Supplementary information

Supplementary figure and movie legends

Supplementary Figure 1. Various membrane damaging agents induce ESCRT proteins recruitment to the site of rupture. *D. discoideum* expressing GFP-Vps32 or Vps4-GFP were treated with digitonin, GPN, LLOMe, purified recombinant ESAT-6 or medium (control) and visualized over time. Still images show representative cells in phase-contrast and fluorescence at 0, 5, 10 and 15 min after treatment. On the right, magnification of one of the images per treatment. Red arrows point to GFP-Vps32 and Vps4-GFP structures at the sites of damage. Scale bars 10 μm.

Supplementary Figure 2. Spatial association of GFP-Vps32 with damaged lysosomes. *D. discoideum* expressing GFP-Vps32 were incubated with TRITC-Dextran (red) (A) or Alexa Fluor 647 Dextran (red) (B) for at least 3 h to label all endosomes, treated with LLOMe or GPN, respectively, and monitored by time-lapse microscopy. Kymographs generated by a repeated linescan through a representative cell show the sustained association of GFP-Vps32 structures with the lysosomes and endosomes (black and white arrows). In B, the compound was added immediately before imaging started.

Supplementary Figure 3. Ultrastructural appearance of the escape site of *M. marinum* in several ESCRT mutants. *D. discoideum* were infected with *M. marinum* and fixed for TEM at 24 hpi. *M. marinum* ("*M.m.*" labelled in red) accessed the cytosol in wt (A and D), *tsg101*- (B and C), *alix*- (E) and *alg2a-/b*- (F). Sites of membrane disruption are highlighted with blue arrows. (C) High magnification inset of the region of interest in (B). Scale bars, 1 μm.

Supplementary Figure 4. Ubiquitination and GFP-Plin recruitment as readout of *M. marinum* cytosolic access. (A-B) *D. discoideum* wt or mutant (*atg1*-, *tsg101*- or *atg1- tsg101*-) were fixed for immunofluorescence. Post-lysosomes were labelled with p80 (green), nuclei were labelled with DAPI (blue), ubiquitin was in green and Atg8 in red. (C-D) *D. discoideum* wt or mutant (*atg1*-, *tsg101*-, or *atg1-tsg101*-) were infected with *M. marinum* (blue) and fixed for immunofluorescence. Both

ubiquitinin and Atg8 (green) decorated the bacteria when the MCV (p80, red) was disrupted. Scale bars 1 µm. (E) *D. discoideum* wt or mutant (*atg1-*, *tsg101-*, or *atg1-tsg101-*) expressing GFP-Plin (green) were infected with *M. marinum* for live microscopy. All mutants showed an increase of GFP-Plin recruitment on the bacteria (red). (F) Proportion of the *M. marinum* bacteria or microcolonies decorated with GFP-Plin. The plot shows the mean and standard deviation (WT N=4, n=139; *atg1-* N=3, n=148; *tsg101-* N=3, n=98; *atg1- tsg101-* N=3, n=191). Two-tailed *t*-tests were performed.

Supplementary Figure 5. AlxA and Alg2a/b do not impact *M. marinum* intracellular replication. (A) *D. discoideum* wt or mutant (*alxA-* or *alg2-/b-*) were infected with *M. marinum* and fixed for immunostaining at 8 hpi (*M. marinum* in red, ubiquitin in green, DAPI in blue). Arrows point to ubiquitinated bacteria. Scale bars, 10 µm and 5 µm for the insets. (B) Quantification of the proportion of ubiquitinated bacteria or bacterial microcolonies. The plot shows the mean and standard deviation [WT (JH10) N=4, n=144; *alxA-* (JH10) N=3, n=134; *alg2a-/b-* (JH10) N=3, n=266]. (C) *D. discoideum* wt or mutant (*alxA-* or *alg2-/b-*) were infected with *M. marinum* and fixed for immunostaining at 8 hpi (*M. marinum* in red, Atg8 in green, DAPI in blue). Arrows point to bacteria decorated with Atg8. Scale bars, 10 µm and 5 µm for the insets. (D) Quantification of the proportion of bacteria or bacterial microcolonies decorated with Atg8. The plot shows the mean and standard deviation [WT (JH10) N=4, n=275; *alxA-* (JH10) N=4, n=170; *alg2a-/b-* (JH10) N=4, n=448]. Two-tailed *t*-test were performed. (E-F) *D. discoideum* wt or mutant (*alxA-* or *alg2-/b-*) were infected with *M. marinum* (blue) and fixed for immunofluorescence. Both ubiquitinin and Atg8 (green) decorate the bacteria when the MCV (p80, red) was disrupted. Scale bars, 1 µm. (G-H) *D. discoideum* wt or mutant (*alxA-* or *alg2-/b-*) were infected with luminescent *M. marinum* and intracellular bacterial growth was monitored in a plate reader over 72 hpi. There was no significant difference between *M. marinum* growth in wt and mutants. Plots represent the mean and standard deviation of N=3 independent experiments. Two-way ANOVA was performed.

Supplementary Movie 1. The ultrastructure of the MCV at the site of *M. marinum* escape. FIB-SEM stack and corresponding 3D reconstruction of the damaged MCV shown in Fig. 1D. "*M.m.*", *Mycobacterium marinum* (in dark blue); "MCV", *Mycobacterium*-containing vacuole (in light blue); electron-dense material surrounding the cytosolic bacteria, in yellow.

Supplementary Movie 2. GFP-Vps32 forms rings in the vicinity of *M. marinum.* Time-lapse of a *D. discoideum* cell expressing GFP-Vps32 and infected with *M. marinum* wt (red). Arrows show GFP-Vps32 rings which formed and seemed to move along the bacterium. Scale bar, 10 μm.

Supplementary Movie 3. Dynamics of GFP-Vps32 recruitment in the vicinity of the MCV. Time-lapse of a *D. discoideum* cell expressing GFP-Vps32 and AmtA-mCherry and infected with *M. marinum* wt (red). Scale bars, 10 μm.

Supplementary Movie 4. Localisation of ESCRT and autophagy components upon digitonin treatment. Time-lapse imaging of *D. discoideum* cells expressing GFP-Tsg101, GFP-Vps32, Vps4-GFP or GFP-Atg8 upon digitonin treatment. Scale bar, 10 μm.

Supplementary Movie 5. Localisation of ESCRT and autophagy components upon LLOMe treatment. Time-lapse imaging of *D. discoideum* cells expressing GFP-Tsg101, GFP-Vps32, Vps4-GFP or GFP-Atg8 upon LLOMe treatment. Cells were incubated with fluorescent dextran (red) for at least 3 h prior to the treatment. Scale bar, 10 μm.

Supplementary Movie 6. Annexin V labels membrane damage upon digitonin treatment. Time-lapse imaging of *D. discoideum* cells that were incubated with Annexin V Alexa Fluor 594 conjugate in the presence of Ca^{2+} and then treated with digitonin. Arrowheads point to the site of membrane disruption. Scale bar, 10 μm.

Supplementary Movie 7. Related to Figure 5G and Figure 5H. Lysosomal leakage upon LLOMe treatment. Time-lapse imaging of *D. discoideum* wt, *tsg101-* and *atg1-* that were incubated with the 10 kDa Alexa Fluor 647 dextran (red) and the 0.5 kDa soluble pH indicator HPTS (green) and then treated with LLOMe. Scale bar, 10 μm.

Supplementary references

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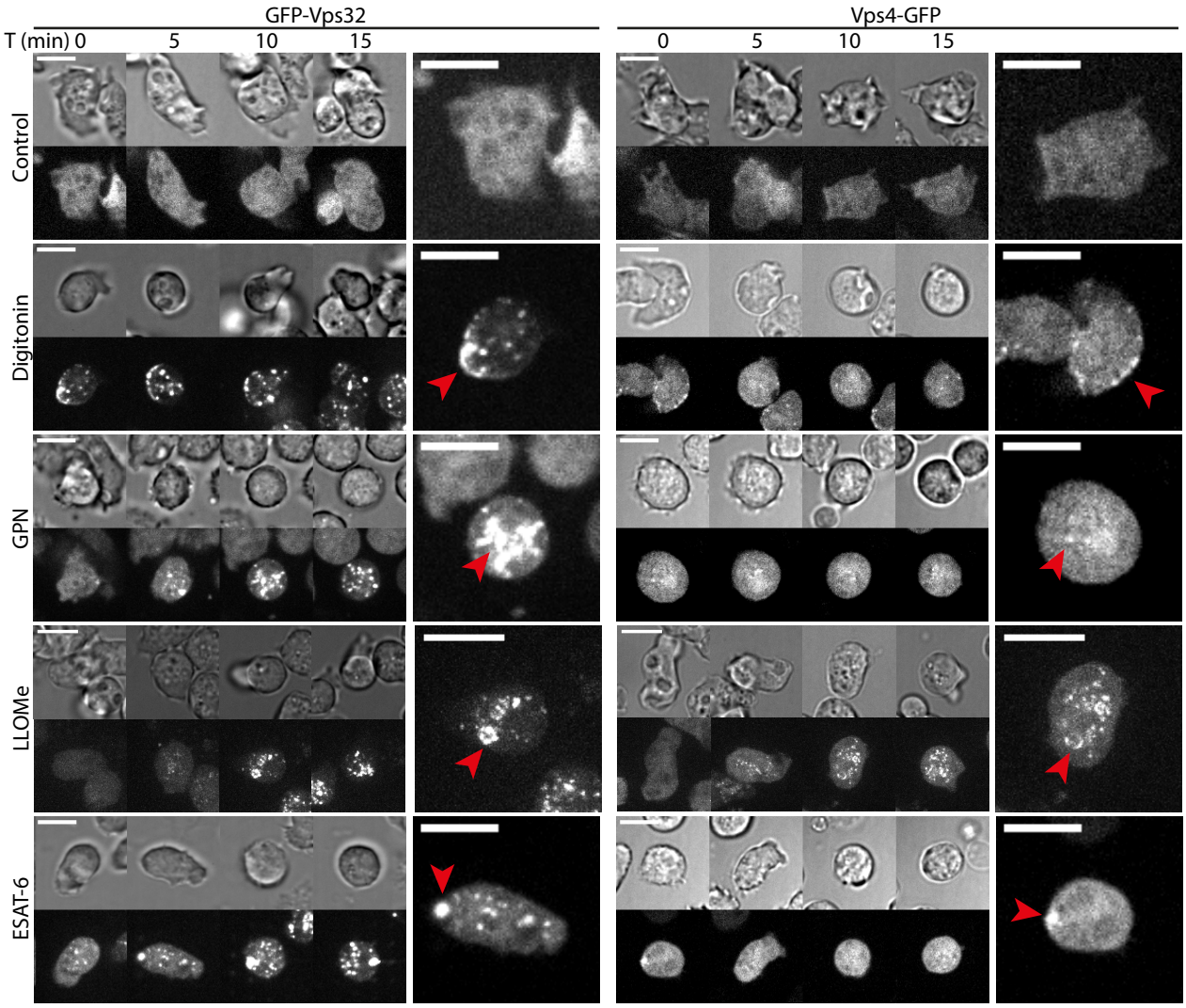
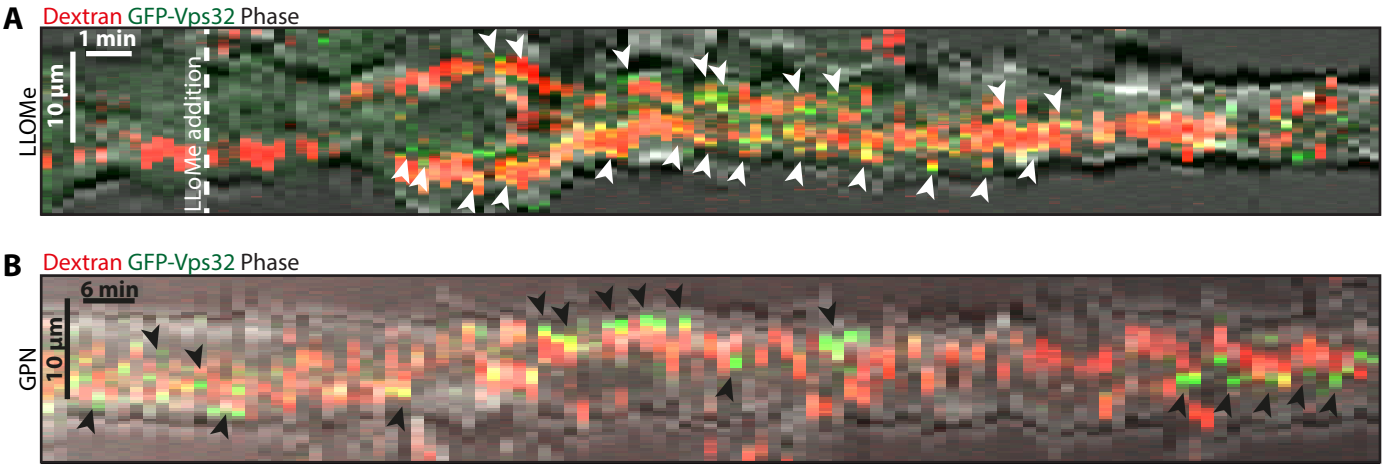
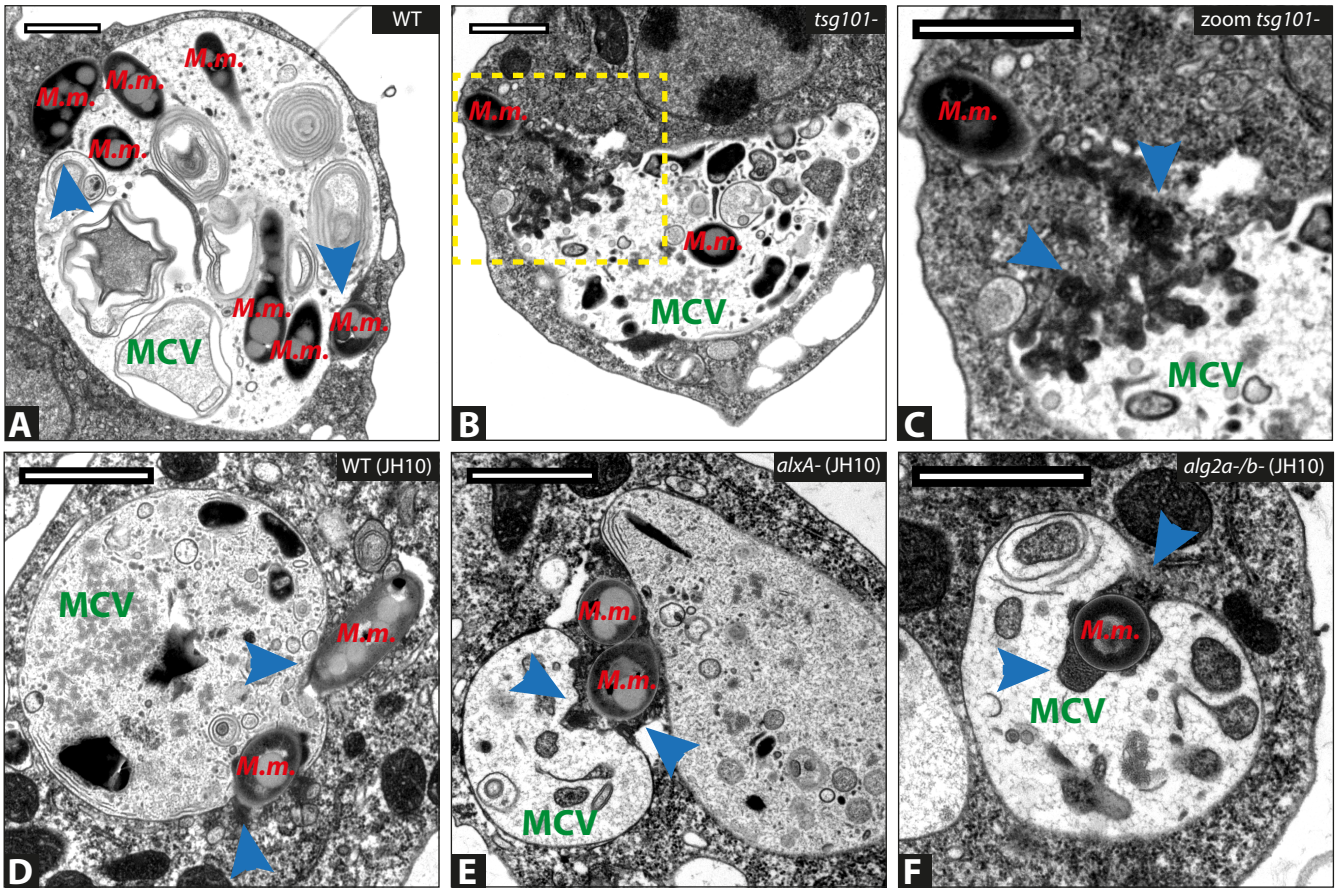
Supplementary Figure 1



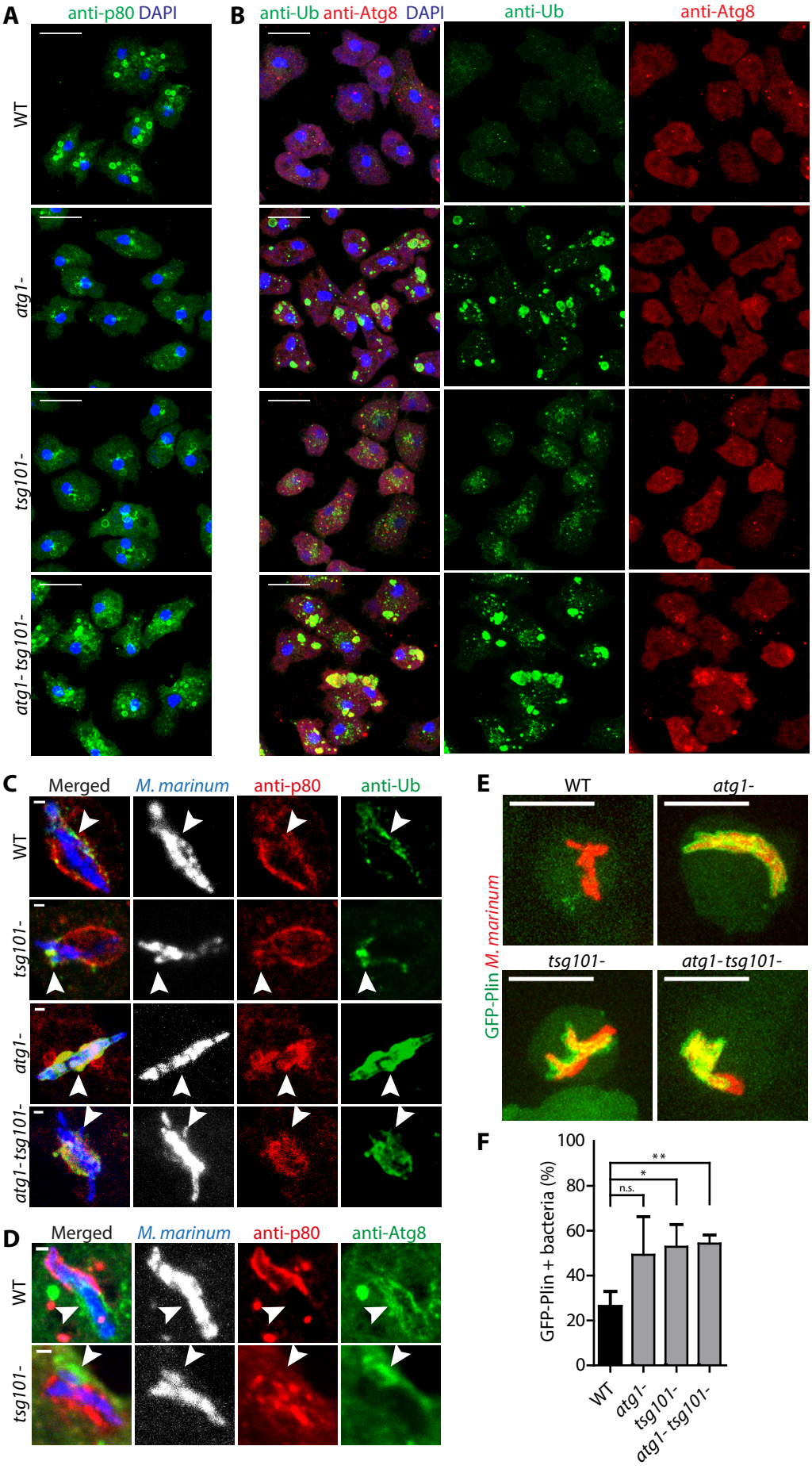
Supplementary Figure 2



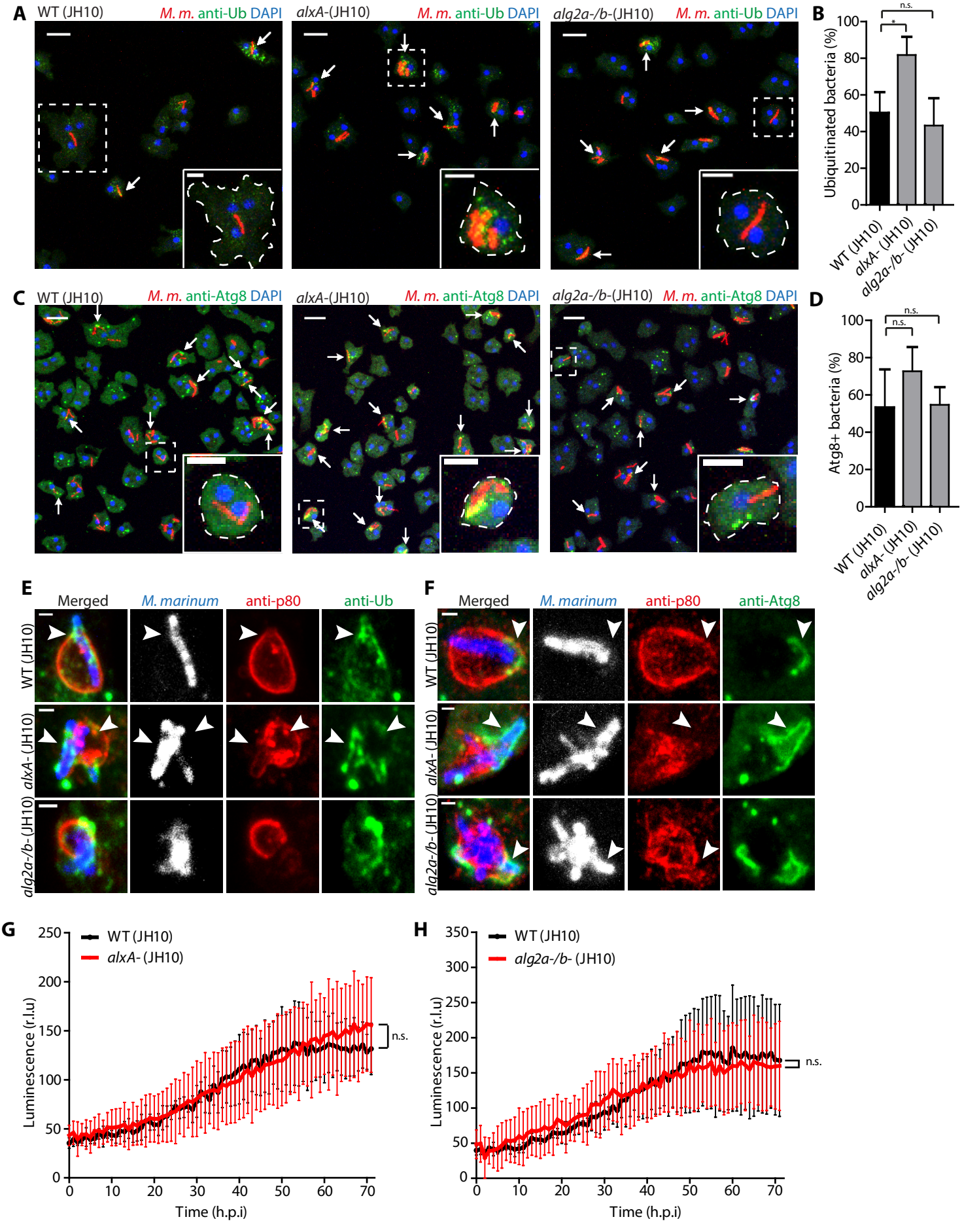
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Table 1.

Strain/Plasmid	Relevant characteristics	Source/Reference
D. discoideum		
Ax2(Ka)	wt	
JH10	wt	
Ax2(Ka) *atg1-*	KO	1
Ax2(Ka) *tsg101-*	KO	This study
Ax2(Ka) *atg1- tsg101-*	KO	This study
JH10 *alxA-*	KO	2
JH10 *alg2a/b-*	KO	3
M. marinum		
M strain	wt	L. Ramakrishnan (Washington University)
ΔRD1	KO	L. Ramakrishnan (Washington University)
D. discoideum plasmids		
pDM317		4
pDM323		1
pJSK500	GFP-Atg8a	5
GFP-Tsg101	*tsg101* cDNA (DDB_G0286797) in pDM317	This study
GFP-Vps32	*vps32* cDNA (DDB_G0275573) in pDM317	This study
Vps4-GFP	*vps4* cDNA (DDB_G0284347) in pDM323	This study
pDNeoGFP-Plin	GFP-Plin	6
AmtA-mCherry	*amtA* cDNA(DDB_G0277503) in pDM1044	7
Mycobacteria plasmids		
pCherry10	mCherry under control of the G13 promoter, Hyg^r	8
pMV306**::***lux*	bacterial luciferase under control of the G13 promoter, Kan^r	9