Atg13 HORMA domain recruits Atg9 vesicles during autophagosome formation

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During autophagosome formation, autophagosome-related (Atg) proteins are recruited hierarchically to organize the preautophagosomal structure (PAS). Atg13, which plays a central role in the initial step of PAS formation, consists of two structural regions, the N-terminal HORMA (from Hop1, Rev7, and Mad2) domain and the C-terminal disordered region. The C-terminal disordered region of Atg13, which contains the binding sites for Atg1 and Atg17, is essential for the initiation step in which the Atg1 complex is formed to serve as a scaffold for the PAS. The N-terminal HORMA domain of Atg13 is also essential for autophagy, but its molecular function has not been established. In this study, we searched for interaction partners of the Atg13 HORMA domain and found that it binds Atg9, a multispanning membrane protein that exists on specific cytoplasmic vesicles (Atg9 vesicles). After the Atg1 complex is formed, Atg9 vesicles are recruited to the PAS and become part of the autophagosomal membrane. HORMA domain mutants, which are unable to interact with Atg9, impaired the PAS localization of Atg9 vesicles and exhibited severe defects in starvation-induced autophagy. Thus, Atg9 vesicles are recruited to the PAS via the interaction with the Atg13 HORMA domain. Based on these findings, we propose that the two distinct regions of Atg13 play crucial roles in distinct steps of autophagosome formation: In the first step, Atg13 forms a scaffold for the PAS via its C-terminal disordered region, and subsequently it recruits Atg9 vesicles via its N-terminal HORMA domain.

autophagy | Atg13 | Atg9 | HORMA domain

Autophagy is a degradation pathway that is conserved among eukaryotes (1). During this process, a cup-shaped membrane structure emerges in the cytoplasm and expands to sequester cytoplasmic components. This structure eventually is sealed to form a double-membrane structure called the autophagosome. The autophagosome then fuses with a lytic compartment (vacuoles in yeasts and plants; lysosomes in metazoans) to degrade its contents. Autophagy is induced in response to nutrient starvation and is required for cell survival under those conditions (2, 3). A number of studies have shown that autophagy also plays a role in intracellular clearance of proteins and organelles, development, antiaging, pathogen clearance, and antigen presentation (4).

Previous studies in yeast identified 38 autophagy-related (Atg) proteins (5). Among them, 19 Atg proteins are required for starvation-induced autophagy. These 19 Atg proteins can be classified into the following six functional groups (6): (*i*) the Atg1 complex, composed of Atg1, Atg13, Atg17, Atg29, and Atg31; (*ii*) Atg9 vesicles; (*iii*) the PI 3-kinase (PI3K) complex, composed of Atg14, Vps30/Atg6, Vps34, Vps15, and Atg38; (*iv*) the Atg2–Atg18 complex; (*v*) the Atg12 conjugation system; and (*vi*) the Atg8 conjugation system. These Atg proteins are recruited hierarchically proximal to the vacuole and organize the preautophagosomal structure (PAS) that is essential for autophagosome formation (7, 8).

The initial step of PAS formation is assembly of the Atg1 complex (9, 10). In response to starvation, Atg1, Atg13, and the Atg17–Atg29–Atg31 complex form the Atg1 complex, which serves as a scaffold of the PAS (11, 12). Recently, we elucidated the molecular details of Atg1 complex formation. Atg13 plays central roles in the starvation-induced assembly of the Atg1 complex (13). Atg13 consists of two distinct domains, the N-terminal HORMA (from Hop1, Rev7, and Mad2) domain and the C-terminal disordered region (Fig. 1*A*). The N-terminal HORMA domain is essential for autophagy but is not involved in Atg1 complex formation (14). The C-terminal disordered region of Atg13 is responsible for binding to Atg1 and Atg17, which is required for the assembly of the Atg1 complex. Under nutrient-rich conditions, the C-terminal disordered region is hyperphosphorylated by the target of rapamycin complex 1 (TORC1) kinase. Under starvation conditions, TORC1 kinase is inactivated, and Atg13 is dephosphorylated immediately. Dephosphorylated Atg13 interacts more strongly with both Atg1 and Atg17, thereby promoting the formation of the Atg1 complex. Thus, Atg13 governs the formation of the Atg1 complex in a dephosphorylation-dependent manner (13).

After the Atg1 complex is formed, the next step is recruitment of Atg9-containing cytoplasmic vesicles (Atg9 vesicles), which are thought to play an essential role in the nucleation step of autophagosome formation (15). In response to starvation, Atg9 vesicles are recruited to the PAS and become part of the autophagosomal membrane. The recruitment of Atg9 vesicles is one of the crucial steps of autophagosome formation, but its molecular mechanism has not been elucidated. In this study, we found that

Significance

Autophagy is a highly conserved degradative process in eukaryotes. In response to starvation, a number of autophagosome-related (Atg) proteins are recruited, and these proteins govern the process of autophagosome formation. Atg9 vesicles are thought to play an essential role in the nucleation step, but it remains unclear how Atg9 vesicles are localized to the site of autophagosome formation. In this study, we found that Atg9 interacts with the HORMA (from Hop1, Rev7, and Mad2) domain of Atg13. Atg13 mutants lacking the Atg9-binding region fail to recruit Atg9 vesicles to the site of autophagosome formation and exhibit severe defects in autophagy. Thus, the HORMA domain of Atg13 facilitates recruitment of Atg9 vesicles during autophagosome formation. Our studies provide a molecular insight into how Atg9 vesicles become part of the autophagosomal membrane.

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The authors declare no conflict of interest.

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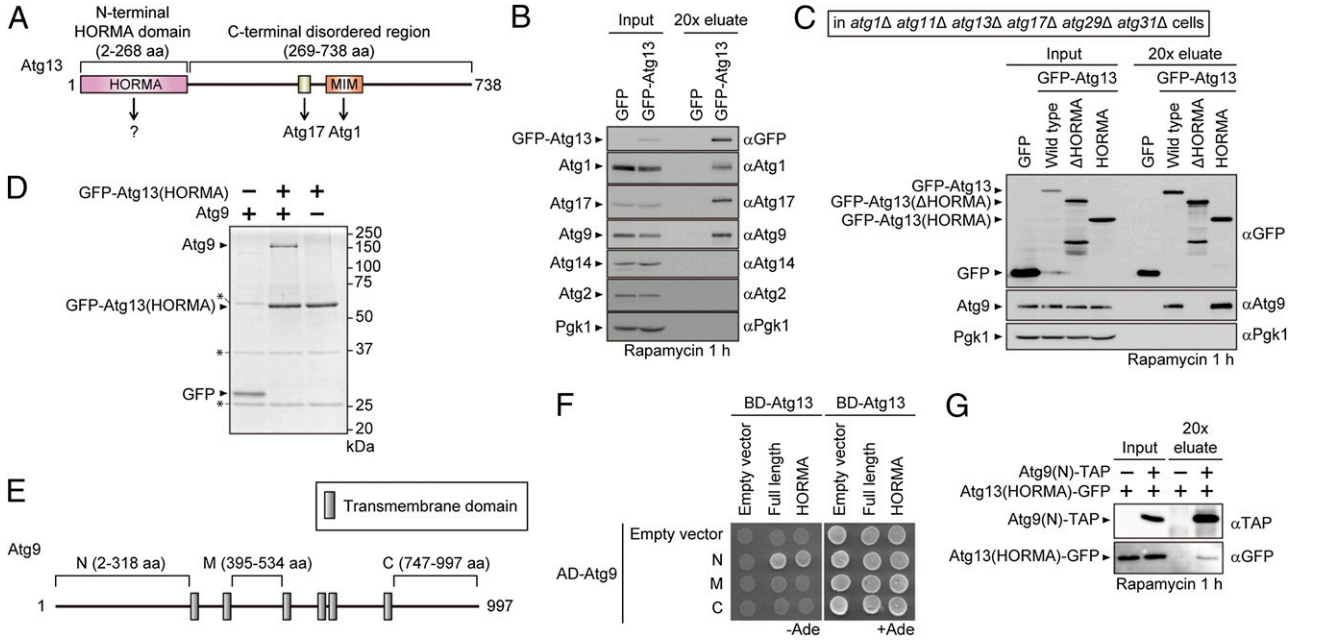

Fig. 1. The HORMA domain of Atg13 binds Atg9. (*A*) Schematic diagram of Atg13. (*B*) Atg13 interacts with Atg9. *atg13*Δ cells expressing GFP or GFP-Atg13 were treated with rapamycin for 1 h. The cells were disrupted using a Multi-beads shocker and were solubilized with 0.1% Nonidet P-40. After a centrifugation at 17,400 × *g* for 10 min, the supernatants were subjected to immunoprecipitation using anti-GFP magnetic beads. Bound materials were eluted with SDS/PAGE sample buffer and subjected to immunoblotting with antibodies against GFP, Atg1, Atg17, Atg9, Atg14, Atg2, and phosphoglycerate kinase 1 (Pgk1). (*C*) The Atg13–Atg9 interaction in vivo. *atg1*Δ *atg11*Δ *atg13*Δ *atg17*Δ *atg29*Δ *atg31*Δ cells expressing GFP, GFP-Atg13, GFP-Atg13^{HORMA} (residues 2–268), or GFP-Atg13$^{\Delta HORMA}$ (residues 281–738) were treated with rapamycin for 1 h and then were subjected to immunoprecipitation as in *B*. Bound materials were eluted with SDS/PAGE sample buffer and subjected to immunoblotting with antibodies against GFP, Atg9, and Pgk1. (*D*) The Atg13 HORMA domain interacts directly with Atg9 in vivo. Cells overexpressing both GFP-Atg13^{HORMA} and Atg9 were treated with rapamycin for 1 h and then were subjected to immunoprecipitation as in *B*. Bound materials were eluted with SDS/PAGE sample buffer and subjected to SDS/PAGE followed by Coomassie Brilliant Blue (CBB) staining. Asterisks indicate a nonspecific binding protein on anti-GFP magnetic beads. (*E*) Schematic diagram of Atg9. (*F*) Yeast two-hybrid analysis of the Atg13–Atg9 interaction. The yeast indicator strain AH109 was transformed with plasmids expressing a transcription activation domain (AD) fused with the N region of Atg9 (residues 2–318), the M region of Atg9 (residues 395–534), or the C region of Atg9 (residues 747–997) and plasmids expressing a DNA-binding domain (BD) fused with full-length Atg13 or the HORMA domain of Atg13. These strains were grown on synthetic dextrose-Leu-Trp (SC-LW) (+Ade) and synthetic dextrose-Leu-Trp-Ade (SC-LWA) (–Ade) agar plates. (*G*) The Atg9^{N}–Atg13^{HORMA} interaction in vivo. Cells expressing tandem affinity purification tag fused Atg9^{N} (Atg9^{N}-TAP) and Atg13^{HORMA}-GFP under control of their own promoters were treated with rapamycin for 1 h and then were subjected to immunoprecipitation using IgG-coated epoxy Dynabeads. Bound materials were eluted with SDS/PAGE sample buffer and subjected to immunoblotting with antibodies against TAP and GFP.

the HORMA domain of Atg13 binds Atg9. Mutational analysis revealed that the Atg13^{HORMA}–Atg9 interaction is essential for the recruitment of Atg9 vesicles. Thus, Atg13 plays an essential role not only in the initial step of the PAS formation but also in the subsequent step of the PAS scaffold formation.

Results

The HORMA Domain of Atg13 Binds Atg9. The N-terminal HORMA domain of Atg13 (residues 2–268) is essential for autophagy, but its molecular function remains unknown (Fig. 1*A*). Because the HORMA domain has been proposed to participate in a protein–protein interaction (16), we performed an affinity-binding screen aimed to identify its interaction partners. We expressed a GFP-fused Atg13 HORMA domain in yeast cells and then immunoisolated the fusion protein using anti-GFP magnetic beads (Fig. S1). Coprecipitated proteins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using this approach, we identified Atg9 as a candidate interaction partner. To confirm that Atg13 interacts with Atg9, we performed immunoprecipitation experiments. When we immunoprecipitated GFP-Atg13 from yeast cell lysates, in addition to the known Atg1 complex components Atg1 and Atg17, Atg9 also coprecipitated (Fig. 1*B*). Because Atg9 interacts with Atg1, Atg11, and Atg17 (17–19), we also examined the Atg13–Atg9 interaction in cells lacking these proteins. Atg9 still coprecipitated with GFP-Atg13 even in cells lacking Atg1, Atg11, and Atg17 (Fig. 1*C*). To determine whether the HORMA domain of Atg13 is sufficient to interact with Atg9, we constructed GFP-Atg13^{HORMA} (residues 2–268) and GFP-Atg13$^{\Delta HORMA}$ (residues 281–738) and expressed them in yeast cells. Atg9 coprecipitated with GFP-Atg13^{HORMA} but not with GFP-Atg13$^{\Delta HORMA}$ (Fig. 1*C*), indicating that the HORMA domain of Atg13 is necessary and sufficient for the interaction with Atg9. When we immunoprecipitated GFP-Atg13^{HORMA} from cells overexpressing GFP-Atg13^{HORMA} and Atg9, only Atg9 coprecipitated (Fig. 1*D*). These results suggested that Atg13 interacts directly with Atg9 via its HORMA domain. A previous study reported that the HORMA domain of Atg13 is required for the recruitment of the PI3K complex (14); however, Atg14, a subunit of the PI3K complex, did not coprecipitate with GFP-Atg13 (Fig. 1*B*).

We next performed yeast two-hybrid analyses to investigate which region of Atg9 interacts with Atg13 (Fig. 1*E*). As shown in Fig. 1*F*, an indicator strain coexpressing the N-terminal cytoplasmic region of Atg9 (Atg9^{N}; residues 2–318) fused to the Gal4 transcription activation domain and the Atg13 HORMA domain fused to the Gal4 DNA-binding domain could grow on agar plates lacking adenine. Furthermore, Atg13^{HORMA}-GFP coprecipitated with Atg9^{N}-TAP (Fig. 1*G*). From these results, we concluded that the HORMA domain of Atg13 binds the N-terminal cytoplasmic region of Atg9.

The Atg13^{HORMA}–Atg9 Interaction Is Essential for Autophagy. To address the role of the Atg13^{HORMA}–Atg9 interaction in autophagosome formation, we tried to obtain Atg13 mutants that abolish the interaction with Atg9. A previous study reported that mitotic arrest deficient 2 (Mad2) also contains the HORMA domain, and this domain interacts with Mad1 via its β-strands 4–6 and hinge loop (20). Therefore, we mutated conserved residues of Atg13 proximal to these structures (Fig. 2*A*) and found that Atg13^{E81L}, Atg13^{D203A}, and Atg13^{I208E} failed to interact with Atg9 (Fig. 2*B*). The interaction with Atg9 also was abolished in Atg13^{R120D} and Atg13^{R213D} (*Discussion*). We also confirmed by the yeast two-hybrid analysis that the Atg13^{HORMA}–Atg9 interaction was abolished by *atg13^{E81L}* and *atg13^{I208E}* mutations (Fig. 2*C*). To investigate the effect of these mutations on the formation of the Atg1 complex, we examined binding of the mutant proteins to Atg1 and Atg17. Atg13^{D203A} interacted with Atg1 and Atg17 as efficiently as the wild-type Atg13 (Fig. S2*A*). In *atg13^{D203A}* mutant cells, phosphorylation of Atg13 occurred normally (Fig. S2*B*). Furthermore, Atg17-GFP showed the PAS localization in these mutant cells (Fig. S2*C*). These results demonstrated that Atg13^{D203A} specifically decreased the affinity for Atg9 without affecting the formation of the Atg1 complex. Next, we examined the autophagic activity of these mutants by alkaline phosphatase (ALP) assay (21). When yeast cells were transferred to starvation medium, autophagic activity increased in cells expressing wild-type Atg13 (Fig. 2*D*). In contrast, cells expressing Atg13^{E81L}, Atg13^{D203A}, and Atg13^{I208E} exhibited severe autophagic defects. We also investigated the autophagic activity by using a GFP-Atg8 processing assay. GFP-Atg8 attaches to the autophagosomal membrane, and this protein is transported to the vacuole via autophagy. HORMA domain mutants showed a defect in the vacuolar transport of GFP-Atg8 (Fig. S2*D*). Furthermore, we examined accumulation of autophagic bodies by using vacuolar protease-deficient yeast cells (BJ3505). Accumulated autophagic bodies were observed in the vacuole in wild-type cells but not in *atg13^{E81L}* or *atg13^{I208E}* mutant cells (Fig. S2*E*). These results suggest that the Atg13^{HORMA}–Atg9 interaction is essential for autophagy.

The HORMA Domain of Atg13 Facilitates Atg9 Vesicle Recruitment During Autophagosome Formation. Next, we sought to determine which step in the process of Atg protein recruitment requires the HORMA domain of Atg13. In response to starvation, Atg proteins are recruited to the PAS hierarchically (Fig. 3*A*). We investigated whether the HORMA domain of Atg13 is required for the PAS localization of Atg9 vesicles. In cells expressing wild-type Atg13, Atg9-2×GFP colocalized with the PAS marker Atg17-2×mCherry at high frequency (87.6%) (Fig. 3*B*). In contrast, the D203A mutation decreased the frequency of colocalization (26.7%). We also observed that the PAS localization of Atg9 vesicles was severely impaired by D203A mutation in *atg2*Δ cells (Fig. S3*B*). From these observations, we concluded that

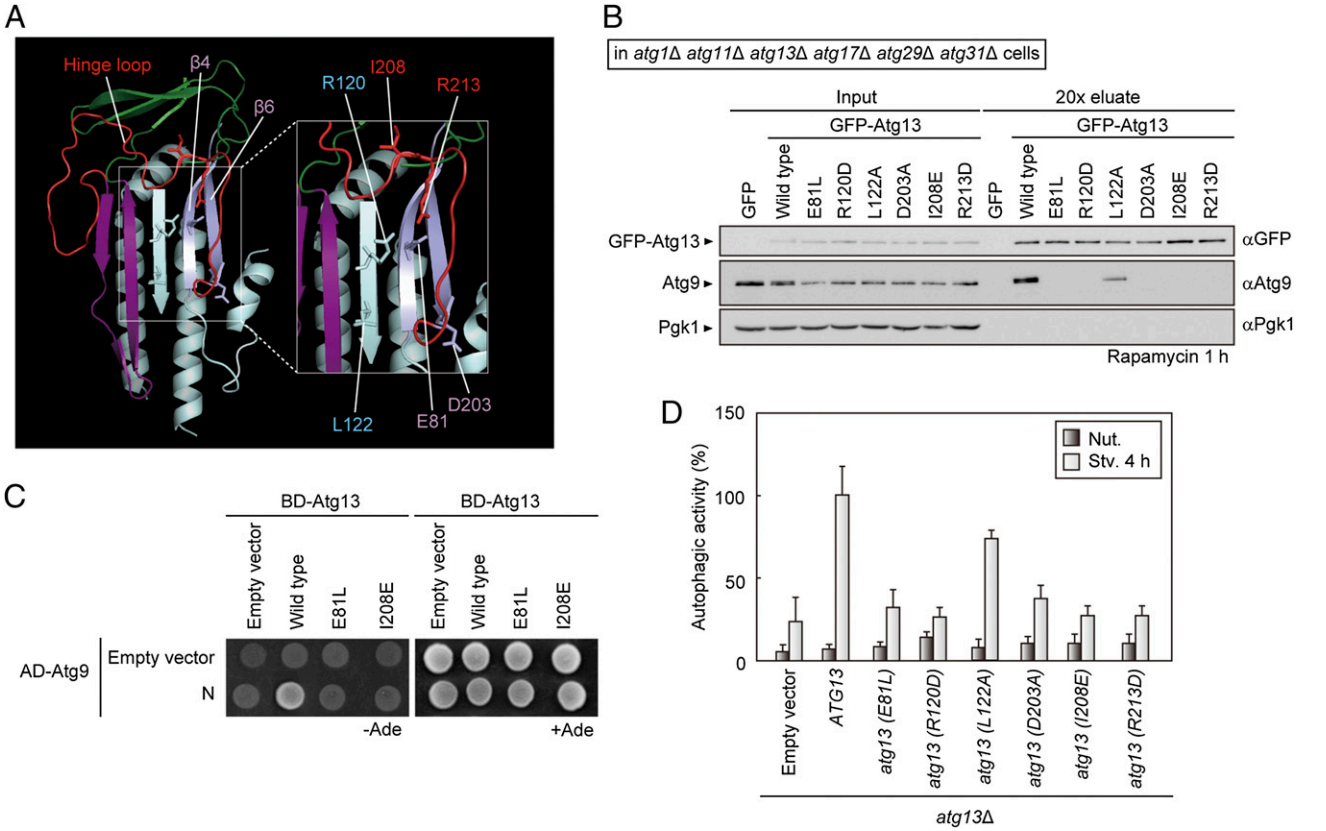

Fig. 2. The Atg13^{HORMA}–Atg9 interaction is essential for autophagy. (*A*) Mutation sites used in this study are shown on the crystal structure of the HORMA domain of Atg13 [Protein Databank (PDB) ID code: 4J2G]. (*B*) The interaction with Atg9 of HORMA domain mutants. *atg1*Δ *atg11*Δ *atg13*Δ *atg17*Δ *atg29*Δ *atg31*Δ cells expressing GFP-Atg13 mutants were treated with rapamycin for 1 h and then were subjected to immunoprecipitation as in Fig. 1*B*. Bound materials were eluted with SDS/PAGE sample buffer and subjected to immunoblotting with antibodies against GFP, Atg9, and Pgk1. (*C*) Yeast two-hybrid analysis of the Atg13–Atg9 interaction. The yeast indicator strain AH109 was transformed with plasmids expressing a transcription activation domain (AD) fused with the N region of Atg9 and plasmids expressing a DNA-binding domain (BD) fused with Atg13 mutants. These strains were grown on SC-LW (+Ade) and SC-LWA (−Ade) agar plates. (*D*) Autophagic activity of *atg13* mutant cells was quantitated by ALP assay. Dark gray bars and light gray bars indicate nutrient-rich (Nut.) and starvation (Stv. 4 h) conditions, respectively. Error bars indicate SD (n = 3).

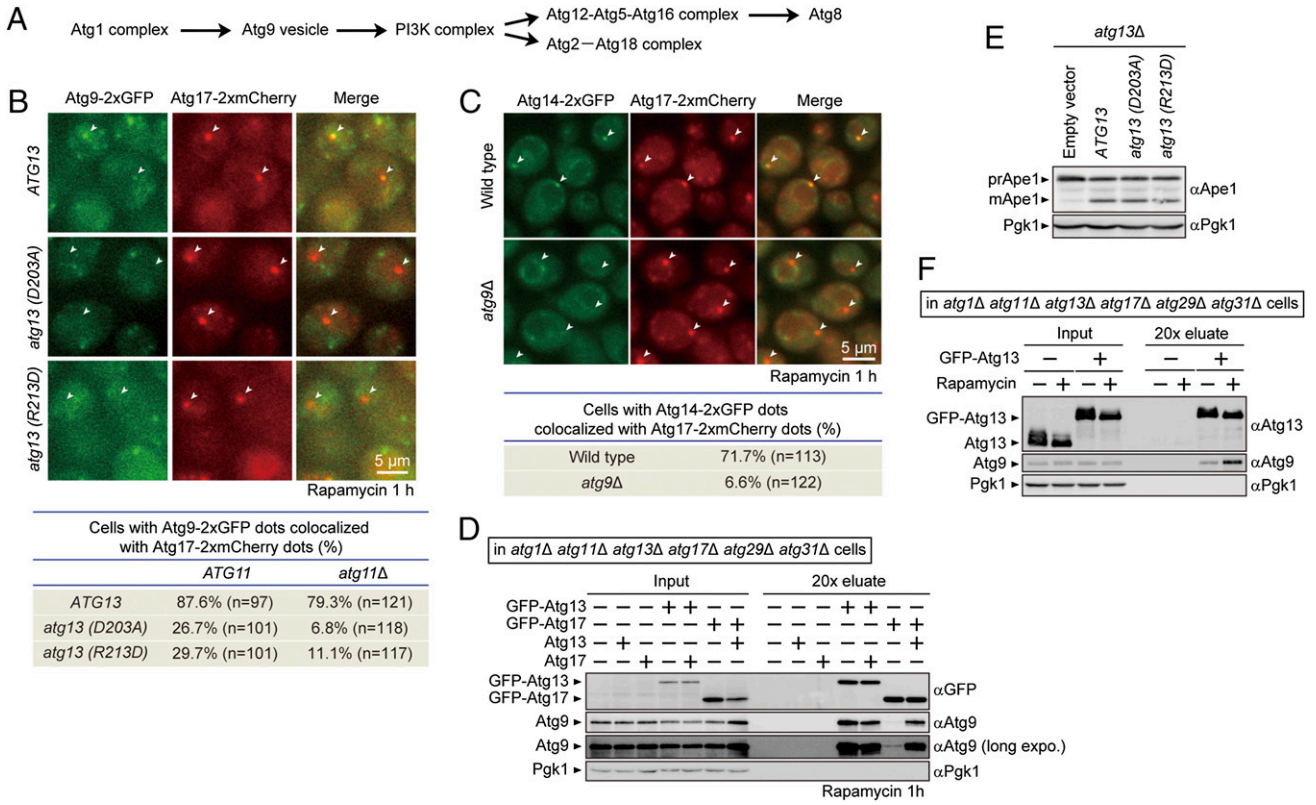

Fig. 3. The HORMA domain of Atg13 facilitates Atg9 vesicle recruitment during autophagosome formation. (*A*) Schematic diagram of the hierarchy of Atg protein recruitment to the PAS. (*B*) PAS localization of Atg9 vesicles in *atg13* mutant cells. *ATG9-2×GFP ATG17-2×mCherry atg13*Δ cells expressing *atg13* mutants were treated with rapamycin for 1 h and then were observed by fluorescence microscopy. Atg17-2×mCherry was used as a PAS marker. Arrowheads indicate the PAS marked by Atg17-2×mCherry. Green and red fluorescence signals were acquired concurrently. The percentages of cells in which Atg9-2×GFP colocalized with Atg17-2×mCherry dots in *B* (*ATG11*) and Fig. S3*A* (*atg11*Δ) are shown. (*C*) PAS localization of the PI3K complex in *atg9*Δ cells. *ATG14-2×GFP ATG17-2×mCherry* or *ATG14-2×GFP ATG17-2×mCherry atg9*Δ cells were treated with rapamycin for 1 h and then were observed by fluorescence microscopy. Arrowheads indicate the PAS marked by Atg17-2×mCherry. Green and red fluorescence signals were acquired concurrently. The percentage of cells in which Atg14-2×GFP colocalized with Atg17-2×mCherry dots is shown. (*D*) The Atg17–Atg9 interaction in *atg13*Δ cells. *atg1Δ atg11Δ atg13Δ atg17Δ atg29Δ atg31Δ* cells expressing GFP-Atg13, GFP-Atg17, Atg13, and/or Atg17 were treated with rapamycin for 1 h. The cells were subjected to immunoprecipitation as in Fig. 1*B*. Bound materials were eluted with SDS/PAGE sample buffer and subjected to immunoblotting with antibodies against GFP, Atg9, and Pgk1. (*E*) The Cvt pathway in HORMA-domain mutant cells. Total cell lysates were prepared from *atg13*Δ cells expressing *atg13* mutants grown to log phase and then were subjected to immunoblotting with antibodies against Ape1 and Pgk1. prApe1 and mApe1 indicate the precursor and mature forms of Ape1, respectively. (*F*) The Atg13–Atg9 interaction is promoted by rapamycin treatment. *atg1Δ atg11Δ atg13Δ atg17Δ atg29Δ atg31Δ* cells expressing GFP-Atg13 were grown to log phase (rapamycin −) and then were treated with rapamycin for 1 h (rapamycin +). The cells were subjected to immunoprecipitation as in Fig. 1*B*. Bound materials were eluted with SDS/PAGE sample buffer and subjected to immunoblotting with antibodies against Atg13, Atg9, and Pgk1.

Atg9 vesicles are recruited to the PAS via the interaction with the Atg13 HORMA domain.

Jao et al. (14, 22) reported that the HORMA domain of Atg13 mediates recruitment of the PI3K complex to the PAS, but no interaction between the Atg13 HORMA domain and the PI3K complex has been observed. Hence, it remains unclear how the Atg13 HORMA domain mediates the recruitment of the PI3K complex to the PAS. In fact, the frequency of PAS localization of Atg14 (a subunit of the PI3K complex) was lower in the *atg13*^{D203A} mutant than in cells expressing wild-type Atg13 (wild type: 54.0%, D203A: 17.9%) (Fig. S3*C*). To investigate the relationship between the PI3K complex and the Atg9 vesicles, we examined the cellular localization of the PI3K complex in *atg9*Δ cells. PAS localization of Atg14 was impaired severely in the absence of Atg9 (wild type: 71.7%, *atg9*Δ: 6.6%) (Fig. 3*C*), implying that the recruitment of the PI3K complex depends on Atg9. From these results, we concluded that the Atg13 HORMA domain recruits Atg9 vesicles, leading to the PAS localization of the PI3K complex.

We previously proposed that Atg9 is recruited to the PAS via the Atg17–Atg9 interaction (19). However, the *atg13*^{D203A} mutant exhibited impaired PAS localization of Atg9 vesicles (Fig. 3*B*), although Atg17 still localized at the PAS. Hence, the Atg17–Atg9 interaction is not sufficient for recruitment of Atg9 to the PAS. Instead, Atg9 vesicles are recruited through the direct interaction with the Atg13 HORMA domain (Fig. 3*B*). To examine whether the Atg17–Atg9 interaction depends on Atg13, we expressed GFP-Atg17 in yeast cells and performed immunoprecipitation experiments. Atg13 interacts with Atg9 independently of Atg17 (Fig. 1*C*). In contrast, Atg9 hardly coprecipitated with GFP-Atg17 in *atg13*Δ cells (Fig. 3*D*), indicating that Atg17 interacts with Atg9 mainly through Atg13. Based on these findings, we propose that the PAS recruitment of Atg9 vesicles is mediated mainly by the Atg13–Atg9 interaction rather than by the Atg17–Atg9 interaction.

The HORMA Domain of Atg13 Is Required for Starvation-Induced Autophagy but Not for the Cytoplasm-to-Vacuole–Targeting Pathway. Under nutrient-rich conditions, a vacuolar amino peptidase Ape1 is transported by selective autophagy called the cytoplasm-to-vacuole–targeting (Cvt) pathway (23, 24). During this process, Atg11 is required for the recruitment of Atg9 vesicles to the PAS

(17). We investigated whether the HORMA domain of Atg13 also is required for this pathway. The Cvt pathway can be monitored by the appearance of the mature form of aminopeptidase 1 (mApe1). Because Atg13 is essential for the Cvt pathway, mApe1 was not observed in *atg13*Δ cells (Fig. 3*E*). In contrast, mApe1 was observed in Atg13^{D203A}-expressing cells, implying that the Atg13^{HORMA}–Atg9 interaction is not required for the Cvt pathway.

Next, we examined whether the Atg13^{HORMA}–Atg9 interaction is regulated under autophagy-induced conditions. Immunoprecipitation experiments revealed that the Atg13^{HORMA}–Atg9 interaction was promoted by rapamycin treatment (Fig. 3*F*). Based on these observations, we conclude that under nutrient-rich conditions Atg11 facilitates recruitment of Atg9 vesicles for the Cvt pathway, but under starvation conditions the Atg13 HORMA domain strengthens its interaction with Atg9, leading to the recruitment of Atg9 vesicles.

Discussion

In this study, we found that the N-terminal HORMA domain of Atg13 binds the N-terminal cytoplasmic region of Atg9. Mutational analysis revealed that Atg9 vesicles are recruited to the PAS via the interaction with the HORMA domain of Atg13. On the other hand, the C-terminal region of Atg13 is required for Atg1 complex formation but not for the interaction with Atg9. Based on these observations, we proposed that Atg13 plays crucial roles in two distinct steps in autophagy and that these roles are mediated by distinct regions. In the first step, Atg13 promotes formation of the Atg1 complex, which serves as a scaffold for the PAS, via the C-terminal disordered region. In the next step, Atg13 recruits Atg9 vesicles via the N-terminal HORMA domain (Fig. 4*A*).

Jao et al. (14) reported that HORMA domain mutants (*atg13*^{R120D} and *atg13*^{R213D}) abolished the PAS localization of the PI3K complex, implying that the HORMA domain of Atg13 mediates recruitment of the PI3K complex to the PAS. In this study, however, we demonstrated that the HORMA domain of Atg13 binds Atg9, which is required for the recruitment of the PI3K complex to the PAS. Hence, we propose that the HORMA domain of Atg13 recruits Atg9 vesicles rather than the PI3K complex. Consistent with this idea, the Atg13^{R213D} mutant used by Jao et al. failed to interact with Atg9 (Fig. 2*B*), and cells expressing this mutant exhibited impaired PAS localization of Atg9 vesicles (Fig. 3*B*).

Importantly, HORMA domain mutants exhibited a severe defect in autophagy but not in the Cvt pathway, and the interaction with Atg9 was promoted by rapamycin treatment. From these observations, we proposed the following model for the recruitment of Atg9 vesicles (Fig. 4*B*): Under nutrient-rich conditions Atg11 recruits Atg9 vesicles to the PAS for the Cvt pathway (17), but under starvation conditions the HORMA domain of Atg13 strengthens its interaction with Atg9, thereby promoting recruitment of Atg9 vesicles to the PAS and facilitating the progression of autophagy. Thus, depending on nutrient conditions, two different molecules, Atg11 and Atg13, mediate the recruitment of Atg9 vesicles for two distinct pathways, the Cvt pathway and autophagy.

In cells expressing Atg13^{D203A}, Atg9 vesicles still colocalized with Atg17-2×mCherry (26.7% of Atg13^{D203A}-expressing cells). This observation raises a question: How do Atg9 vesicles localize

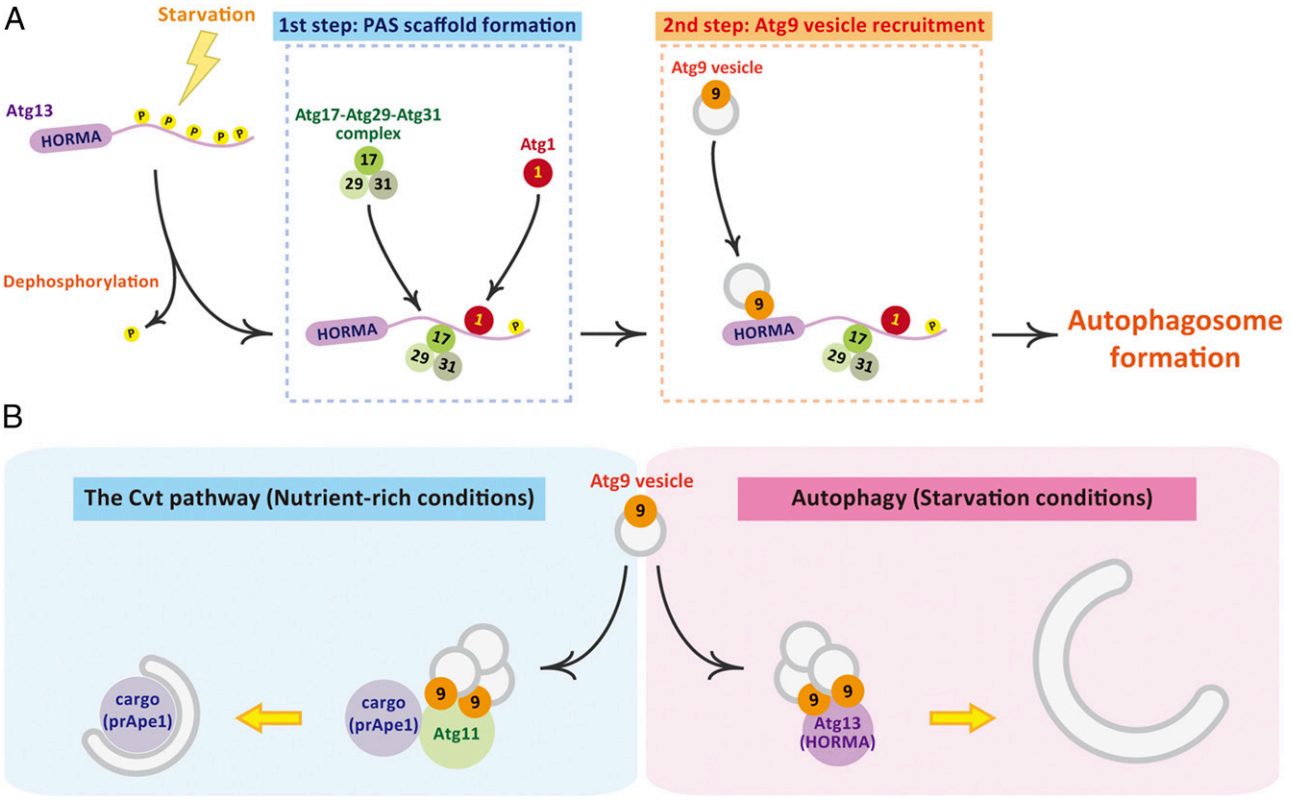

Fig. 4. Recruitment of Atg9 vesicles to the PAS by the Atg13 HORMA domain. (*A*) Two distinct regions of Atg13 play two crucial roles at the initiation of autophagosome formation. In the first step in PAS formation, Atg13 is dephosphorylated rapidly in response to starvation, promoting the interaction with Atg1 and Atg17 and leading to the formation of the PAS scaffold via the C-terminal disordered region. (The N-terminal HORMA domain is not required for this step.) In the second step, Atg13 interacts with Atg9 via its N-terminal HORMA domain to recruit Atg9 vesicles to the PAS. (*B*) Atg9 vesicle recruitment is mediated by two different molecules depending on nutrient conditions. Under nutrient-rich conditions, Atg11 recruits Atg9 vesicles for the Cvt pathway. Under starvation conditions, Atg9 vesicles are recruited to the PAS via the interaction with the Atg13 HORMA domain.

at the PAS, when Atg13 fails to interact with Atg9? The PAS localization of Atg9 was severely impaired in *atg13*^{D203A} *atg11*Δ cells (6.8%) (Fig. 3*B* and Fig. S3*A*) as compared with *atg13*^{D203A} *ATG11* cells (26.7%) (Fig. 3*B*). This result suggests that Atg11 plays some role in the PAS localization of Atg9 vesicles even under starvation conditions. However, autophagic activity was abolished almost completely in Atg13^{D203A}-expressing cells (Fig. 2*D*), implying that the Atg11–Atg9 interaction is not sufficient for the progression of autophagy.

As shown in Fig. 3*F*, the Atg13^{HORMA}–Atg9 interaction is promoted by rapamycin treatment, but it is unclear how this interaction is regulated. Interestingly, the Atg13 HORMA domain enhances the interaction with Atg9 by rapamycin treatment (Fig. S4). Previously, we examined phosphorylation sites of Atg13 by LC-MS/MS (13), but no phosphorylation sites were detected in the N-terminal HORMA domain, although we identified more than 40 phosphorylation sites in the C-terminal disordered region. These observations raise the possibility that posttranslational modification of Atg9 regulates binding with the Atg13 HORMA domain. Recent studies revealed that Atg9 is phosphorylated by Atg1 (18), but Atg1 kinase activity is dispensable for the PAS localization of Atg9 (15). Hence, another kinase might be involved in regulating the Atg13^{HORMA}–Atg9 interaction. A putative phosphate sensor of the Atg13 HORMA domain may function in the interaction with phosphorylated Atg9 (14). Further investigation will be necessary to resolve this issue.

To examine the conservation of the Atg13^{HORMA}–Atg9 interaction, we performed a homology analysis of mammalian Atg13 (mAtg13). The results of this analysis suggested that mAtg13 also contains a HORMA domain in its N-terminal region (residues 2–200) (Fig. S5). Alignment with yeast Atg13 revealed that the residues constituting β4, β6, and the hinge loop, all of which are important for the interaction with Atg9, are conserved. This feature raises the possibility that mAtg13^{HORMA} also interacts with mAtg9. Another possibility is that mAtg13^{HORMA} might interact with other autophagy-related proteins. Determining the interaction partner of mAtg13 HORMA domain and how this interaction contributes to autophagy in mammalian cells are important issues to be resolved.

Experimental Procedures

S. cerevisiae strains used in this study are listed in Table S1. Plasmids used in this study are listed in Table S2. Standard protocols were used for yeast manipulation (25). Cells were cultured at 30 °C in SD/CA medium [0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.5% casamino acids, and 2% (wt/vol) glucose] supplemented with the appropriate nutrients. Autophagy was induced by transferring the cells to SD-N medium [0.17% yeast nitrogen base without amino acids and ammonium sulfate, and 2% (wt/vol) glucose]. Otherwise, autophagy was induced by treating cells with 0.2 μg/mL rapamycin (Sigma-Aldrich).

Cells expressing GFP- and mCherry-tagged proteins with a 17-residue linker (GGAAGGSSASGASGASG) were generated using the pYM series of plasmids (26) by a PCR-based gene modification method (26) with minor modifications. Gene deletions were performed using the pFA6a-kanMX6 series, as described previously (26).

Further information about plasmid construction of yeast expression vectors, immunoblotting, antibodies, immunoprecipitation, fluorescence microscopy, yeast two-hybrid analysis, and the ALP assay is provided in *SI Experimental Procedures*.

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