

Recruitment of the autophagic machinery to endosomes during infection is mediated by ubiquitin

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Although ubiquitin is thought to be important for the autophagic sequestration of invading bacteria (also called xenophagy), its precise role remains largely enigmatic. Here we determined how ubiquitin is involved in this process. After invasion, ubiquitin is conjugated to host cellular proteins in endosomes that contain *Salmonella* or transfection reagent-coated latex (polystyrene) beads, which mimic invading bacteria. Ubiquitin is recognized by the autophagic machinery

independently of the LC3–ubiquitin interaction through adaptor proteins, including a direct interaction between ubiquitin and Atg16L1. To ensure that invading pathogens are captured and degraded, Atg16L1 targeting is secured by two backup systems that anchor Atg16L1 to ubiquitin-decorated endosomes. Thus, we reveal that ubiquitin is a pivotal molecule that connects bacteria-containing endosomes with the autophagic machinery upstream of LC3.

Introduction

Autophagy is a membrane trafficking process in which double membrane-bound spherical structures called autophagosomes deliver cytosolic contents to lysosomes/vacuoles for degradation. In addition to the well-understood physiological role of autophagy in recycling intracellular materials in response to starvation, there is increasing evidence that autophagy is involved in diverse physiological processes such as cellular immunity. Autophagy specifically targets invading bacteria to restrict their growth (also called xenophagy; Mizushima et al., 2008).

Autophagosome formation is mediated by at least 18 core autophagy-related (Atg) proteins, which comprise the following six functional units (Suzuki and Ohsumi, 2010): (1) the uncoordinated 51-like kinase (ULK)–Atg1 protein kinase complex, (2) the autophagy-specific phosphatidylinositol 3-kinase (PI3K) complex, (3) the phosphatidylinositol 3-phosphate (PI3P)–binding protein complex, (4) Atg9L1, (5) the LC3 (mammalian homologue of yeast Atg8) system, and (6) the Atg12 system. Two ubiquitin (Ub)-like molecules, LC3 and Atg12, are covalently conjugated to phosphatidylethanolamine (PE) and Atg5, respectively. The Atg12–Atg5 conjugate associates with Atg16L1 to form a dimeric complex (referred to as the Atg16L1 complex; Mizushima et al., 2003; Fujita et al., 2009), and functions as an E3-like factor in the LC3 system (Fujita et al., 2008b). ULK1

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Abbreviations used in this paper: EBSS, Earle's balanced salt solution; FIP200, FAK family-interacting protein of 200 kD; MEF, mouse embryonic fibroblast; PE, phosphatidylethanolamine; *Salmonella*, *Salmonella enterica* serovar Typhimurium; Ub, ubiquitin; ULK, uncoordinated 51-like kinase.

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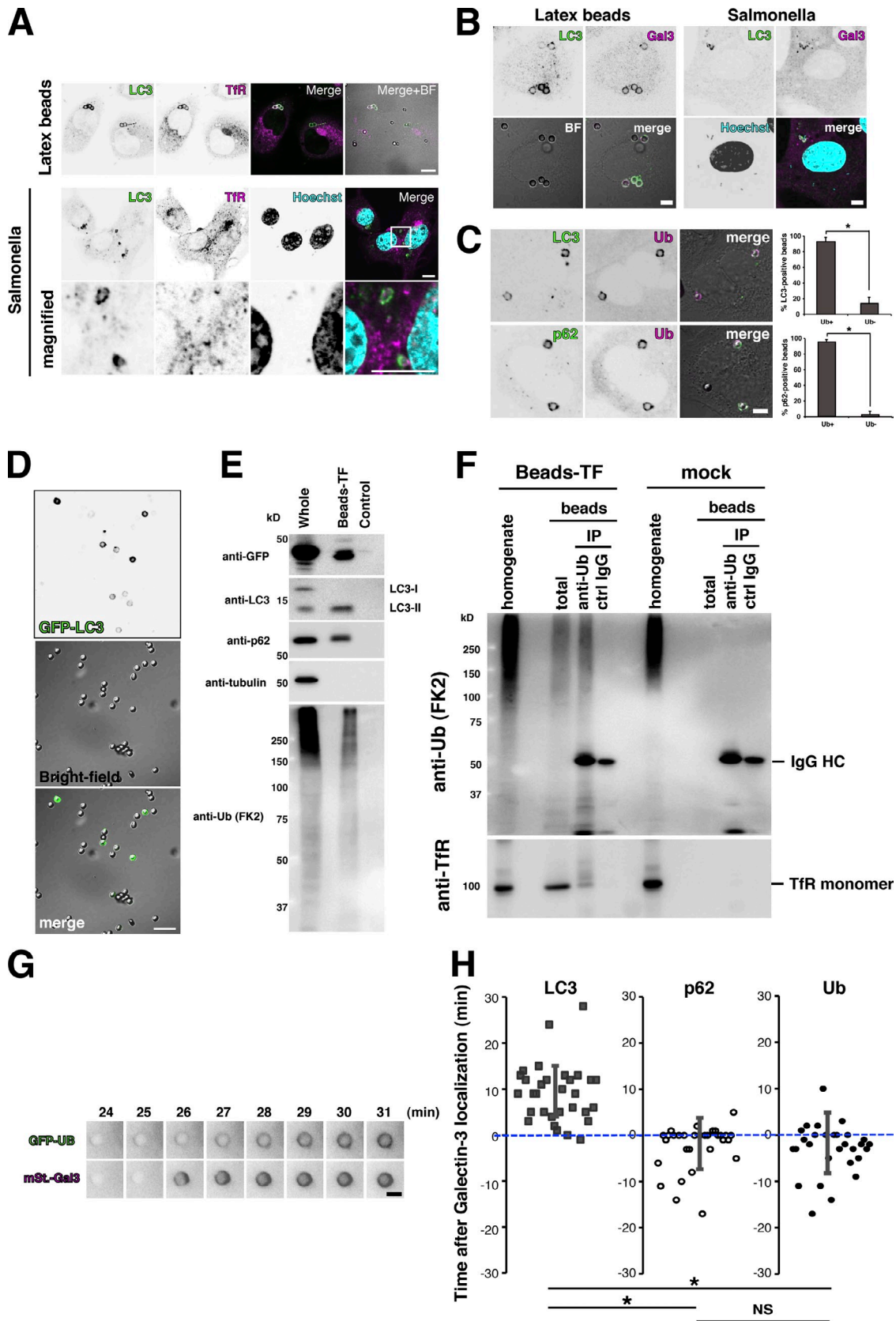


Figure 1. **Ub-positive endosomes containing *Salmonella* or beads are targeted by autophagy.** (A) HeLa cells were infected with *S. Typhimurium* (*Salmonella*) for 1 h or transfected with Effectene-coated latex beads for 3 h and then subjected to immunocytochemistry for LC3 and transferrin receptor (TfR). Bar, 10 μ m. (B) HeLa cells were infected with *Salmonella* for 1 h or transfected with Effectene-coated latex beads for 3 h and then subjected to immunocytochemistry for LC3 and galectin3. Bar, 5 μ m. (C) HeLa cells were transfected with Effectene-coated latex beads for 3 h and subjected to immunocytochemistry for LC3 and Ub (top) or LC3 and p62 (bottom). Bar, 5 μ m. The percentages of LC3- or p62-positive beads per Ub-positive (Ub+) or Ub-negative (Ub-) beads were

and ULK2, the mammalian homologues of yeast Atg1, are serine/threonine protein kinases that form a large protein complex with Atg13, a FAK family-interacting protein of 200 kD (FIP200), and Atg101 (referred to as the ULK1 complex; Mizushima, 2010). The ULK1 complex plays an essential role in initiating autophagosome formation (Mizushima, 2010).

Ubiquitination is thought to play important roles during xenophagy because LC3-positive bacteria are also decorated with Ub. However, it is largely unknown how Ub contributes to the autophagic response against invading bacteria (Fujita and Yoshimori, 2011). Furthermore, the target of ubiquitination is unknown, although plausible candidates are bacterial surface proteins.

How Ub is linked to autophagosome formation is still problematic, although it is widely accepted that adaptor proteins bridge ubiquitinated substrates and autophagosomal membranes by binding to both Ub and LC3, which localizes to autophagosomal membranes. The adaptors p62, NDP52, and optineurin possess both a Ub-binding domain and LC3-binding domain and are required for efficient selective autophagy against bacteria (Thurston et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009; Wild et al., 2011). However, it is still debated whether the selectivity in autophagy against bacteria is attributed only to LC3–adaptor interactions. We recently reported that the LC3 system is dispensable to localize other Atg proteins and to form the autophagic double membrane in response to invading bacteria (Kageyama et al., 2011). These findings suggest that the selectivity of autophagy is not accounted for by the interaction between adaptors and LC3 and other mechanisms that recruit other Atgs could be responsible for Ub selectivity. We also confirmed that both LC3 lipidation, which is required for autophagosome binding, and LC3 recruitment to the target depend on recruitment of the Atg16L1 complex, even if adaptors exist (Kageyama et al., 2011).

In this study, we propose a novel model for autophagy against bacteria using *Salmonella enterica* serovar Typhimurium (*S. Typhimurium* or *Salmonella*) and transfection reagent-coated latex beads as model substrates (Kobayashi et al., 2010). The ubiquitination of host proteins within bacteria- or bead-containing endosomes plays a role in induction of selective autophagy. Furthermore, we show that Atg16L1, one of the earliest recruited and upstream Atgs, both directly and indirectly recognizes the Ub-decorated endosomes through multiple pathways.

Results

Proteins on bead- or *Salmonella*-containing endosomes are ubiquitinated

It was previously suggested that *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is targeted by autophagy before

escaping from endosomes. (Birmingham and Brumell, 2006; Zheng et al., 2009; Kageyama et al., 2011; Thurston et al., 2012). In addition, it has been recently reported that polystyrene beads coated with transfection reagents are selectively targeted by LC3-positive autophagosomes after being endocytosed into cells, although uncoated beads are not sequestered by autophagy even if internalized into endosomes (Kobayashi et al., 2010). Presumably, damage of endosomes by transfection reagents triggers autophagy. Because LC3 colocalized with transferrin receptor in both *Salmonella*-infected and bead-transfected cells (Fig. 1 A), both *Salmonella* and the beads are targeted by autophagy while within endosomes. Furthermore, LC3-positive *Salmonella* and beads were galectin3-positive (Fig. 1 B). Galectin3 is a β -galactose-binding lectin and a good marker of damaged endosomes because the luminal glycochain becomes accessible to cytosolic galectin3 (Paz et al., 2010). These results suggest that autophagosomes form in response to *Salmonella*- or bead-containing endosomes whose membranes are broken and permeabilized, perhaps by the *Salmonella* type III secretion system or transfection reagents, respectively. In addition, Ub clearly surrounded the LC3-positive or p62-positive transfected beads in the same manner as during autophagy against invading bacteria (Fig. 1 C; Yoshikawa et al., 2009; Zheng et al., 2009).

To examine the targets of ubiquitination, we purified beads surrounded by autophagosomal membranes using density gradient centrifugation (Fig. 1 D). As a result, ubiquitinated proteins were detected in the bead-containing autophagosome fraction, which contains lipidated LC3 and p62 (Fig. 1 E). Because the artificial latex beads do not have any proteins on their surface, host cellular proteins must be ubiquitinated in the process. The most plausible ubiquitinated targets are endosomal proteins because autophagosomes sequester the beads within endosomes. In fact, the purified bead–autophagosome fraction contained ubiquitinated transferrin receptor, which is not typically ubiquitinated because it is a recycling receptor (Fig. 1 F). We reasoned that any endosomal protein could be a ubiquitination target. From these results, we conclude that endosomal membranes, which are damaged by the bacteria or beads within the endosomes, are ubiquitinated and targeted by autophagy.

We next asked the order of events among endosomal rupture, ubiquitination, and LC3 recruitment using dual-color live-cell imaging analysis. Ub appeared around the transfected beads nearly concomitantly with galectin3. p62 also appeared together with galectin3. By contrast, LC3 was always recruited after galectin3, and localized around the beads 0–15 min after galectin3 (Fig. 1, G and H; Videos 1 and 2; and unpublished data). Presumably, endosomal rupture rapidly triggers the ubiquitination of intrinsic proteins, after which LC3 is slowly recruited to the endosomes.

enumerated. Statistical analysis was performed by Student's unpaired *t* test. *, $P < 0.01$. (D and E) HeLa cells stably expressing GFP-LC3 were transfected with Effectene-coated latex beads for 3 h. Bead–autophagosomes were fractionated as described in Materials and methods. The bead–autophagosome fraction was observed by confocal microscopy (D; bar, 10 μ m) or lysed with RIPA buffer and subjected to Western blot analysis using the indicated antibodies (E). In a control sample, scraped cells were mixed with Effectene-coated beads and immediately homogenized. (F) The bead–autophagosome fraction was lysed and subjected to immunoprecipitation with an anti-Ub IgG antibody (FK2) or control IgG. Co-immunoprecipitated molecules were examined by Western blotting using the indicated antibodies. (G and H) NIH3T3 cells stably expressing mStrawberry (mStr)-Gal3 and GFP-LC3, mStr-Gal3, and GFP-p62, or mStr-Gal3 and GFP-Ub were transfected with Effectene-coated latex beads for 30 min and then washed. Live cells were observed at 1-min intervals by fluorescence microscopy. Bar, 3 μ m. The time after galectin3 localization was measured for at least 30 cases for each combination (H). Statistical analysis was performed by Student's unpaired *t* test. *, $P < 0.05$; NS, not significant.

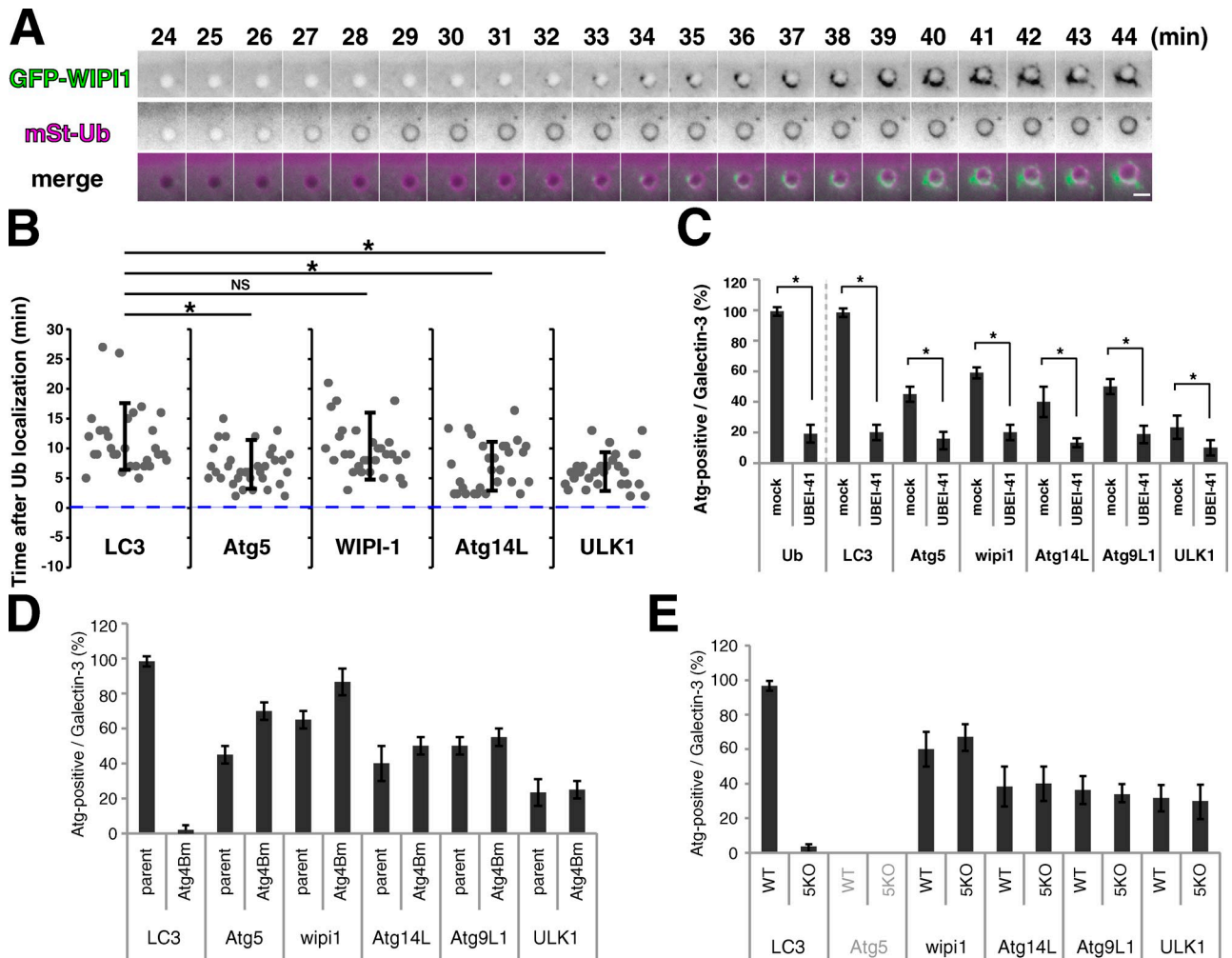


Figure 2. Ubiquitination and recruitment of Atg proteins. (A and B) NIH3T3 cells stably expressing mStr-Ub and GFP-tagged LC3, Atg5, WIPI-1, Atg14L1, or ULK1 were transfected with Effectene-coated latex beads for 30 min. Then, live cells were observed at 1-min intervals by fluorescence microscopy. Bar, 3 μ m. The time after Ub localization was measured for at least 30 cases for each combination. Each value represents the mean \pm SD. Statistical analysis was performed by Student's unpaired *t* test. *, $P < 0.05$; NS, not significant. (C) NIH3T3 cells stably expressing GFP-tagged Ub, LC3, Atg5, WIPI-1, Atg14L1, Atg9L1, or ULK1 were transfected with Effectene-coated beads for 3 h in the presence or absence (mock) of 30 μ M UBEI-41 (a ubiquitin E1-specific inhibitor) and subjected to immunocytochemistry for galectin3. The percentages of GFP-positive per galectin3-positive beads were enumerated. At least 30 beads were counted ($n = 3$). The values are the mean \pm SD. Statistical analysis was performed by Student's unpaired *t* test. *, $P < 0.05$. (D and E) Parent NIH3T3 cells, Atg4B mutant overexpressing NIH3T3 cells (D), wild-type MEFs, and Atg5-KO MEFs (E) stably expressing GFP-tagged LC3, Atg5, WIPI-1, Atg14L1, Atg9L1, or ULK1 were transfected with Effectene-coated latex beads for 3 h and subjected to immunocytochemistry for galectin3. The percentages of Atg-positive per galectin3-positive beads were enumerated. At least 30 beads were counted ($n = 3$). The values are the mean \pm SD.

Ubiquitination is required to recruit Atg proteins

To determine the order in which Ub as well as LC3 and other Atg proteins are recruited to the transfected beads, we performed live-cell imaging of bead-transfected NIH3T3 cells stably expressing mStr-tagged Ub and the GFP-tagged Atg proteins LC3, Atg5, WIPI1, Atg14L, and ULK1 (Fig. 2, A and B; and Videos 3–7). The trafficking of each Atg protein greatly differed (Videos 3–7). The time lag between the localization of each Atg protein and Ub was quantified (Fig. 2 B). All of the Atg proteins were recruited to the transfected beads after Ub recruitment. Remarkably, LC3 and WIPI1 localized to the beads after the other Atg proteins, suggesting that the autophagic machinery recognizes the ubiquitinated substrate independently of an LC3-mediated mechanism.

Next, to directly demonstrate the necessity of ubiquitination, we used a Ub-activating enzyme (E1)-specific inhibitor (UBEI-41) because it enables blockade of ubiquitination instantly in cultured cells (Yang et al., 2007). As expected, the number of Ub-positive beads among galectin3-positive beads was drastically reduced upon UBEI-41 treatment (Fig. 2 C, Ub). Galectin3 was recruited to transfected beads even in UBEI-treated cells, indicating that internalization of beads and endosomal rupture occurred in our experimental condition. We also found that administering UBEI-41 strongly inhibited LC3 recruitment (Fig. 2 C, LC3). Moreover, the localization of all of the other representative Atg proteins was severely affected by UBEI-41 treatment. These results indicate that ubiquitination plays an important role in recruiting not only LC3 but also other Atg proteins to invading pathogens.

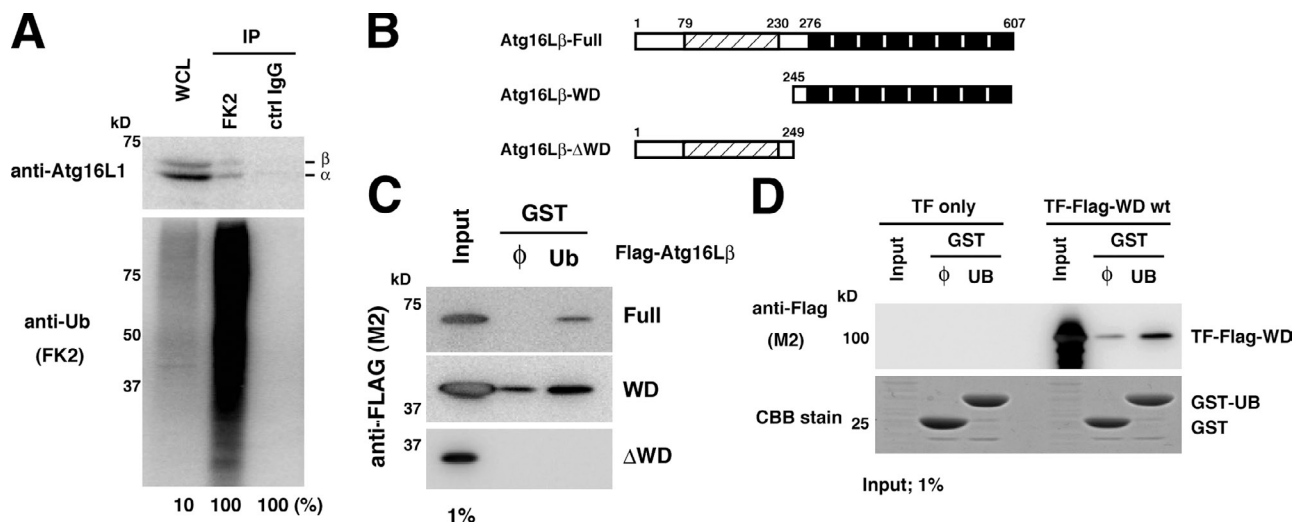


Figure 3. The WD β -propellers of Atg16L1 directly interact with Ub. (A) HeLa cells were lysed with 1% Triton X-100 lysis buffer and subjected to immunoprecipitation with FK2- or control IgG-immobilized beads. The coimmunoprecipitated molecules were examined by Western blotting using the indicated antibodies. (B) Schematic diagram of Atg16L1 (Full) and its deletion constructs. (C) Purified recombinant GST or GST-Ub-immobilized glutathione Sepharose beads were incubated with lysates from HEK293T cells transiently expressing FLAG-tagged Atg16L1 constructs for 1 h at 25°C with gentle agitation. The beads were washed three times with ice-cold PBS and the bound complexes were eluted with 50 mM reduced glutathione and then subjected to Western blotting for FLAG. (D) Purified GST or GST-Ub-immobilized glutathione Sepharose beads were incubated with lysates from bacteria expressing trigger factor (TF) or TF-FLAG-WD β -propellers for 1 h at 25°C with gentle agitation. Washing and elution were performed as described in C. The eluted samples were subjected to SDS-PAGE and Western blotting for FLAG.

Likewise, we systematically quantified the recruitment of Atg proteins to galectin3-positive structures in autophagy-deficient cells (Atg5-KO, Atg14L-KO, Atg9L1-KO, and FIP200-KO cells as well as cells expressing an Atg4B mutant in which the LC3 system is blocked) stably expressing a series of GFP-tagged Atg proteins (Fig. 2, D and E; and Fig. S1; Kuma et al., 2004; Fujita et al., 2008a; Hara and Mizushima, 2009; Matsunaga et al., 2009; Saitoh et al., 2009). The ULK1 complex, Atg9L1, and the Atg16L1 complex were recruited to galectin3-positive structures independently of other Atg proteins (Fig. 2, D and E; and Fig. S1), which is consistent with autophagy against *Salmonella* and Parkin-mediated mitophagy (Itakura et al., 2012; Kageyama et al., 2011), and our live-cell imaging data (Fig. 2, A and B). Altogether, it is clear that before LC3 recruitment, the three core autophagy machineries, notably the Atg16L1 complex, the ULK1 complex, and Atg9L1, independently recognize the ubiquitinated endosomes containing bacteria or beads.

Ub moieties vary from mono-Ub to extended chains, linked through seven lysine residues in Ub or via the N-terminal methionine residue (linear; Iwai and Tokunaga, 2009). It has been recently reported that K63- and linear-linked Ub chains clearly colocalize with invading *Salmonella* (van Wijk et al., 2012). To examine the presence of K48- and K63-linked Ub chains to the invading *Salmonella* or transfected beads, we used K48- or K63-specific antibodies (Newton et al., 2008). Both K48- and K63-linked Ub signals were well colocalized with poly-Ub signals (Fig. S2 A). This result and the previous report show that different Ub linkages, including K48, K63, and linear, could be part of the Ub coat on the invading *Salmonella* and transfected beads.

It remains enigmatic which linkage of Ub is required for selective autophagy. To test loss of function of K63-linked or linear-linked Ub chains, Ubc13^{fl/fl}, HOIL-1L KO, and their control

mouse embryonic fibroblasts (MEFs) were challenged with *Salmonella* (Yamamoto et al., 2006; Tokunaga et al., 2009). Ubc13 is an E2 enzyme crucial for generating K63-linked chains (Hofmann and Pickart, 1999), and HOIL-1L is an accessory protein of the LUBAC E3 ligase complex that is very important for efficient formation of linear-linked Ub chains (Tokunaga et al., 2009). We found that neither Ubc13^{fl/fl} nor HOIL-1L KO blocked LC3 recruitment to the Ub-positive *Salmonella* (Fig. S2, B and C). Collectively, these results indicate that autophagic machinery targeting did not depend solely on K63- or linear-Ub linkages.

The WD β -propellers of Atg16L1 directly interact with Ub

How do the three core autophagy machineries recognize the Ub-positive substrate? We focused on Atg16L1 because it contains a C-terminal WD β -propeller domain, whose function is unknown, but the same domain in several other proteins reportedly functions as an Ub-binding domain (Pashkova et al., 2010). Therefore, we tested the possibility that Ub directly interacts with the WD β -propellers of Atg16L1. First, we examined whether endogenous Atg16L1 coimmunoprecipitated with polyubiquitinated proteins. As shown in Fig. 3 A, Atg16L1 was detected in FK2 (a monoclonal antibody against poly Ub)-immunoprecipitated samples, but not in a control sample immunoprecipitated with IgG. To examine the interaction more directly, a GST pull-down experiment was performed. Purified GST or GST-Ub was immobilized to glutathione Sepharose beads (Fig. S3 A) and incubated with lysates of HEK293T cells transiently expressing a series of FLAG-tagged Atg16L1 constructs, including full-length Atg16L1, the WD β -propellers alone, or Atg16L1 lacking the complete WD region (Δ WD; Fig. 3 B). Full-length and the WD β -propellers bound to GST-Ub more efficiently than to

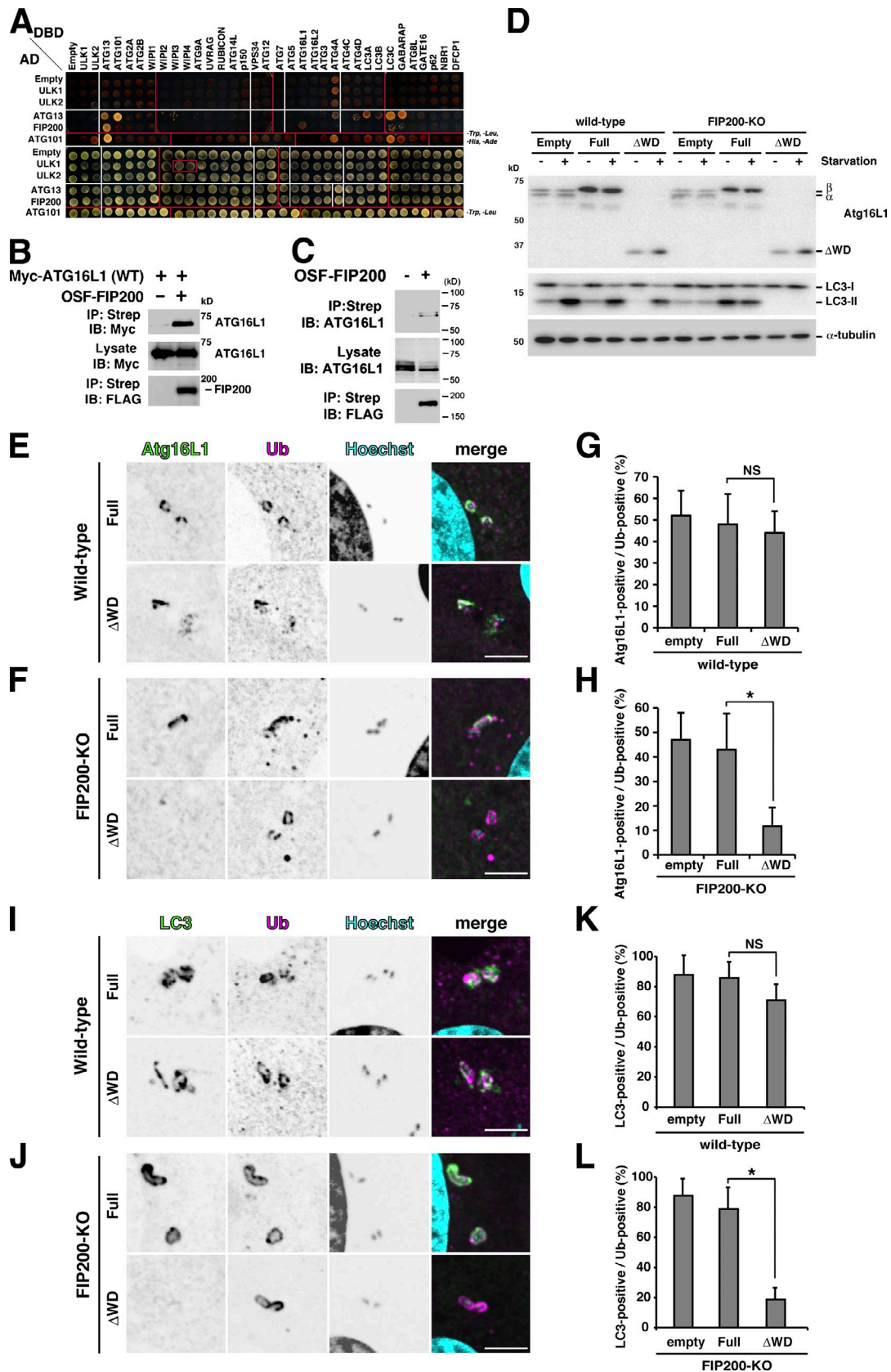


Figure 4. The WD β -propellers of Atg16L1 recruit LC3 in the absence of FIP200. (A) Yeast two-hybrid interactions of the ULK1 complexes with other human Atg proteins. ULK1, ULK2, Atg13, FIP200, and Atg101-AD fusions (rows 2–6) or control AD constructs (row 1, Empty) were coexpressed with control DBD constructs (column 1, Empty) or Atg protein–DBD fusions (columns 2–35) and tested for positive yeast two-hybrid interactions (top) and cotransformation (control, bottom). White lines divide images derived from the same plate, and red lines divide images from different plates. Atg16L1 positively interacted with FIP200 in this assay. (B) FIP200 binds to exogenously expressed Atg16L1. Myc-Atg16L1 coprecipitations with an empty vector control (lane 1) or OneStRep-FLAG

GST alone. In contrast, the Δ WD mutant did not bind to GST-Ub (Fig. 3 C). Next, we prepared recombinant trigger factor-FLAG-tagged WD β -propellers of Atg16L1 using bacteria (Fig. S3 B). A GST pull-down experiment was performed with these recombinant proteins. The WD β -propellers bound to GST-Ub more efficiently than to GST alone (Fig. 3 D). From these results, we concluded that there is a direct interaction between the WD β -propellers of Atg16L1 and Ub.

Atg16L1 is recruited by binding to Ub and FIP200

Although Atg16L1 could bind to Ub, deleting the WD β -propellers only minimally affected Atg16L1 recruitment to invading *Salmonella* (Fujita et al., 2009), suggesting that binding to Ub is not sufficient for Atg16L1 recruitment and additional interacting partner(s) functions in the recruitment. Thus, we searched for the Atg16L1 complex-interacting proteins using mass spectrometry. We found that ULK1 and FIP200 coimmunoprecipitated with the Atg16L1 complex, which consists of Atg16L1, Atg12, and Atg5 (Table S1). Because ULK1 forms a large protein complex including ULK1/2, Atg13, FIP200, and Atg101 (Mizushima, 2010), we performed a direct yeast two-hybrid assay with these proteins and found that Atg16L1 directly interacts with FIP200 (Fig. 4 A). The interaction was also confirmed by immunoprecipitation experiments (Fig. 4, B and C). Thus, we found that Atg16L1 binds to FIP200 in addition to Ub.

Next, to test the roles of these interactions in autophagy, we generated Atg16L1-replaced wild-type and FIP200-KO cells (Fig. 4 D). We previously reported that stably expressing an exogenous Atg16L1 construct can replace the endogenous Atg16L1 protein with the exogenous one (Fujita et al., 2009; see legend of Fig. 4 for more details). In sharp contrast to wild-type cells, in FIP200-KO cells the recruitment of Atg16L1 to Ub-positive *Salmonella* and beads depended on the WD β -propellers (Fig. 4, E and H; and unpublished data). Next, we examined the effect of deleting the WD β -propellers on LC3 recruitment to Ub-positive *Salmonella* or beads because the Atg16L1 complex functions as an E3-like factor in the LC3 system (Fujita et al., 2008b). In wild-type cells, LC3 recruitment was not remarkably affected by deleting the WD β -propellers as was previously reported (Fig. 4, I and K; Fujita et al., 2009). On the contrary, deleting the WD β -propellers significantly decreased the localization of LC3 to Ub-positive *Salmonella* and beads in FIP200-KO cells (Fig. 4, J and L; and unpublished data). We also confirmed that LC3 lipidation was totally dependent on the

WD β -propellers of Atg16L1 (Fig. 4 D). These results show that the localization of the Atg16L1 complex to Ub-positive *Salmonella* or beads involves interactions with both Ub via the WD β -propellers and FIP200, resulting in PE conjugation of LC3. Because the ULK1-FIP200 complex recruitment also depends on ubiquitination (Fig. 2 C), Atg16L1 must recognize Ub on the target through two mechanisms, one of which is direct recognition and the other is indirect.

To address the impact of the WDR-mediated mechanisms on the order of the Atg16L1 and ULK1-FIP200 complex recruitment, we quantified the localization of ULK1 and Atg16L1 in Ub-positive *Salmonella* in Atg16L1-reconstituted cells. The deletion of WDR did not affect the percentage of Atg16L1-positive in ULK1-GFP-positive population (Fig. S4 B). In sharp contrast to this, the deletion of WDR significantly decreased the percentage of Atg16L1-positive in ULK1-GFP-negative population (Fig. S4 C). These results suggest that the FIP200 is recruited to the Ub-positive *Salmonella* before Atg16L1 in the absence of WDR-mediated mechanisms.

Atg16L1 recognizes the target via three pathways

We further explored the interaction between Atg16L1 and FIP200. Using a yeast two-hybrid analysis, we found that Atg16L1 bound to both the FIP200-N (1-840) and FIP200-C (1276-1591) fragments (Fig. 5 A), whereas the Atg16L1 Δ WD mutant (1-246) bound only to FIP200-C (Fig. 5, B and E). We decided to focus on the interaction between Atg16L1 and the FIP200-C fragment in order to determine why Δ WD can fulfill autophagic functions in the presence of FIP200. In a yeast two-hybrid analysis, the FIP200-C fragment bound to the Atg16L1 (1-246) fragment but not to the Atg16L1 (1-230) fragment, suggesting the importance of residues 230-246 for this interaction (Fig. 5 B). Thus, we performed alanine scanning in this region, and found that residues 239-246 were important for this interaction (Fig. 5, C and D). We confirmed the impact of 239-242A mutations within Atg16L1 on its ability to interact with the FIP200-C fragment in an immunoprecipitation experiment (Fig. 5 F). Thus, we obtained an Atg16L1 mutant that lacks affinity for FIP200.

We then generated a series of cells reconstituted with various Atg16L1 mutants in which Atg16L1 Δ /MEFs stably express the above-mentioned Atg16L1 constructs (Fig. 6 A; and Fig. S5 A). The Atg16L1-reconstituted cells were challenged with *Salmonella* or transfected with beads, and the percentages of LC3- or Atg16L1-positive per Ub-positive were counted. The

(OSF)-FIP200 (lane 2). (C) FIP200 binds endogenous Atg16L1. Atg16L1 coprecipitations with an empty vector control (lane 1) or OSF-FIP200 (lane 2). (D) Wild-type or FIP200-KO MEFs stably expressing the empty vector, full-length Atg16L1, or Δ WD mutant were incubated in growth medium (-) or Earle's balanced salt solution (EBSS) (+) for 1 h and examined by Western blotting using the indicated antibodies. We previously reported that stable expression of an exogenous Atg16L1 construct can be used to replace the endogenous Atg16L1 protein with an exogenous Atg16L1 protein (Fujita et al., 2009) because free Atg16L1 molecules not complexed with the Atg12-Atg5 conjugate are preferentially degraded by the ubiquitin-proteasome system. In control cells (Empty), two Atg16L1 splicing variants, the α - and β -forms, were detected. In full-length β -form-replaced cells (Full), the α -form was not detected, whereas in Δ WD-replaced cells (Δ WD) both the α - and β -forms were minimally detected. Thus, we successfully obtained cells that express only endogenous levels of the full-length or Δ WD mutant. (E-L) Atg16L1-replaced wild-type or FIP200-KO MEFs were infected with *Salmonella* (MOI = 10) for 1 h and subjected to immunocytochemistry for Atg16L1 and Ub (E and F) or LC3 and Ub (I and J). The percentages of Atg16L1-positive (G and H) or LC3-positive per Ub-positive bacteria were enumerated (K and L). At least 50 *Salmonella* were counted. The average \pm SD is shown for three independent experiments. Statistical analysis was performed by Student's *t* test. *, *P* < 0.05; NS, not significant. Bar, 5 μ m.

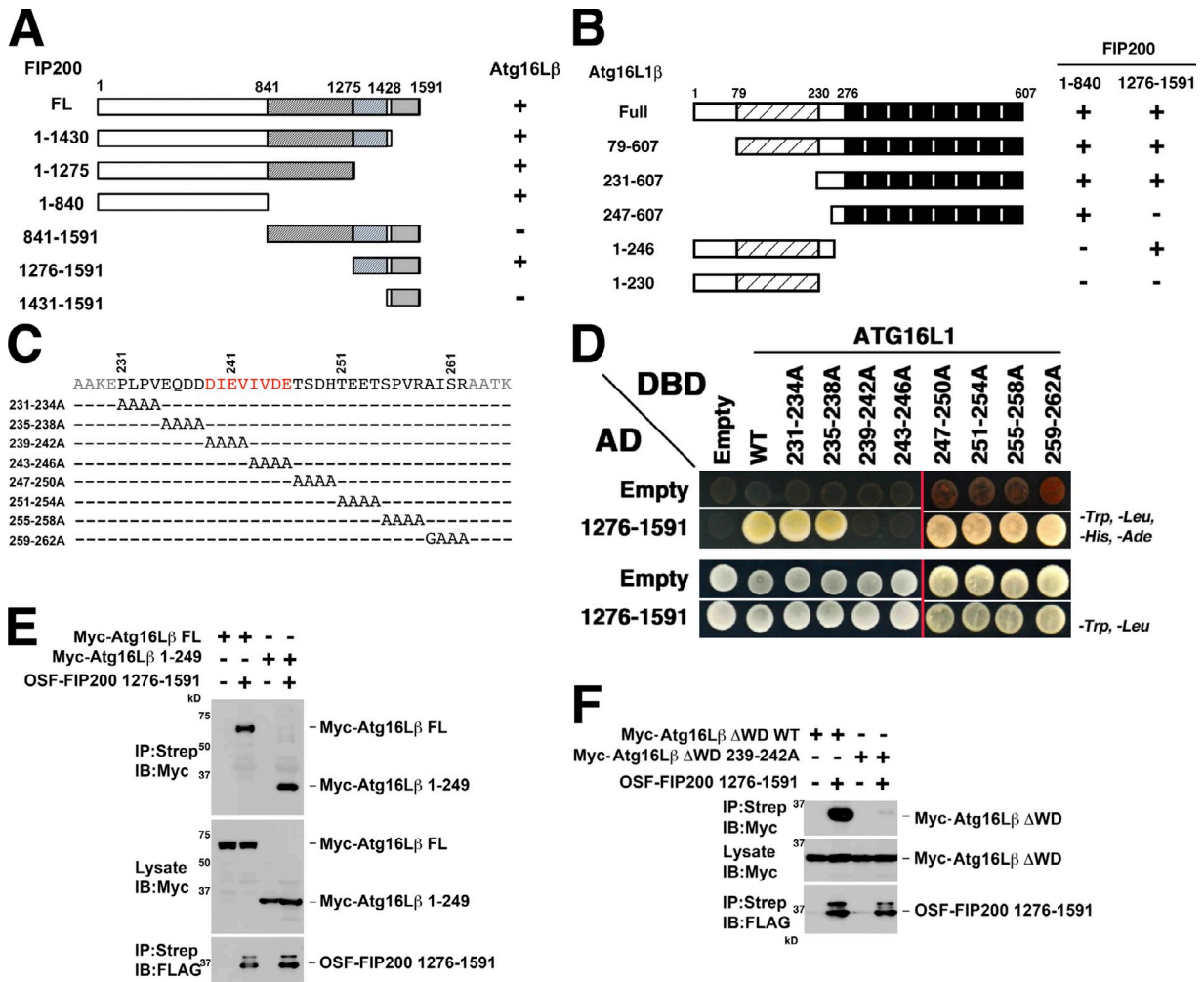


Figure 5. **Atg16L1-FIP200 interacting domains.** (A) Mapping of the Atg16L1-binding site on FIP200. Yeast two-hybrid mapping experiments showing that Atg16L1 binds to both the N-terminal and C-terminal coiled-coil regions of FIP200. (B) Mapping of the FIP200-binding site within Atg16L1. Yeast two-hybrid mapping experiments showing that the 1–840 fragment of FIP200 binds to the C-terminal 247–607 region, while the 1276–1591 fragment of FIP200 binds to the N-terminal 1–264 region of Atg16L1. (C and D) Additional yeast two-hybrid mapping experiments showing that the binding site of the FIP200 1276–1591 fragment maps to residues 239–246 of Atg16L1. White lines divide images derived from the same plate, and red lines divide images from different plates. (E) The N-terminal 1–249 fragment of Atg16L1 is sufficient to interact with FIP200. Full-length (lanes 1 and 2) or the N-terminal 1–249 fragments (lanes 3 and 4) of Myc-Atg16L1 coprecipitates with empty vector controls (lanes 1 and 3) or OSF-FIP200 1276–1591 (lanes 2 and 4). (F) Co-precipitation experiments confirming that OSF-FIP200 1276–1591 coprecipitates with wild-type (WT) Myc-Atg16L1 (1–249) fragments, but not with analogous fragments containing a mutant FIP200-binding site (Myc-Atg16L1 Δ WD 239–242A).

localization of LC3 or Atg16L1 to invading bacteria and transfected beads was slightly affected by the Δ WD + 239–242A mutation, but not significantly abolished (Fig. 6, B–E; and Fig. S5, C–F). This suggests that, besides interactions with FIP200 and Ub, another mechanism functions to localize the Atg16L1 complex in selective autophagy. We previously reported that residues 193–230 in Atg16L1 are essential for the inhibitory effects of excess Atg16L1 on autophagy (Itoh et al., 2008). Thus, we further explored this region and found that substituting residues W194 and M195 with alanine (194–195A) synergistically affected the function of Atg16L1 in both canonical and selective autophagy (Fig. 6 and Fig. S5). Although the W194–M195 mutations alone slightly affected autophagic activity, a combination of 239–242A, 194–195A, and the Δ WD mutation synergistically reduced the recruitment of LC3 and Atg16L1 to the ubiquitinated substrate. Thus, we propose that the Atg16L1

complex localizes and directs LC3 to ubiquitinated substrates through these three independent mechanisms.

Intriguingly, in contrast to selective autophagy, starvation-induced nonselective autophagy requires only Ub and FIP200 binding of Atg16L1 (Fig. 6 F and Fig. S5). Full-length 239–242A minimally affected both PE conjugation and LC3 puncta formation in starvation-induced autophagy (Fig. 6 F; and Fig. S5, A and G). Furthermore, deleting the WD β -propellers in Atg16L1 minimally affected autophagosome formation and p62 degradation (Fig. 6 F; and Fig. S5, B and G), as we previously reported (Fujita et al., 2009). In sharp contrast to these mutants, a combination of the Δ WD and 239–242A mutations almost completely reduced LC3 puncta formation under starvation conditions (Fig. 6 F). We also confirmed that the Δ WD + 239–242A mutation significantly reduced the number of Atg16L1 puncta (Fig. S5 H). Therefore, FIP200 binding and the WD β -propellers of Atg16L1

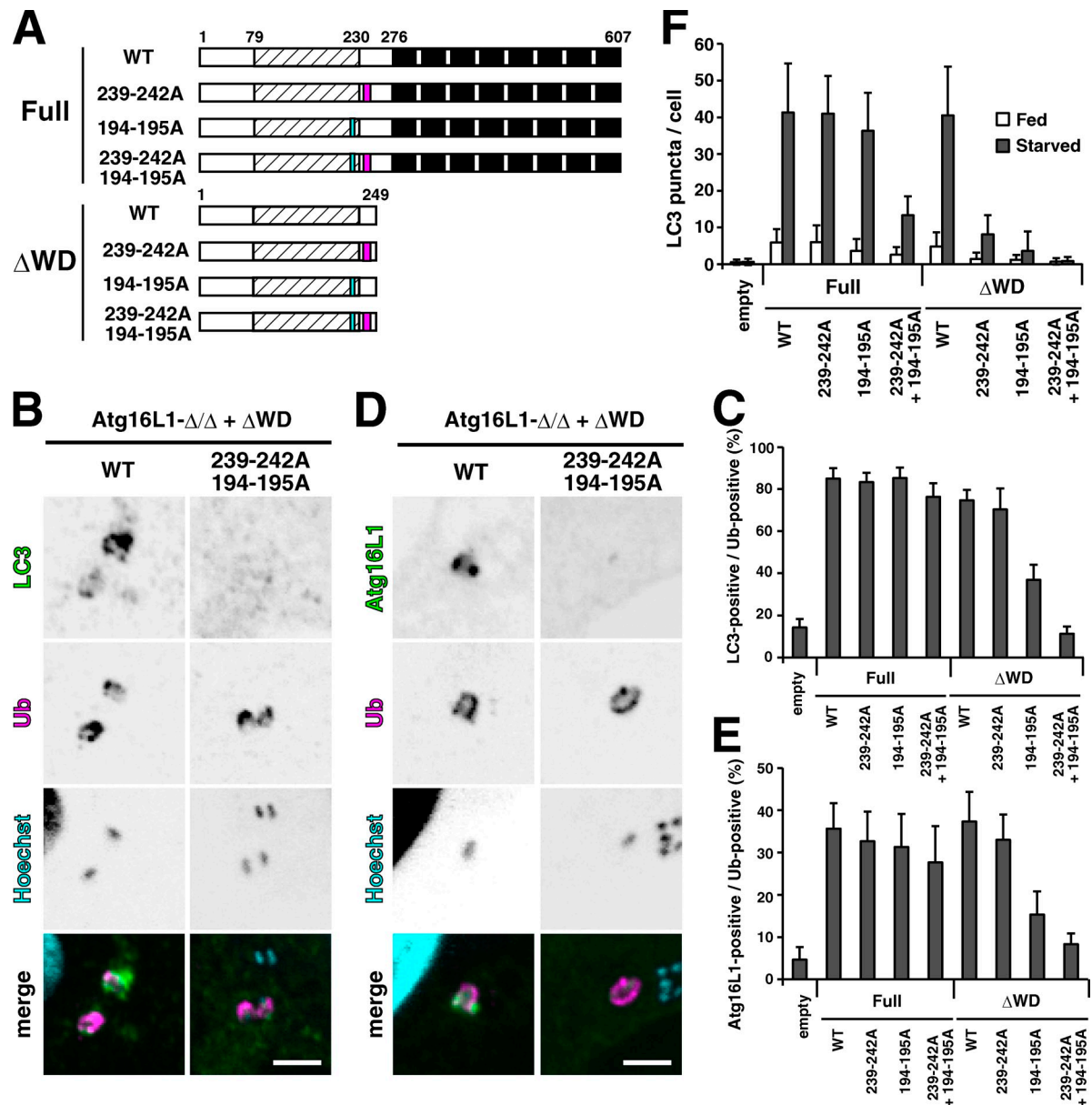


Figure 6. **Effects of Atg16L1 mutations on autophagy against *Salmonella*.** (A) Schematic diagram of Atg16L1 and various mutants. The magenta box indicates 239–242A and the cyan box indicates 194–195A. (B–E) Atg16L1-Δ/Δ MEFs stably expressing the indicated constructs were infected with *Salmonella* (MOI = 10) for 1 h and then analyzed by immunocytochemistry for LC3 (B) or Atg16L1 (D). Bar, 5 μm. The percentage of LC3- or Atg16L1-positive *Salmonella* per Ub-positive *Salmonella* per Ub-positive *Salmonella* was enumerated by fluorescence microscopy (C and E). At least 50 bacteria were counted. The average ± SD is shown for three independent experiments. (F) Atg16L1-Δ/Δ MEFs stably expressing the indicated constructs were cultured in growth medium (Fed) or EBSS (Starved) for 1 h and then subjected to immunocytochemistry using an anti-LC3 antibody. The number of LC3 puncta in each cell was counted for more than 50 cells. The average ± SD is shown for three independent experiments.

are sufficient to localize the Atg16L1 complex under starvation conditions, indicating that there are different mechanisms in selective and nonselective autophagy.

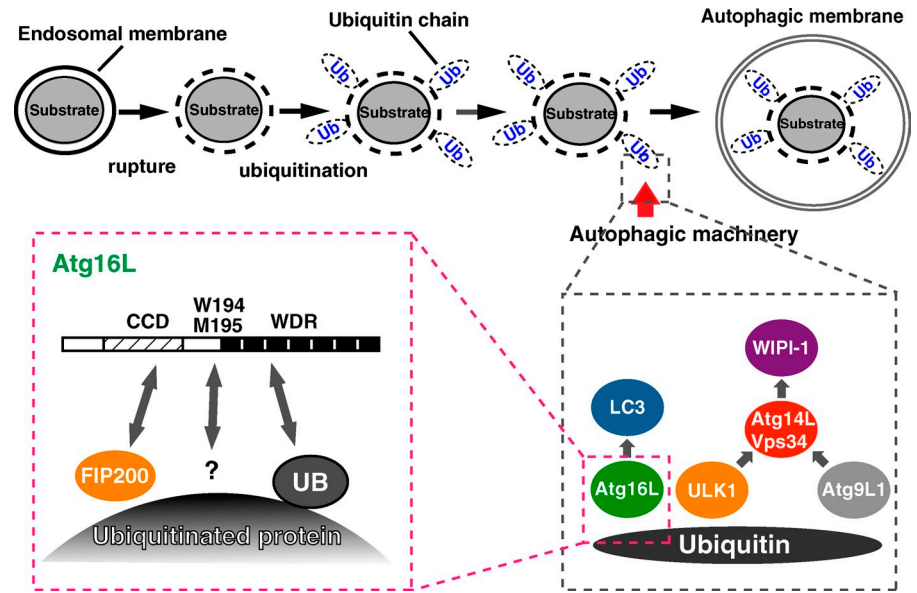
Discussion

In this paper, we revealed that ubiquitination plays an important role in the autophagic response to invading pathogens, notable *Salmonella*. First, we have shown that the endosomal membrane surrounding *Salmonella* and latex beads is ubiquitinated. Second, ubiquitination is necessary for recruiting three pivotal components of the autophagic machinery, notably the Atg16L1 complex,

the ULK1 complex, and Atg9L1. Third, Atg16L1 directly binds to Ub and FIP200. These interactions are redundant in both starvation-induced and autophagy against *Salmonella*/transfected bead. Thus, we have provided molecular evidence of the ubiquitinated target and determined how ubiquitination recruits the autophagic machinery (Fig. 7).

Our model is significantly different from the current prevailing model in which bacteria that have escaped into the cytosol are ubiquitinated and targeted by autophagy (Thurston et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009; Wild et al., 2011). Consistent with our model, electron microscopic analysis showed that a significant fraction of bacteria within cells is

Figure 7. **A possible mechanism of Ub-mediated autophagic sequestration of invading bacteria and transfection reagent-coated latex beads.** After internalization and endosomal rupture, Ub is conjugated to host cellular proteins in endosomes that contain *Salmonella* or transfection reagent-coated latex beads (top). Ub would be recognized by three pivotal components of the autophagic machinery, notably the Atg16L1 complex, the ULK1 complex, and Atg9L1 (bottom right). Atg16L1 localizes to the ubiquitinated target through three mechanisms, the interaction of coiled-coil domain (CCD) with FIP200, the interaction of coiled-coil domain (CCD) with FIP200, the interaction of WD repeats (WDR) with Ub, and the 194–195 region-mediated mechanism (bottom left).



contained within multilamellar compartments (Zheng et al., 2009; Kageyama et al., 2011). Our model is also consistent with these two recent papers that diacylglycerol (DAG) is involved in selective autophagy (Shahnazari et al., 2010), as the localization of DAG surrounding *Salmonella* indicates the existence of a membrane, and galectin8 targets damaged endosomes to autophagy via an NDP52-mediated mechanism (Thurston et al., 2012). It is still unclear how host endosomal proteins are ubiquitinated. Upon membrane rupture, the luminal side of endosomal proteins is exposed to the cytosol, which may lead to their ubiquitination. Alternatively, a drastic change in the endosomal ion concentrations may trigger ubiquitination. In any case, host cells likely have a simple and common mechanism that targets invading pathogens for selective autophagy, despite differences in the invading bacterial species. If bacterial proteins are directly ubiquitinated, diverse E3 ligases are required to recognize and target diverse bacterial species and it will be important to identify the E3 ligase(s) that function in selective autophagy. Furthermore, we do not exclude the possibility that bacterial proteins are ubiquitinated; however, our data indicate that neither bacterial escape from endosomes nor the ubiquitination of bacteria are essential for selective autophagy. It was previously shown that membrane remnants resulting from the endosomal escape of *Shigella flexneri* in infected cells colocalized with polyubiquitinated proteins and were targeted for autophagic degradation (Dupont et al., 2009). These membrane remnants were likely targeted by a similar mechanism that is used to degrade other invading bacteria. All bacteria that enter the cytosol have to cross a membrane, which possibly involves membrane rupture. Therefore, the recognition of Ub-positive damaged membranes might be a general mechanism that can respond to different types of invading bacteria.

Ub has been generally proposed to play a role in selective autophagy. Very recently, it has been reported that LRSAM1 functions as the E3 ligase responsible for Ub-mediated anti-*Salmonella* autophagy (Huett et al., 2012). In this study, we showed that blocking ubiquitination by an E1-specific inhibitor

leads to a defect in selective autophagy using latex beads and a galectin3 marker system. We further substantiated these findings by discovering that Atg16L1 directly binds Ub. In the absence of ubiquitination, the recruitment of other pivotal Atg units, notably the ULK1 complex and Atg9L1, was disrupted (Fig. 2 C). It has been proposed that adaptor molecules, such as p62, NDP52, and optineurin, bridge ubiquitinated substrates and autophagosomal membranes through LC3 (Thurston et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009; Wild et al., 2011). This model predicts that adaptors recruit the autophagic machinery as an LC3-positive structure. In this study, we systematically explored the hierarchy of Atg proteins in selective autophagy and found that three Atg functional units, the ULK1 complex, Atg9L1, and the Atg16L1 complex localized to ubiquitinated substrates independently of other Atg proteins (Fig. 2, D and E; Fig. S1; and Fig. 7). Furthermore, we showed that LC3 localized to these substrates after the appearance of these three units. Our data indicate that LC3 is dispensable for the initial stage of selective autophagy. However, we note that it is possible that the adaptor-LC3 interaction might function in other aspects, such as a zipper between ubiquitinated substrates and autophagosomal membranes to facilitate efficient enveloping.

Although we used an E1 inhibitor to block ubiquitination, the drug blocks all of the ubiquitination reactions in the cells nonspecifically, which might affect Atg protein recruitment indirectly. LRSAM1 contributes the ubiquitination to invading bacteria. However, invading bacteria are still ubiquitinated even in LRSAM1-deficient cells (Huett et al., 2012), indicating that other E3 enzyme(s) besides LRSAM1 is also involved in the ubiquitination accompanying bacteria invasion. To test the contribution of ubiquitination to selective autophagy more directly, a set of E3 enzymes should be identified in the future studies.

Our data and the previous report indicate that at least K48-, K63-, and linear-linked Ub chains localize to the invading *Salmonella* (Fig. S2 A; van Wijk et al., 2012). And in vitro *Salmonella* ubiquitination reaction by LRSAM1 predominantly induces K6- and K27-linked Ub chains (Huett et al., 2012).

These evidences indicate that different Ub-linkages could be part of the Ub-coat on the invading *Salmonella*. Further, we showed that autophagic machinery targeting did not depend solely on K63- or linear-Ub linkages (Fig. S2, B and C). These results suggest that autophagic machinery could target different Ub linkages, or other linkages besides K63 and linear might be specifically favored by autophagic machinery.

Atg16L1 directly interacts with ubiquitin and FIP200 through the WD β -propellers and residues 239–242, respectively (Figs. 3–5). The WD β -propellers of Atg16L1 are required to localize the Atg16L1 complex and LC3 to ubiquitinated substrates in the absence of FIP200 (Fig. 4, E–L). This result explains our previous observation that LC3 localized to a single membrane in *Salmonella*-infected FIP200-KO cells (Kageyama et al., 2011). Because the WD β -propellers have an affinity for Ub (Fig. 3) and host cellular proteins are ubiquitinated upon bacterial invasion (Fig. 1 and Fig. S1), PE conjugation of LC3 could occur in Ub-positive endosomal membranes in FIP200-KO cells. We do not have a clear answer as to why Atg16L1 has dual (or triple) recruiting mechanisms. It is noteworthy that Atg16 in simple eukaryote-like budding yeast lacks the WD β -propellers. One interesting possibility is that higher eukaryotes such as mammals have evolved to use autophagy to clear invading pathogens, and to secure such a complex process Atg16L1 may be involved in a back-up system. Interestingly, the WD β -propellers also seem to be involved in starvation-induced autophagy. p62-positive foci are observed at the autophagosome formation site (Itakura and Mizushima, 2010). We also observed Ub-positive signals at the starvation-induced autophagosome formation site (unpublished data). Thus, mammals may be evolved to use the Ub-binding capacity of Atg16L1 in starvation-induced autophagy.

A combination of Atg16L1 239–242A and Δ WD mutations severely impaired starvation-induced autophagy (Fig. S5), while selective autophagy against invading pathogens still occurred (Fig. 6; and Fig. S5, C–F). This indicates that interactions between Atg16L1 and FIP200 or Ub are not sufficient to localize the Atg16L1 complex in selective autophagy. We identified other important amino acids, 194–195 in Atg16L1 (Fig. 6 and Fig. S5). Because the Δ WD, 239–242A, and 194–195A mutations synergistically reduced the recruitment of Atg16L1 to the substrate (Fig. 6 and Fig. S5), we propose that all of these regions help localize the Atg16L1 complex to the ubiquitinated substrate (Fig. 7). Because residues 194–195 of Atg16L1 do not appear to be involved in FIP200 binding, Atg16L1 dimerization, or interactions with other Atg proteins (unpublished data), the region could interact with an unidentified factor. Interestingly, the Atg16L1 Δ WD construct contains the 194–195 region, although expression of the Δ WD mutant severely blocked LC3 recruitment in FIP200-KO cells (Fig. 4, J and L). The absence of a double membrane in these cells may lead to loss of the interaction partner for the 194–195 region, although we do not know the target(s) of this region.

In this study, we have uncovered the mechanisms by which selective autophagy responds to invading pathogens and shown that ubiquitinated substrates are recognized by the Atg16L1 complex independently of an LC3-mediated mechanism. These

discoveries are similar to another Ub-mediated endosomal process, the multi-vesicular body pathway. Cargo proteins are ubiquitinated and the molecular machinery involved in this pathway, called ESCRT, is recruited by directly binding to Ub (Katzmann et al., 2001). Furthermore, our model may be applicable to Parkin-mediated autophagy against mitochondria, in which mitochondria are also decorated with Ub (Youle and Narendra, 2011). Thus, we provide a novel role for Ub among its many diverse functions. Our findings will open up new avenues in the study of autophagy.

Materials and methods

Reagents and antibodies

Cell culture reagents were purchased from Invitrogen. The following antibodies were used: anti-mouse Atg16L1 (Mizushima et al., 2003), anti-p62 (MBL), anti-LC3 (MBL), anti-transferrin receptor (Invitrogen), anti-poly Ub (clone FK2; BIOMOL), anti-K48 linked Ub (clone Apu2; EMD Millipore), anti-K63 linked Ub (clone Apu3; EMD Millipore) anti-galectin3 (Santa Cruz Biotechnology, Inc.), anti-Lamp1 (Santa Cruz Biotechnology, Inc.), anti-FLAG (clone M2; Sigma-Aldrich), anti-GFP (Roche), anti-Myc (clone 9E10), and anti- α -tubulin (clone B5-1-2; Sigma-Aldrich). All other reagents were purchased from Sigma-Aldrich.

DNA engineering and recombinant retroviruses

The pMRX-IRES-puro and pMRX-IRES-blast vectors were gifts from S. Yamaoka (Tokyo Medical and Dental University, Tokyo, Japan; Saitoh et al., 2003). To generate recombinant retroviruses, cDNAs corresponding to mStrawberry (mStr)-tagged galectin3, mStr-Ub, GFP-Ub, and GFP-p62 were subcloned into the pMRX-IRES-puro vector. Various human Atg16L1 β (isoform-1) mutants were cloned into the pMRX vector, including full-length (1–607), Δ WD repeat domain (1–249), and the Crohn's disease-associated mutant (T300A; Fujita et al., 2009). In addition, pMRX constructs were generated to encode various GFP-tagged Atg proteins, including LC3 (N terminus), Atg5 (N terminus), WIPI1 (N terminus), Atg14L (N terminus), Atg9L1 (C terminus), and ULK1 (C terminus; Kageyama et al., 2011), as previously reported. Recombinant retroviruses were prepared as previously described (Saitoh et al., 2003). To prepare recombinant proteins, cDNA corresponding to Ub was subcloned into the pGEX6P-1 vector, and cDNA corresponding to FLAG-tagged wild-type or the Crohn's disease-associated mutant of the WD β -propellers of Atg16L1 was subcloned into the pCold-TF vector (Takara Bio Inc.).

Cell culture and retroviral infections

Plat-E cells were provided by T. Kitamura (The University of Tokyo, Tokyo, Japan; Morita et al., 2000). HeLa cells, HEK293T cells, NIH3T3 cells, wild-type MEFs, and autophagy-deficient MEFs (Atg5-KO, Atg14L-KO, Atg9L1-KO, Atg16L1- Δ/Δ , and FIP200-KO) were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 U/ml penicillin, and 50 U/ml streptomycin in a 5% CO₂ incubator at 37°C (Kuma et al., 2004; Fujita et al., 2008a; Saitoh et al., 2008, 2009; Hara and Mizushima, 2009; Matsunaga et al., 2009; Nishimura et al., 2013). Stable transformants were selected in growth medium with 1 μ g/ml puromycin or 5 μ g/ml blasticidin (Invivogen).

Western blotting

Samples were subjected to SDS-PAGE, and transferred to polyvinylidene fluoride membranes. The membranes were blocked with TBST (TBS and 0.1% Tween 20) containing 1% skim milk and were then incubated overnight at 4°C with primary antibodies 1,000–3,000 \times diluted in the blocking solution. Membranes were washed three times with TBST, incubated for 1 h at room temperature with 10,000 \times dilutions of HRP-conjugated secondary antibodies (GE Healthcare) in the blocking solution, and washed five times with TBST. Immunoreactive bands were then detected using ECL plus (GE Healthcare) and a chemiluminescence detector (LAS-3000, Fujifilm; Kimura et al., 2009).

Bacterial infections and bead transfections

Salmonella enterica serovar Typhimurium (SR-11 x3181) was provided by the Research Institute of Microbial Disease, Osaka University (Osaka, Japan). Bacteria were grown overnight at 37°C, and then sub-cultured at

1:33 for 3 h in LB without antibiotics. The bacterial inocula were prepared by pelleting at 10,000 *g* for 2 min, and were then added to host cells at a multiplicity of infection (MOI) of 10–100 at 37°C with 5% CO₂ (Kageyama et al., 2011). Bead transfections were performed as previously reported (Kobayashi et al., 2010). Transfection reagent-coated beads were prepared by mixing the beads (NH₂, 17145–5; PolySciences, Inc.) with Effectene transfection reagent (301425; QIAGEN), according to the manufacturer's instructions except that bead suspension was used instead of DNA solution. The resulting bead mixture (~100 μ l) was further mixed with 1 ml of growth medium, and added to cells by replacing the medium. After incubation with the bead mixture for 1 h at 37°C in a CO₂ incubator, the cells were washed twice with fresh growth medium to remove unattached beads, and further incubated for the time indicated in each experiment.

Immunofluorescence and microscopy

Cells were cultured on coverslips, fixed with 3% PFA in PBS for 10 min, and permeabilized with 50 μ g/ml digitonin in PBS for 5 min. Cells were then treated with 50 mM NH₄Cl⁻/PBS for 10 min at room temperature and blocked with PBS containing 3% BSA for 15 min. Primary antibodies were diluted 1:500 or 1:1,000, and Alexa Fluor-conjugated secondary antibodies (Invitrogen) were diluted 1:1,000 in PBS containing 3% BSA. Coverslips were incubated with primary antibodies for 60 min, washed six times with PBS, and incubated with secondary antibodies for 60 min. Samples were mounted using Slow Fade Gold and observed with an laser confocal microscope (FV1000; Olympus). The microscope images were taken using the FV1000 confocal laser-scanning microscope system equipped with a 100 \times /NA 1.40 oil immersion objective lens. Fluorochromes associated with the secondary antibodies were Alexa Fluor 405, 488, 568, or 594. Image acquisition software used was Fluoview (Olympus). The images were adjusted using Photoshop CS4 software (Adobe). For live-cell imaging, cells were grown in DMEM D6434 (Sigma-Aldrich) supplemented with 10% FBS with antibiotics on a glass-bottom dish (D310300; Matsunami Glass) and transfected with Effectene-coated latex beads for 30 min as previously described (Kobayashi et al., 2010). After beads transfection, the glass-bottom dish was mounted onto the microscope stage, which was equipped with a humidified environment chamber (MH-BC; Olympus) that maintained the dish at 37°C with 5% CO₂. Images were acquired using an inverted microscope (model IX81; Olympus) equipped with a 60 \times /1.40 NA oil immersion objective (Olympus), a xenon lamp, a cooled charge-coupled device camera (CoolSNAP HQ; Roper Scientific), and a ZDC system under the control of MetaMorph v7.6.5.0 (Molecular Devices, MDS Analytical Technologies).

GST pull-down assay

GST and GST-Ub were purified from *Escherichia coli* lysates over glutathione Sepharose 4B (GE Healthcare) and dialyzed in PBS. 30 μ g of each GST protein immobilized on 30 μ l of glutathione Sepharose 4B was used per binding reaction. Mammalian cell lysates or bacterial cell lysates were used as the input proteins. HEK293T cells were transiently transfected with plasmids encoding the indicated Atg16L1 constructs, homogenized in PBS containing protease inhibitors (complete protease inhibitor cocktail; Roche), and then cleared by centrifugation and filtration (0.22 μ m). Alternatively, *E. coli* BL-21 (DE3) cells expressing trigger factor-fused FLAG-WD β -proteins of Atg16L1 were sonicated in PBS containing 0.01% Triton X-100 and protease inhibitors and cleared by centrifugation and filtration (0.22 μ m). Immobilized beads and cell lysates were incubated in a total volume of 500 μ l PBS containing 0.1 mg/ml BSA and 0.01% Triton X-100 with gentle agitation for 1 h at 25°C. The beads were washed three times with ice-cold PBS and the bound complexes were eluted with 50 mM reduced glutathione in PBS and then subjected to SDS-PAGE and Western blot analyses.

Fractionation of bead-autophagosomes

Bead-autophagosomes were isolated using a modification of the method described by Desjardins et al. (1994). HeLa cells were transfected with Effectene-coated beads and incubated for 3 h. The cells were harvested with a silicone rubber scraper and homogenized in buffer (20 mM Hepes-KOH, pH 7.4, 80 mM sucrose, 220 mM mannitol, 10 mM *N*-ethylmaleimide, 1 mM PMSF, and a protease inhibitor cocktail [Roche]) by repeatedly passing (~15 times) through a 1-ml syringe with a 25-gauge needle. The homogenate was centrifuged at 1,000 *g* for 10 min to remove the cytosol and microsomes. The bead-autophagosomes were isolated by flotation on a discontinuous gradient composed of 1 ml each of increasing concentrations of sucrose solutions (60, 40, 35, 30, 25, 20, 15, 10, and 5%; all sucrose solutions were wt/wt in 3 mM imidazole, pH 7.4). The resulting pellet was resuspended in a 25% sucrose solution and loaded on top of a

30% sucrose solution. The samples were centrifuged in a swinging bucket rotor (SW41Ti; Beckman Coulter) for 1 h at 100,000 *g* at 4°C. The bead-autophagosome-containing fraction was collected from the interface of the 10 and 15% sucrose solution. The bead-autophagosomes were resuspended in 2 ml of ice-cold PBS and pelleted by centrifuging for 10 min at 20,000 *g* at 4°C.

Yeast two-hybrid assays

Yeast two-hybrid assays were performed using the Matchmaker GAL4 Yeast Two Hybrid 3 system (Takara Bio Inc.) as previously reported (Langelier et al., 2006). *Saccharomyces cerevisiae* AH-109 was cotransformed with the cloning vectors, pGADT7 or pGBKT7, containing the inserts of interest. The transformed yeast cells were incubated on yeast nitrogen base and glucose with minus Leu, minus Trp selection for 3 d at 30°C. 10–100 colonies were resuspended in a liquid culture of Sabouraud dextrose broth (minus Leu, minus Trp), and incubated on Sabouraud dextrose broth (minus Leu, minus Trp, minus Ade, minus His) plates for 3 d.

Identification of co-purifying proteins by mass spectrometry

HEK293T cells were seeded (3 \times 10⁶ cells/55-cm² dish) and cotransfected with 3 μ g each of plasmids encoding members of the Atg16L1 complex (Atg12, Atg5, and Atg16L1) using polyethylenimine (25,000 kD; Poly-sciences, Inc.) as previously described (Durocher et al., 2002). The cells were harvested 48 h after transfection by incubating in 300 μ l lysis buffer (50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with proteinase inhibitor cocktail (Roche) and 1% Triton X-100. Lysates were clarified by centrifugation (18,000 *g*, 10 min, 4°C) and incubated with Strep-Tactin Sepharose (30 μ l slurry, 2 h, 4°C; IBA GmbH). The matrix was washed four times in wash buffer (20 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with 0.1% Triton X-100, and the purified Atg16L1 complexes were eluted with 2.5 mM desthiobiotin. The co-purified proteins were identified by mass spectrometry. Briefly, the co-purified proteins were identified after SDS-PAGE and band excision. The proteins were digested with trypsin and identified by separating the peptide mixtures using nano-flow liquid chromatography with online tandem mass spectrometry (LC-MS/MS). Tandem mass spectra were acquired automatically and then searched against a nonredundant human database from the NCBI database with the Mascot Server (Matrix Science).

Statistics

All values in the figures are shown with standard deviation. Statistical analyses were performed using a two-tailed unpaired *t* test. *P* values <0.05 were considered statistically significant.

Online supplemental materials

Fig. S1 shows the result of hierarchical analysis of Atg proteins in bead-transfected cells. Fig. S2 shows localization of K48- and K63-linked Ub chains to the invading *Salmonella* or transfected beads, and loss of function effect of K63- or linear-linked Ub chains on selective autophagy. Fig. S3 shows the purity of the recombinant proteins for the GST pull-down assay. Fig. S4 shows the effect of deletion of WDR in Atg16L1 on the order of the Atg16L1 and ULK1-FIP200 complex recruitment to Ub-positive *Salmonella*. Fig. S5 shows the effect of Atg16L1 mutations on canonical and selective autophagy against transfected beads. Videos show dynamics of LC3 and galectin3 (Video 1), Ub and galectin3 (Video 2), LC3 and Ub (Video 3), Atg5 and Ub (Video 4), WIPI-1 and Ub (Video 5), Atg14L and Ub (Video 6), or ULK1 and Ub (Video 7) in autophagy against transfected beads. Table S1 provides a list of FIP200 peptides detected by affinity purification with the Atg16L1 complex and mass spectrometry analysis. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201304188/DC1>.

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