

Quo vadis? Interferon-inducible GTPases go to their target membranes via the LC3-conjugation system of autophagy

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ABSTRACT

Many intracellular pathogens survive and replicate within vacuole-like structures in the cytoplasm. It has been unclear how the host immune system controls such pathogen-containing vacuoles. Interferon-inducible GTPases are dynamin-like GTPases that target the membranes of pathogen-containing vacuoles. Upon their oligomerization on the membrane, the vacuole structure disintegrates and the pathogen gets exposed to the hostile cytoplasm. What has been obscure is how the immune system detects and directs the GTPases to these pathogen shelters. Using a common protist parasite of mice, *Toxoplasma gondii*, we found that the LC3 conjugation system of autophagy is necessary and sufficient for targeting the interferon-inducible GTPases to membranes. We dubbed this process Targeting by Autophagy proteins (TAG). In canonical autophagy, the LC3 conjugation system is required to form membrane-bound autophagosomes, which encircle and deliver cytosolic materials to lysosomes for degradation. In TAG, however, the conjugation system is required to mark the membranes of pathogen-containing vacuoles with ubiquitin-like LC3 homologs, which function as molecular beacons to recruit the GTPases to their target membranes. Our data suggest that the LC3 conjugation system of autophagy plays an essential role in detecting and marking pathogen-containing vacuoles for immune effector targeting by the host immune system.

ARTICLE HISTORY

Received 14 June 2016
Revised 8 July 2016
Accepted 11 July 2016

KEYWORDS

autophagy; GBP; IFN; IRG;
LC3; TAG; targeting;
Toxoplasma gondii; ubiquitin

Enemy in disguise: How are pathogen-containing vacuoles targeted?

Many intracellular pathogens survive and replicate within vacuole-like structures, which are usually made by pathogens through reorganization of existing cellular membrane structures.¹ This so-called pathogen-containing vacuole provides pathogens not only a shelter from the host immune defense system but also a base to exploit the host cells. Thus, for the fitness and survival of the host, the operating base of pathogens has to be detected and destroyed by the immune defense system.² The immune system may have evolved to recognize these abnormally reorganized membrane structures as pathogen-associated molecular patterns, yet it has been obscure whether such pattern recognition receptor exists against pathogen-containing vacuoles.²

What has been known are effector proteins used by the host immune system to fight these vacuolar pathogens; immunity related GTPases (IRGs) and guanylate-binding proteins (GBPs) are interferon-inducible, dynamin-like GTPases that destroy these pathogen shelters.^{3,4} Upon their

induction, preferentially by interferon-gamma (IFNG), these effectors rapidly accumulate on the membranes of pathogen-containing vacuoles. The targeted membranes are subsequently vesiculated and eventually rupture, exposing the resident pathogens to the host cytoplasm. The consequences of such exposure include inhibition of the pathogen replication, activation of cytosolic pathogen sensors, and subsequent death of the pathogens and/or the host cells.⁵⁻⁷ In contrast to these outcomes, it has been poorly understood how the IRGs and GBPs are directed to the membrane of these vacuoles.⁸

To explain the targeting mechanism of the IRGs and GBPs, a prevalent model in the field has been the “guard” model (also similarly known as “missing-self” model).^{2,9,10} The gist of the “guard” model is a set of “guard” proteins that mark and protect the host cell membranes from the “executer” IFN-inducible GTPases. The IRG family is subdivided into GMS IRGs and GKS IRGs, based on their sequence in a conserved GTPase domain.¹¹ Membrane-bound GMS IRGs (e.g. IRGM1, IRGM2, and IRGM3/IGTP) are considered the “guard” proteins and

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predominantly cytosolic GKS IRGs (e.g., IRGA6/IIGP1 and IRGB6/TGTP1) function as the “executer” proteins: the “guard” GMS IRGs act as guanosine nucleotide dissociation inhibitor (GDI) of “executer” GKS IRG proteins, keeping the GKS IRGs in their inactive GDP-bound form.^{9,11} In the case of *Toxoplasma gondii*, the parasitophorus vacuole membrane (PVM) of *T. gondii* is derived from the host plasma membrane while *T. gondii* removes most (if not all) of the membrane-associated host proteins.¹² This suggests that the PVM of *T. gondii* may not be protected with the “guard” proteins and thus may be identified as a target membrane for the “executer” proteins. The GKS IRGs can translocate to the PVM by simple diffusion and can be activated to bind GTP in the absence of the “guard.” Subsequently, their GTP-dependent oligomerization may lead to the vesiculation and consequent disruption of the parasitophorus vacuole of *T. gondii*.^{13–16} Further, the IRG system has been shown to control the localization of GBPs onto the PVM through ubiquitination, although the mechanism is still not completely understood.^{17–19} Therefore, the “guard” model predicted that any endomembrane structure without the protective “guard” can be targeted spontaneously by GKS IRGs and subsequently GBPs.²

Intriguingly, several groups found that *Atg5*, an essential autophagy gene, is required to target these GKS IRGs and GBPs to the membranes of vacuoles containing pathogens like *Chlamydia trachomatis* and *T. gondii*.^{10,15,20,21} Without *Atg5*, GKS IRGs and GBPs are induced normally by IFNG, but they form aggregates in the cytoplasm rather than targeting pathogen-containing vacuoles.²² Since the major function of autophagy is to deliver cytoplasmic materials to lysosomes for degradation²³ and the cytoplasmic aggregates of GKS IRGs in *Atg5* knockout cells were composed of GTP-bound active forms,^{20,24} it was proposed that the degradative autophagy pathway might be required to maintain a functional pool of the IFN-inducible GTPases by removing falsely aggregated GTPases.²⁰

Targeting by Autophagy proteins (TAG): LC3 homologs mark membranes to be targeted

Using a well-established murine model of protist *T. gondii* infection, we examined the role of the autophagy pathway in proper targeting of IFN-inducible GTPases to the membranes of pathogen-containing vacuoles. Contrary to the expected, lysosomal degradation through autophagy did not affect targeting of GKS IRGs and GBPs to the PVM of *T. gondii* and subsequent control of *T. gondii* replication by IFNG.²⁵ Pharmacological induction or inhibition of the autophagy pathway also did not play any role in the targeting process. Further, genetic ablation of other essential autophagy genes (e.g., *Ulk1*, *Ulk2*, *Atg14*) had no effect on targeting of IFN-inducible GTPases. These data clearly

demonstrated that the targeting process is independent of the degradative autophagy pathway but dependent on *Atg5*.²⁵

Atg5 is an essential gene for the formation of double-membrane-bound autophagosomes, which sequester and transport cytosolic materials to lysosomes.²⁶ Autophagosome formation requires the conjugation of ubiquitin-like microtubule-associated-protein-1-light-chain-3 (LC3) and its homologs to phosphatidylethanolamine (PE) on membranes, which is essential for the extension of the membrane and the completion of the globular autophagosome. For the conjugation of LC3 homologs, ATG5 forms a protein complex with ATG12 and ATG16L1, and they function as an E3-like ligase complex with an E1-like activating enzyme, ATG7, and an E2-like conjugating enzyme, ATG3.^{23,27} We found that not only ATG5 but the entire LC3 conjugation system (ATG7, ATG3, and ATG12–ATG5–ATG16L1 complex) of autophagy is necessary to target LC3, GKS IRGs and GBPs to the PVM of *T. gondii* and subsequent control of *T. gondii* infection *in vitro* and *in vivo* by IFNG,²⁵ which is consistent with recent findings from other groups.^{21,28} Collectively, we found that the targeting process of GKS IRGs and GBPs is governed by a non-canonical and non-degradative function of the LC3 conjugation system of autophagy.²⁵

Since the only known function of the entire LC3 conjugation system is indeed to conjugate LC3 homologs to a membrane,²⁹ we further examined whether the conjugation of LC3 homologs is required for the targeting process. Multiple LC3 homologs exist in mammalian systems, and they act in different stages of autophagosome formation.³⁰ The LC3 subfamily (LC3A and LC3B in mice) functions in elongation of the autophagosomal membrane and the GABARAP subfamily (GABARAP, GABARAPL1, and GABARAPL2) works in a later stage of autophagosome completion.³¹ Recent studies further established the difference between these 2 subfamilies with respect to their interaction partners.^{32–35} In spite of these distinct autophagic functions of LC3 homologs, we found that all LC3 homologs play an essential but overlapping function for targeting of the GKS IRGs and GBPs to the PVM and subsequent control of *T. gondii* infection by IFNG.³⁶ That is, either LC3 or GABARAP subfamily alone was sufficient for proper targeting of GKS IRGs and GBPs, and only in the absence of both subfamilies the targeting process was disrupted. Our data suggest that both subfamilies of LC3 homologs function analogously in recruiting the IFN-inducible GTPases to the PVM of *T. gondii*.³⁶ The 2 subfamilies share a ubiquitin-like core domain but possess dissimilar N-termini, which are known to be essential for their distinct autophagic functions.³⁰ Thus, their overlapping function in TAG suggest that the shared ubiquitin-like domain may play a crucial role in the targeting process, while the difference at the N-

termini may be removed by a potential post-translational modification during the process.

A crucial question was whether the LC3 conjugation system is not only necessary but also sufficient for targeting the GKS IRGs and GBPs to a membrane. That is, can the LC3 conjugation system define the targeting site of GKS IRGs and GBPs? Since the E3-like ATG12–ATG5–ATG16L1 complex specifies the conjugation site of LC3 homologs,²⁹ to examine this possibility, we relocated the ATG12–ATG5–ATG16L1 complex to plasma membrane or mitochondria outer membrane, using the KRAS-CAAX motif and a modified anchor-away system, respectively.^{29,37} In these settings, the IFN-inducible GTPases relocated to the plasma membrane and mitochondria outer membrane, where the LC3 and the conjugation system relocated.³⁶ These data clearly showed that the LC3 conjugation system indeed can specify the target membrane of the GKS IRGs and GBPs and further suggest that LC3 homologs on the PVM of *T. gondii* are the factors that recruit the IFN-inducible GTPases specifically to the target membrane.

How do LC3 homologs recruit the IFN-inducible GTPases to the target membrane?

Since LC3 localizes on the outer (cytosolic side) membrane of cellular autophagosome,³⁸ in theory LC3-decorated autophagosomes would be targeted and disrupted by the GKS IRGs and GBPs upon their induction by IFN, if LC3 on the membrane is the only necessary signal to recruit them. In fact, IRGs have been shown to interact with autophagy proteins and to be involved in canonical degradative autophagy.^{39–42} In these reports, however, IRGs stimulate autophagy rather than interfere, and furthermore we have not observed any significant effect of IFNG on canonical degradative autophagy.⁴³ Although we cannot exclude the possibility that the special nature of the autophagosome (e.g. double-membrane) prevents it from being disrupted by GKS IRGs and GBPs upon their targeting, similar to the lysosome,⁴⁴ our data suggest that the IFN-inducible GTPases do not target and affect the autophagosomes decorated with LC3.

One relevant observation to these outstanding questions is that *T. gondii* infection differentially affected the recruitment of the GKS IRGs and GBPs to the membrane where LC3 localized.³⁶ In the absence of *T. gondii* infection, induction of GKS IRGs and GBPs by IFNG was not sufficient to send them noticeably to the mitochondria outer membrane marked with LC3 via the anchor-away system. In contrast, upon *T. gondii* infection of the IFNG-treated cells, both GTPases were substantially recruited to the LC3-marked mitochondria membrane.³⁶ Intriguingly, the recruitment of GKS IRGs to the plasma

membrane marked with LC3 via the KRAS-CAAX system was not dependent on *T. gondii* infection.³⁶ In this regard, it is interesting to note that there is no known GMS IRGs on the plasma membrane⁴⁵ whereas IRGM1 localizes on mitochondria.⁴⁶ Thus, *T. gondii* infection may affect the recruitment of, at least, GKS IRGs to the LC3-marked membranes by altering the localization of GMS IRGs. Further, we also observed that stably expressed IFN-inducible GTPase did not go to the PVM of *T. gondii* unless the infected cells were activated with IFNG (unpublished). It is also noteworthy that the localization of LC3 homologs on the PVM of *T. gondii* was not dependent on IFNG signal but was substantially enhanced upon IFNG treatment,³⁶ indicating a potential role of IFNG in modifying the function of the LC3 conjugation system and/or the LC3 homologs on the membrane. Taken together, these data suggest that cellular events induced by IFNG and *T. gondii* infection may substantially modify the interaction between LC3 homologs and the IFN-inducible GTPases.

Both IFNG treatment and *T. gondii* infection can induce substantial changes of gene expressions and signaling pathways in target cells.^{47–49} Further, LC3 homologs can be post-translationally modified in various ways.^{50–53} Therefore, it is tempting to speculate a ‘triple-check’ model of IFN, LC3, and infection to explain how LC3 homologs recruit the IFN-inducible GTPases to the target membrane: we hypothesize that IFN enables LC3 homologs on a membrane to function as ‘guanine nucleotide exchange factor (GEF)’ for the local activation of the IFN-inducible GTPases and *T. gondii* infection causes the LC3 homolog-marked membranes to be free of GMS IRGs that function as ‘GDI’ for the inactivation of the GTPases.⁹ Such ‘IFN-activation’ of LC3 homologs may work as direct post-translational modifications of the LC3 homologs or through targeting of additional factors (e.g., ubiquitin^{18,19}) that can work with the LC3 homologs. As a necessary corollary, the GKS IRGs and GBPs would be activated and multimerize on a membrane with GEF, ‘IFN-activated’ LC3 homologs, and without GDI, GMS IRGs.

This model predicts that expression of GKS IRGs or GBPs,⁴⁴ especially their activated forms,⁵⁴ over a control capacity of GMS IRGs may simply override such ‘triple-check’ restriction. Likewise, this ‘triple-check’ model can also explain a previous finding on the recruitment of GKS IRGs and GBPs to lipid droplet (LD) in the absence of *T. gondii* infection.^{44,54} In wild type cells, LDs are marked with IRGM1, IRGM3 and some LC3.⁵⁴ However, in the absence of *Irgm1* and *Irgm3*, LC3 accumulates substantially on LDs and the GKS IRGs and GBPs are targeted to the LDs.⁵⁴ Since GMS IRGs are not on the LDs, ‘IFN-activated’ LC3 on the LD might be sufficient to recruit the GKS IRGs and GBPs in

the absence of *T. gondii* infection, just like the LC3-marked plasma membrane.³⁶

For the case of autophagosomes, we speculate that an IFN-mediated change of LC3 may not occur due to the potential inaccessibility of LC3 upon its occupation by other autophagic process related proteins³⁸ or due to some other special nature of the autophagosome.⁵⁵ In this regard, it is noteworthy that the LC3 on autophagosomes is partially removed by the deconjugating enzyme ATG4 via some incompletely understood mechanism,^{56,57} suggesting a limited accessibility of the LC3 on autophagosome for modification. Since the membrane of autophagosomes is derived from various sources of endomembranes,⁵⁸ autophagosomal membranes are likely to be associated with GMS IRGs.⁴⁴ Further, both mouse IRGM1 and its human homolog IRGM interact with many key autophagy proteins, and at least partial localization of them on autophagosomes were reported.^{41,59,60} Thus, autophagosomes may be heavily associated with GMS IRGs to the extent which *T. gondii* infection may not considerably alter GMS localization. Further studies will illuminate the functional mechanism of the TAG process, including this ‘triple-check’ model of IFN, LC3, and infection.

Does the E3-like ATG12–ATG5–ATG16L1 complex detect pathogen-containing vacuoles?

If the LC3 homologs can specify where the GKS IRGs and GBPs go and the E3-like ATG12–ATG5–ATG16L1 complex determines where the LC3 homologs are conjugated to, then what brings the ATG12–ATG5–ATG16L1 complex to the membrane of pathogen-containing vacuoles? In fact, we were able to detect the complex on the PVM of *T. gondii* in as early as 2 minutes-post-infection of *T. gondii*,³⁶ which is similar kinetics to the initiation of canonical autophagy.⁶¹ Such swift recruitment suggests that detection of *T. gondii* invasion occurs quickly without transcriptional or translational change in the infected cells. In theory, the ATG12–ATG5–ATG16L1 complex may go to the target membrane, directly by recognizing the abnormally reorganized membrane structure of *T. gondii* PV or indirectly by another upstream sensor that recognizes the structure as a pathogen-associated molecular pattern.

ATG5 can bind membranes without ATG12 and ATG16L1.⁶² Recent data from the yeast ATG12–ATG5–ATG16 complex further showed that the direct membrane binding activity of ATG5 is inhibited by ATG12 conjugation and the inhibition is relieved upon its binding to ATG16.⁶³ Thus, the ATG12–ATG5–ATG16 complex can directly bind to membranes through ATG5, yet the complex doesn’t significantly associate with membranes *in vivo* without proautophagic stimulus.⁶⁴ These data suggest that the direct membrane binding activity of ATG5 in the complex is

further restricted by currently unknown factors. Thus, we speculate that the absence of an unknown inhibitory signal on the PVM of *T. gondii*, at least transiently, may lead to the recruitment of the ATG12–ATG5–ATG16L1 complex to the membrane via ATG5, as proposed in the “guard” model or “missing-self” model.^{2,9,10}

Alternatively, but not exclusively, the complex may be recruited to the PVM of *T. gondii* via an interaction partner of ATG16L1 on the membrane. In canonical autophagy, the ATG12–ATG5–ATG16L1 complex is recruited to the site of autophagosome initiation via WIPI2b (WD repeat domain, phosphoinositide interacting 2b). WIPI2b can bind to phosphatidylinositol 3-phosphate (PtdIns3P) at the initiation site and bring the complex to the site via its interaction with the coiled-coil domain of ATG16L1.⁶⁵ The coiled-coil domain of ATG16L1 is essential for autophagosome formation *in vivo*, through oligomerization of ATG16L1 and interaction with upstream autophagy genes.^{64–67} Intriguingly, the coiled-coil domain of ATG16L1 is also required for the IFNG-mediated control of *T. gondii* infection,³⁶ although we did not observe any significant role of PtdIns3P in the control of *T. gondii* by IFNG.²⁵ These data may suggest that targeting process require the oligomerization of ATG16L1, but the coiled-coil domain of ATG16L1 may be required to bring the ATG12–ATG5–ATG16L1 complex through its interaction with a potential sensor on the PVM of *T. gondii*. In this regard, it is worth noting that the PVM of *T. gondii* was rapidly marked with GFP fused to the pleckstrin homology domain of AKT, which recognizes a membrane containing PtdIns(3,4,5)P₃ or PtdIns(3,4)P₂.⁴⁹ Taken together, the ATG12–ATG5–ATG16L1 complex may be recruited to the PVM of *T. gondii* via another protein that can bind both the phosphorylated derivatives of phosphatidylinositol on the PVM and ATG16L1, in a similar fashion to the initiation of canonical autophagosome formation.

A current working model for the TAG-mediated control of *T. gondii* infection

We found that the LC3 conjugation system of autophagy marks the membrane of pathogen-containing vacuole to be targeted and disrupted by the IFN-inducible GTPases. Importantly, IFN is not required for the LC3 conjugation system to mark the membrane;³⁶ that is, with or without the activation of cells with IFN, pathogen-containing vacuoles get marked with the LC3 homologs. However, we also observed substantially enhanced localization of LC3 homologs on the PVM of *T. gondii* upon IFNG treatment.³⁶ Only upon activation of cells with IFN, the GKS IRGs and GBPs are induced and then targeted to

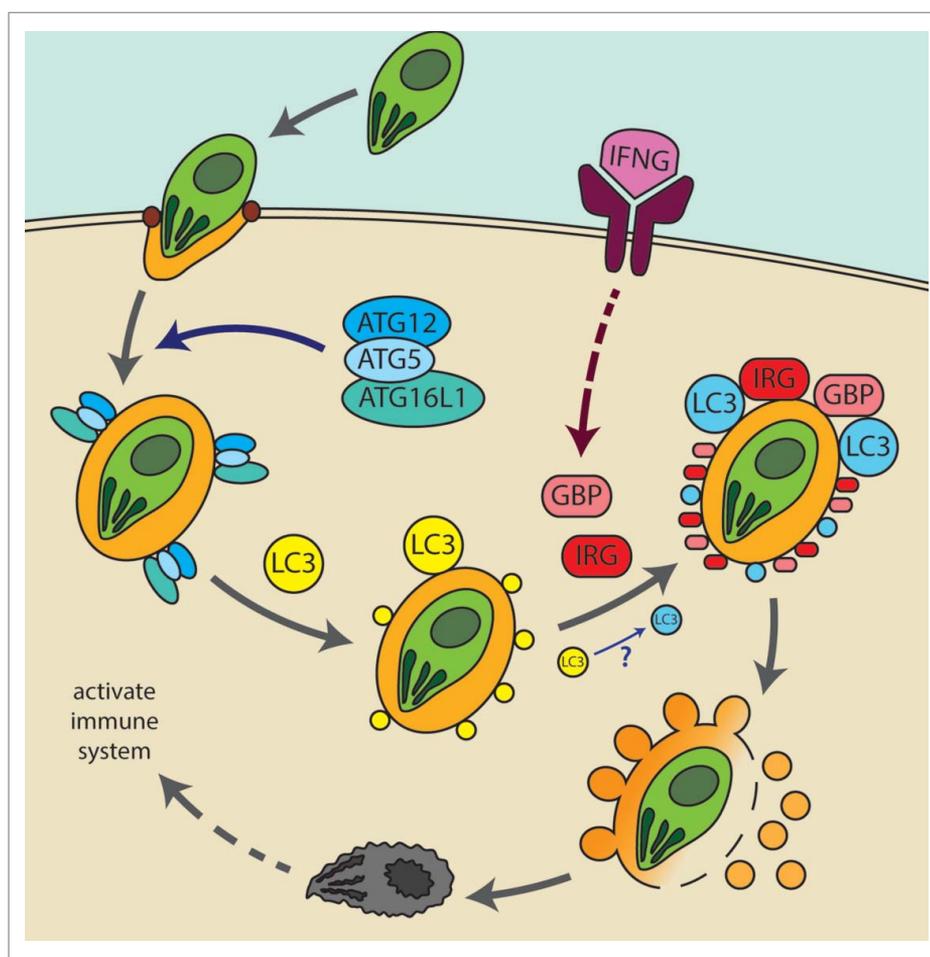


Figure 1. A current working model for the TAG-mediated control of *T. gondii* infection. Upon invasion and formation of the PV of *T. gondii*, the ATG12–ATG5–ATG16L1 complex is recruited to the PVM. The complex conjugates the LC3 homologs on the PVM of *T. gondii*, and the conjugated LC3 homologs on the PVM are activated by IFNG (e.g. post-translational modification of the LC3 homologs or through targeting of additional factors [e.g., ubiquitin^{18,19}] that can work with the LC3 homologs) and then recruit the GKS IRGs (for simplicity, just indicated as IRG in the figure) and GBPs upon their induction by IFNG. GKS IRGs and GBPs on the PVM disrupt the membrane by vesiculation, and *T. gondii* exposed to cytoplasm upon the PV disintegration gets killed and further activates the immune system.

the LC3-marked membranes for their effector function. Based on our data, we propose the following working model for the TAG-mediated control of *T. gondii* infection (Fig. 1): 1) upon invasion and formation of the PV of *T. gondii*, the ATG12–ATG5–ATG16L1 complex is recruited to the PVM, 2) the complex conjugates the LC3 homologs on the PVM of *T. gondii* and dissociates, 3) the conjugated LC3 homologs on the PVM are ‘IFN-activated’ and recruit induced GKS IRGs and GBPs upon the activation of cells with IFNG, 4) GKS IRGs and GBPs on the PVM get activated in the absence of GMS IRGs and disrupt the membrane by vesiculation, and 5) *T. gondii* exposed to cytoplasm upon the PV disintegration is killed and further activates the immune system. There are many remaining questions to be answered in order to understand the TAG of IFN-inducible GTPases. How the ATG12–ATG5–ATG16L1 complex is involved in

sensing the invasion of vacuolar pathogens and how the LC3 homologs bring the GKS IRGs and GBPs specifically to the target membrane will be the next key questions to be tackled.

The host immune system has evolved a defense strategy to sense and attack abnormally reorganized endo-membrane structures as pathogen-associated molecular patterns. Understanding this immune defense strategy of the host and potential evasion strategies of pathogens would allow us to develop more effective therapeutics against the diseases caused by vacuolar pathogens.

Abbreviations

ATG	autophagy related
GABARAP	GABA type A receptor-associated protein
GBP	guanylate binding protein

IFN	interferon
IGTP	interferon gamma induced GTPase
IIGP1	interferon inducible GTPase 1
IRG	immunity-related GTPase
LC3	microtubule associated protein 1 light chain 3
PE	phosphatidylethanolamine
PVM	parasitophorous vacuole membrane
TAG	targeting by autophagy proteins
TGTP1	T cell specific GTPase 1
ULK	uncoordinated 51-like kinase
WIPI2b	WD repeat domain, phosphoinositide interacting 2b.

Funding

This work was supported by startup fund to S.H., and in part by Brinson Foundation Junior Investigator Grant, Cancer Research Foundation, an Institutional Research Grant (IRG-58-004-53-IRG) from the American Cancer Society, the University of Chicago Comprehensive Cancer Center Support Grant (P30 CA14599), the University of Chicago Digestive Diseases Research Core Center (NIDDK P30DK42086), and Cancer Center Core facilities (DNA sequencing and genotyping, flow cytometry, integrated microscopy, and monoclonal antibody). S.B.B. was supported by Molecular and Cellular Biology Training Grant (T32 GM007183).

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