論文の内容の要旨

論文題目　Identification of TEX264 as a novel receptor for ER autophagy
(小胞体選択的オートファジーの新規レセプターTEX264 の同定)

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Autophagy is an intracellular degradation pathway in which cytoplasmic components are degraded by the lysosome. Previously, autophagy was thought to be a non-selective bulk degradation pathway; however, it is now clear that some proteins and damaged organelles are selectively recognized and degraded by autophagy. Identification and characterization of substrates and receptors for selective autophagy will accelerate our understanding of mechanisms and significance of selective autophagy.

Typical substrates/receptors of selective autophagy are directly or indirectly recognized by Atg8 or its homologs present on the autophagic membranes. In mammals, the Atg8 homologs are classified into two subfamilies: the microtubule-associated protein light chain 3 (LC3) and gamma-aminobutyric acid receptor-associated protein (GABARAP) families. Selective substrates that have LC3-interacting regions (LIRs) or GABARAP-interacting motifs (GIMs) can be directly recognized by autophagic membranes.

Autophagic degradation of endoplasmic reticulum (ER) fragments, which is termed ER-phagy, is a selective type of autophagy. In mammals, four ER-phagy receptors have been identified so far: FAM134B, RTN3, CCPG1, and SEC62. Their functions may be different spatiotemporally. However, the known ER-phagy receptors are required only partially, implying that there might be an unidentified functionally redundant molecule involved.

In this study, to identify selective autophagy substrates or receptors/adaptors, My collaborators Drs. Natsume and Hatta performed differential interactome analysis using a wild-type (WT) LC3B and a LIR recognition-defective LC3B mutant. Among these proteins detected by differential interactome screening, I focused on testis expressed 264 (TEX264) because it showed a high binding score. TEX264 was originally identified as a testis-expressing membrane protein. However, the function of TEX264 remains unknown. Immunoprecipitation analysis confirmed that TEX264 interacted with LC3B. TEX264 contains an evolutionarily conserved putative LIR motif (FEEL) in the C-terminal (amino acids 273–276) regions. Substitution of putative LIR with alanine completely abolished the interaction with LC3B. These data suggest that TEX264 interacts with LC3B via the LIR motif.

To investigate the role of TEX264 in autophagy, I first monitored the intracellular localization of TEX264. TEX264-green fluorescent protein (GFP) and endogenous TEX264

showed a reticular pattern under nutrient-rich conditions and colocalized with the ER marker. These data suggest that TEX264 is present throughout the ER. Under starvation condition, TEX264-GFP formed punctate structures. Most of these structures colocalized with LC3 suggesting that TEX264 closely associates with the autophagic membrane. On the other hand, TEX264 LIR mutant, in which FEEL was replaced with AAAA, did not form punctate structures. In addition, some of the TEX264-positive puncta colocalized with LAMP1. These results suggest that TEX264 associates with autophagic membranes in a LIR-dependent manner and is delivered to lysosomes via autophagy.

I tested whether endogenous TEX264 was degraded by autophagy. Upon induction of autophagy by amino acid starvation, the amount of TEX264 was reduced. This reduction was restored by the lysosome inhibitor. Such reduction in TEX264 level was not observed in autophagy-deficient FIP200-knockout (KO) cells, even under starvation conditions. These data suggest that TEX264 is degraded by the autophagy-lysosome pathway.

Next, I compared the expression levels of TEX264 in various tissues between WT and autophagy-deficient mice. TEX264 accumulated heavily in all of the autophagy-deficient tissues tested. These data suggest that TEX264 is degraded by autophagy in both cultured cells and mouse tissues.

Given that TEX264 is an ER transmembrane protein and delivered to lysosomes, I hypothesized that TEX264 functions as a receptor for ER-phagy. I developed a doxycycline-inducible ER-phagy reporter that has an N-terminal ER signal sequence followed by tandem monomeric red fluorescent protein (RFP) and GFP sequences and the ER retention sequence KDEL. When ER-phagy was activated by starvation, the RFP fragment appeared as a result of degradation of the reporter inside the ER because the linker between RFP and GFP was cleaved, thus producing RFP, which is relatively stable in lysosomes.

Next, I determined the role of TEX264 in ER-phagy using the ER-phagy reporter. I generated TEX264-KO HeLa cells using the CRISPR-Cas9 method. In TEX264-KO cells, the production of the RFP fragment was significantly impaired under both nutrient-rich and starvation conditions. Cleavage of the reporter was restored by re-expression of exogenous TEX264 but not by the LIR mutant. These data suggest that TEX264 and its LIR motif are important for ER-phagy.

ER-phagy activity was also monitored by fluorescence microscopy using the same reporter. This reporter should appear yellow (green and red) in the ER matrix. When it is transported to lysosomes by autophagy, it becomes red because GFP but not RFP is quickly quenched in the acidic environment. Hence, the total RFP intensity of these red puncta should indicate the amount of the ER-phagy reporter delivered to lysosomes. The RFP intensity in red puncta was lower in TEX264-KO cells than in WT cells under both nutrient-rich and starvation

conditions. The reduction in the RFP intensity was restored by re-expression of exogenous TEX264 but not by the TEX264 LIR mutant. Thus, TEX264 is a novel ER-phagy receptor.

Four ER-phagy receptors, namely, FAM134B, CCPG1, RTN3, and SEC62, have been reported in mammals as already mentioned. To determine the relative contributions of these known ER-phagy receptors and TEX264, the endogenous level of each receptor was monitored during autophagy. The levels of TEX264 and CCPG1 reduced during starvation. The level of FAM134B was unchanged, probably because both degradation and synthesis were enhanced during starvation; treatment with the lysosome inhibitor bafilomycin A_1 caused accumulation of FAM134B. By contrast, the levels of RTN3 and SEC62 were stable irrespective of bafilomycin A_1 treatment. These data suggest that large amounts of TEX264, FAM134B, and CCPG1 are subjected to autophagic degradation, and so I hypothesized that these three proteins are major receptors for general ER-phagy.

The relative importance of these receptors was further determined by small interfering RNA (siRNA)-mediated knockdown of four of the five receptors. Depletion of FAM134B, CCPG1, RTN3, and SEC62 (except TEX264) caused a partial reduction in the cleavage of the ER-phagy reporter under basal and starvation conditions, but a significant level (>50%) of ER-phagy activity remained. By contrast, knockdown of all ER-phagy receptors including TEX264 suppressed the reporter cleavage to a level comparable with that in FIP200-depleted cells. These data suggest that, although there is some redundancy, TEX264 is a major ER-phagy receptor.

To compare the binding efficiency of each ER-phagy receptor with LC3/GABARAP family proteins, I determined the pull-down efficiency of all binding pairs. TEX264 interacted strongly with LC3A and GABARAPL1. FAM134B and CCPG1 interacted preferentially with GABARAP family proteins. However, their binding efficiencies were weaker than those of TEX264-LC3A and TEX264-GABARAPL1 interactions. These data suggested that TEX264 interacts most efficiently with LC3/GABARAP family proteins among the ER-phagy receptors.

To further determine the function of these ER-phagy receptors, I generated TEX264, FAM134B, and CCPG1 triple-KO HeLa cells. ER-phagy activity was almost completely suppressed in triple-KO cells although bulk autophagy and lysosome function were not affected. Additional knockdown of SEC62 or RTN3 in triple-KO cells did not further suppress reporter cleavage under these experimental conditions. ER-phagy in triple-KO cells was rescued by exogenous expression of TEX264, although more efficiently than that by FAM134B or CCPG1. Moreover, although autophagic degradation of TEX264 was observed in almost all tissues, that of the other receptors was observed in only some tissues. Taken together, these data suggest that TEX264 contributes most to ER-phagy among the ER-phagy receptors.

During ER-phagy, rough ER membranes associate with autophagic membranes.

However, the two membranes are not directly attached because ribosomes exist between them. TEX264 contains a long intrinsically disordered region (IDR) near the LIR. I hypothesized IDR which does not fold into a compact structure and is rather dynamic and flexible is required for ER-phagy receptor function. I generated TEX264 mutants with different deletions in the IDR. When lacking almost the entire IDR but retained the LIR, TEX264 failed to colocalize with LC3. When I inserted an IDR sequence from human ATG13, LC3 colocalization was rescued. These results indicate that IDR rather than a specific amino acid sequence is critical for the association between TEX264 and the autophagic membranes.

Finally, I tested whether the IDR is required for the ER-phagy receptor function of TEX264. Expression of the IDR full-deletion mutant failed to restore ER-phagy in TEX264-KO cells. Furthermore, expression of the TEX264 mutant replaced with the IDR from ATG13 was also functional. These data suggest that the long IDR in TEX264 functions as a bridge between the ER and autophagosomal membranes to act as an ER-phagy receptor.

In summary of this study, I identified TEX264 as a novel ER-phagy receptor. This study has provided the following novel insights into this topic. First, I analyzed the relative importance of the five ER-phagy receptors including TEX264. TEX264 is ubiquitously expressed in all tissues that I have tested. The binding affinity of TEX264 to LC3/GABARAP family proteins is stronger than that of the other four receptors; thus, TEX264 is the most efficient of the known receptors. Furthermore, our results suggest that deletion of TEX264 causes the largest inhibition of ER-phagy at least in HeLa cells. Based on these data, I hypothesized that, although there is redundancy, TEX264 contributes the most among the ER-phagy receptors.

Second, TEX264 has a long IDR domain, which is required for its ER-phagy receptor function. IDRs are contained in the other ER-phagy receptors, and so this may be a common feature of ER-phagy receptors. I hypothesize that IDRs are required to bridge the long distance between ER and autophagosome membranes because ribosomes exist on ER membranes that attach to autophagic membranes.

Third, I generated a novel ER-phagy reporter, with which I could quantitatively measure ER-phagic flux. Using our new reporter, I could quantify ER-phagy activity not only during starvation but also under basal conditions, and found that TEX264 was required for both.