Identification of CUL4A-DDB1-WDFY1 as an E3 ubiquitin ligase complex involved in initiation of lysophagy

Highlights

- CUL4A-DDB1-WDF1 E3 ubiquitin ligase complex regulates lysophagy

- LAMP2 is K48-linked poly-ubiquitinated by the CUL4A complex upon lysosomal damage

- Ubiquitinated LAMP2 acts as a tag of damaged lysosomes to initiate lysophagy

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In brief

Teranishi et al. show the mechanism involved in selective autophagy recognizing and removing damaged lysosomes, termed lysophagy. The CUL4A-DDB1-WDFY1 E3 ubiquitin ligase complex mediates K48-linked poly-ubiquitination of LAMP2 upon lysosomal damage, which recruits the autophagic machinery. These findings reveal how cells selectively tag damaged lysosomes to initiate lysophagy.
Identification of CUL4A-DDB1-WDFY1 as an E3 ubiquitin ligase complex involved in initiation of lysophagy

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SUMMARY

Macroautophagy is a bulk degradation system in which double membrane-bound structures called autophagosomes to deliver cytosolic materials to lysosomes. Autophagy promotes cellular homeostasis by selectively recognizing and sequestering specific targets, such as damaged organelles, protein aggregates, and invading bacteria, termed selective autophagy. We previously reported a type of selective autophagy, lysophagy, which helps clear damaged lysosomes. Damaged lysosomes become ubiquitinated and recruit autophagic machinery. Proteomic studies using transfection reagent-coated beads and further evaluations reveal that a CUL4A-DDB1-WDFY1 E3 ubiquitin ligase complex is essential to initiate lysophagy and clear damaged lysosomes. Moreover, we show that LAMP2 is ubiquitinated by the CUL4A E3 ligase complex as a substrate on damaged lysosomes. These results reveal how cells selectively tag damaged lysosomes to initiate autophagy for the clearance of lysosomes.

INTRODUCTION

Macroautophagy is a bulk degradation system in which double membrane-bound structures called autophagosomes to deliver cytosolic materials to lysosomes (Lamb et al., 2013). Various substances, including toxins, lipids, β-amyloid (Halle et al., 2008), and crystals (Homung et al., 2008) (cholesterol, urate, silica), can rupture endosomes/lysosomes, leading to dysfunction of lysosomes. The damage can cause oxidative stress, inflammation, and cell death (Boya and Kroemer, 2008; Repnik et al., 2017) and can lead to various illnesses such as neurodegenerative diseases. When the lysosomal membrane is damaged by the lysosomotropic compound L-leucyl-L-leucine methyl ester (LLOMe), damaged lysosomes are ubiquitinated and selectively sequestered by autophagosomes, termed lysophagy (Franco et al., 2017; Fujita et al., 2013; Hung et al., 2013; Maejima et al., 2013; Papadopoulos et al., 2017, 2020; Yoshida et al., 2017). Likewise, other groups and we have shown that endosomes containing invading bacteria also become a target of selective autophagy (Birmingham et al., 2008; Fujita et al., 2013; Kageyama et al., 2011; Thurston et al., 2012; Zheng et al., 2009). Similarly, when polystyrene beads coated with transfection reagents are endocytosed into cells, those endosomes with beads are damaged and selectively sequestered by autophagosomes, mimicking xenophagy (Fujita et al., 2013; Kobayashi et al., 2010). In these cases of selective autophagy, ubiquitination of substrates is essential for tagging the damaged materials and bringing autophagic machinery to initiate autophagy. Some studies have identified E3 ligases involved in xenophagy (Begun et al., 2015; Huett et al., 2012; Kimura et al., 2015; Manzanillo et al., 2013), yet it remains unclear if these E3 ligases recognize damaged endosomes or bacteria themselves. For lysophagy, the SCFFBXO27 ubiquitin ligase complex, which is expressed mainly in the brain and muscle, ubiquititates damaged lysosomal proteins by binding luminal glycoproteins (Yoshida et al., 2017). However, a ubiquitously expressed E3 ligase involved in lysophagy remains unclear. How cells recognize damaged membranes and the subsequent ubiquitination mechanisms have not been fully elucidated.

RESULTS

Identification of CUL4A E3 ligase as a regulatory protein in lysophagy

To search for proteins playing an essential role in the initial damage recognition step of lysophagy, we performed proteomic analysis using different types of transfection reagent-coated polystyrene...
A

GFP-ATG5

LOMε

siLUC

siWDFY1

siCUL4A

siATG16

B

Galectin3

hours after J.CME washout

1 h

4 h

10 h

siLUC

siATG16

siCUL4A

siDDB1

siNEDD8

siWDFY1

C

Galectin3

CUL4A

siLUC

siCUL4A #1

siCUL4A #2

siWDFY1

siWDFY1 #1

siWDFY1 #2

D

FLAG-WDFY1

HA-TR-TUBE

WB: DDB1

WB: DDB1

WB: FLAG

WB: FLAG

Input

E

substrate

Ub

E2

DDB1

NEDD8

ROC

CUL4A

(legend on next page)
beads. When beads are coated with effectene, endocytosed beads induce endosomal membrane damage and are ubiquitinated, as previously reported (Kobayashi et al., 2010) (Figure S1A). On the other hand, beads coated with polyethyleneimine (PEI) did not trigger severe endosomal membrane damage or induce ubiquitination (Figure S1A). The bead-containing autophagosome fraction can be isolated using density gradient centrifugation (Fujita et al., 2013). We purified fractions and analyzed them using mass spectrometry. Using these two transfection reagents, we compared proteins recruited around beads, and 974 proteins were detected only in the damaged endosomes fraction (Table S1; Pride database: PXD0035151). To focus on proteins involved in the early steps of lysophagy, cells internalizing effectene-coated beads were treated with bafilomycin A1 to exclude proteins involved in autophagosome-lysosome fusion steps. As a result of these screens, 123 proteins with high scores were analyzed for Gene Ontology enrichment (Table S1) and taken for further analysis.

To evaluate if any of the 123 proteins were involved in an early step of lysophagy, small interfering RNA (siRNA) knockdown of these candidates using a solid-phase transfection method was performed (Erfle et al., 2008), and the GFP-Atg5 dot formation was monitored in response to lysosomal damage using LLOMe (Table S2). LC3-II is a well-known marker for autophagosomes and autolysosomes, marking from the beginning of the formation of autophagosomes through their fusion with lysosomes. On the other hand, Atg5 is a better marker for the early steps of the autophagosome formation process, as once the autophagosome closes, the Atg5 complex is released from the autophagosomal membrane (Mizushima et al., 2001). Thus, observation of Atg5 indicates the formation of autophagosome sites. Atg16L1 siRNA was used as a positive control because Atg16L1 directly binds to ubiquitin chains and recruits Atg5 as an Atg12-5-16L1 complex (Fujita et al., 2013). The number of GFP-Atg5 dots was significantly decreased in ATG16L1 knockdown cells compared with control cells upon LLOMe treatment (Figures 1A, S1B, and S1D). Knockdown of the candidate E3 ligase, Cullin 4a (CUL4A), showed a significant decrease in Atg5 recruitment on damaged lysosomes (Figures 1A, S1B, and S1C). We further performed a lysophagy assay to monitor the clearance of damaged lysosomes. Galectin3 (Gal3) is a marker for damaged endo-lysosomes, as previously shown (Maejima et al., 2013). Gal3 is a cytosolic β-galactose-binding lectin, and Gal3 shows cytosolic localization under normal conditions. However, upon lysosomal damage, Gal3 can access the lumen of lysosomes and shows punctate localization via binding to the lumenal glycochain. When cells were treated with LLOMe for 1 h, Gal3 heavily stained damaged lysosomes, and after washout of LLOMe, these Gal3 puncta eventually disappeared over time, as seen in siLUC as a negative control (Figure 1B). In contrast, ATG16L1 knockdown led to significant numbers of Gal3 dots remaining even after 10 h of LLOMe washout, indicating the clearance of damaged lysosomes by autophagy (Maejima et al., 2013) (Figures 1B and S1D). In this lysophagy assay, CUL4A knockdown caused a delay in Gal3 dot clearance resembling that seen in ATG16L1 knockdown cells (Figures 1B and S1D). With the Atg5 localization results, CUL4A seems to be involved in clearing damaged lysosomes.

**CUL4A-DDB1-WDFY1 complex is involved in lysophagy**

CUL4A is one of the Cullin-RING-based E3 ligases that constitute a significant subclass of RING finger E3 ligases (Petroski and Deshaies, 2005) and forms a complex. The C terminus of CUL4A interacts with the RING finger protein (ROC1) to recruit the E2 ubiquitin-conjugating enzyme, and the N terminus interacts with its adaptor protein, DNA damage-binding protein 1 (DDB1). DDB1 binds to DDB1-CUL4A-associated factors (DCAFs), which act as specific substrate receptors (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006). There are more than 60 DCAFs, implying that CUL4A E3 ligase has broad cellular functions, but details are unknown. Although the CUL4A E3 ligase component candidates NEDD8, DDB1, and one of the DCAFs (WDFY1) were not listed as top 123 proteins but still were detected in proteomic analysis, we investigated their role in lysophagy by using a lysophagy assay and examining Atg5 recruitment (see MS detection sheet in Table S1). They are also expressed ubiquitously in various tissues. The efficiency of Gal3 dot clearance in cells with knockdown of DDB1, NEDD8, and WDFY1 was just as low as seen in Atg16L1 knockdown and CUL4A knockdown cells (Figures 1B, S1B, and S1D), and the recruitment of GFP-Atg5 was significantly lowered in WDFY1 knockdown cells compared with the control cells (Figures 1A, S1B, and S1C). We further found that the clearances of Gal3-positive lysosomes in...
CUL4A or WDFY1 siRNA knockdown cells were restored by the expression of a siRNA-resistant protein (Figure 1C). These results indicate that CUL4A-DDB1 seems to be involved in lysophagy with substrate receptor WDFY1 as a complex. To test the binding between WDFY1 and DDB1, we performed an immunoprecipitation experiment using FLAG M2 agarose and found that endogenous DDB1 was co-immunoprecipitated with FLAG-WDFY1 (Figure 1D), although the interaction was independent of LLOMe treatment. We further found that MLN4924, an inhibitor of NEDD8-activating enzymes, inhibited the clearance of Gal3-positive damaged lysosomes (Figure S2), suggesting that the neddylation of CUL4A is essential for the function. Our results show that WDFY1 is a part of a DDB1-CUL4A complex, and the proteins function together when lysophagy is induced (Figure 1E).

**CUL4A complex preferentially functions in lysophagy**

In addition, we checked the involvement of CUL4A in effectencoeated beads induced lysophagy using GFP-RFP-Gal3 (tGal3) expressing HeLa cells (Maetani et al., 2013). When lysophagy occurs in these cells, the RFP/GFP ratio of tGal3 on damaged endosomes increases because the GFP signals are preferentially quenched in the acidic degradative environment (Kimura et al., 2007) (Figure S3A). As a result, 6 h after transfection with the beads, the RFP/GFP ratio around the beads in the CUL4A knockdown cells was close to 1 (almost no quenching of GFP), almost at the same level as in Atg16L knockdown cells, suggesting that CUL4A is also involved in bead-induced lysophagy (Figure S3B).

We next examined if CUL4A is involved in canonical autophagy using a GFP-RFP-LC3 (tLC3 autophagy flux) assay (Kimura et al., 2007). No significant differences were found in starvation-induced autophagy in both control and CUL4A knockdown cells, indicating that CUL4A is not involved in canonical autophagy (Figure S4). Also, translocation of LC3 from the nucleus (Huang et al., 2015) was observed in both control and CUL4A knockdown cells, supporting the idea that CUL4A is not involved in canonical starvation-induced autophagy. To test the effect of CUL4A or WDFY1 siRNA knockdown on other selective autophagy, we monitored mitophagy after treatment with valinomycin, an ionophore that induces mitophagy (Figure S5A). As a result, 6 h after transfection with the beads, the RFP/GFP ratio around the beads in the CUL4A knockdown cells was close to 1 (almost no quenching of GFP), almost at the same level as in Atg16L knockdown cells, indicating that CUL4A is also involved in bead-induced lysophagy (Figure S5B). Mitophagy using a GFP-RFP-LC3 (tF LC3 autophagy flux) assay (Kimura et al., 2007) (Figure S3A). As a result, 6 h after transfection with the beads, the RFP/GFP ratio around the beads in the CUL4A knockdown cells was close to 1 (almost no quenching of GFP), almost at the same level as in Atg16L knockdown cells, suggesting that CUL4A is also involved in bead-induced lysophagy (Figure S3B).

We observed the involvement of CUL4A-DDB1-WDFY1 E3 ubiquitin ligase in recognizing damaged lysosomes using a GFP-RFP-LC3 (tLC3 autophagy flux) assay (Kimura et al., 2007). No significant differences were found in starvation-induced autophagy in both control and CUL4A knockdown cells, indicating that CUL4A is not involved in canonical autophagy (Figure S4). Also, translocation of LC3 from the nucleus (Huang et al., 2015) was observed in both control and CUL4A knockdown cells, supporting the idea that CUL4A is not involved in canonical starvation-induced autophagy. To test the effect of CUL4A or WDFY1 siRNA knockdown on other selective autophagy, we monitored mitophagy after treatment with valinomycin, an ionophore that induces mitophagy. However, these knockdowns did not inhibit the valinomycin-induced mitophagy (Figure S5A). This result suggests that CUL4A complex preferentially functions in lysophagy. We further tested the effect of knocking down the ligase on LC3 lipidation during lysosomal damage response. However, CUL4A or WDFY1 siRNA knockdown did not affect LC3 lipidation, probably because of autophagy-independent LC3 lipidation (Figure S5B) (Nakamura et al., 2020).

**CUL4A regulates K48-linked poly-ubiquitination**

Previous reports have shown that recruitment of both K48-linked and K63-linked poly-ubiquitin chains to damaged endosomes/lysosomes, and these modifications are necessary for the activity of lysophagy (Franco et al., 2017; Fujita et al., 2013; Papadopoulos et al., 2017; Yoshida et al., 2017). Consistent with these reports, LLOMe-induced damaged lysosomes were ubiquitinated by K48-linked and K63-linked poly-ubiquitin chains in control siLUC knockdown cells (Figure 2). In contrast, a significant reduction of K48 poly-ubiquitin intensity on GFP-Gal3 dots was observed in both CUL4A and WDFY1 siRNA knockdown cells, whereas K63 poly-ubiquitin modification occurred almost at the same level as in siLUC knockdown cells (Figures 2 and S1E). These results suggest that the CUL4A-DDB1-WDFY1 complex is necessary for K48-linked poly-ubiquitin modification but not for K63-linked poly-ubiquitin modification. The ubiquitin modification was also examined on bead-induced lysophagy. A similar phenotype was observed: K63 poly-ubiquitin modification occurred at the same level as in control cells, but there was much reduced K48 poly-ubiquitin modification in both CUL4A and WDFY1 siRNA knockdown cells (Figure S6).

**CUL4A complex recognizes damaged lysosomes via WDFY1**

We observed the involvement of CUL4A-DDB1-WDFY1 E3 ubiquitin ligase complex in ubiquitinating damaged lysosomes. Does the CUL4A-DDB1-WDFY1 complex also have a role in recognizing damaged lysosomes? To examine how the CUL4A-DDB1-WDFY1 complex recognizes damaged lysosomes, we checked the localization of WDFY1, DDB1, and CUL4A upon LLOMe treatment. WDFY1 was localized to early endosomes via its FYVE domain under the normal condition, as previously reported (Ridley et al., 2001) (Figure 3A). Interestingly, in addition to early endosomes (blue arrowheads), WDFY1 was also found on damaged lysosomes upon LLOMe treatment (Figures 3A and 3B, green arrowheads), indicating that WDFY1 can localize on damaged lysosomes. Consistently, CUL4A and DDB1 localized on damaged lysosomes after LLOMe treatment (Figure 3C). It is known that the CUL4A-DDB1 complex recognizes specific substrates via DCAF (Jin et al., 2006; Higa et al., 2006; Jin et al., 2006). We next examined whether the CUL4A-DDB1-WDFY1 complex recognizes damaged lysosomes via WDFY1. As expected, CUL4A localization to damaged lysosomes was significantly reduced in WDFY1 knockdown cells compared with control cells upon LLOMe treatment (Figure 3C). Contrarily, DDB1 localization to damaged lysosomes was unaffected in CUL4A knockdown cells, supporting the idea that WDFY1 and DDB1 are the key molecules to take CUL4A to the damaged lysosomes (Figure 3C). Last, a CUL4A mutant (H2-H5) that cannot bind to DDB1 (He et al., 2006) was not recruited to damaged lysosomes compared with wild-type CUL4A (Figure S7). These results suggest that the CUL4A-DDB1-WDFY1 complex plays a role in recognizing damaged lysosomes via WDFY1.

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**Figure 2. CUL4A-DDB1-WDFY1 complex mediates K48-linked, but not K63-linked, poly-ubiquitination of damaged lysosomes**

HeLa cells expressing GFP-galectin3 were treated with LLOMe for 1 h. After LLOMe washout, the cells were incubated for 1 h and subjected to immunostaining with antibodies specific to K48- or K63-linked ubiquitin chains. The intensity of ubiquitin staining on galectin3 dots was quantified for at least 100 cells for each experiment. The data represent mean ± SD from more than 4 independent experiments. One-way ANOVA was used to test the significant difference. Scale bar: 25 µm. See also Figure S6.
(legend on next page)
The CUL4A complex ubiquitinates LAMP2 upon lysosomal damage

What does WDFY1 recognize on damaged lysosomes? To clarify the target of the CUL4A-DDB1-WDFY1 complex on damaged lysosomes, we searched for WDFY1 interacting proteins on damaged lysosomes. We focused on abundant lysosomal proteins, LAMP1, LAMP2, and LIMP2 (Saftig and Klumperman, 2009), and performed precipitation analysis. Only LAMP2 was co-immunoprecipitated with WDFY1 (Figure 4A). The amount of co-precipitated LAMP2 was increased upon LLOMe treatment and decreased upon MLN4924 treatment. These results suggest that LAMP2 is a substrate of WDFY1, and the neddylation of CUL4A is essential for its binding. To analyze ubiquitination further, we transiently expressed FLAG-TR-TUBE to stabilize ubiquitinated substrates (Yoshida et al., 2015, 2017). As reported previously (Yoshida et al., 2017), we confirmed the ubiquitination of LAMP2 upon LLOMe treatment (Figure 4B). We did not observe a prominent elevation of ubiquitinated LAMP1 level, suggesting that ubiquitination of LAMP2 is predominant upon lysosomal membrane damage. Next, we asked whether the CUL4A complex regulates this ubiquitination against LAMP2. We found that CUL4A knockdown decreased the amount of ubiquitinated LAMP2 compared with samples treated with siLUC and LLOMe (Figure 4B). Last, K48-linked
ubiquitination efficiency was examined in LAMP2 knockdown cells. The intensity of K48 labeling on Gal3 dots was much lower in LAMP2 knockdown cells, and there were hardly any changes with K63-linked ubiquitination (Figure 5). Therefore, we concluded that LAMP2 is the substrate that gets K48-linked ubiquitination by the CUL4A-DDB1-WDFY1 E3 ubiquitin ligase complex on damaged lysosomes.

**LAMP2 is essential for recruiting CUL4A complex to damaged lysosomes and lysophagy**

Next, to examine whether WDFY1 recruitment to damaged lysosomes depends on LAMP2, the localization of WDFY1, CUL4A, or DDB1 was observed in LAMP2 knockdown cells. The recruitment of WDFY1, CUL4A, and DDB1 to damaged lysosomes were significantly decreased in LAMP2 knockdown cells compared with siLUC control cells (Figure 6A), suggesting that LAMP2 is essential for the recruitment of CUL4A complex to damaged lysosomes. We next tested the effect of LAMP2 knockdown on lysophagy. A significant delay in Gal3 dots clearance was observed in LAMP2 knockdown cells compared with siLUC control cells, indicating significant inhibition of lysophagy in LAMP2 knockdown cells, comparable with autophagy-deficient cells (Figure 6B). These results indicate that LAMP2 is required for lysophagic activity. Gal3 dots were not observed before LLOMe treatment and only after treatment with LLOMe in LAMP2 knockdown cells at the same level as in siLUC control.
Figure 6. LAMP2 recruits WDFY1 to lysosomes upon lysosomal membrane damage

(A) HeLa cells expressing GFP-WDFY1, GFP-CUL4A, or GFP-DDB1 were transfected with indicated siRNAs. The cells were treated with LLOMe and stained with an anti-galectin3 antibody. The intensity of GFP-tagged protein on galectin3 dots was quantified from 92 cells for each experiment. The data represent mean ± SD from more than 3 independent experiments. An unpaired t test was used to test the significant difference. Scale bar: 20 μm.

(B) LAMP2 is required for the elimination of damaged lysosomes. HeLa cells were treated with LLOMe for 1 h and washed out. The cells were fixed at the indicated time and stained with an anti-galectin3 antibody. The number of endogenous galectin3 puncta per cell was quantified from 100 cells for each experiment. The data represent mean ± SD from more than 3 independent experiments. Two-way ANOVA was used to test the significant difference. Scale bar: 25 μm.
cells (Figure 6B), indicating that LAMP2 knockdown alone did not damage the lysosomal membrane or affect the efficiency of lysosomal damage by LLOMe.

**The lumen side of LAMP2 is essential for WDFY1 recruitment during lysophagy**

Last, LAMP1-LAMP2 chimera proteins were constructed and used to determine which domain is essential for interaction with WDFY1 (Figure 7). LAMP2 and LA1-C/LA2-L chimeric protein expression showed WDFY1 recruitment to damaged lysosomes, whereas LA2-C/LA1-L chimeric protein expression significantly reduced WDFY1 recruitment (Figure 7). RFP plasmid was co-transfected with siRNA to show which cells were knocked down with siLAMP2. These results indicate that the lumen side of LAMP2 is essential for the WDFY1 recruitment during lysophagy.

**DISCUSSION**

In selective autophagy, ubiquitination is the critical step in connecting substrate recognition with autophagy through the interaction with autophagy receptor/adaptor proteins (Kirkin et al., 2009). In this study, we identified the CUL4A-DDB1-WDFY1 E3 ligase complex as a lysophagy regulator and found that this complex, upon lysosomal damage, ubiquitinates LAMP2. The CUL4A complex may contribute to the recruitment of autophagy receptor/adaptor proteins and autophagy-related proteins such as LC3 through LAMP2-ubiquitination.

This finding develops the understanding of molecular mechanisms in lysophagy, but there are still remaining questions. First, although we found that CUL4A ubiquitinated LAMP2, we could not exclude the possibility that other E3 ubiquitin ligase(s) also contribute to the recognition of damaged lysosomes. CUL4A knockdown decreased ~40% of K48-linked poly-ubiquitinated protein signals, as shown in Figures 2 and 4B. These results suggest that other enzymes ubiquitously expressed in various tissues or cell lines may have a redundant function together with CUL4A. Along this line, several E3 ligases might recognize different conditions of endosomal/lysosomal damages. Size, degree, or amount of membrane rupture may trigger different types of membrane repair machinery through recruiting E3 ligases or other related proteins. Second, we could not investigate which region of LAMP2 was ubiquitinated because of technical issues, although we showed that the lumen side of LAMP2 is essential for the WDFY1 recruitment during lysophagy. However, ~92% of LAMP2 locates in the lysosomal lumen, and ~4% is in a trans-membrane region. In the remaining ~4% of the cytosolic region, there is only one lysine residue that is a potential target of K48-linked ubiquitination. In the lumen region of LAMP2, there are a total of 16 lysine residues. Thus, we assume that the lumen side of LAMP2 has more chance of being ubiquitinated by the CUL4A complex. Further studies must clarify which amino acid or domain the CUL4A complex targets. The third question is whether only LAMP2 is a ubiquitination target during lysophagy. The previous study observed a slight increment of ubiquitinated LAMP1 level upon LLOMe treatment. LAMP1, other lysosomal proteins, or other materials can potentially be ubiquitinated (Otten et al., 2021).

In intact cells, the CUL4A complex locates in the cytosol, which spatially separates from the lumen region of LAMP2. In our model, once the lysosomal membrane is injured, the lumenal side would be exposed to the cytosol and targeted for ubiquitination. This study contributes to understanding a mechanism of how cells tag only damaged lysosomes.

**Limitations of the study**

It was challenging to decide where the cut-off was for proteomic studies; CUL4A was included in 123 top hits but not the other components of the CUL4A complex. However, WDFY1, DDB1, and NEDD8 were detected below 123 proteins, so we still have good candidates out of the top 123 hits. Although CUL4A, DDB1, and WDFY1 are expressed ubiquitously in various tissues and many cell lines, we did not address the physiological role(s) in various tissues in this study.

Further work is needed to reveal how the CUL4A complex recognizes LAMP2, where the precise amino acids of LAMP2 are ubiquitinated, and where ubiquitination occurs within the lumen or on flip-flopped membrane.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**Figure 7. The luminal region is required for WDFY1 recruitment**

Top: Schematic diagram of LAMP1-LAMP2 chimeras. Bottom: Endogenous LAMP2 was replaced by exogenous LAMP2 or its chimera proteins, LA1-C/LA2-L and LA2-C/LA1-L. The cells were treated with LLOMe for 1 h and stained with anti-galectin3 antibody. RFP plasmid was transfected with LAMP2 or chimera plasmid as a transfection control. Cell areas were labeled with dashed lines. The intensity of GFP-WDFY1 on galectin3 dots was quantified. The data represent mean ± SD from 8 independent experiments. An unpaired t test was used to test the significant difference. Scale bar: 25 μm.
**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111349.

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**AUTHOR CONTRIBUTIONS**

H.T., K.T., T.Y., and M.H. designed the experiments and analyzed the data. H.T., K.T., M.S., T.U., and K.Y. performed the experiments. T.O. suggested a protein to analyze. T.H. and T.N. performed mass spectrometry. K.Y. helped analyze data. H.T., K.T., and M.H. wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**

Angers, S., Li, T., Yi, X., MacCoss, M.J., Moon, R.T., and Zheng, N. (2008). Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. Nature 443, 590–593. https://doi.org/10.1038/nature05175.

Begun, J., Lassen, K.G., Jijon, H.B., Baxt, L.A., Goel, G., Heath, R.J., Ng, A., Tam, J.M., Kuo, S.Y., Villablanca, E.J., et al. (2015). Integrated genomics of work flow for multiplexing siRNA assays by solid-phase reverse transcription. Proc. Natl. Acad. Sci. USA 112, 17003–17010. https://doi.org/10.1073/pnas.1504529112.

Birmingham, C.L., Smith, A.C., Bakowski, M.A., Yoshimori, T., and Brumell, J.H. (2006). Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J. Biol. Chem. 281, 11374–11383. https://doi.org/10.1074/jbc.M509157200.

Boya, P., and Kroemer, G. (2008). Lysosomal membrane permeabilization in cell death. Oncogene 27, 6434–6451. https://doi.org/10.1038/onc.2008.310.

Erfle, H., Neumann, B., Rogers, P., Bulkescher, J., Ellenberg, J., and Peppercorn, R. (2008). Work flow for multiplexing siRNA assays by solid-phase reverse transfection in multiwell plates. J. Biomol. Screen 13, 575–580. https://doi.org/10.1177/1087057108320133.

Franco, L.H., Nair, V.R., Scharn, C.R., Xavier, R.J., Torrealba, J.R., Shilooh, M.U., and Levine, B. (2017). The ubiquitin ligase Smurf1 functions in selective autophagy of Mycobacterium tuberculosis and anti-tuberculous host defense. Cell Host Microbe 21, 59–72. https://doi.org/10.1016/j.chom.2016.11.002.

Fujita, M., Morita, E., Itoh, T., Tanaka, A., Nakaoaka, M., Osada, Y., Umemoto, T., Saitoh, T., Nakatogawa, H., Kobayashi, S., et al. (2013). Recruitment of the autophagic machinery to endosomes during infection is mediated by ubiquitin. J. Cell Biol. 203, 115–128. https://doi.org/10.1083/jcb.201304188.

Halle, A., Hormung, V., Petzold, G.C., Stewart, C.R., Monks, B.G., Reinheckel, T., Fitzgerald, K.A., Latz, E., Moore, K.J., and Golenbock, D.T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat. Immunol. 9, 857–865. https://doi.org/10.1038/ni.1636.

He, Y.J., McCall, C.M., Hu, J., Zeng, Y., and Xiong, Y. (2006). DBB1 functions as a linker to recruit receptor WD40 proteins to CUL4-Roc1 ubiquitin ligases. Genes Dev. 20, 2949–2954. https://doi.org/10.1101/gad.1483206.

Higa, L.A., Wu, M., Ye, T., Kobayashi, R., Sun, H., and Zhang, H. (2006). CUL4-DBB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. Nat. Cell Biol. 8, 1277–1283. https://doi.org/10.1038/ncb1490.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., Fitzgerald, K.A., and Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. 9, 847–856. https://doi.org/10.1038/ni.1631.

Huang, R., Xu, Y., Wan, W., Shou, X., Qian, J., You, Z., Liu, B., Chang, C., Zhou, T., Lippincott-Schwartz, J., and Liu, W. (2015). Deacetylation of nuclear LC3 drives autophagy initiation under starvation. Mol. Cell 57, 456–466. https://doi.org/10.1016/j.molcel.2014.12.013.

Huang, Y.H., Chen, L.M.W., Yang, J.Y., and Yang, W.Y. (2013). Spatiotemporally controlled induction of autophagy-mediated lysosome turnover. Nat. Commun. 4, 2111. https://doi.org/10.1038/ncomms2111.

Jin, J., Arias, E.E., Chen, J., Harper, J.W., and Walter, J.C. (2006). A family of diverse Cull4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. Mol. Cell 22, 709–721. https://doi.org/10.1016/j.molcel.2006.08.010.

Kageyama, S., Omori, H., Saitoh, T., Sone, T., Guan, J.L., Akira, S., Imamoto, F., Noda, T., and Yoshimori, T. (2011). The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against Salmonella. Mol. Biol. Cell 22, 2290–2300. https://doi.org/10.1091/mbc.E10-11-0893.

Kimura, S., Noda, T., and Yoshimori, T. (2007). Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3, 452–460. https://doi.org/10.4161/aut.3451.

Kimura, T., Jain, A., Choi, S.W., Mandell, M.A., Schroder, K., Johansen, T., and Deretic, V. (2015). TRIM-mediated precision autophagy targets cytoplasmic regulators of innate immunity. J. Cell Biol. 210, 973–989. https://doi.org/10.1083/jcb.201503023.

Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. (2009). A role for ubiquitin in selective autophagy. Mol. Cell 34, 259–269. https://doi.org/10.1016/j.molcel.2009.04.026.

Kobayashi, S., Kojidani, T., Osakada, H., Yamamoto, A., Yoshimori, T., Hiraoa, Y., and Haraguchi, Y. (2010). Artificial induction of autophagy around polystyrene beads in nonphagocytic cells. Autophagy 6, 36–45. https://doi.org/10.4161/auto.6.1.10324.

Lamb, C.A., Yoshimori, T., and Tooze, S.A. (2013). The autophagosome: origins unknown, biogenesis complex. Nat. Rev. Mol. Cell Biol. 14, 759–774. https://doi.org/10.1038/nrm3696.

Maejima, I., Takahashi, A., Omori, H., Kimura, T., Takabatake, Y., Saitoh, T., Yamamoto, A., Hamasaki, M., Noda, T., Isaka, Y., and Yoshimori, T. (2013). Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury. EMBO J. 32, 2336–2347. https://doi.org/10.1038/emboj.2013.171.

Manzello, P.S., Ayres, J.S., Watson, R.O., Collins, A.C., Souza, G., Rae, C.S., Schneider, D.S., Nakamura, K., Shilooh, M.U., and Cox, J.S. (2019). The
ubiquitin ligase parkin mediates resistance to intracellular pathogens. Nature 501, 512–516. https://doi.org/10.1038/nature12566.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. (2001). Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. J. Cell Biol. 152, 657–668. https://doi.org/10.1083/jcb.152.4.657.

Morita, S., Kojima, T., and Kitamura, T. (2000). Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. 7, 1063–1066. https://doi.org/10.1038/sj.gt.3301206.

Nakamura, S., Shigeyama, S., Minami, S., Shima, T., Akayama, S., Matsuda, T., Esposito, A., Napolitano, G., Kuma, A., Namba-Hamano, T., et al. (2020). LC3 lipidation is essential for TFEB activation during the lysosomal damage response to kidney injury. Nat. Cell Biol. 22, 1252–1263. https://doi.org/10.1038/s41556-020-00583-9.

Otten, E.G., Werner, E., Crespiillo-Casado, A., Boyle, K.B., Dharamdasani, V., Pathe, C., Santhanam, B., and Randow, F. (2021). Ubiquitylation of lipopolysaccharide by RNF213 during bacterial infection. Nature 594, 111–116. https://doi.org/10.1038/s41586-021-03566-4.

Papadopoulos, C., Kirchner, P., Bug, M., Grum, D., Koerver, L., Schulze, N., Poehler, R., Dressler, A., Fengler, S., Arhazaouy, K., et al. (2017). VCP/p97 cooperates with YOD1, UBXD1 and PLAA to drive clearance of ruptured lysosomes by autophagy. EMBO J. 36, 135–150. https://doi.org/10.15252/embj.201695148.

Papadopoulos, C., Kravic, B., and Meyer, H. (2020). Repair or lysophagy: dealing with damaged lysosomes. J. Mol. Biol. 432, 231–239. https://doi.org/10.1016/j.jmb.2019.08.010.

Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. Nat. Rev. Mol. Cell Biol. 6, 9–20. https://doi.org/10.1038/nrm1547.

Repnik, U., Borg Distefano, M., Speth, M.T., Ng, M.Y.W., Progida, C., Hoflack, B., Gruenberg, J., and Griffiths, G. (2017). L-leucyl-L-leucine methyl ester does not release cysteine cathepsins to the cytosol but inactivates them in transiently permeabilized lysosomes. J. Cell Sci. 130, 3124–3140. https://doi.org/10.1242/ijcs.204529.

Ridley, S.H., Ktistakis, N., Davidson, K., Anderson, K.E., Manifava, M., Elison, C.D., Lipp, P., Bootman, M., Coadwell, J., Nazarian, A., et al. (2001). FENS-1 and DFCP1 are FYVE domain-containing proteins with distinct functions in the endosomal and Golgi compartments. J. Cell Sci. 114, 3991–4000.

Saftig, P., and Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. Nat. Rev. Mol. Cell Biol. 10, 623–635. https://doi.org/10.1038/nrm2745.

Tabata, K., Prasad, V., Paul, D., Lee, J.Y., Pham, M.T., Twu, W.I., Neufeldt, C.J., Cortese, M., Cerikan, B., Stahl, Y., et al. (2021). Convergent use of phosphatidic acid for hepatitis C virus and SARS-CoV-2 replication organelle formation. Nat. Commun. 12, 7276. https://doi.org/10.1038/s41467-021-27511-1.

Thurston, T.L.M., Wandel, M.P., von Muhlinen, N., Foeglein, A., and Randow, F. (2012). Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial infection. Nature 482, 414–418. https://doi.org/10.1038/nature10744.

Yoshida, Y., Saeki, Y., Murakami, A., Kawakami, J., Tsuchiya, H., Yoshihara, H., Shindo, M., and Tanaka, K. (2015). A comprehensive method for detecting ubiquitinated substrates using TR-TUBE. Proc. Natl. Acad. Sci. USA 112, 4630–4635. https://doi.org/10.1073/pnas.1422313112.

Yoshida, Y., Yasuda, S., Fujita, T., Hamasaki, M., Murakami, A., Kawakami, J., Iwai, K., Saeki, Y., Yoshimori, T., Matsuda, N., and Tanaka, K. (2017). Ubiquitination of exposed glycoproteins by SCF(FBXO27) directs damaged lysosomes for autophagy. Proc. Natl. Acad. Sci. USA 114, 8574–8579. https://doi.org/10.1073/pnas.1702615114.

Zheng, Y.T., Shahnazari, S., Brech, A., Lamark, T., Johansen, T., and Brumell, J.H. (2009). The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. J. Immunol. 183, 5909–5916. https://doi.org/10.4049/jimmunol.0900441.
### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE                             | IDENTIFIER   |
|---------------------|------------------------------------|--------------|
| **Antibodies**      |                                    |              |
| Mouse monoclonal anti-LAMP1 (H4A3) | Santa Cruz Biotechnology          | sc-20011; RRID: AB_626853 |
| Mouse monoclonal anti-LAMP2 (H4B4) | Santa Cruz Biotechnology          | sc-18822; RRID: AB_626858 |
| Rabbit polyclonal anti-LIMP2 | NOVUS Biologicals                | NB400-129; RRID: AB_2301298 |
| Rabbit polyclonal anti-CUL4A | Cell Signaling technology         | 2699; RRID: AB_2086563 |
| Mouse monoclonal anti-DDB1 (2B12D1) | Thermo Fisher Scientific  | 37-6200; RRID: AB_2533331 |
| Rabbit polyclonal anti-NEDD8 | Cell Signaling technology         | 2745; RRID: AB_10695300 |
| Rabbit monoclonal anti-Atg16L1 | Cell Signaling technology         | 8089; RRID: AB_10950320 |
| Rabbit polyclonal anti-LC3 | MBL                               | PM036; RRID: AB_2274121 |
| Rabbit polyclonal anti-GFP | MBL                               | 598; RRID: AB_591816 |
| Mouse monoclonal anti-α-tubulin | Sigma-Aldrich                     | T5168; RRID: AB_477579 |
| Mouse monoclonal anti-E1E1 | BD transduction laboratories      | 610456; RRID: AB_397829 |
| Rat monoclonal anti-Galectin3 (M3/38) | Santa Cruz Biotechnology  | sc-23938; RRID: AB_627658 |
| Rabbit monoclonal anti-Ubiquitin, K48-specific (Apu2) | EMD Milipore               | 05-1307; RRID: AB_1587578 |
| Rabbit monoclonal anti-Ubiquitin K63-specific (Apu3) | EMD Milipore               | 05-1308; RRID: AB_1587580 |
| Rabbit polyclonal anti-p62 | MBL                               | PM045; RRID: AB_1279301 |
| Mouse monoclonal anti-FLAG (M2) | Sigma-Aldrich                     | F3165; RRID: AB_259529 |
| Goat-anti-Rabbit(H + L)-HRP | Jackson Immuno Research Laboratories, Inc. | 111-035-003; RRID: AB_2313567 |
| Goat-anti-Mouse(H + L)-HRP | Jackson Immuno Research Laboratories, Inc. | 115-035-003; RRID: AB_10015289 |
| Alexa Fluor 488 donkey anti-rabbit IgG | Thermo Fisher Scientific      | A-21206; RRID: AB_2535792 |
| Alexa Fluor 488 donkey anti-mouse IgG | Thermo Fisher Scientific      | A-21202; RRID: AB_141607 |
| Alexa Fluor 488 donkey anti-mouse IgG2a | Thermo Fisher Scientific      | A-21131; RRID: AB_2535771 |
| Alexa Fluor 568 donkey anti-rabbit IgG | Thermo Fisher Scientific      | A-10042; RRID: AB_2534017 |
| Alexa Fluor 568 donkey anti-mouse IgG | Thermo Fisher Scientific      | A-10037; RRID: AB_2534013 |
| Alexa Fluor 568 donkey anti-mouse IgG1 | Thermo Fisher Scientific      | A-21124; RRID: AB_2535766 |

| **Chemicals, peptides, and recombinant proteins** |                                    |              |
|---------------------------------------------------|------------------------------------|--------------|
| Dulbecco’s modified Eagle’s medium (DMEM) | Sigma-Aldrich                      | D6429        |
| Fetal bovine serum (FBS) | Thermo Fisher Scientific          | 10270-106    |
| Penicillin-Streptomycin | Sigma-Aldrich                      | P4333        |
| L-Glutamine Solution | Sigma-Aldrich                      | G7513        |
| Trypsin/EDTA | Sigma-Aldrich                      | T4174        |
| Earle’s Balanced Salts (EBSS) | Sigma-Aldrich                      | E2888        |
| Bafilomycin A1 | Sigma-Aldrich                      | B1793        |
| 4%-Paraformaldehyde Phosphate Buffer Solution | Nakarai Tesque                   | 09154-85     |
| DAPI Solution | Nakarai Tesque                    | 19178-91     |
| Lipofectamine RNAiMAX Transfection Reagent | Thermo Fisher Scientific      | 13778150     |
| lipofectamin 2000 | Thermo Fisher Scientific          | 11668019     |
| PEI MAX - Transfection Grade Linear Polyethyleneimine Hydrochloride (MW 40,000) | PSI | 24765-100 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Opti-MEM reduced serum Medium | Thermo Fisher Scientific | 31985070 |
| Polybead Amino Microspheres 3.00μm | Polysciences | 17145-5 |
| Mounting Medium | VECTASHIELD | H-1000 |
| Complete, EDTA-free Protease Inhibitor Cocktail | Roche | 4693132001 |
| Immobilon Forte Western HRP substrate | Merck | WBLUF0500 |
| Leu-Leu methylester hydrobromide (LLOME) | Sigma-Aldrich | L7393 |
| MLN4924 | Sigma-Aldrich | 505477 |
| MG132 | Sigma-Aldrich | M7449 |
| valinomycin | Sigma-Aldrich | V0627 |
| anti-FLAG M2 agarose | Sigma-Aldrich | A2220 |
| Mitophagy detection kit | Dojindo | MD01 |
| Strep-tactin XT 4Flow high capacity | iBa | 2-5030-002 |

Critical commercial assays

- Mitophagy dye | Dojindo | 349-92051 |

Deposited data

- Mass spectrometry data used in this study | the Pride database (Project accession: PXD035151) |

Experimental models: Cell lines

| Cell line | Source |
|-----------|--------|
| HeLa Kyoto | N/A |
| PlatE | N/A |

Oligonucleotides

- siRNAs, see Table S3 | This study |
- Cloning primers, See Table S3 | This study |

Recombinant DNA

| DNA | Source |
|-----|--------|
| pMRX-ires-puro-EGFP-Atg5 | Maejima et al. (2013) |
| pcDNA3.1-FLAG-WDFY1 | This study |
| pMRX-ires-puro-EGFP-Galectin3 | Fujita et al. (2013) |
| pMRC-ires-puro-mStrawberry-DDB1 | This study |
| pMRC-ires-puro-mStrawberry-CUL4A | This study |
| pMRX-ires-puro-EGFP-WDFY1 | This study |
| pMRX-ires-puro-EGFP-CUL4A | This study |
| pMRX-ires-puro-EGFP-WDFY1 siRNA-resistant | This study |
| pMRX-ires-puro-EGFP-EGFP-WDFY1 siRNA-resistant | This study |
| pMRX-ires-puro-EGFP-WDFY1 siRNA-resistant | This study |
| pmRFP | Clontech |
| pcDNA3.1-LAMP2 | This study |
| pcDNA3.1-LA2-L/LA1-C | This study |
| pcDNA3.1-LA1-L/LA2-C | This study |
| tLC3 | Kimura et al. (2007) |
| pCAG-One strep-tag-FLAG (OSF)-WDFY1 | This study |
| FLAG-TR-TUBE | Yoshida et al. (2015) |
| HA-TR-TUBE | Yoshida et al. (2015) |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maho Hamasaki (hamasaki@fbs.osaka-u.ac.jp).

**Material availability**
All stable materials and reagents generated in this study are available from the lead contact upon request under a universal MTA.

**Data and code availability**
- Mass spectrometry data used in this study have been deposited into the Pride database (Project accession: PXD035151).
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Cell lines and culture conditions**
HeLa cells and Plat-E (Morita et al., 2000) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM D6429; Sigma-Aldrich) containing 10% fetal bovine serum (FBS), 5 U/mL penicillin, and 50 U/mL streptomycin at 37°C with 5% CO₂. For nutrient starvation, cells were cultured in EBSS (Sigma-Aldrich).

**Transfections and generation of stable cell lines**
For transient expression, plasmid transfection was carried out using the Lipofectamine 2000 reagent (Invitrogen) or PEI MAX according to the manufacturer’s protocol. Retrovirus production was performed as described earlier (Bhargava et al., 2020). For stable expression, retrovirus infected stable transformants were selected in a growth medium with 1 μg/mL puromycin or 5 μg/mL blasticidin (Invitrogen).

**METHOD DETAILS**

**Reagents and antibodies**
All reagents, resources, and antibodies used in this study are listed in Key resources table. Leu-Leu methylester hydrobromide (LLOMe), Bafilomycin A1 (BafA1), valinomycin, and MG132 were dissolved in DMSO for stock solutions. MLN4924 was dissolved in ethanol for stock solution.

**RNA interference**
Validated siRNAs (Invitrogen) were used for siRNA screening. Solid-phase siRNA transfection was performed as described (Erfle et al., 2008). For validation experiments, siRNA sequences used in this study are listed in Key resources table, siRNAs were transfected to cells using RNAiMAX (Invitrogen) at 20 nM according to the manufacturer’s instructions. The cells were analyzed 72 h after transfection.

**Plasmid construction**
All plasmids used in the study were listed in Key resources table. The pMRX-IRES-puro vector was kindly provided by Toshio Kitamura (University of Tokyo, Tokyo, Japan) (Morita et al., 2000). Human full-length WDFY1, DDB1, and CUL4A were amplified with...
specific primers described in key resources table using HeLa cDNA and cloned into pMRX-GFP, pMRX-mStrawberry (mSt), pcDNA3.1-GFP, pcDNA3.1-mSt or pcDNA3.1-FLAG vectors. In addition, pMRX vectors encoding GFP-Atg5, GFP-RFP-LC3, or GFP-GaIII were constructed to encode sequences as previously described (Fujita et al., 2013; Kimura et al., 2007; Maejima et al., 2013).

**Beads transfection**

Beads transfection was performed as previously reported (Kobayashi et al., 2010). In brief, transfection reagent-coated beads were prepared by mixing the beads (NH2-, 17145–5; PolySciences, Inc.) with transfection reagent according to the manufacturer’s instructions, except that bead suspension was used instead of DNA solution. The bead mixture was mixed with 1 mL of growth medium and added to cells in a 96-well plate by replacing the medium. After incubation for 3 h at 37°C in a CO2 incubator, the cells were washed with a fresh growth medium to remove unattached beads and further incubated for the time indicated in each experiment.

**Fluorescence and immunofluorescence microscopy**

Cells were cultured on coverslips or glass-bottomed 96 well plate (Greiner), fixed with 3% PFA in PBS for 20 min, and permeabilized with 50 μg/mL digitonin in PBS for 7 min. Cells were then blocked with PBS containing 0.2% gelatin for 30 min and incubated with the indicated primary antibodies diluted with blocking buffer. After washing with PBS, the samples were incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies) diluted with blocking buffer. Images were acquired using an inverted microscope (IX83; Olympus) equipped with a 60x/1.40 NA oil immersion objective (Olympus) or a confocal quantitative image cytometer (CQ1; Yokogawa).

**Western blotting**

Western blotting was performed as described previously (Tabata et al., 2021). Samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 1–5% skim milk containing TBST (0.1% Tween 20 in TBS) and incubated with primary antibodies overnight at 4°C. Membranes were washed with TBST and incubated with HRP-conjugated secondary antibodies (GE Healthcare). The proteins were detected using a chemiluminescence detector (Bio-Rad).

**Immunoprecipitation and immunoblotting**

Forty-eight hours post-transfection, HeLa cells were used for immunoprecipitation experiments. For immunoprecipitation for FLAG-WDFY1 or OSF-WDFY1, cells were lysed with lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor mixture (Roche). For immunoprecipitation for FLAG-TR-TUBE, cells were lysed with lysis buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5% NP-40) containing a protease inhibitor mixture. After lysis, the cell lysates were centrifuged at 20,000 x g for 10 min at 4°C. The supernatants were subjected to immunoprecipitation using FLAG M2 agarose beads or Streptactin beads for 2 h at 4°C. The beads were then washed with 1 mL lysis buffer five times, and immune complexes were eluted by 2× protein sample buffer (100 mM Tris-HCl pH 6.8, 5 mM EDTA, 0.1% bromophenol blue, 20% glycerol, 4% SDS, 12% β-mercaptoethanol). After heating samples for 5 min at 95°C, input cell lysates and immunoprecipitants were subjected to western blotting analyses.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All values in experiments were presented as means ± S.D. as indicated in the figure legends. An unpaired student’s t-tests, ordinary one-way ANOVA, or 2-way ANOVA was performed using Prism8 or 9 software (GraphPad). A p value <0.05 was considered statistically significant.