolic proteins to the extracellular milieu. Importantly, many neurodegenerative disease-associated neurotoxic proteins such as Tau, α-Syn, and SOD1 are clients of this pathway (8, 10), which suggests a possible connection between defects in MAPS and neuronal dysfunction.

Mechanistically, MAPS can be initiated by an endoplasmic reticulum (ER)-associated deubiquitinase, USP19, which also has a unique chaperone activity. USP19 uses this chaperone activity to recruit misfolded or unassembled cytosolic proteins to the ER surface. Subsequently, these cargoes are detected entering late endosomes, which are often in close contact with the ER (8, 10) (see Fig. 1). The mechanism by which cargoes enter late endosomes is unclear, but this process appears to be guided by an HSC70 cochaperone named DNAJC5, which is localized to late endosomes (7, 10).

Besides MAPS, it was shown previously that certain misfolded cytosolic proteins bearing a so-called CMA motif can be targeted to lysosomes for degradation (11). Intriguingly, in CMA, cytosolic substrates undergo a membrane translocation process reminiscent of that in the MAPS pathway. This process

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2 The abbreviations used are: MAPS, misfolding-associated protein secretion; CMA, chaperone-mediated autophagy; α-Syn, α-synuclein; LAMP2, lysosome-associated membrane protein 2; HEK, human embryonic kidney; DNAJC5, DnaJ heat shock protein family (Hsp40) member C5; ER, endoplasmic reticulum; DHFR, dihydrofolate reductase; co-IP, coimmunoprecipitation; EGFP, enhanced GFP; LC3, light chain 3; DMEM, Dulbecco’s modified Eagle’s medium; mCh, mCherry; mLAMP2, mouse LAMP2; mCMA, mutant CMA; HSP, heat shock protein.
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Figure 1. A comparison of the MAPS and CMA pathways. In both pathways, cytosolic cargoes need to be translocated into the lumen of a late endocytic compartment following recognition by a chaperone. In MAPS, USP19 is one of the substrate recognition molecules that enrich substrates on the surface of the ER (1). Subsequently, substrates are transferred to DNAJC5 on late endosome (LE) (2) for translocation across the membrane (3). In CMA, HSC70 recognizes substrates via a CMA motif and then targets them to LAMP2a for entry into lysosomes. PM, plasma membrane.

requires substrate recognition by the heat shock protein HSC70, which targets CMA clients to a lysosome-localized single-spanning membrane protein named LAMP2a. Subsequently, clients are unfolded while being translocated into the lumen of lysosomes via a channel formed by LAMP2a (12) (Fig. 1).

Given that late endosomes and lysosomes overlap significantly in mammalian cells, we sought to determine whether secretion through MAPS involves CMA-dependent translocation of cytosolic proteins into a prelysosomal compartment. We studied the dependence of MAPS on substrate unfolding, serum deprivation, CMA motif, and LAMP2, hallmarks of the CMA pathway (11). We found that secretion through MAPS does not require protein unfolding, is inhibited by serum starvation, and is not dependent on the CMA motif. Interestingly, secretion of MAPS cargoes depends on LAMP2, but LAMP2 does not seem to function as the previously proposed protein channel in MAPS. Instead, it seems to be indirectly involved in this process. We conclude that higher eukaryotic cells have a novel mechanism that imports misfolded cytosolic proteins into late endosomes.

Results

Secretion through MAPS does not require protein unfolding

In CMA, substrate translocation into lysosomes requires unfolding. To determine whether this is also true for MAPS, we first generated a construct expressing α-Syn fused with a tightly folded domain from dihydrofolate reductase (DHFR), which is a reductase for dihydrofolate acid (13) (Fig. 2A). As α-Syn is an intrinsically misfolded protein secreted through MAPS, the fusion protein was similarly secreted as demonstrated by immunoblotting analysis of conditioned medium collected from 293T cells transiently expressing α-Syn–DHFR (Fig. 2B).

Like α-Syn, α-Syn–DHFR secretion was enhanced when USP19 was overexpressed (Fig. 2B). The DHFR domain is known to fold into a tightly packed structure in the presence of its substrate, folic acid (13), which prevents DHFR from unfolding and thus alters its protease sensitivity (Fig. 2C). If protein unfolding was required for α-Syn to enter the MAPS pathway, incubation of cells with aminopterin, a derivative of folic acid, should diminish α-Syn secretion. However, we found that α-Syn–DHFR was secreted similarly regardless of whether aminopterin was present (Fig. 2D). This result suggests that protein unfolding is not necessary for cargo transit through the MAPS pathway.

To further test the role of protein unfolding in MAPS, we generated another α-Syn variant that contained bacterial β-gal (LacZ) at the N terminus (Fig. 2A). Because spontaneous refolding of LacZ in medium was inefficient (Fig. 2E), if α-Syn was secreted in an unfolded form, it should not be detected by the β-gal assay. However, using the β-gal assay, we consistently detected LacZ–α-Syn in conditioned medium harvested from cells overexpressing LacZ–α-Syn (Fig. 2F), suggesting that at least a fraction of the secreted LacZ–α-Syn was folded. As expected, the secretion of LacZ–α-Syn was also stimulated by USP19 and DNAJC5 (Fig. 2F) and was diminished by ~50% when USP19 was inactivated in cells by CRISPR-mediated gene editing (Fig. 2G). The incomplete secretion blockade by USP19 depletion suggests possible redundancy with an unknown gene(s) (7). Together, these results confirmed that the fusion protein was still secreted through MAPS.

To quantify the level of folded LacZ–α-Syn in medium, we collected conditioned medium from cells expressing LacZ–α-Syn either by itself or together with USP19 or DNAJC5. We then divided each sample into two equal portions and measured the level of LacZ–α-Syn by both the β-gal assay and an immunobased ELISA. The former only measured the amount of folded LacZ–α-Syn, whereas the latter detected LacZ–α-Syn regardless of its folding state. In cells expressing only LacZ–α-Syn, LacZ–α-Syn secretion measured by the β-gal assay was almost identical to that measured by ELISA, suggesting that secreted LacZ–α-Syn contains 100% folded β-gal. For medium collected from cells coexpressing USP19 or DNAJC5, we found that more than 80% of the secreted protein contained folded β-gal (Fig. 2H). Collectively, these data strongly suggest that protein unfolding is dispensable for secretion through MAPS.

Serum starvation inhibits MAPS

CMA is activated under stress conditions (e.g., serum starvation) (14). If MAPS used a similar mechanism to move substrates into late endosomes, the secretion of α-Syn should be increased under serum starvation conditions. We thus treated α-Syn–expressing cells with serum-free medium or, as a control, with regular medium. We then measured the secretion of α-Syn, the canonical ER secretory cargo Clusterin, or the
Figure 2. Secretion via MAPS does not require substrate unfolding. A, a schematic illustration of constructs used in this study. B, α-Syn–DHFR–FLAG secretion is stimulated by USP19. HEK293T cells were cotransfected with α-Syn–DHFR–FLAG or α-Syn–FLAG together with either an empty vector (E.V.) or a plasmid expressing FLAG-tagged USP19. Medium and lysates were analyzed by immunoblotting using antibodies against the indicated proteins. C, aminopterin changes the conformation of α-Syn–DHFR–FLAG. α-Syn–FLAG or α-Syn–DHFR–FLAG was purified from either control cells or cells treated with aminopterin (10 μM; 5 h). Purified proteins were treated with trypsin as indicated followed by SDS-PAGE and silver staining. D, aminopterin has no effect on secretion of α-Syn–DHFR. Secretion of α-Syn–DHFR–FLAG or α-Syn–DHFR–FLAG from 293T cells transfected as indicated was analyzed by immunoblotting. E, spontaneous refolding of β-gal is negligible. β-Gal treated with 8 M urea (orange bars) or left untreated was diluted in DMEM (1:1000). β-Gal was measured at the indicated time points. F and G, secretion of LacZ–α-Syn is mediated by USP19 and DNAJC5. F, secretion of LacZ–α-Syn from cells transfected with LacZ–α-Syn together with the indicated plasmids was analyzed by the β-gal assay. The level of LacZ–α-Syn secretion was normalized by the level of α-Syn in cell lysate (error bars represent mean ± S.E.; n = 3 independent experiments; *, p value <0.05). G, secretion of LacZ–α-Syn from control or USP19-null CRISPR cells transfected with LacZ–α-Syn was analyzed as in D (error bars represent mean ± S.E.; n = 3 independent experiments; *, p value <0.05). H, secreted LacZ–α-Syn is mostly folded. Cells were transfected as in D. LacZ–α-Syn in medium was determined by both β-gal assay and ELISA (error bars indicate variations of two independent experiments). Med., medium; Lys., lysate; A.U., arbitrary units.
exosome–dependent cargoes HSC70 and HSP90. Immunoblotting analyses showed that a small amount of HSP90 and HSC70 was secreted from cells cultured in serum-free medium (Fig. 3B), consistent with reports that serum starvation activates exosome–mediated secretion of these chaperones (15, 16). By contrast, the secretion of Clusterin was modestly inhibited (Fig. 3A), whereas the secretion of α-Syn was more dramatically reduced (~5-fold) under serum-starved condition (Fig. 3, A and B).

Because α-Syn secretion can be stimulated by USP19 and DNAJC5, we tested whether these factors could restore α-Syn secretion under the serum starvation condition. We found that, compared with cells cultured in normal medium, α-Syn secretion was consistently diminished under serum starvation condition regardless of whether or not these factors were expressed (Fig. 3B). Together, these results suggest that MAPS is inhibited by serum starvation, further differentiating this process from CMA.

**Secretion of α-Syn is independent of the CMA motif**

CMA-dependent degradation of α-Syn requires a CMA motif (VKKDQ), which is recognized by HSC70 for lysosomal targeting (17). We therefore evaluated whether this motif is required for α-Syn secretion. To this end, we mutated this motif by substituting DQ with AA (Fig. 4A). This mutant is defective in CMA as shown previously (17). We expressed this mutant (mCMA) either by itself or together with USP19 or DNAJC5. In parallel, wildtype (WT) α-Syn was transfected as a control. Immunoblotting analyses showed that the secretion of this mutant was comparable to that of WT α-Syn under all conditions (Fig. 4, B and C). These results further support the notion that MAPS does not use the CMA pathway to import cargoes into late endosomes.

**Role of LAMP2 in MAPS**

Another key feature of the CMA pathway is its dependence on LAMP2a, a type I membrane protein encoded by an alternatively spliced LAMP2 gene. This isoform is distinct from other variants in the C-terminal 10 amino acids, which form a short tail facing the cytosol. In CMA, HSC70 delivers cargo to LAMP2a by interacting with this C-terminal tail (12). Cargoes are then imported into lysosomes for degradation.

We tested whether DNAJC5 can target MAPS cargoes to LAMP2 for translocation into late endosomes analogously to the function of HSC70 in CMA. First, we examined whether LAMP2 might interact with DNAJC5 using both imaging and coimmunoprecipitation (co-IP). Confocal microscopy showed that DNAJC5 was colocalized extensively with LAMP2 (Fig. 5A, top panels). As expected, most LAMP2-positive vesicles could be labeled with mCherry–Rab9 (mCh-Rab9), but a small subset of Rab9-positive vesicles did not contain LAMP2 (middle panels). Likewise, many mCherry–Rab9–positive vesicles could be stained by a LysoTracker dye, which is known to label acidic compartments, but some Rab9-containing endosomes were either not stained by LysoTracker or only stained weakly (bottom panels). These observations suggest that the colocalization of DNAJC5 with LAMP2 occurs at an acidic compartment downstream of Rab9 recruitment. Intriguingly, despite such colocalization, co-IP experiments failed to detect a significant interaction between LAMP2 and DNAJC5 even when DNAJC5 was overexpressed (Fig. 5B). We also failed to detect an interaction between USP19 and LAMP2, although the association of DNAJC5 and USP19 with their cognate chaperone partners (HSC70 for DNAJC5 and HSP90 for USP19) was readily detectable (Fig. 5B). Furthermore, immunoprecipitation of α-Syn from cells overexpressing FLAG–tagged α-Syn failed to detect any interaction between α-Syn and LAMP2.

**Figure 3.** Serum starvation inhibits α-Syn secretion. A, serum starvation inhibits α-Syn secretion. 293T cells transfected with an empty vector (lane 1) or α-Syn–FLAG (lanes 2 and 3) were incubated with regular serum (lanes 1 and 2) or serum-free medium (lane 3) for 4 h. Conditioned medium and cells lysate were analyzed by immunoblotting with antibodies against the indicated proteins. B, serum starvation inhibits USP19- and DNAJC5-induced α-Syn–FLAG secretion. Cells transfected with α-Syn–FLAG and the indicated plasmids were incubated with regular medium (lanes 1–3) or serum-free medium (lanes 4–6) for 4 h prior to immunoblotting analysis. The graph shows quantification from three independent experiments (error bars, mean ± S.E.; n = 3; **, p value <0.005; **, p value <0.01; *, p value <0.05). Ctrl, control; Med., medium; Lys., lysate; A.U., arbitrary units.

**Figure 5.** LAMP2 associates with DNAJC5 but not with USP19. A, representative images from co-IP experiments using α-Syn–FLAG, DNAJC5–FLAG, and USP19–FLAG. The FLAG–USP19 and DNAJC5–FLAG fusion proteins (red) were coexpressed with α-Syn–FLAG (green) in 293T cells, and lysates were immunoprecipitated with anti-α-Syn antibody. B, bar graph showing mean and standard deviation of α-Syn–FLAG levels (arbitrary units) in lysates (Lys.) and conditioned medium (Med./Lys.) for control (Ctrl.), USP19, and DNAJC5 conditions (lanes 1–3). Value was calculated by using ImageJ software following densitometric analysis of immunoblotting and arbitrary unit (A.U.) is shown.
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(Fig. 6, C and D). Furthermore, re-expression of mouse LAMP2a that is resistant to siRNA in LAMP2a knockdown 293T cells failed to rescue the secretion defect (Fig. 6, E and F). By contrast, we found that expression of mouse LAMP2a inhibited basal secretion of α-Syn (Fig. 6, E and F), likely because of a dominant-negative effect that impairs endogenous LAMP2 function. This phenotype is not unique to LAMP2a as expression of LAMP2b resulted in a similar degree of MAPS inhibition (Fig. 6G). Collectively, these observations suggest that, although the function of LAMP2 is required to maintain a normal level of α-Syn secretion, it might not function as a protein-translocation channel because its function can be bypassed by overexpression of DNAJC5, a substrate-recruiting factor in MAPS, and because its activity is independent of the C terminus of LAMP2a.

We reasoned that knockdown of LAMP2 might impair certain endolysosome function(s) essential for MAPS. In an attempt to identify such dysfunction, we stained control and LAMP2 knockdown cells with a LysoTracker dye, but we observed no obvious difference in either the number or morphology of the stained vesicles (Fig. 7A). However, immunoblotting analysis showed that LAMP2 knockdown cells had a slightly higher level of Rab9 compared with control cells. The difference, albeit small, was reproducible and statistically significant (Fig. 7, A–C). In addition, the level of DNAJC5 in LAMP2 knockdown cells was ~20% lower than that in control cells (Fig. 7C). Although these results did not pinpoint precisely the defect in LAMP2 knockdown cells causal to reduced MAPS, they support the possibility that LAMP2 is critical for maintaining the functional integrity of a prelysosomal compartment, possibly by influencing the stability/abundance of factors important for MAPS.

MAPS substrates are not degraded by lysosomes

Because MAPS cargoes are found in acidified endosomes decorated with Rab9, we wished to determine whether these endosomes could target a fraction of cargoes to lysosomes for degradation. We first treated 293T cells stably expressing EGFP-tagged α-Syn with bafilomycin A or chloroquine, both known to disrupt lysosomal degradation (18, 19). Accumulation of either full-length EGFP–α-Syn or a degradation intermediate would indicate lysosomes as a destination for at least a fraction of EGFP–α-Syn. However, immunoblotting with GFP antibodies did not detect any difference in EGFP–α-Syn between untreated and treated cells, although the accumulation of LC3II, a lipid-modified LC3 variant that constantly undergoes lysosome-dependent degradation, was readily observed (Fig. 8A). Thus, it seems that MAPS substrate is not degraded by lysosomes.

The lack of obvious degradation of α-Syn might be because only a small fraction of α-Syn is disposed by this process as shown previously (7). We therefore determined whether these lysosome inhibitors affect protein secretion via MAPS. We reasoned that if fusion with lysosome and plasma membrane represents two alternative trafficking routes for cargoes entering late endosomes, then inhibition of lysosomal degradation should increase cargo flux toward plasma membrane and therefore result in more protein secretion. We measured the secre-
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tion kinetics for two MAPS cargoes (α-Syn and GFP(1–10)), but we found that lysosomal inhibitors neither increase nor inhibit the secretion (Fig. 8, B and C). Collectively, these data suggest that, although MAPS cargoes can reach a late endocytic compartment, they are exempted from degradation by lysosomes.

**Discussion**

In this study, we demonstrated that the MAPS pathway utilizes a mechanism distinct from that used by the CMA pathway to direct misfolded cytosolic cargoes toward the extracellular milieu. In MAPS, misfolded proteins are translocated into the lumen of a subset of endosomes. The majority of endosomes carrying MAPS substrates are decorated with Rab9 and LAMP2 (7, 10), marking them as late endosomes or lysosomes. In the endocytic trafficking pathway, endosomes usually mature and fuse with lysosomes to degrade luminal materials. However, several lines of evidence suggest that MAPS substrates are not degraded by lysosomes. First, a significant fraction of endosomes bearing misfolded MAPS substrates cannot be labeled or degraded by lysosomes. This process may use a yet-to-be-discovered protein translocation machinery at this unconventional protein secretion path that imports cytosolic proteins into the membrane of late endosomes or lysosomes as a dedicated secretory compartment. Alternatively, MAPS substrates may traffic to a subset of “nondegradative lysosomes” that are dedicated to protein secretion.

The entry of misfolded proteins into late endosomes in the MAPS pathway is to some extent analogous to CMA in which translocation of cytosolic proteins into the lumen of lysosomes occurs. Both processes use chaperones to target soluble proteins to the membranes, resulting in their translocation into the lumen of a membrane-encircled compartment. However, several distinctions suggest that they use different mechanisms to move proteins across the membranes. First, lysosome translocation in CMA requires protein unfolding, whereas secretion through MAPS is independent of protein unfolding. Second, CMA requires a CMA motif for substrate recognition, but secretion via MAPS is not dependent on the CMA motif. Finally, CMA-mediated degradation, but not MAPS, can be enhanced by serum starvation. These results clearly indicate the existence of a previously unknown protein translocation path that imports cytosolic proteins into late endosomes/lysosomes. This process may use a yet-to-be-discovered protein translocation machinery in the membrane of late endosomes or lysosomes. Because complete protein unfolding is dispensable for secretion through the MAPS pathway, the translocation machinery, if it exists in MAPS, should be large enough to accommodate folded protein domains. Why serum starvation diminishes MAPS is unclear. One possible explanation is that serum starvation evokes a perinuclear positioning of late endosomes/lysosomes (20–22), hindering their transportation to cell periphery and subsequent fusion with the plasma membrane.

Despite the apparent distinctions between MAPS and CMA, both processes require the function of LAMP2. In the case of the CMA pathway, only a specific isoform, LAMP2a, is involved as it has a unique C-terminal tail required for HSC70 interaction. Intriguingly, we showed that knockdown of LAMP2 attenuates MAPS, but in this case, LAMP2 probably serves a function distinct from that in CMA because the secretion defect caused by LAMP2 knockdown can be rescued by overexpression of DNAJC5, which functions as a substrate-recruiting factor in MAPS. In addition, no significant interaction between LAMP2 and DNAJC5 or α-Syn could be detected. Because LAMP2 is an abundant glycoprotein coating the luminal surface of late endosome/lysosome, its depletion might disrupt structural integrity of this compartment. Alternatively, as LAMP2 was recently shown to bind cholesterol (23), its depletion is expected to perturb lipid homeostasis in cells. One way or another, depletion of LAMP2 might compromise substrate recruitment by DNAJC5 in MAPS, which would explain why overexpression of DNAJC5 rescues the secretion defect caused by LAMP2 depletion. It is currently unclear which LAMP2 isoform is involved in MAPS. Because they are almost identical in amino acid sequence and molecular weight, these variants cannot be distinguished by antibodies. The similarity in the coding genes also makes it difficult to target a specific isoform by siRNA.

In summary, our study establishes a subset of a nondegradative endosomes/lysosomes as a dedicated secretory compartment for unconventional secretion of misfolded proteins. After being targeted to the surface of this compartment, misfolded proteins can enter the lumen of this compartment at least in a partially folded state. This process utilizes a mechanism distinct from that used by cargoes of the CMA pathway. Future studies will be required to elucidate the nature of the protein translocation machinery at this unconventional protein secretion compartment.

**Materials and methods**

**Cell lines, plasmids, siRNAs, and reagents**

HEK293T and COS-7 cells were purchased from ATCC. USP19 CRISPR null and control cells were described previously (7). Cells were maintained in DMEM (Corning Cellgro) containing 10% fetal bovine serum and penicillin-streptomycin. Cells recovered from liquid nitrogen freezing were maintained for at least 2 weeks before being used in the secretion experi-

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**Figure 5. LAMP2 colocalizes but does not interact with DNAJC5. A, subcellular localization of LAMP2, DNAJC5, and Rab9. Top panels, COS-7 cells were fixed and stained with LAMP2 (green) and DNAJC5 (red) antibodies. Scale bars, 5 μm. Middle panels, COS-7 cells transfected with mCh-Rab9 (red) were stained with LAMP2 antibodies (green). Bottom panels, COS-7 cells transfected with mCitrine-Rab9 (mCitrine-Rab9, green) were stained with LysoTracker Red for 5 min and imaged. The insets for the top two rows show enlarged views of regions of interest of a different cell not shown in this field. The boxes in the bottom row highlight examples of Rab9-positive vesicles stained with a LysoTracker dye. B, LAMP2 does not interact with DNAJC5–FLAG or FLAG–USP19. Cells transfected with the indicated plasmids were lysed in LCHAPS buffer. FLAG-tagged proteins were pulled down (PD) by FLAG-agarose beads. The precipitated samples were analyzed by immunoblotting with antibodies against the indicated proteins. C, LAMP2 does not interact with α-Syn. C is the same as B except that a plasmid expressing α-Syn–FLAG was transfected. E.V., empty vector.**

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**Chaperone-mediated autophagy is not involved in MAPS**
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A

| M(kDa) | Medium | Lysate |
|-------|--------|--------|
| 25    | α-Syn-FLAG | Clusterin |
| 37    | α-Syn-FLAG |
| 25    | LAMP2    |
| 25    | USP19    |

B

α-Syn secretion (Med/lyg) (%)

C

| M(kDa) | Medium | Lysate |
|-------|--------|--------|
| 25    | DNAJC5-FLAG | DNAJC5-FLAG |
| 25    | α-Syn-FLAG |
| 25    | Ponceau S. |
| 100   | LAMP2    |
| 50    | DNAJC5-FLAG |
| 25    | α-Syn-FLAG |

D

α-Syn secretion (Med/lyg) (A.U.)

E

| siRNA | Control | LAMP2A siRNA |
|-------|---------|--------------|
| mLAMP2A-FLAG | α-Syn-FLAG |
| Clusterin |
| HSP90    |
| HSC70    |

F

α-Syn secretion (Med/lyg) (A.U.)

G

α-Syn secretion (Med/lyg) (A.U.)
ments. LAMP2 antibody was purchased from Santa Cruz Biotechnology (SC-18822). DNAJC5 antibody was purchased from LSBio (LS-C22593). Bafilomycin A1 and chloroquine were purchased from Sigma.

The following plasmids (pCMV6-H9251-Syn-FLAG, pRK5-FLAG-USP19, pRK5-FLAG-GFP1–10, pcDNA3-FLAG-DNAJC5, and mCh-Rab9) were reported previously (7, 10). The pCMV6-α-Syn-mCMA plasmid was generated by site-directed mutagen-

**Figure 6.** Secretion of α-Syn depends on LAMP2. A, secretion of α-Syn from 293T cells transfected with the indicated siRNA was analyzed by immunoblotting. B, the graph shows the quantification of three independent experiments in A. C and D, DNAJC5 overexpression rescues secretion of α-Syn in LAMP2 knockdown cells. The graph in D shows the quantification of three independent experiments in C (error bars represent mean ± S.E.; n = 3; *** p value <0.005; *, p value <0.05; NS, not significant). E, expression of mouse LAMP2 inhibits α-Syn secretion. 293T cells treated with either control (Ctrl.) or LAMP2 siRNA were transfected with either an empty vector (E.V.) or mLAMP2a together with α-Syn. Conditioned medium and cell lysates were analyzed by immunoblotting. F, quantification of the experiment in E. G, expression of either LAMP2a or LAMP2b inhibits α-Syn secretion. G is the same as E, excepted that WT cells were used, and cells were transfected with either mLAMP2a or mLAMP2b. Med., medium; Lys., lysate; A.U., arbitrary units; hLAMP, human LAMP.

**Figure 7.** LAMP2 knockdown alters lysosome homeostasis. A and B, LAMP2 knockdown affects the levels of Rab9 and DNAJC5. A, knockdown of LAMP2 does not affect lysosome morphology. A fraction of control and LAMP2 knockdown cells was analyzed by immunoblotting to confirm knockdown efficiency (left panels). The remaining cells were stained with LysoTracker Red dye (right panels). B, control (Ctrl.) or LAMP2 knockdown cells transfected with mCitrine-Rab9 (mCi-Rab9) were subjected to biochemical fractionation. The resulting membrane (Mem.) and cytosol (Cyto.) fractions were analyzed by immunoblotting. The numbers show the fold change of the GFP-Rab9 level. C, whole-cell extracts of control and LAMP2 knockdown cells were analyzed by immunoblotting. Asterisk, non-specific band. The graphs show the quantification from four independent experiments (error bars represent mean ± S.E.; n = 4; *** p value <0.005; *, p value <0.05). A.U., arbitrary units; WCE, whole-cell extracts.
esis. pCMV-mLAMP2a and pCMV-LAMP2b were purchased from Origene. To generate pcDNA-LacZ–H9251-Syn, the following primers were used to amplify H9251-Syn cDNA: forward primer, 5'-ATCGATCGGCAGATGTATTCATGAAAGGAGT-3'; reverse primer, 5'-TACGAACCTGAAGCCTAAAACCTGGTATGC-3'. The PCR fragment was digested by NotI and AgeI and then cloned into the vector pcDNA3.1/V5-His/LacZ (Invitrogen).

To make plasmid expressing H9251-Syn–DHFR, Escherichia coli DHFR sequence was amplified from DHFR-myc, which was a gift from Carolyn Bertozzi (Addgene plasmid 20214) (24), using PCR. The PCR fragment was added at the C terminus of H9251-Syn in pCMV6-α-Syn-FLAG using MluI and NotI restriction sites.

siRNAs were purchased from Invitrogen. The targeting sequences are: siRNA-LAMP2 number 1, 5'-GCACCAUUAAGUUAUCUGA-3'; siRNA-LAMP2 number 2, 5'-GCAAGUAAUCACUUU-3'; and siRNA-LAMP2 number 3, 5'-GGAUGGCAUUUACCAA-3'.

Transfections in 293T cells were performed with TransIT-293 (Mirus), and those in COS-7 cells were performed with Lipofectamine 2000 (Invitrogen). For gene silencing experiments, Lipofectamine RNAiMAX was used according to the manufacturer’s protocol.

**Immunoblotting, protein level measurements, and statistical analyses**

Immunoblotting was performed using standard protocols. To quantify proteins secreted into the media, 15 μl of conditioned medium was mixed with 4× sample buffer and analyzed by immunoblotting using horseradish peroxidase–conjugated secondary antibodies. Immunoblotting signal was detected by the enhanced chemiluminescence method (Millipore, WBKLS0100) and recorded by a Fuji LAS-4000 imager. The intensities of protein bands were quantified by Image Gauge v3.0. Protein secretion efficiency was determined by normalizing the level of secreted proteins by the amount of expressed proteins in cell lysates, which was determined by immunoblotting with fluorescence-labeled secondary antibodies. For immunoblotting using fluorescence-labeled secondary antibodies, blots were scanned by a LI-COR Biosciences Odyssey scanner, and the intensities of protein bands were determined by Odyssey software.

**Coimmunoprecipitation under native conditions**

To perform co-IP experiments, HEK293T cells (~5 × 10^6) were seeded and grown in a 6-well plate for 24 h and then transfected with 1 μg of plasmid expressing proteins as indicated in the figure legends. Cells were collected 24 h post-transfection and lysed in 350 μl of LCHAPS buffer (1% CHAPS, 30
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Protein secretion analysis

To measure the secretion of misfolded substrates, cells (2.5 × 10^5) seeded in a poly-d-lysine–coated 12-well plate were grown for 24 h and then transfected with 250 ng of plasmid expressing a MAPS substrate together with 250 ng of plasmid expressing a MAPS regulator as indicated in the figure legends. At 24 h post-transfection, we replaced the medium with 1.5 ml of fresh DMEM. Cells were grown for another 16 h before we harvested conditioned medium. To test secretion under serum starvation condition, we removed old medium 30 h post-transfection. We incubated cells with fresh medium with or without fetal bovine serum for 4 h before we harvested medium and prepared cell lysates. The medium was subjected to sequential centrifugation, first at 1,000 g for 5 min to remove contaminated cells and then at 10,000 g for 30 min to remove cell debris. Cleared supernatant was mixed with 4× sample buffer and heated before immunoblotting analyses. Cells were lysed in 200 μl of LNP lysis buffer (0.5% Nonidet P-40, 50 mM Tris/ HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA). To test the role of LAMP2 in MAPS, we seeded 293T cells at 6.0 × 10⁵/well in a regular 6-well tissue culture plate on day 0. On day 1, cells were transfected with siRNA (60 pmol) using RNAiMAX following the manufacturer’s instructions. On day 2, cells were subcultured at a 1:8 ratio and incubated with fresh medium for 6 h. Plasmid expressing α-Syn was then transfected, and cells were maintained for 24 h before medium change. After medium change, cells were then incubated for another 16 h before conditioned medium was harvested for analyses by immunoblotting.

Biochemical fractionation

HEK293T cells (1 × 10^⁷) were collected and washed with ice-cold phosphate-buffered saline (PBS). Cells were then treated with 800 μl of LHP buffer (10 mM Tris-HCl, pH 7.4, 10 mM potassium chloride, 2 mM magnesium chloride, 1 mM DTT) containing a protease inhibitor mixture on ice for 10 min before being homogenized by a Dounce homogenizer. Sucrose was then added to 250 mM to prevent damage of subcellular organelles and membrane vesicles. Homogenized cells were subjected to centrifugation at 1,000 × g for 5 min to remove unbroken cells and nuclei. The supernatant fractions were further centrifuged at 100,000 × g for 30 min to sediment total membrane vesicles. The membranes were washed with the PB buffer (115 mM potassium acetate, 5 mM sodium acetate, 25 mM HEPES, pH 7.3, 2.5 mM magnesium chloride, 0.5 mM EGTA) containing 250 mM sucrose before SDS-PAGE analysis.

Limited trypsin digestion

HEK293T cells transfected with 1 μg of α-Syn–FLAG or α-Syn–DHFR–FLAG were treated with either DMSO or 10 μM aminopterin for 5 h. Cells were lysed with LNP lysis buffer with 10 μM aminopterin without protease inhibitor mixture or DTT. The lysates were centrifuged at 10,000 × g for 20 min, and the supernatants were collected. To purify α-Syn proteins, the supernatants were incubated with 40 μl of prewashed anti-FLAG M2-agarose gel (Sigma, A2220) for 2 h at 4 °C. The bound proteins were washed with WNP wash buffer (0.05% Nonidet P-40, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA) three times and further eluted with 150 μl of 3× FLAG peptide solution (Sigma, F4799; 200 μg/ml). The eluates (18 μl) were incubated with various concentrations of trypsin for 30 min at 37 °C in a total volume of 20 μl. The reactions were quenched by addition of preheated 2× Laemmli buffer, and the eluates (10 μl) were resolved by SDS-PAGE. The gel was stained using a silver staining kit (Invitrogen, 45-1001) according to the manufacturer’s protocol.

Immunostaining and confocal microscopy

To characterize protein colocalization, COS-7 cells were seeded at 1.5 × 10⁵/well in a 35-mm μ-dish (ibidi GmbH, Germany) coated with fibronectin (Sigma, F1141; 10 μg/ml). Where indicated, 100 ng of mCh-Rab9 plasmid was transfected using Lipofectamine 2000 following the manufacturer’s instructions. At 18 h post-transfection, cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS. Fixed cells were washed and stained with antibodies (LAMP2, 1:200; DNAJC5, 1:50) in PBS containing 5% fetal bovine serum and 0.2% saponin. Cells were imaged on an LSM 880 confocal microscope (Carl Zeiss Microscopy). The objective lens used was the Zeiss Plan-Apo 63×/1.4 oil differential interference contrast.

To stain lysosomes, LysoTracker Red DND-99 (Invitrogen, L7528) was used to stain the cells for 5 min according to the manufacturer’s instructions. Images were acquired using Zen (Zeiss) and processed using Photoshop (Adobe).

Statistics and reproducibility

All gels shown are representatives of at least three independent replicates. The n values in the graphs indicate the number of independent experiments. Error bars show mean ± S.E. Unless specified, p values were calculated using two-tailed, paired Student’s t test.
Chaperone-mediated autophagy is not involved in MAPS

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