

# Studies on Membrane Recognition Specificity of Vacuolar Phospholipase Atg15

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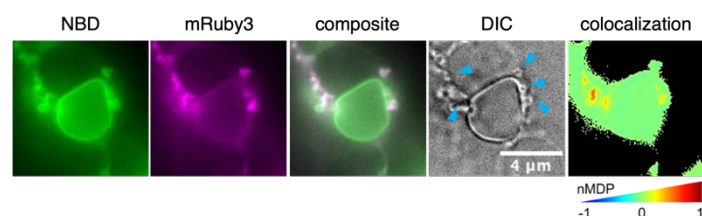
## Introduction

Macroautophagy (hereafter referred to as "autophagy") is a vital intracellular degradation pathway that plays a crucial role in maintaining cellular homeostasis. In *Saccharomyces cerevisiae*, when autophagy is induced under conditions such as nutrient starvation, autophagy-related (Atg) proteins accumulate at a specific region of the vacuolar membrane. This leads to the formation of the isolation membrane, which expands to encapsulate cellular components targeted for degradation, ultimately forming the autophagosome that fuses with the vacuole. The inner vesicle, known as the autophagic body (AB), is released into the vacuole, where its membrane is degraded by lipases, and its contents are broken down by hydrolases. Atg15, a vacuolar phospholipase, is necessary to degrade the membrane of AB (Epple et al., 2001; Teter et al., 2001). It is not yet known how Atg15 recognizes its substrates, phospholipids and membranes. Therefore, in this study, I aimed to investigate the membrane binding properties of Atg15 and its underlying molecular mechanisms.

## Results

### 1. Positive correlation between Atg15 membrane binding and membrane curvature

To investigate the effect of membrane curvature on Atg15 binding to membranes, I prepared liposomes with diameters ranging from several hundred nm to several thousand nm, and examined the binding affinity of Atg15 to these liposomes. As a result, purified recombinant Atg15 protein strongly bound to liposomes with diameters ranging from tens to two hundred nm, while it showed little binding to larger liposomes.

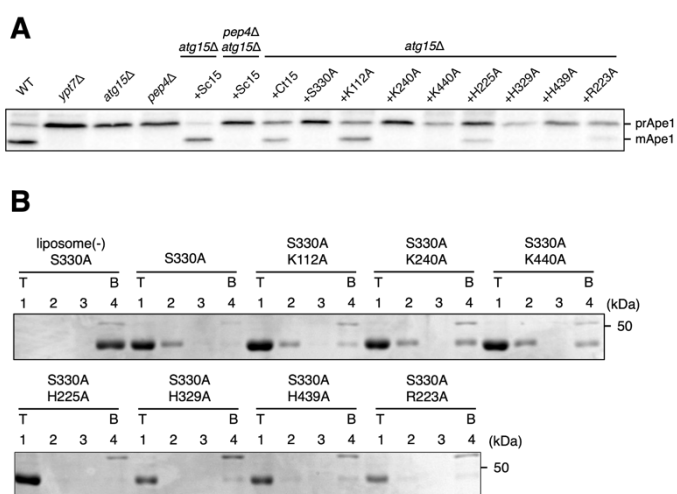


**Fig. 1 Atg15 prefers to bind to smaller liposomes**

The purified mRuby3-Atg15 recombinant protein was incubated with GUV and smaller liposomes labeled with NBD-PE at 25°C. The blue arrowheads indicate smaller liposomes.

## 2. Basic amino acid residues around the active center in Atg15

Structural predictions using ColabFold suggest that the region around the active center of Atg15 is rich in positive charge (Watanabe et al., 2023). To investigate the role of this positively charged region in Atg15, I generated mutants, in which each of the seven basic amino acid residues (K112, R223, H225, K240, H329, H439, and K440) in this region, was replaced with alanine. I then evaluated the ability of these mutants to degrade Cvt bodies (a specific type of autophagic bodies that enclose precursor aminopeptidase 1 (prApe1) inside) *in vivo* and their binding affinity to liposomes. To prevent liposome disruption during the binding experiments between the Atg15 recombinant protein and liposomes, the enzyme was inactivated by replacing the active center, S330, with alanine. As a result, all mutants, except for K112A, exhibited either reduced or completely inhibited degradation of Cvt bodies (Fig. 2a). However, all mutants bound to liposomes at levels comparable to S330A (Fig. 2b). These findings indicate that the basic amino acid residues forming the positively charged region around the active center are essential for the degradation activity of Atg15.



**Fig. 2 Basic amino acids residues around active center in Atg15**

(A) *In vivo* activity of Atg15 was estimated by the maturation of Ape1 by western blotting analysis. prApe1 and mApe1 indicate precursor Ape1 and mature Ape1. (B) A flotation assay was performed using mutant Atg15 recombinant proteins. Liposomes were composed of DOPC/DOPE (60:40).

## Conclusion

In this study, I demonstrated that the membrane binding affinity of Atg15 correlates positively with membrane curvature and that the basic amino acids forming the positively charged region near the active center do not contribute to membrane binding but are essential for the degradation activity of Atg15. These findings are expected to provide insight into the substrate recognition mechanism of Atg15. In particular, the question of why the lipase Atg15 does not degrade the limiting membrane of the vacuole may be explained by the idea that Atg15 specifically targets membranes with relatively large positive curvature.