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Targeting by AutophaGy proteins (TAG): Targeting of IFNG-inducible GTPases to membranes by the LC3 conjugation system of autophagy

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ABSTRACT

LC3 has been used as a marker to locate autophagosomes. However, it is also well established that LC3 can localize on various membranous structures other than autophagosomes. We recently demonstrated that the LC3 conjugation system (ATG7, ATG3, and ATG12–ATG5-ATG16L1) is required to target LC3 and IFNG (interferon, gamma)-inducible GTPases to the parasitophorus vacuole membrane (PVM) of a protist parasite *Toxoplasma gondii* and consequently for IFNG to control *T. gondii* infection. Here we show that not only LC3, but also its homologs (GABARAP, GABARAPL1, and GABARAPL2) localize on the PVM of *T. gondii* in a conjugation-dependent manner. Knockout/knockdown of all LC3 homologs led to a significant reduction in targeting of the IFNG-inducible GTPases to the PVM of *T. gondii* and the IFNG-mediated control of *T. gondii* infection. Furthermore, when we relocated the ATG12–ATG5-ATG16L1 complex, which specifies the conjugation site of LC3 homologs, to alternative target membranes, the IFNG-inducible GTPases were targeted to the new target membranes rather than the PVM of *T. gondii*. These data suggest that the localization of LC3 homologs onto a membrane by the LC3 conjugation system is necessary and sufficient for targeting of the IFNG-inducible GTPases to the membrane, implying Targeting by AutophaGy proteins (TAG). Our data further suggest that the conjugation of ubiquitin-like LC3 homologs to the phospholipids of membranes may change the destiny of the membranes beyond degradation through lysosomal fusion, as the conjugation of ubiquitin to proteins changes the destiny of the proteins beyond proteasomal degradation.

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Introduction

MAP1LC3/LC3 (microtubule associated protein 1 light chain 3) has been the most frequently used marker to locate autophagosomes, which sequester and deliver cytoplasmic materials to lysosomes for degradation.[1] In canonical macroautophagy (henceforth autophagy), LC3 and its homologs play crucial roles in specific capture of autophagosomal cargoes, formation of globular autophagosomes, and delivery of autophagosomes to lysosomes through microtubule tracks.[2-4] Intriguingly, recent studies show that LC3 can be also associated with nonautophagosomal membranous structures, such as phagosomes and secretory vesicles, but the function of LC3 on such structures is poorly understood.[5-8] Moreover, the involvement of LC3 homologs and their potential functions in those nonautophagosomes are unclear.[9]

The ubiquitin-like LC3 and its homologs in the mammalian system (e.g., LC3A, LC3B, GABARAP, GABARAPL1, and GABARAPL2 in the murine system) are conjugated primarily to phosphatidylethanolamine in lipid bilayer membranes, through the sequential action of the E1-like activating enzyme ATG7, E2-like conjugating enzyme ATG3, and E3-like ligase ATG12–ATG5-ATG16L1 complex in vivo.[2] It is the ATG12–ATG5-ATG16L1 complex that specifies the conjugation site of LC3 and its homologs, so a plasma membrane localizing version of this complex results in LC3 conjugation on the plasma membrane rather than the autophagosomal membrane.[10]

We recently showed that the LC3 conjugation system is required for targeting of LC3 and IFNG-inducible GTPases to the parasitophorus vacuole membrane (PVM) of the protist parasite *Toxoplasma gondii* and subsequent control of *T. gondii* infection by IFNG.[11] In contrast, neither lysosomal degradation through the autophagy pathway nor upstream autophagy genes (e.g., *Ulk1, Ulk2, Atg14*) are required for the targeting process or the IFNG-mediated control of *T. gondii* infection, signifying the noncanonical and nondegradative function of the LC3 conjugation system.[11]

The parasitophorus vacuole (PV) is a single-membrane cytoplasmic vacuole that contains the invaded *T. gondii* and is formed from the cellular plasma membrane during the active invasion of *T. gondii*.[12] Since the PV does not fuse with endosomes or lysosomes, it provides a shelter for *T. gondii* to replicate safely while evading the hostile immune surveillance and defense system in the cytoplasm of host cell.[13,14] Upon the

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stimulation of the cells with IFNG (interferon, gamma), however, IFNG-inducible GTPases, such as immunity-related GTPases (IRGs) and guanylate binding proteins (GBPs), are induced and targeted to the PVM of *T. gondii*.[15,16] Targeting of IRGs and GBPs to the PVM leads to the disruption of the PVM structure and subsequent cytoplasmic exposure followed by the death of *T. gondii*.[15,16] We found that LC3 localizes on the PVM of *T. gondii* in both unstimulated and IFNG-stimulated cells, suggesting that LC3 localization on the PVM is not dependent on IFNG stimulation and it may function in targeting the IFNG-inducible GTPases to their target membrane.[11]

Here we show that all known LC3 homologs localized on the PVM of *T. gondii* in a conjugation-dependent manner and were required for proper targeting of the IFNG-inducible GTPases and inhibition of *T. gondii* infection. Further, the LC3 conjugation system was not only necessary, but also sufficient for targeting of the IFNG-inducible GTPases to a target membrane. Our data suggest that the LC3 conjugation system may target specific effector molecules to a membranous structure by conjugating ubiquitin-like LC3 homologs to the phospholipids of the membrane, implying TAG.

Results

LC3 homologs localize on the parasitophorus vacuole membrane of T. gondii in a conjugation-dependent manner

We previously detected endogenous LC3 on the PVM of *T. gondii*.[11] We also detected early recruitment of the ATG12–ATG5-ATG16L1 complex (as shown by ATG5) to the PVM of *T. gondii* (Fig. S1). Therefore, the other LC3 homologs might also localize on the PVM of *T. gondii*. The antibody that we used was known to detect the major form of LC3 (LC3B) preferentially.[11] To investigate the potential localization of LC3 homologs on the PVM of *T. gondii* specifically, we cloned all known protein coding transcripts of LC3 homologs in the murine system (namely LC3A, LC3B, LC3B-1, LC3B-2, GABARAP, GABARAP-1, GABARAPL1, GABARAPL2) into a lentiviral plasmid that expresses N-terminal FLAG and HA epitope-tagged proteins (Fig. 1A). Upon transduction and selection of wild-type mouse embryonic fibroblasts (WT MEFs) with lentiviruses expressing individual LC3 homologs, we examined the expression of the LC3 homologs and their localization with regard to the PVM of *T. gondii*. Three of the 8 cloned LC3 homologs were not expressed well for some unclear reason (Fig. S2A). However, the other 5 cloned LC3 homologs were expressed reliably in the transduced MEFs (Fig. 1B). Consistent with our previous observation of the endogenous LC3,[11] all LC3 homologs were detected on the PVM of *T. gondii* in the absence of IFNG-mediated activation of the cells (Fig. S2B). In the cells activated with IFNG, they colocalized with the IFNG-inducible GTPases (TGTP1/IRGB6 as a representative of IRGs) on the PVM of *T. gondii* (Fig. 1C). Although there was a concern that epitope-tagging of LC3 might affect its localization on the PVM,[11] at least our tagging system that adds ~40 amino acids at the N terminus did not significantly affect the localization of LC3B on the PVM of *T. gondii* (Fig. S2C). Furthermore, IFNG augmented the localization of epitope-tagged LC3 homologs on the PVM of *T. gondii* in the transduced MEFs, as it does for endogenous LC3 (Fig. S2D).[11] These data demonstrated that not only the major LC3B, but also all LC3 and GABARAP subfamily members localized on the PVM of *T. gondii*.

LC3 can be associated with a membrane structure in both conjugation-dependent and independent manners.[17] To test the role of conjugation for the localization of LC3 homologs on the PVM of *T. gondii*, we first stably expressed a nonconjugatable mutant of LC3B (LC3B^{G120A}) using the lentiviral transduction system in *Lc3b* knockout (KO) MEFs.[6] As expected, the phospholipid-conjugated form of LC3B (the faster moving form of WT LC3B) disappeared in the MEFs expressing the LC3B^{G120A} mutant (Fig. 2A). Although its expression level was comparable to that of WT (Fig. 2A), LC3B^{G120A} did not localize on the PVM of *T. gondii* in contrast to the WT LC3B (Fig. 2B). Further, we generated WT MEFs stably expressing the nonconjugatable mutants of GABARAP (GABARAP^{G116A}), GABARAPL1 (GABARAPL1^{G116A}), and GABARAPL2 (GABARAPL2^{G116A}). Similar to LC3B^{G120A}, they were expressed well but did not localize on the PVM of *T. gondii* (Fig. 2A and 2B). Collectively, these data suggest that the LC3 homologs localize on the PVM of *T. gondii* through their conjugation to the phospholipids in the membrane of PV.

LC3 homologs are required for targeting of the IFNG-inducible GTPases and consequent control of T. gondii infection by IFNG

Only the LC3 conjugation system of autophagy (ATG7, ATG3, and ATG12–ATG5-ATG16L1), not the lysosomal degradation nor upstream autophagy initiation genes, was required for targeting of the IFNG-inducible GTPases to the PVM and consequent control of *T. gondii* infection by IFNG.[11] Since the only known function of the whole LC3 conjugation system in vivo is indeed to conjugate LC3 homologs to the phospholipids in a membrane, these data suggest that the conjugation of LC3 homologs to the PVM may be also required for the targeting process and subsequent control of *T. gondii* infection by IFNG. However, there is a significant discrepancy between LC3 and the IFNG-inducible GTPases localizing on the PVM of *T. gondii* (for example, compare Fig. 3A and B).[11] These data further suggest that the recruitment of LC3 and the IFNG-inducible GTPases to the PVM of *T. gondii* might be spatially and temporally regulated for efficient targeting and disruption of the membrane structure of the pathogens.[11] Therefore, we next examined the role of LC3 homologs in targeting of the IFNG-inducible GTPases to the PVM and consequent control of *T. gondii* infection by IFNG. Since only *Lc3b* knockout cells are currently available,[18] we performed knockdown of the remaining LC3 homologs stably with small hairpin RNA (shRNA) and transiently with small interfering RNA (siRNA). First, to investigate the role of the LC3 subfamily, we transduced *Lc3b* WT and KO MEFs with lentiviruses expressing shRNA against red fluorescent protein (*Rfp*, as control) or *Lc3a*. Upon drug selection of the transduced cells, the cells were further treated with IFNG and infected with *T. gondii*, and we monitored targeting of the IFNG-inducible GTPases to the PVM of *T. gondii* and control of *T. gondii* infection by IFNG. The substantial knockdown of *Lc3a* transcript and the knockout of *Lc3b* did not significantly affect targeting of the IFNG-inducible

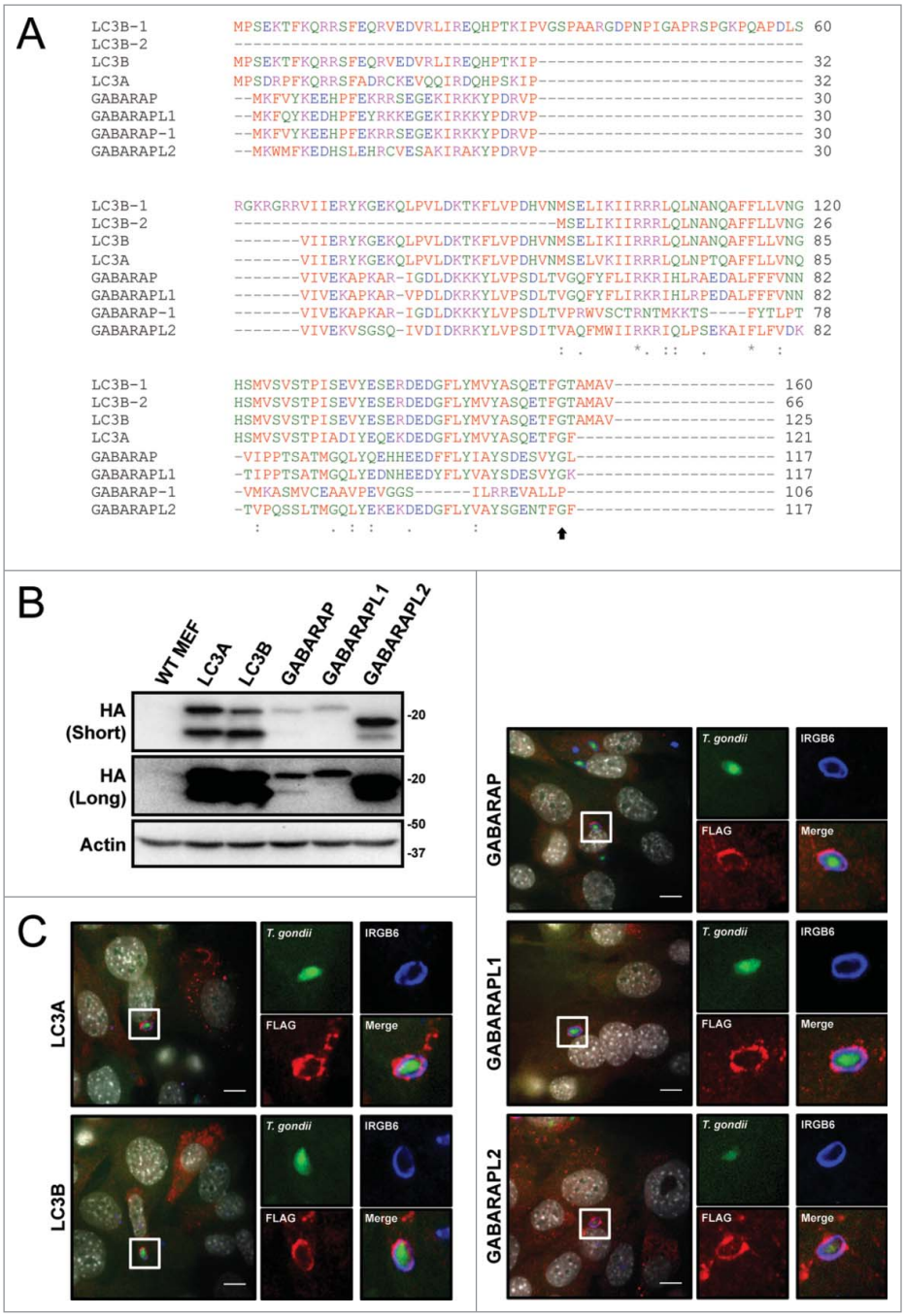

Figure 1. LC3 homologs localized on the PVM of *T. gondii*. (A) Protein sequences of murine LC3 homologs aligned using CLUSTAL 2.1 multiple sequence alignment program. The black arrow indicates the position of a conserved glycine residue for conjugation to the membrane. (B) Western blot assay for the protein expression level of LC3 homologs in the transduced WT MEFs. Short and long indicates exposure time. (C) Immunofluorescence assay for the localization of *T. gondii* (green, GFP), LC3 homologs (red, FLAG), and TGTP1/IRGB6 (blue, TGTP1/IRGB6) in the transduced MEFs, pretreated with 100 U/ml IFNG for 24 h and then infected with *T. gondii* (MOI = 1) for 2 h. Scale bar: 10 μm. Experiments were performed at least 3 times and representative data (B) and images (C) are shown here.

GTPases (TGTP1/IRGB6) to the PVM of *T. gondii* (Fig. 3B) and control of *T. gondii* infection by IFNG (Fig. 3C). Second, to investigate the role of the GABARAP subfamily, we transfected the *Lc3b* WT MEFs with either pooled control siRNAs or pooled siRNAs against *Gabarap, Gabarapl1*, and *Gabarapl2* together. Again, the substantial knockdown of *Gabarap* (reduced to 14.1% ± 4.0), *Gabarapl1* (reduced to 29.1% ± 5.1), and *Gabarapl2* (reduced to 23.7% ± 4.4) transcripts (Fig. 3D) did not significantly affect targeting of the IFNG- induced GTPases (IIGP1/IRGA6 and TGTP1/IRGB6) to the PVM of *T. gondii* (Fig. 3E) and control of *T. gondii* infection by IFNG (Fig. 3F). Of note, none of the knockout and/or knockdown of LC3 homologs significantly affected the infectivity of *T. gondii* for the cells (Fig. S3).

Considering the fact that all LC3 homologs localized on the PVM of *T. gondii* (Fig. 1C), these data suggest that either the LC3 subfamily or GABARAP subfamily of LC3 homologs is sufficient for the targeting process and subsequent control of *T. gondii* infection by IFNG. Thus, to determine the role of the entire group of LC3 homologs, we transfected pooled siRNAs against the *Gabarap* subfamily to the *Lc3b* KO MEFs transduced with shRNA against *Lc3a*. RNA interference against LC3

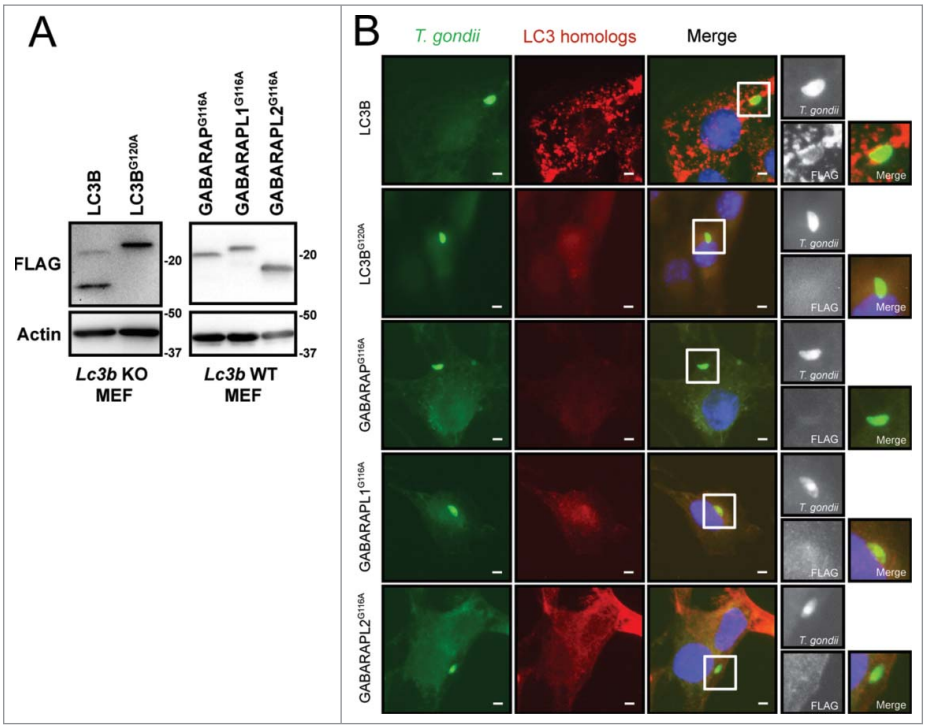

Figure 2. LC3 homologs localized on the PVM of *T. gondii* in a conjugation-dependent manner. (A) Western blot assay for the protein expression level of nonconjugatable LC3 homologs in the transduced *Lc3b* WT and KO MEFs. (B) Immunofluorescence assay for the localization of *T. gondii* (green, GFP) and nonconjugatable LC3 homologs (red, FLAG) in the transduced MEFs, infected with *T. gondii* (MOI = 1) for 2 h. Scale bar: 10 μm. Experiments were performed at least 3 times and representative images are shown here.

homologs led to knockdown of *Lc3a* (reduced to 20.8% ± 3.5), *Gabarap* (reduced to 9.0% ± 1.8), *Gabarapl1* (reduced to 15.4% ± 2.2), and *Gabarapl2* (reduced to 30.7% ± 4.0) transcripts (Fig. 3G). In that condition, we observed significantly less targeting of the IFNG-inducible GTPases (IIGP1/IRGA6 and TGTP1/IRGB6) to the PVM of *T. gondii*, compared to the pooled control siRNAs (Fig. 3H). Consistently, we also observed significantly less control of *T. gondii* infection by IFNG in the cells expressing significantly less LC3 homologs (Fig. 3I). These data suggest that both subfamilies of LC3 homologs function analogously in recruiting the IFNG GTPases to the PVM of *T. gondii* and consequent control of *T. gondii* infection by IFNG. It is noteworthy that both LC3 and GABARAP subfamilies are essential for autophagosome biogenesis but act in different stages of autophagy — the LC3 subfamily in elongation of the phagophore membrane, whereas the GABARAP subfamily in a later stage of autophagosome maturation.[19] In addition, recent studies further support the distinction between those 2 subfamilies with respect to their autophagic functions.[20-23] In contrast to the distinct autophagic functions of LC3 homologs, these data suggest that all LC3 homologs play an essential but overlapping function for targeting of the IFNG GTPases to the PVM and subsequent inhibitory effect on *T. gondii* infection.

The LC3 conjugation system of autophagy is sufficient for targeting the IFNG-inducible GTPases to an alternative target membrane

The membrane-bound LC3 homologs may function as molecular beacons to guide the IFNG-inducible GTPases to the membrane where they locate. We asked whether the localization of LC3 homologs on a membrane, other than the PVM of *T. gondii*, is sufficient to recruit the IFNG-inducible GTPases to that alternative membrane. The most direct way to test this possibility would be to conjugate an LC3 homolog to a target membrane after removing all the other LC3 homologs inside a cell, and to investigate the recruitment of the IFNG-inducible GTPases to that target membrane. Currently, such a cell line lacking all known LC3 homologs does not yet exist. Thus, we reasoned that changing the localization of the ATG12–ATG5-ATG16L1 complex might be the best practical way to test the possibility. The ATG12–ATG5-ATG16L1 complex determines the conjugation site of LC3: its plasma membrane-localizing version, generated by tagging a 17 amino acid KRAS-CAAX motif (KRAS-CAAX: KDGKKKKKKSKTKCVIM) to the C terminus of an ATG16L1 mutant (ATG16L1[1–249]), redirects LC3 conjugation mostly to the plasma membrane where the ATG16L1 mutant predominantly localizes.[10] Therefore, we took the same approach in our system, expressing the ATG16L1 mutants with added C-terminal KRAS-CAAX motifs in *Atg16l1* KO MEFs and examining targeting of the IFNG-inducible GTPases to the plasma membrane.

Mammalian ATG16L1 has 3 major domains: the N-terminal domain for binding to ATG5, the coiled-coil domain for dimerization and multiple protein-protein interactions, and C-terminal WD repeat β-propeller domain for potentially additional protein-protein interactions (e.g., with ubiquitin).[24-28] We previously reported that only the evolutionarily conserved N terminus (i.e., amino acids 1–249) of ATG16L1, including the ATG5-binding and coiled-coil domains, is sufficient for targeting of the IFNG-inducible GTPases to the PVM of *T. gondii*

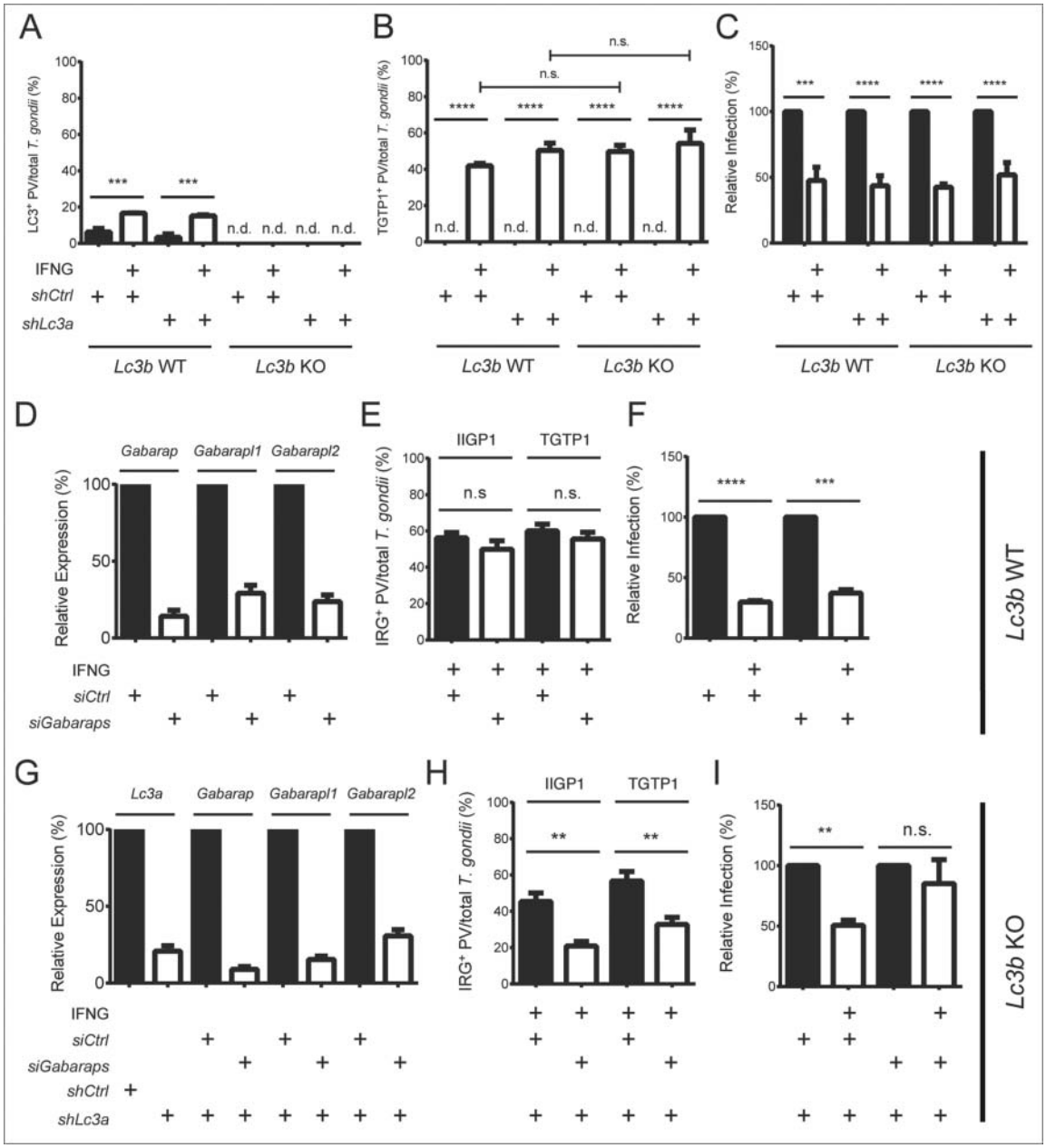

Figure 3. LC3 homologs are required for targeting of the IFNG-inducible GTPases and consequent control of *T. gondii* infection by IFNG. (A-B) Quantification of immunofluorescence assay for LC3 (A) and TGTP1/IRGB6 (B), localized on the PVM of *T. gondii* in the *Lc3b* WT and KO MEFs transduced with either shRNA against *Rfp* (*shCtrl*) or shRNA against *Lc3a* (*shLc3a*) and then infected with *T. gondii* (MOI = 1) for 2 h. When indicated, the cells were pretreated with 100 U/ml IFNG for 24 h. (C) Relative quantification of flow cytometry analysis for *T. gondii* replication after infecting the cells described in (A-B) with *T. gondii* (MOI = 1) for 24 h. When indicated, the cells were pretreated with 100 U/ml IFNG for 24 h. (D) Quantitative PCR analysis for the transcript level of the *Gabarap* subfamily in the *Lc3b* WT MEFs transfected with pooled siRNAs against either control (*siCtrl*) or the *Gabarap* subfamily (*siGabaraps*). (E) Quantification of immunofluorescence assay for IIGP1/IRGA6 and TGTP1/IRGB6 localized on the PVM of *T. gondii* in the *Lc3b* WT MEFs transfected with either *siCtrl* or *siGabaraps*, using the same condition as in (B). (F) Relative quantification of flow cytometry analysis for *T. gondii* replication in the cells described in (E) using the same condition as in (C). (G) Quantitative PCR analysis for the transcript level of the *Gabarap* subfamily in the *Lc3b* KO MEFs transduced with *shLc3a* and transfected with either *siCtrl* or *siGabaraps*. *Lc3a* transcript level was compared to that of *Lc3b* KO MEF transduced with *shCtrl*. (H) Quantification of immunofluorescence assay for IIGP1/IRGA6 and TGTP1/IRGB6 localized on the PVM of *T. gondii* in the *Lc3b* KO MEFs transduced with *shLc3a* and transfected with either *siCtrl* or *siGabaraps* in the same condition as in (E). (I) Relative quantification of flow cytometry analysis for *T. gondii* replication in the cells described in (H) using the same condition as in (F). Experiments were performed 2 to 4 times, and the data were combined for presentation as average ± SEM. Statistical analysis was performed using one-way analysis of variation (Tukey post test) for (A-C) and paired t-test for (E-F) and (H-I). n.s., not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.d., not detected.

and control of *T. gondii* infection by IFNG, as well as LC3 lipidation (i.e., its conjugation to phospholipid).[11] To investigate the role of the ATG12–ATG5-ATG16L1 complex in the targeting process, we constructed the cytoplasmic and membrane-localizing versions of 3 ATG16L1 mutants (Fig. 4A): ATG16L1 (1–79) has the domain for ATG5 binding; ATG16L1(80–249) has the domain for the dimerization of ATG16L1 and its interaction with other proteins such as upstream autophagy proteins; ATG16L1(1–249) has both domains and is necessary and sufficient for the lipidation of LC3 homologs in vivo.[27] As expected, overexpression of the N-terminal RFP-fused cytoplasmic version of those ATG16L1 mutants led to their localization mostly in the cytoplasm and on some punctate structures (Fig. 4B). In contrast, upon tagging them with C-

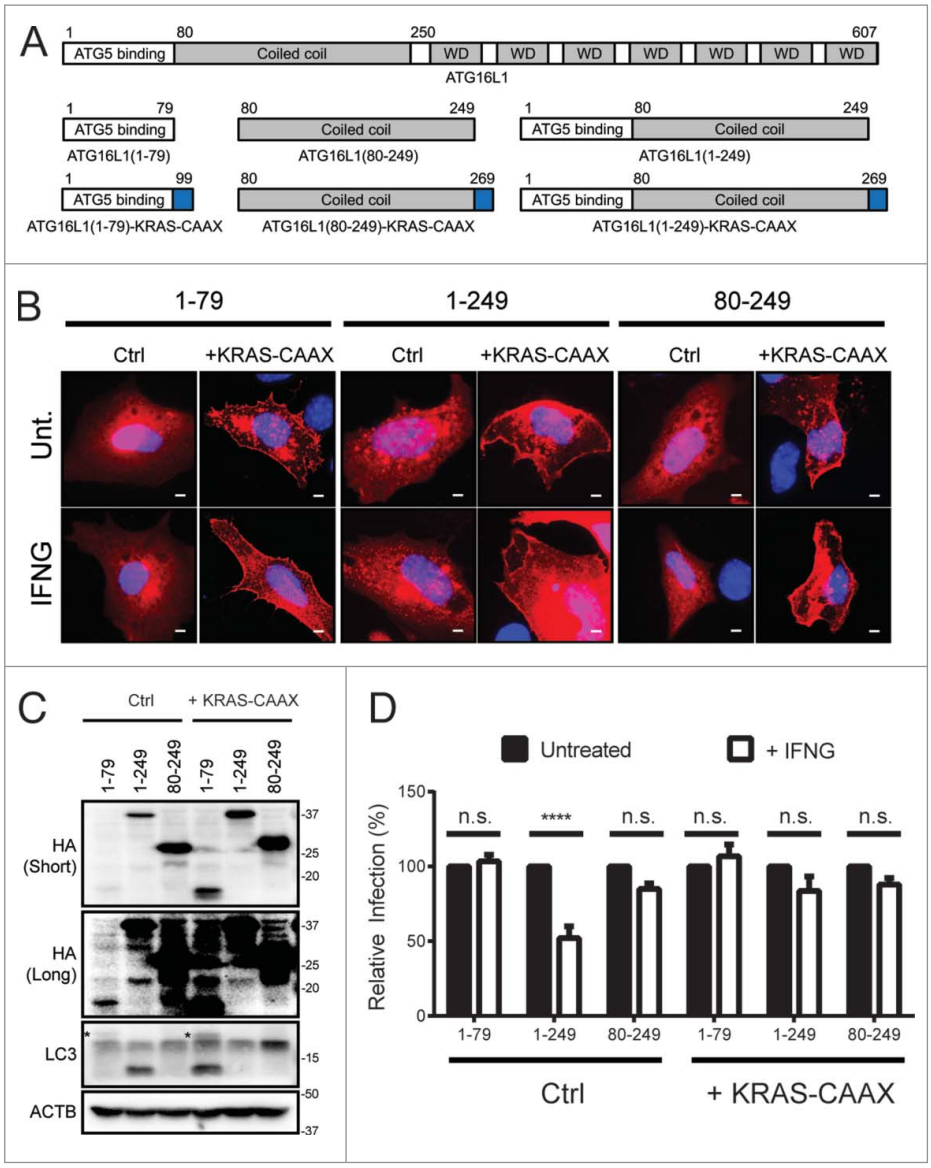

Figure 4. Cytoplasmic and membrane-anchored versions of ATG16L1 mutants. (A) Schematic diagram of murine ATG16L1 wild type (WT) and deletion mutants. KRAS-CAAX motif at the C terminus is indicated in blue. (B) Fluorescence assay for the localization of N-terminal RFP-fused cytoplasmic control (Ctrl) and membrane-anchored (+ KRAS-CAAX) versions of ATG16L1 mutants in untreated (Unt.) and IFNG-treated MEFs. (C) Western blot assay for the protein expression level of cytoplasmic control and membrane-anchored versions of ATG16L1 mutants as depicted in Fig. 4A in *Atg16l1* KO MEFs. Asterisk indicates remaining pre-probed HA signal of ATG16L1(1–79) mutants. (D) Relative quantification of flow cytometry analysis for *T. gondii* replication after pretreating the cells described in (C) with none or 100 U/ml IFNG for 24 h and then infecting them with *T. gondii* (MOI = 1) for 24 h. Experiments were performed at least 3 times, and the data were combined for presentation as average ± SEM. Statistical analysis was performed using one-way analysis of variation (Tukey post test). ****, $p < 0.0001$; n.s., not significant. Scale bar: 10 μm.

terminal KRAS-CAAX,[27,29] a substantial portion of the mutants localized on the plasma membrane (Fig. 4B). IFNG had no effect on the localization of both cytoplasmic and membrane-localizing versions of those ATG16L1 mutants (Fig. 4B).

We transduced and selected *Atg16l1* KO MEFs with lentiviruses expressing N-terminal FLAG- and HA epitope-tagged ATG16L1 mutants. The cytoplasmic versions of the ATG16L1 (1–249) mutant restored LC3 lipidation, but neither the ATG16L1(1–79) nor the ATG16L1(80–249) could restore lipidation, confirming that the lipidation of LC3 requires a complex of ATG16L1 with the ATG12–ATG5 conjugate and the dimerization/interaction capacity of ATG16L1 (Fig. 4C). Interestingly, when the same mutants were expressed with the C-terminal KRAS-CAAX motif in *Atg16l1* KO MEFs, the lipidation of LC3 was detected constantly only in the cells with ATG16L1 (1–79)-KRAS-CAAX (Fig. 4C). In contrast, we did not find a substantial restoration of LC3 lipidation by the ATG16L1 (1–249)-KRAS-CAAX mutant through a whole cell population analysis using western blot. It was previously shown that the transient overexpression of the ATG16L1(1–249)-KRAS-CAAX mutant substantially increases the lipidation of LC3 in wild-type cells, especially with additional ATG12–ATG5.[10] The discrepancy between the 2 systems might be explained by the heterogeneous low-level expression of the lentivirally-transduced genes in the polyclonal cell population. Alternatively, the KRAS-CAAX-mediated membrane anchorage of ATG16L1 (1–249) might significantly decrease the dimerization/interaction capacity of ATG16L1 in the absence of wild-type ATG16L1 or its function to bring the ATG12–ATG5 conjugate and the associated E2 ATG3-LC3 to a target membrane in

the absence of extra ATG12–ATG5. Conversely, the C-terminal KRAS-CAAX may anchor the ATG5 binding domain (1–79) of ATG16L1 on a membrane in a conformation that enables the domain to skip the necessary protein-protein interactions for the LC3 conjugation.

We next examined whether the ATG16L1 mutants can restore the control of *T. gondii* by IFNG and targeting of the IFNG-inducible GTPases to the PVM of *T. gondii* in the transduced *Atg16l1* KO MEFs. Both cytoplasmic control and membrane-anchored versions of ATG16L1(1–79) and ATG16L1(80–249) were unable to restore the IFNG-mediated control of *T. gondii* infection in the transduced *Atg16l1* KO MEFs significantly, demonstrating that both the ATG5 binding and coiled-coil domains are required for the control (Fig. 4D). As it restored the lipidation of LC3, the cytoplasmic ATG16L1(1–249) restored the IFNG-mediated control of *T. gondii* infection (Fig. 4D) and targeting of the IFNG-inducible GTPases (TGTP1/IRGB6 as a representative) to the PVM of *T. gondii* (Fig. 5A). In contrast, the mostly membrane-anchored ATG16L1(1–249)-KRAS-CAAX did not restore the control of *T. gondii* infection by IFNG (Fig. 4D) and restored inefficiently targeting of LC3 and the IFNG-inducible GTPases (TGTP1/IRGB6 and GBP1 through GBP5) on the PVM of *T. gondii* in *Atg16l1* KO MEFs (Fig. 5B), probably because it was anchored on membranes other than the PVM of *T. gondii* (Fig. 5C). Nevertheless, the ATG16L1(1–249)-KRAS-CAAX mutant was able to recruit both LC3 and the IFNG-inducible GTPases to the plasma membrane, where it is anchored (Fig. 5A and B). Further, there was substantial overlap between the LC3 and the IFNG-inducible GTPase on the plasma membrane (Fig. 5C and D). These data suggest that the mutant could restore the lipidation of LC3 at least locally even in the absence of substantial restoration of LC3 lipidation at a whole population level (Fig. 4C). Taken together, these data demonstrated that the LC3 conjugation system of autophagy is not only necessary for targeting of the IFNG-inducible GTPases to the PVM of *T. gondii* but also sufficient to target them to an alternative membrane.

IFNG treatment and T. gondii infection influence targeting by the LC3 conjugation system of autophagy

To further confirm the directing role of the LC3 conjugation system in targeting the IFNG-inducible GTPases independently, we introduced the anchor-away system, which can control the location of a protein of interest (the target) through conditional tethering of the protein to an abundant protein in a specific location (the anchor) by rapamycin-dependent heterodimerization.[30] The modified anchor-away system enabled us to monitor the translocation of ATG16L1(1–249) from the cytoplasm to the mitochondrial outer membrane in the same cell, in contrast to the KRAS-CAAX approach that allowed us to compare the cytoplasmic and membrane-anchored mutants of ATG16L1 in 2 different cells. We developed a mitochondrial anchor by fusing TOMM20 (translocase of outer mitochondrial membrane 20)[31] to the FKBP12-rapamycin-binding (FRB) domain and green fluorescent protein (GFP) (Fig. 6A). As the target, we constructed a fusion protein of ATG16L1(1–249) with FK506 binding protein (FKBP) domain and RFP. Since the FKBP and FRB domains form a heterodimer in the presence of rapamycin,[30] the target with FKBP domains translocates to the mitochondria outer membrane where the anchor with FRB domains localizes. Thus, the cytoplasmic target (RFP-ATG16L1[1–249]-4×FKBP) translocated to the mitochondria upon its heterodimerization with the anchor (TOMM20-2×FRB-GFP) on mitochondria by rapamycin treatment (Fig. 6B).

When we expressed the target and the anchor in *Atg16l1* KO MEFs, endogenous LC3 occasionally colocalized with the target in the cytoplasm even in the absence of rapamycin (Fig. 7A), as shown previously.[10] Rapamycin treatment recruited the target to mitochondria and also brought LC3 onto mitochondria (Fig. 7A and B). As a control, in the absence of the target, we did not detect any colocalization of LC3 with the anchor on mitochondria (Fig. S4A). Intriguingly, we observed a significant increase of LC3 localization on mitochondria upon IFNG treatment and *T. gondii* infection, which is reminiscent of the increased localization of LC3 on the PVM of *T. gondii* upon IFNG treatment[11] and also the augmented localization of LC3 on the plasma membrane (Fig. 5B). These data suggest that the interaction between the ATG12–ATG5-ATG16L1 complex with LC3 and/or the conjugation of LC3 on a membrane by the ATG12–ATG5-ATG16L1 complex can be substantially enhanced by IFNG treatment and *T. gondii* infection. By comparison, IRG (IIGP1/IRGA6 as a representative) did not colocalize with the target in the cytoplasm upon induction by IFNG, but occasionally colocalized with the target on mitochondria upon treatment with rapamycin. It was upon *T. gondii* infection that IIGP1/IRGA6 significantly colocalized with the target on mitochondria, suggesting that targeting of the IRGs to a membrane by the LC3 conjugation system was modulated by *T. gondii* infection (Fig. 7A and B). Although the same effect of *T. gondii* infection was not observed in the KRAS-CAAX-mediated plasma membrane localization of IRG (TGTP1/IRGB6), the localization of GBP1 through GBP5 on mitochondria in the anchor-away system (Fig. 7B) and on the plasma membrane in the KRAS-CAAX system (Fig. 5B) were also augmented by *T. gondii* infection. Consistent with LC3, in the absence of the target, we did not detect any colocalization of the IFNG-inducible GTPases with the anchor on mitochondria (Fig. S4A). In addition, we did not detect any significant effect of rapamycin treatment on the expression of the IFNG-inducible GTPases in our experimental condition (Fig. S4B). Taken together, our data clearly demonstrated that the LC3 conjugation system could direct LC3 and the IFNG-inducible GTPases to a target membrane, and further suggest that the targeting is augmented by IFNG and *T. gondii* infection.

Discussion

Here we show that all known LC3 homologs localized on the PVM of *T. gondii* in a conjugation-dependent manner and were required for targeting of the IFNG-inducible GTPases to the PVM, and inhibition of *T. gondii* infection by IFNG. Further, the ubiquitin-like LC3 conjugation system was not only necessary but also sufficient for targeting of the IFNG-inducible GTPases to target membranes. This is clearly a nondegradative

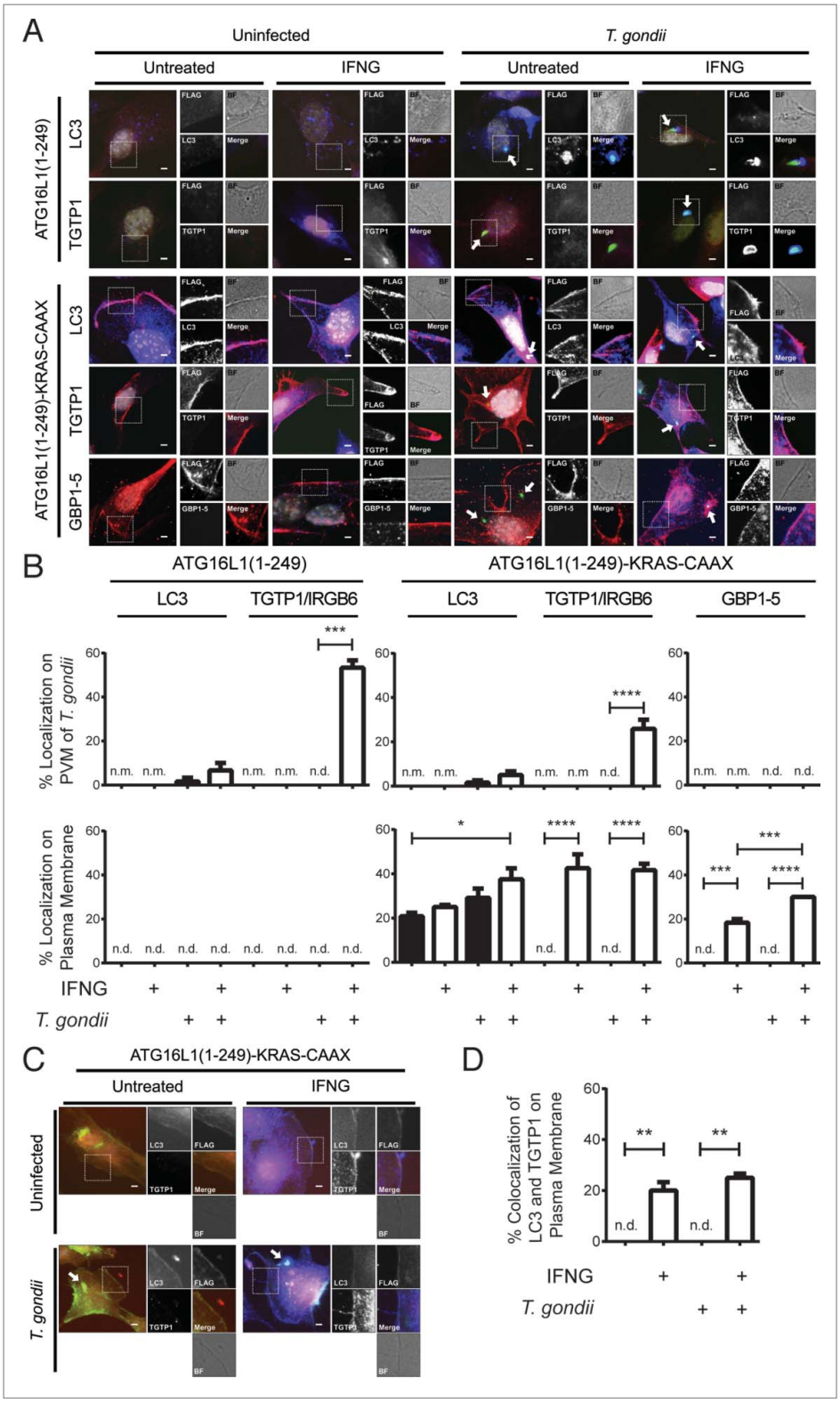

Figure 5. The LC3 conjugation system of autophagy is sufficient for targeting of the IFNG-inducible GTPase to an alternative target membrane. (A) Immunofluorescence assay for the localization of *T. gondii* (green, GFP), cytoplasmic (top) and membrane-anchored (bottom) versions of ATG16L1(1–249) (red, FLAG), and LC3-TGTP1/IRGB6-GBP1 through GBP5 (GBP1-5; blue) in the transduced MEFs. When indicated, cells were pretreated with 100 U/ml IFNG for 24 h and then infected with *T. gondii* (MOI = 1) for 2 h. (B) Quantification of immunofluorescence assay for LC3, TGTP1/IRGB6 and GBP1 through GBP5 localized on the PVM of *T. gondii* or the plasma membrane in the condition as described in (A). (C) Immunofluorescence assay for the colocalization of LC3 (red) and TGTP1/IRGB6 (blue) on the plasma membrane in the *Atg16l1* KO MEFs transduced with the membrane-anchored versions of ATG16L1(1–249) (green, FLAG). When indicated, cells were pretreated with 100 U/ml IFNG for 24 h and then infected with *T. gondii* (MOI = 1) for 2 h. (D) Quantification of immunofluorescence assay for the colocalization of LC3 and TGTP1/IRGB6 in the cells described in (C). Scale bar: 10 μm. Experiments were performed 2 to 4 times and representative images are shown here. The data were combined for presentation as average $\pm$ SEM. Statistical analysis was performed using one-way analysis of variation (Tukey post test). n.s., not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.d., not detected; n.m., not monitored.

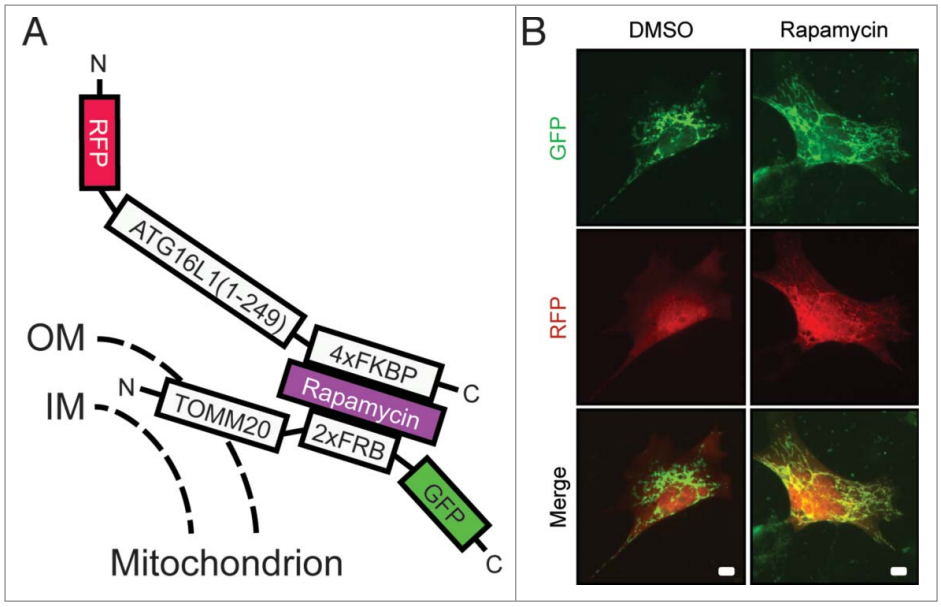

Figure 6. The anchor-away system for translocation of ATG16L1(1–249) to mitochondria. (A) Schematic diagram of the anchor-away system used in this study. The anchor (TOMM20 + 2 copies of FRB domain + GFP) can bring the target (ATG16L1[1–249] + 4 copies of FKBP domain + RFP) to the mitochondria outer membrane upon their heterodimerization by rapamycin (Rapa). (B) Fluorescence assay for the localization of the anchor (GFP) and the target (RFP) in the cotransfected *Atg16l1* KO MEFs upon treatment with rapamycin or vehicle control (DMSO) for 2 h. Scale bar: 10 μm. Experiments were performed at least 3 times and representative images are shown here.

function of the LC3 conjugation system of the autophagy pathway. In canonical autophagy, the LC3 conjugation system is required to elongate the phagophore membrane while capturing cytoplasmic cargoes and eventually to form a closed globular autophagosome.[32] In this noncanonical function, the same functional module is required to mark a membrane structure of an intracellular pathogen with the ubiquitin-like LC3 homologs and to recruit effector proteins to the "LC3-marked" membrane. Our data clearly demonstrated that the LC3 conjugation system of autophagy can function not only for a degrading process of proteins through lysosomal fusion but also for a targeting process of proteins to a specific membrane structure, which we term as TAG.

To fully understand the molecular mechanism of TAG for targeting of the IFNG-inducible GTPases to the PVM of *T. gondii*, 2 critical questions remain to be answered. The first question is how the PV is recognized as a target membranous structure for the ATG12–ATG5-ATG16L1 complex to conjugate LC3 homologs. Since ATG12–ATG5-ATG16L1 specifies the conjugation site of LC3 homologs,[10] the complex must be recruited to the PVM of *T. gondii* before the conjugation of the LC3 homologs. The ATG12–ATG5-ATG16L1 complex may be associated with the membrane of the PV of *T. gondii* directly through the membrane-binding activity of ATG5[33] and/or indirectly through potential interaction partners of ATG16L1 on the membrane.[27,28] For canonical autophagy, WIPI2B (WD repeat domain, phosphoinositide interacting 2B) recruits the ATG12–ATG5-ATG16L1 complex to the site of autophagosome initiation via its binding to phosphatidylinositol 3-phosphate (PtdIns3P) on the site and its interaction with ATG16L1.[28] Although we did not observe any significant role of PtdIns3K in the control of *T. gondii* by IFNG upon its inhibition with wortmannin or LY294002 in our previous study,[11] it is noteworthy that the PVM of *T. gondii* can be rapidly bound by GFP fused to the pleckstrin homology (PH) domain of AKT, which can recognize a membrane containing PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2.[34] These data suggest that the phosphorylated derivatives of phosphatidylinositol in the PVM of *T. gondii*, presumably other than PtdIns3P, may recruit the ATG12–ATG5-ATG16L1 complex onto the membrane through effector proteins that can bind to the phosphoinositides and interact with the complex, similar to canonical autophagy initiation.

Another potential explanation for recruitment of the ATG12–ATG5-ATG16L1 complex to the PVM is the "missing-self" hypothesis.[35] The PVM of *T. gondii* is derived from the host plasma membrane by *T. gondii*, which removes most (if not all) of the membrane-associated host proteins.[13] A prevalent theory in the field to explain the targeting of the IFNG-inducible GTPases to the PVM has been the "guard" theory.[35-37] The gist of the "guard" theory is that a set of "guard" proteins mark and protect host cell membranes from effector IFNG-inducible GTPases (e.g. IIGP1/IRGA6, TGTP1/IRGB6). The ATG12–ATG5-ATG16L1 complex may be prevented from binding to cellular membrane structures by membrane-associated cellular "guard" proteins, and the absence of the "guard" on the PVM of *T. gondii* may lead to the recruitment of the complex to the membrane.

The second question is how the IFNG-inducible GTPases are targeted specifically to the LC3-marked PV, for instance, instead of being targeted to LC3-decorated autophagosomes. If the IFNG-inducible GTPases are targeted to the LC3-decorated autophagosomes, they may disrupt the autophagosomes and consequently inhibit the degradative autophagy process. In fact, there have been a couple of reports showing that the mouse and human homologs of IRGs are associated with autophagy proteins and involved in canonical degradative autophagy.[38-41] However, the IRGs in those reports promote the formation of autophagosomes rather than disrupting the autophagosomal membrane. Since the human IRG system differs significantly from the mouse

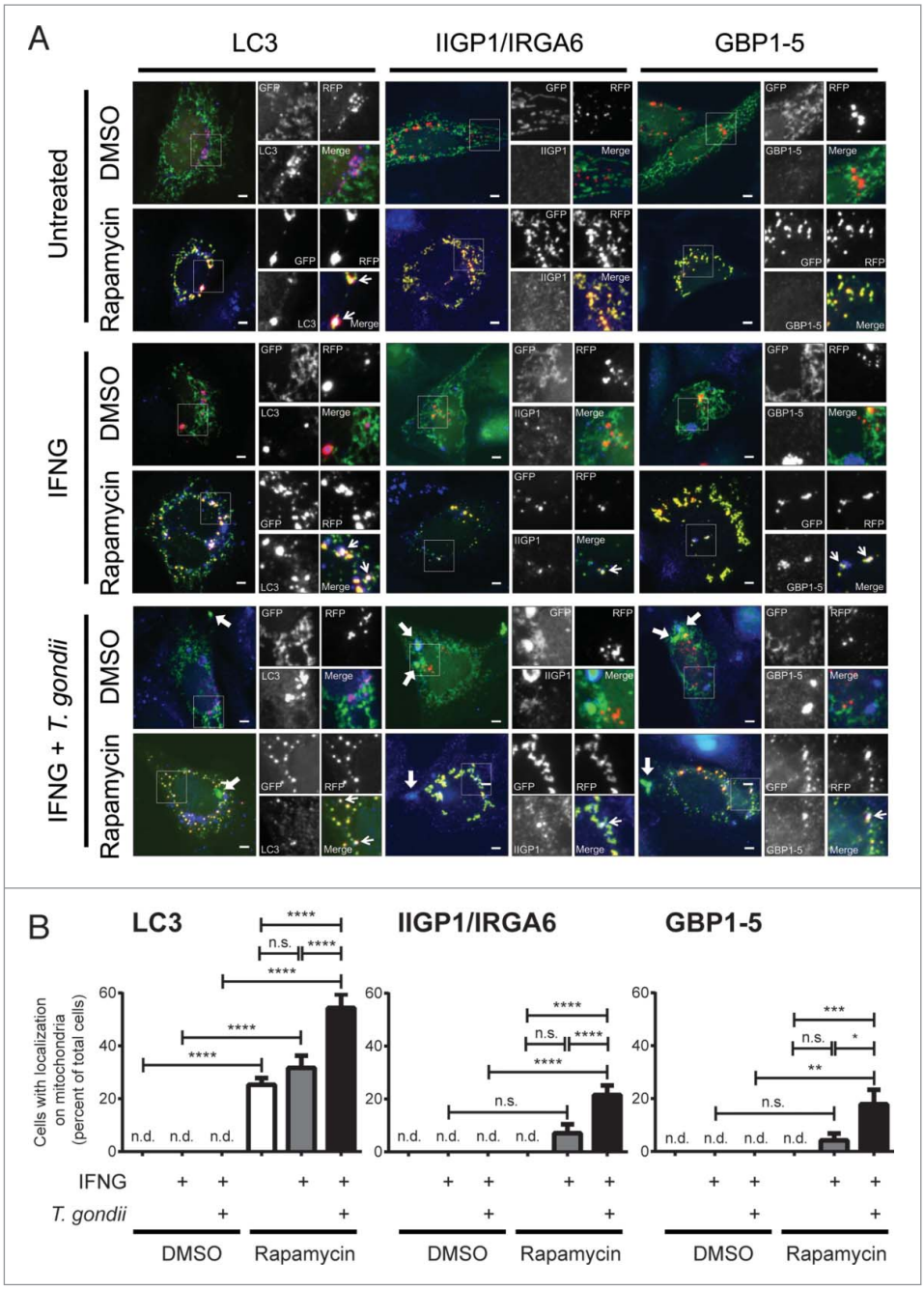

Figure 7. IFNG treatment and *T. gondii* infection influences targeting of the IFNG-inducible GTPases to an alternative target membrane by the LC3 conjugation system. (A) Immunofluorescence assay for the localization of the anchor (TOMM20-2xFRB-GFP), the target (RFP-ATG16L1[1–249]-4xFKBP), and LC3 (blue, left), IIGP1/IRGA6 (blue, center), or GBP1 through GBP5 (GBP1-5; blue, right) in the cotransfected *Atg16l1* KO MEF cells upon treatment with rapamycin or vehicle control (DMSO) for 2 h. When indicated, cells were pretreated with 100 U/ml IFNG for 24 h and/or infected with *T. gondii* (MOI = 1) for 2 h. Scale bar: 10 μm. Block arrows in the merged images of whole cells indicate *T. gondii*. Arrows in the merged images of insets indicate colocalization of the anchor, the target, and LC3, IIGP1/IRGA6, or GBP1 through GBP5. (B) Quantification of triple colocalization on mitochondria of the anchor (GFP), the target (RFP), and LC3 (left), IIGP1/IRGA6 (center), or GBP1 through GBP5 (right) as shown in (A). Experiments were performed 6 times and representative images are shown here; a total of 39∼54 cells that express both anchor and target in each condition were counted for quantification; the data were combined for presentation as average ± SEM. Statistical analysis was performed using one-way analysis of variation (Tukey post test). n.s., not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; n.d., not detected.

IRG system, it has been unclear whether the human IRGs play a role in controlling *T. gondii* infection similar to the mouse IRGs.[42-44] In contrast, the role of mouse IRGs in disrupting the PV of *T. gondii* has been clearly demonstrated by many groups,[16] and as shown here the targeting of the mouse IRGs can be determined by the LC3 conjugation system. Nevertheless, we have not observed any significant colocalization of mouse IRGs with autophagosomes (unpublished) and any significant effect of IFNG on canonical degradative autophagy,[45] suggesting that mouse IRGs are not targeted to autophagosomes. However, we cannot completely rule out the possibility that the mouse IRGs are indeed targeted to LC3-decorated autophagosomes but do not disrupt the autophagosomes due to the physical difference between the double membrane of the autophagosome and the single membrane of the PV of *T. gondii*.

Bioinformatics analysis of the IFNG-inducible GTPases revealed a potential LC3-interacting region[46] around their core GTPase domain (e.g., [EYD]FFII in IIGP1/IRGA6 and [EDI]FGPL in GBP2). We occasionally detected weak interaction between the IFNG-inducible GTPases and LC3 homologs only in IFNG-treated and *T. gondii*-infected cells (unpublished). Our data shown in Fig. 7 clearly demonstrated that the recruitment of the IFNG-inducible GTPases by the LC3 conjugation system to a certain target membrane was significantly augmented by *T. gondii* infection. Further, we also observed that expression of individual IFNG-inducible GTPases alone did not lead to their targeting to the PV of *T. gondii* (unpublished), suggesting that IFNG-inducible changes, more than simple expression of the IFNG-inducible GTPases, are necessary for proper targeting of the IFNG effectors onto the PVM of *T. gondii*. However, since the fully functional IRG system (so consequently the GBP system too) requires the expression of most (if not all) IRG proteins,[35-37] we cannot rule out the possibility that IFNG may be simply required to induce the expression of those GTPases. Nevertheless, these data suggest that cellular signaling events induced by IFNG and *T. gondii* infection may substantially modify the interaction between the LC3 homologs and the IFNG-inducible GTPases. IFNG treatment and *T. gondii* infection modulate many cellular signaling pathways and induce a large transcriptional change in the treated and infected cells.[15,34,47] Further, various post-translational modifications of LC3 homologs have been reported.[48-51] We speculate that LC3 homologs are post-translationally modified upon IFNG treatment and *T. gondii* infection so that they can specifically recruit the IFNG-inducible GTPases to the membrane of the PV either directly or indirectly.

LC3 was initially thought to be a marker only of autophagic degradation because it was conjugated only on phagophore membranes, which are destined to be delivered to the lysosome and to be degraded following lysosomal fusion. Now LC3 has been shown to be conjugated on many non-autophagosomal membrane structures, which are involved in trafficking of cargoes to more than the lysosome.[6,7,52] Furthermore, our data suggest that LC3 can serve as a molecular beacon to recruit effector proteins to the membrane where it is conjugated and thus to modify the fate of the membrane itself rather than the cargoes inside the membranous structures. Simply, LC3 conjugation to phospholipids on a membrane may modify how the membrane interacts with other molecules in a cell. As far as we know, LC3 homologs are the only known proteins that are directly conjugated to phospholipids on a membrane and consequently modify the membrane. Thus, it is very tempting to speculate that the conjugation of ubiquitin-like LC3 homologs to phospholipids (in membranes) can function like the conjugation of ubiquitin to proteins. Ubiquitination was initially known as the marker of protein degradation through the proteasome.[53] Since then, ubiquitination has been shown to modify the stability, trafficking, and protein-protein interaction of ubiquitinated proteins.[54] Future studies will illuminate the parallel and divergent natures of the LC3 and ubiquitin conjugation systems, as well as shed light on other, yet undiscovered participants.

In summary, we showed here that the ubiquitin-like LC3 conjugation system of autophagy can target proteins to specific membrane structures. The contribution of the autophagy pathway to the host immune defense has been studied mostly in the context of lysosomal degradation, and the function of individual proteins has not been clearly distinguished from that of the whole degradative autophagy pathway.[55] Considering the essential role of the LC3 conjugation system, but insignificant role of the degradative activity of the autophagy pathway in controlling the infection of *T. gondii* in vivo,[11] it is crucial to elucidate the functional mechanism of the LC3 conjugation system-mediated detection and disintegration of the parasitophorus vacuole of *T. gondii* and further to investigate the potential role of the LC3 conjugation system in controlling other intracellular vacuolar pathogens.

Materials and methods

Cells, transfection, and transduction

Cells were grown in DMEM (Mediatech, 10–013) supplemented with 10 mM HEPES (Mediatech, 25-060-CI), 1 × MEM nonessential amino acids (Mediatech, 25-025-CI), 100 U/ml each of penicillin and streptomycin (Mediatech, 30-002-CI) and 10% fetal bovine serum (Biowest, US1520) at 37°C under 5% CO_2. MEFs were transduced with lentiviral vectors for 2 d and polyclonal populations of the transduced cells were selected with puromycin (Sigma-Aldrich, P9620) at 3 ug/ml as previously described.[11] For transient transfection of plasmids, Lipofectamine 2000 Transfection Reagent (Invitrogen, 11668–019) was used according to the manufacturer's instruction. For transduction, lentivirus was generated in 293T cells by transfecting the lentiviral vector plasmids with packaging vector (psPAX2; Addgene, 12260) and pseudotyping vector (pMD2.G; Addgene, 12259) using the calcium phosphate precipitation method.[11,45] Produced lentivirus was filtered through a 0.45 -μm syringe filter and added onto the cells.

Construction of plasmids

All LC3 homologs in the murine system as shown in Fig. 1A, except *Gabarap-1*, were PCR amplified using mouse cDNA from primary bone marrow-derived macrophages as a template and cloned into a modified entry vector of the Gateway system (Life Technologies), pENTR-SH (Fig. S5). *Gabarap-1* was synthesized through the IDT miniGene service and cloned into pENTR-SH. The nonconjugatable forms of *Lc3* homologs were generated using primers with the mutated sequences and also cloned into pENTR-SH. The *Atg16l1* mutants were generated by PCR and cloned into either pENTR-SH as control or pENTR-kras-CAAX to add a C-terminal KRAS-CAAX motif for plasma membrane localization. The *Atg16l1(1–249)* was also cloned into pENTR-4xFKBP-N1 to add C-terminal quadruple FKBP domains for the anchor-away assay. *Atg5*[45] was also cloned into pENTR-SH. For stable expression, the cloned genes in the pENTR vectors were transferred through an LR clonase reaction (Life Technologies, 11791–020) to a lentiviral destination vector of the Gateway system, pHNHF (Fig. S5), which stably expresses a gene of interest with N-terminal FLAG and HA epitopes in transduced cells.[56] For localization

monitoring of the genes, the cloned genes in the pENTR vectors were transferred to a home-made destination vector, pDRFP (modified from pTAG[57]), which expresses a gene of interest with N-terminal RFP in transfected cells. To generate a mitochondrial anchor for the anchor-away assay (pEGFP-N1-TOMM20-2xFRB), murine TOMM20 and duplicate FRB domains were cloned into the pEGFP-N1 (Clontech, 6085–1) vector. The sequence of all PCR amplified DNAs and cloned vectors were verified by sequencing. The full DNA sequences of plasmids and cloned genes used in the study are provided as supplementary information (Fig. S5).

Infection with T. gondii

Tachyzoites of the type II *T. gondii* strain expressing GFP (PTG) were maintained in human foreskin fibroblasts (ATCC, CRL-1634) as described previously.[11] Infected human foreskin fibroblasts were harvested when they were 10∼50% lysed using a cell scraper and 26G needle to release *T. gondii* tachyzoites. Tachyzoites were collected by centrifugation at 400 xg for 10 min at 18°C and resuspended in culture medium. Infection was set up by adding the prepared tachyzoites to cells at a multiplicity of infection (MOI) of 1 and incubating them at 37°C. After 1 h incubation, the inoculum was discarded and the infected cells were washed twice with phosphate-buffered saline (PBS; Mediatech, 21–040). After addition of fresh medium, cells were further incubated at 37°C until harvest (2 h post-infection [hpi] for immunofluorescence assay and 24 hpi for flow cytometry). For synchronized infection, a potassium buffer shift was used as described previously.[58] Wherever indicated, cells were pre-treated with murine IFNG (Peprotech, 315–05) at 100 U/ml (50 ng/ml) for 24 h before *T. gondii* infection.

Western blot, immunofluorescence, flow cytometry and antibodies

For western blot (WB), total cellular proteins were harvested with sample buffer (0.1 M Tris-HCl, pH 6.8, [Fisher Scientific, BP1521, A144-212], 4% SDS [Sigma-Aldrich, L3771], 4 mM EDTA [Sigma-Aldrich, E4884], 286 mM 2-mercaptoethanol [Sigma-Aldrich, M3148], 3.2 M glycerol [Fisher Scientific, BP229-1], 0.05% bromophenol blue [Sigma-Aldrich, B5525]) and analyzed as previously reported.[11,45] For immunofluorescence (IF) and flow cytometry (FC), cells were fixed with 2% formaldehyde (Ted Pella, 18505) in PBS for 10 min at room temperature, permeabilized with 0.05% saponin (IF) (Thermo Scientific, 419231000) or 0.2% Triton X-100 (FC) (Sigma-Aldrich, T8787) in PBS, and analyzed as previously reported.[11,45] For IF, nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Molecular Probes, D3571) and *T. gondii* was detected with GFP expressed by the PTG strain. The images were acquired using the EVOS FL Cell Imaging System or Olympus DSU Spinning Disk confocal (Integrated Light Microscopy Core Facility, The University of Chicago). For FC, the data were acquired using FACSCanto or LSRFortessa flow cytometers (Flow Cytometry Core Facility, The University of Chicago) and analyzed using FlowJo software. Uninfected samples were used as gating control and infected samples without IFNG-treatment were used as a normalization control to calculate the relative infection (%) of IFNG-treated samples. Antibodies used in this study are as follows: Actin (Santa Cruz Biotechnology, sc-47778HRP), FLAG (Sigma-Aldrich, F7425), GBP1 through GBP5 (Santa Cruz Biotechnology, sc-166960), HA (The Frank W. Fitch Monoclonal Antibody Facility, The University of Chicago, 12CA5), IIGP1/IRGA6 (kind gift from Dr. Jonathan C. Howard, University of Cologne),[59] TGTP1/IRGB6 (Santa Cruz Biotechnology, sc-11079), and LC3 (Sigma-Aldrich, L7543 for WB; MLB, PM036 for IF).

Knockdown and quantitative PCR

For shRNA-mediated stable knockdown, pLKOpuro-based lentiviral shRNA constructs against RFP and LC3A were obtained from the Washington University Genome Institute and lentiviral transduction was performed as described above. For siRNA-mediated transient knockdown, we purchased the following products from GE Dharmacon: siGENOME non-targeting siRNA Pool #1 (D-001206-13-05), SMARTpool siGENOME siRNA against *Gabarap* (M-041776-01-0005), *Gabarapl1* (M-040444-01-0005), and *Gabarapl2* (M-059605-01-0005). All siRNAs were dissolved in siRNA buffer (GE Healthcare, B-002000-UB-100) and transfected into cells using DharmaFECT 1 transfection reagent (GE Healthcare, T-2001-02). Specific target sequences of all shRNA and siRNAs used in this study will be available upon request. For quantification of transcripts upon knockdown, RNAs were extracted using TRI reagent (Sigma-Aldrich, T9424) and reverse transcription was performed using IMPROM-II reverse transcriptase (Promega, A3803) according to the manufacturer's instruction. Primers for qPCR are as follows: *Lc3a* (5′-gaccgctgtaaggaggtgc-3′, 5′-cttgaccaactcgctcatgtta-3′), *Gabarap* (5′-aagaggagcatccgttcgaga-3′, 5′-gctttgggggctttttccac-3′), *Gabarapl1* (5′-ggaccacccccttcgagtatc-3′, 5′-cctcttatccagatcagggacc-3′), *Gabarapl2* (5′-ggaacacagatgcgtggaatc-3′, 5′-gatgtccgaggggaccaagta-3′).

Anchor-away assay

A plasmid expressing mitochondrial anchor (pEGFP-N1-TOMM20-2xFRB) was cotransfected with another plasmid expressing target (pDRFP-Atg16L1/1-249-4xFKBP) into *Atg16l1* KO MEFs. At 24 h after cotransfection of both plasmids with or without treatment of IFNG at 100 U/ml (50 ng/ml), the transfected cells were infected with *T. gondii* as described above and treated with 50 nM rapamycin (LC laboratories, R5000) or vehicle control (DMSO; Mediatech, 25-950-CQC) for 2 h and fixed for immunofluorescence analysis.

Quantitative analysis of image data

For the quantitative analysis of LC3 homologs, IRGs, GBP1 through GBP5, and ATG5 localization on the PVM of *T. gondii*, at least 30 infected *T. gondii* were counted for each experiment unless otherwise indicated. For the quantification of LC3, TGTP1/IRGB6, and GBP1 through GBP5 localization on the plasma membrane, 30 transduced cells expressing ATG16L1 (1–249)-KRAS-CAAX (by FLAG staining) on the plasma

membrane were counted for each experiment. For the quantification of anchor-away, only the cells containing the anchor and the target (whether it is in the cytoplasm [DMSO] or on mitochondria [rapamycin]) as well as *T. gondii* (if infected) were counted (39–54 cells per each condition in total), and we scored the cell as positive only if there was a triple colocalization (anchor, target, and LC3-IIGP1/IRGA6-GBP1 through GBP5).

Statistical analysis

Statistical analysis of data was performed using GraphPad Prism with paired t-test, unpaired t-test, or one-way analysis of variation (with multiple comparison using Tukey post test) as indicated in the figure legends. Unless otherwise stated, all experiments were performed at least 3 times, and the data were combined for presentation as average ± SEM. All differences not specifically indicated to be significant were not significant (n.s., $p > 0.05$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Abbreviations

ATG	autophagy related
FKBP	FK506 binding protein
FRB	FKBP12-rapamycin-binding
GABARAP	GABA type A receptor-associated protein
GBP	guanylate binding protein
GFP	green fluorescent protein
IFN	interferon
IIGP1	interferon inducible GTPase 1
IRG	immunity-related GTPase
KO	knockout
LC3	microtubule-associated protein 1 light chain 3
MEF	mouse embryonic fibroblast
MOI	multiplicity of infection
PBS	phosphate-buffered saline
PE	phosphatidylethanolamine
PVM	parasitophorous vacuole membrane
RFP	red fluorescent protein
TAG	targeting by autophagy proteins
TGTP1	T cell specific GTPase 1

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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