Autophagy regulates *Wolbachia* populations across diverse symbiotic associations

Denis Voronin, Darren A. N. Cook, Andrew Steven, and Mark J. Taylor[1]

Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

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Wolbachia **are widespread and abundant intracellular symbionts of arthropods and filarial nematodes. Their symbiotic relationships encompass obligate mutualism, commensalism, parasitism, and pathogenicity. A consequence of these diverse associations is that *Wolbachia* encounter a wide range of host cells and intracellular immune defense mechanisms of invertebrates, which they must evade to maintain their populations and spread to new hosts. Here we show that autophagy, a conserved intracellular defense mechanism and regulator of cell homeostasis, is a major immune recognition and regulatory process that determines the size of *Wolbachia* populations. The regulation of *Wolbachia* populations by autophagy occurs across all distinct symbiotic relationships and can be manipulated either chemically or genetically to modulate the *Wolbachia* population load. The recognition and activation of host autophagy is particularly apparent in rapidly replicating strains of *Wolbachia* found in somatic tissues of *Drosophila* and filarial nematodes. In filarial nematodes, which host a mutualistic association with *Wolbachia,* the use of antibiotics such as doxycycline to eliminate *Wolbachia* has emerged as a promising approach to their treatment and control. Here we show that the activation of host nematode autophagy reduces bacterial loads to the same magnitude as antibiotic therapy; thus we identify a bactericidal mode of action targeting *Wolbachia* that can be exploited for the development of chemotherapeutic agents against onchocerciasis, lymphatic filariasis, and heartworm.**

Brugia malayi | innate immunity | chemotherapy | helminth | endosymbiont

Wolbachia is a widespread and abundant endosymbiotic bacterium of arthropods and filarial nematodes that resides in vacuoles of host germline and somatic cells. *Wolbachia* show a diverse variety of symbiotic associations with their host, ranging from obligate mutualism in filarial nematodes to commensal, parasitic, or pathogenic associations in insects and other arthropod hosts (1–5).

In filarial nematodes *Wolbachia* is obligatory for normal larval growth and development, embryogenesis, and survival of adult worms (1). Although the molecular basis of this mutualistic relationship remains unknown, a comparison of host and bacterial genomes suggests that intact biosynthetic pathways for haem, nucleotides, riboflavin, and FAD may be among the contributions of the bacteria to the biology of the nematode host (6–8). The biological processes most sensitive to *Wolbachia* loss include larval growth and development and embryogenesis in adult females. These processes have a high metabolic demand because of the rapid growth, development, and organogenesis of the nematode and are associated with the rapid expansion of *Wolbachia* populations following larval infection of mammalian hosts and in reproductively active adult females (9). Loss of *Wolbachia* results in extensive apoptosis of germline and somatic cells of embryos, microfilariae, and fourth-stage (L4) larvae, presumably because of the lack of provision of an essential nutrient or metabolite required to prevent apoptosis of these cells and tissues (10); thus apoptosis due to loss of *Wolbachia* accounts for some of the antifilarial activities of antibiotic therapy.

Therefore we wished to investigate the mechanisms responsible for the regulation of *Wolbachia* population growth to determine if activation of host nematode defense could be turned against the host's symbiont, targeting *Wolbachia* for chemotherapeutic treatments as an alternative to antibiotics. Our studies revealed that periods of rapid population growth and expansion were accompanied by activation of the autophagy pathway and that chemical and genetic manipulation of this pathway could regulate bacterial populations directly at a level equivalent to that achieved with antibiotic treatment. We then extended our observation to other *Wolbachia* symbiotic relationships and showed that both parasitic and pathogenic strains of *Wolbachia* also could be regulated by insect autophagy, demonstrating that this mechanism is a common one for the control and regulation of *Wolbachia* populations.

Results

Initiation and Activation of Autophagy by *Wolbachia* in *Brugia malayi*. ATG8a is a major autophagosomal maturation marker and serves as a biomarker of autophagy activation in eukaryotic cells. This protein has two main forms: (*i*) a cytosol-associated form, which comprises a reservoir pool of protein, and (*ii*) a cleaved membrane-associated form located on the phagosomal membranes (11, 12). We used antibodies generated to detect human ATG8a (LC3), which has 85.56% homology with the related protein in *Brugia malayi*. We detected no other proteins with the same sequence in the nematode and *Wolbachia* protein databases. ATG8a was observed by confocal microscopy throughout the lateral chord cytoplasm of *B. malayi* adult females and was associated with areas where *Wolbachia* reside (Fig. 1 *A–D*). This pattern of ATG8a distribution was not observed in *Acanthocheilonema viteae*, a *Wolbachia*-free filarial nematode (Fig. 1 *E* and *F*).

Next we studied the expression of ATG8a protein in *Wolbachia*-infected *B. malayi*, tetracycline-treated *B. malayi*, and *A. viteae* (a *Wolbachia*-free filarial nematode) during different life-cycle developmental stages, which experience different rates of *Wolbachia* population growth. Protein extracts from microfilaria or mosquito vector-derived third-stage larvae (L3), the stages that show the lowest ratio and rate of bacterial growth (9), had either no or a minor signal of the cytosol-associated form of ATG8a (Fig. 1*G*). In contrast, both forms of ATG8a were expressed abundantly in 14-d-old L4 larvae and adult stages. Tetracycline depletion of *Wolbachia* resulted in the loss of the abundant cytosolic form of ATG8a, and only activated forms were detected, showing that there was no new production of ATG8a protein following depletion of *Wolbachia* (Fig. 1*G*). In *A. viteae* adult female worms, only minor signals of the cytosolic form of ATG8a were detected.

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[1]To whom correspondence should be addressed. E-mail: mark.taylor@liverpool.ac.uk.

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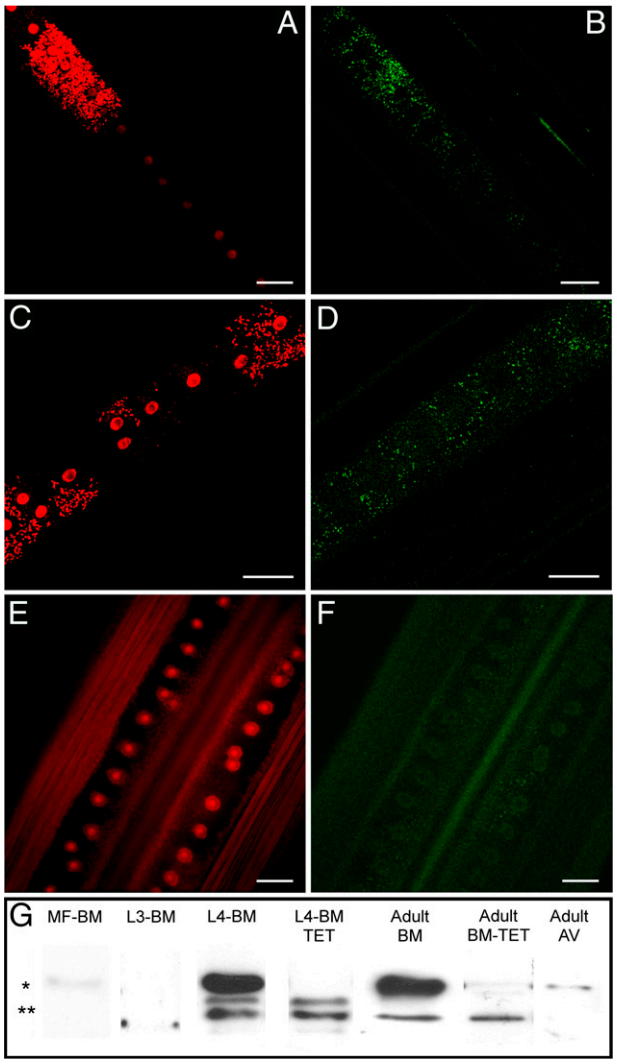

Fig. 1. Association of ATG8a expression and *Wolbachia* in the filarial nematode *B. malayi*. (*A–F*) ATG8a (green in *B*, *D*, and *F*) colocalized with *Wolbachia* clusters (small red spots in *A* and *C*; large red structures are nematode nuclei) throughout the lateral chord cytoplasm of adult female *B. malayi* (*A–D*) and is absent from naturally *Wolbachia*-free *A. viteae* (*E* and *F*). (Scale bars: 50 μm. (*G*) Western blot (composite image) of the ATG8a protein in *B. malayi* (BM) and *A. viteae* (AV). MF-BM, *B. malayi* microfilaria; L3-BM, *B. malayi* L3; L4-BM, *B. malayi* L4; L4-BM-TET, *B. malayi* L4 treated with tetracycline in vivo for 14 d; Adult-BM, protein extract from untreated adult females; Adult-BM-TET protein extract from adult females treated with tetracycline in vivo for 6 wk. *ATG8a cytosolic form; **cleaved membrane-associated forms.

Next we investigated the gene expression of *atg8a*, a major marker of autophagy initiation, during the life-cycle stages [microfilariae, L3, L4 (14-d-old), and adults] of *B. malayi.* No expression of *atg8a* was observed in microfilaria, in which the number and ratio of *Wolbachia* is the lowest of all life-cycle stages (9). Expression of *atg8a* in L3 larvae was detectable and was used as a basal level for comparison with the gene expression in other stages. An 11- to 14-fold increase in *atg8a* expression was observed in L4 (14-d-old) larvae and adult worms compared with L3 larvae ($P < 0.003$) (Fig. S1*A*).

Together these results confirm that the activation of autophagy in *B. malayi* is dependent on the presence of *Wolbachia* and is markedly elevated and activated during periods in which the bacterial population grows rapidly and in the developmental stages with the highest bacterial density.

Regulation of Autophagy Controls *Wolbachia* Populations in *B. malayi*. Next we investigated whether regulators of autophagy affected *Wolbachia* growth in *B. malayi*. Rapamycin, which acts by inhibiting the suppressor target of rapamycin (TOR), was used as an activator of autophagy (13, 14). We treated microfilaria, L3 larvae, and L4 (14-d-old) larvae in vitro with rapamycin (5 μM final concentration) for 5 d. The *Wolbachia* number was lower in all treated stages (39% in microfilaria, 26% in L3 larvae, 41% in L4 larvae) than in DMSO-treated controls (Fig. 2). Treatment of adult female worms for 7 d with rapamycin resulted in more than a two times reduction in *Wolbachia* loads; this reduction is similar in magnitude to that achieved using doxycycline, the current gold standard for antiwolbachial treatment, (Fig. 2*E*).

In parallel we used siRNA silencing (siTOR) designed specifically to inhibit the expression of *B. malayi* target of rapamycin (bmTOR) in the nematode. In adult female worms, a significant reduction ($P < 0.001$) of *Wolbachia* number was observed after 7 d of treatment with siTOR compared with siGFP-treated controls (Fig. 2*E*), showing that suppression of bmTOR and activation of autophagy results in reduced bacterial density. Next we used siRNA to silence ATG1, a key regulator of autophagy initiation. In this experiment silencing of ATG1 and inhibition of

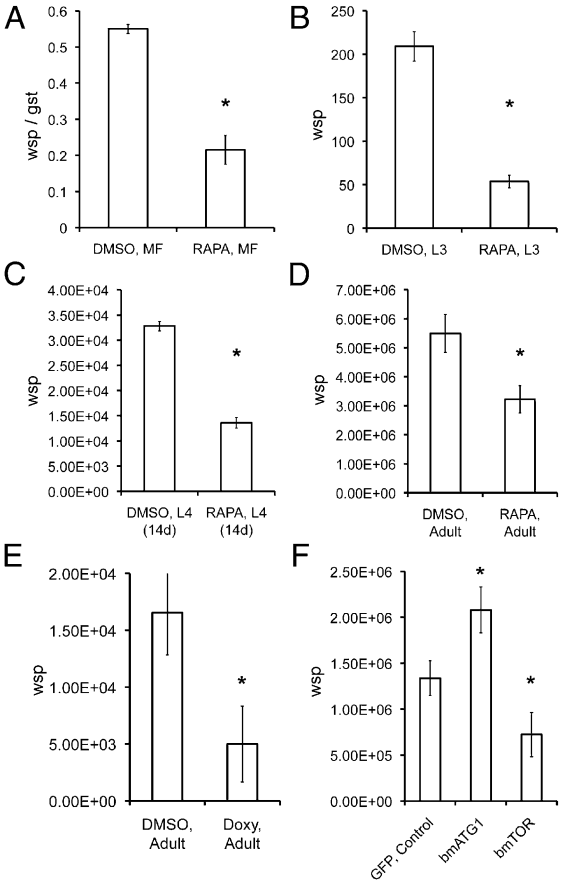

Fig. 2. qPCR analysis of *Wolbachia* numbers in *B. malayi* after in vitro treatment with rapamycin (*A–D*), doxycycline (*E*), or siRNA (*F*). (*A*) Ratio of *wsp*/*gst* in microfilaria after 5 d of treatment with rapamycin (RAPA). (*B*) Number of *wsp* copies in L3 larvae treated for 5 d. (*C*) Number of *wsp* copies in L4 larvae treated for 5 d. (*D*) Number of *wsp* copies per worm in adult females treated with rapamycin or DMSO (control) for 7 d. (*E*) Number of *wsp* copies per worm in adult females treated with doxycycline (Doxy) and DMSO (control). (*F*) Number of *wsp* copies per worm in adult females treated with siRNA (bmTOR or bmATG1) or GFP as a control. *$P < 0.001$.

autophagy led to a significant increase in *Wolbachia* numbers in adult worms (Fig. 2*F*).

Thus, the pharmacological or genetic activation and suppression of autophagy directly regulate *Wolbachia* populations in *B. malayi*.

Cellular Mechanism of bmTOR Inhibition in *B. malayi*. To confirm that autophagy was induced by the inhibition of TOR and to investigate further the mechanism by which *Wolbachia* is eliminated from *B. malayi*, we fixed treated and control adult females for transmission electron microscopy (TEM) 2 d after treatment with rapamycin or siTOR. The cytoplasm of hypodermal chord and embryonic cells contained numerous primary and mature lysosomes and phagolysosomes in samples treated with rapamycin. The number of lysosomes was 3.6 times higher in the rapamycin-treated samples than in the control samples ($P < 0.001$) Fig. 3*A* and Fig. S2*A*). In the cytoplasm of hypodermal chords from treated samples we observed numerous lysosomes surrounding *Wolbachia* and fused with the bacterial vacuole (Fig. 3*B*). These observations were reproduced using siRNA-bmTOR treatment, which inhibits TOR synthesis. Phagolysosomes containing digested material, including bacteria-like structures, were found in the cytoplasm of hypodermal chord cells confirming that bacteria were recognized and digested by the activation of autophagy. Therefore, activation of autophagy increased maturation of phagosomes containing bacteria and resulted in their fusion with lysosomes. Nuclear structure in the hypodermal chord cells remained intact.

Blockage of embryogenesis caused by extensive apoptosis is one of the major biological processes affected by the depletion of *Wolbachia* (10). We observed significant morphological alterations of embryonic cells in adult females treated with rapamycin or siRNA-bmTOR. There were dramatic changes of cytoplasm density, with the presence of large vacuoles, mature lysosomes, and clusters of proteins suggesting active digestive processes (Fig. 3 *D* and *F*); these changes were not observed during filarial embryogenesis in control samples (Fig. 3 *C* and *E*). Eighty percent of the nuclei from treated embryos and stretched microfilaria were fragmented, with condensed chromatin and loss of nuclear membrane integrity, events that occur soon after depletion of *Wolbachia* from *B. malayi* (10), suggesting that activation of apoptotic cell death was induced in the embryos after the treatment with rapamycin (Fig. S2*B*). Such phenotypic outcomes are not observed in *Caenorhabditis elegans* treated with rapamycin, which instead promotes reproductive development and increased lifespan (15), suggesting that our observations in *B. malayi* are caused by *Wolbachia* depletion. In conclusion, inhibition of TOR induced typical intracellular events consistent with the activation of autophagy in *B. malayi* adult females, resulting in a reduction of *Wolbachia* populations and subsequent induction of apoptosis in embryos.

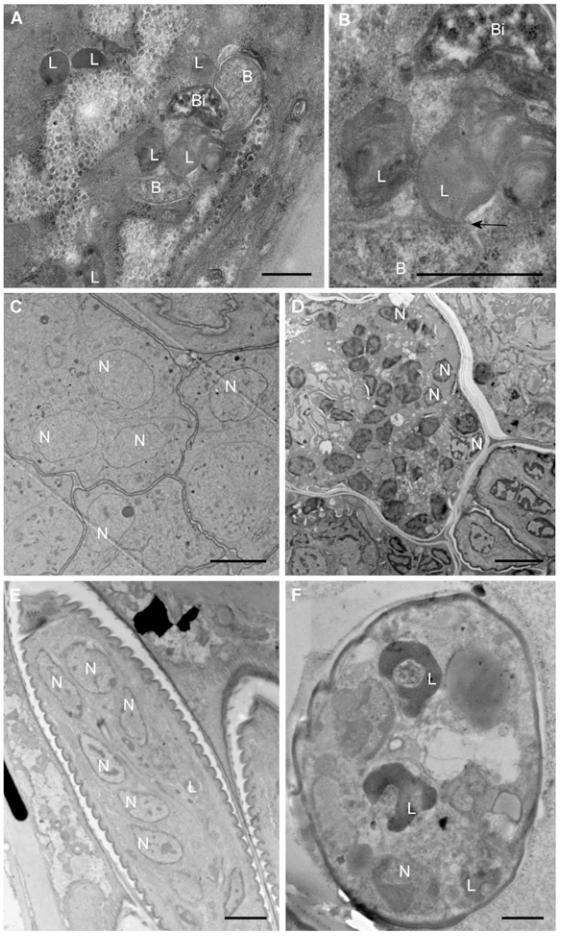

Fig. 3. Morphological effects on *B. malayi* treated with rapamycin. Micrographs of hypodermal chord cells (*A* and *B*), developing embryos (*C* and *D*), and stretched microfilaria (*E* and *F*) in the uterus of adult females treated with rapamycin and control. *A*, *B*, *D*, and *F* show rapamycin-treated samples; *C* and *E* show control samples. The arrow in *B* indicates the fusion of the lysosome and bacteria. B, bacteria; Bi, degenerated bacteria; L, lysosomes; N, nuclei. (Scale bars: 1 μm in *A* and *B*; 15 μm in *C*–*F*.)

ATG8a Localization on the Bacterial Vacuole and in the Cell Wall and Matrix of *Wolbachia*. To establish and maintain population levels necessary for a mutualistic symbiotic relationship, *Wolbachia* must evade or subvert autophagosomal destruction. Immuno-TEM of ATG8a protein localized a single or a few discreet cluster(s) on the vacuoles containing *Wolbachia* (Fig. 4). Immunogold labeling also was localized to the bacterial cell wall (Fig. 4 *B* and *D*) and within the bacterial matrix (Fig. 4 *A*, *C*, *D*, and *E*). This observation suggests a possible mechanism whereby *Wolbachia* either recruits or modifies the ATG8a host nematode protein to promote bacterial survival and evasion of autophagy. A BLAST search of ATG8a peptide against the translated *w*Bm genome revealed no homology to explain cross-reactivity of antibodies or production of a mimic bacterial protein. However, this result does not exclude the possibility of a 3D homolog of ATG8a synthesized by bacteria.

Autophagy Controls *Wolbachia* Populations in Insects. *Autophagy regulates* Wolbachia *from the mosquito* Aedes albopictus. To determine whether regulation of bacterial populations by autophagy extends to other types of *Wolbachia* that parasitize insects and arthropods, we used the mosquito cell line C6/36 infected with *Wolbachia* from the mosquito *Aedes albopictus* (*w*AlbB). We incubated infected C6/36 (*w*AlbB) cells and noninfected C6/36 (NI) cells with compounds overnight and processed samples for immunofluorescent localization of ATG8a. ATG8a was observed in C6/36 (*w*AlbB) cells under standard culture conditions and increased in intensity after induction of autophagy by treatment with rapamycin (Fig. 5 *A* and *B*). ATG8a was not commonly observed in C6/36 (NI) cells during standard culture (Fig. 5*D*) but showed the same pattern of increased intensity after treatment with rapamycin as seen in the infected C6/36 (*w*AlbB) cells (Fig. 5*E*). Suppression of autophagy by treatment with 3-methyladenine (3-MA) almost completely eliminated the signal from the cytoplasm of infected and noninfected mosquito cells (Fig. 5 *C* and *F*). To confirm that induction of autophagy in C6/36 (*w*AlbB) cells by rapamycin led to an increase in the maturation of phagosomes, we calculated the number of cells that displayed lysosomal activity. Approximately 90% of cells treated with rapamycin showed high lysosomal activity, compared with 10% of control cells (Fig. S2*C*).

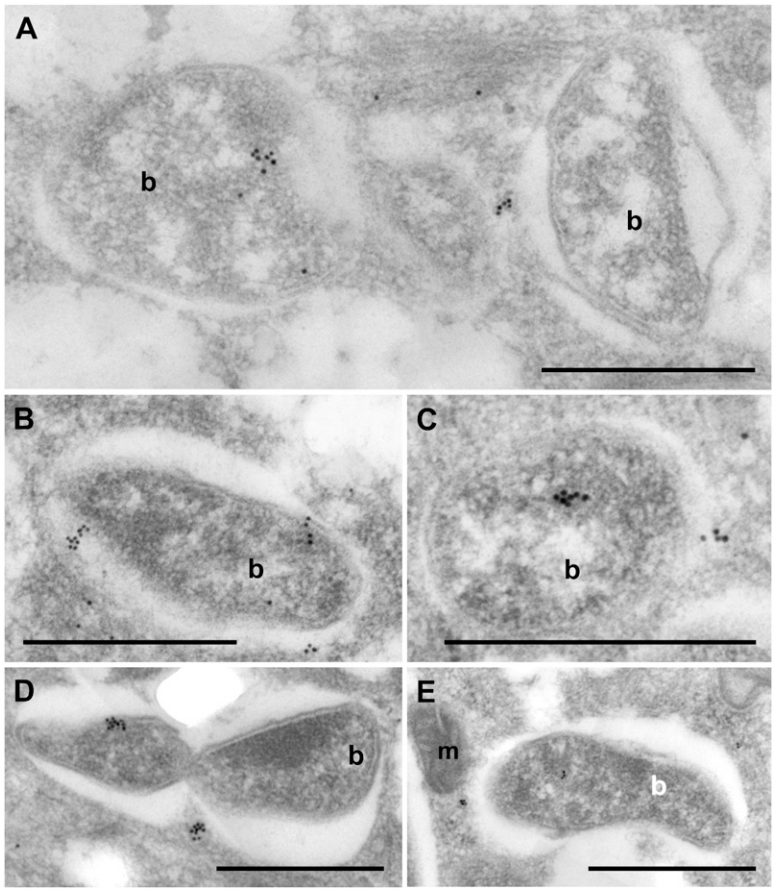

Fig. 4. Ultrastructural localization of ATG8a protein in *B. malayi* and *Wolbachia* on the bacterial vacuole (*A–E*), bacterial cell wall (*B* and *D*), and in the bacterial cell matrix (*A, B, C, E*). b, bacteria; m, mitochondria. (Scale bars: 1 μm.)

Treatment with rapamycin significantly reduced the number of *w*AlbB in C6/36 cells at days 3 and 5 after treatment as compared with the control (Fig. 5*G*). Suppression of autophagy with 3-MA, led to an increase in bacteria numbers that became significant 5 d after treatment (Fig. 5*G*). Using a different approach to induce autophagy, we subjected C3/36 (*w*AlbB) cells to starvation by culture in the absence of FCS supplementation for 2 h every second day of culture over a 5-d period (a starvation that did not affect the rate of mosquito cell growth). Induction of starvation resulted in a significant reduction in numbers of *Wolbachia* (Fig. 5*H*). Addition of inhibitors of autophagy—3-MA or wortmannin—restored the numbers of *Wolbachia* to levels equivalent to those in control cells, confirming that autophagy is responsible for reduced bacterial numbers following starvation (Fig. 5*H*).

Both these approaches confirm that *Wolbachia* populations are regulated by autophagy in C6/36 mosquito cells.

Autophagy regulates* Wolbachia *in* Drosophila *cells. The PC15 cell line infected with the pathogenic strain *w*MelPop was derived from naturally infected *Drosophila melanogaster* (w1118) and was provided by W. Sullivan (University of California, Santa Cruz, CA). We used this cell line for siRNA treatment to block specific autophagy protein synthesis. Cells were treated with siTOR targeted to *Drosophila* TOR, and siATG1 targeted to the ATG1 protein, either singly or in combination. In PC15 (*w*MelPop) cells, *Wolbachia* number was reduced by 45% 5 d after treatment with siTOR (Fig. 6*A*) and by 95% on day 9 after treatment as compared with control samples. Activation of autophagy through inhibition of TOR can be blocked by suppression of ATG1. There was no effect on *Wolbachia* number in *Drosophila* cells after 5 d of treatment with siTOR and siATG1 treatment in combination (Fig. 6*A*). This result confirms that activation of autophagy by TOR inhibition could be suppressed by the absence of a downstream partner (ATG1) in the same signaling pathway. Moreover, *Wolbachia* numbers increased in PC15 cells treated with only siATG1 molecules (Fig. 6*A*). This observation allows us to conclude that *Wolbachia* (*w*MelPop) is under autophagy control during cell-line cultivation and that the suppression of autophagy results in an increase in *w*MelPop populations.

Increased expression of atg8a in* D. melanogaster *infected with wMelPop. *w*MelPop has a pathogenic effect on *D. melanogaster*, shortening its lifespan. Here we investigate the role of autophagy in protecting the host against pathogenic *Wolbachia* in this *Wolbachia*/*Drosophila* association. The expression of the *atg8a* gene in *D. melanogaster* was compared in *w*MelPop-infected *D. melanogaster* (w1118) and *Wolbachia*-free *D. melanogaster* (w1118). A threefold ($P < 0.05$, $n = 3$) increase in *atg8a* gene expression in infected female flies was detected using a housekeeping gene (RP49) for normalization of the data (Fig. S1*B*). All results were confirmed using another housekeeping gene (actin) for normalization of the data.

***Rapamycin decreases* Wolbachia *(wMelPop) populations in* D. melanogaster.** Rapamycin was added to the standard food given to *Drosophila*. *D. melanogaster* females infected with *w*MelPop receiving the rapamycin-supplemented or standard food (as the control) laid new embryos overnight; then the adult flies were eliminated from the vial. The next generation was collected 3 d later, and quantitative PCR (qPCR) was used to determine the number of bacteria, which was reduced by 30% in *Drosophila* treated with rapamycin as compared with the control (Fig. 6*B*).

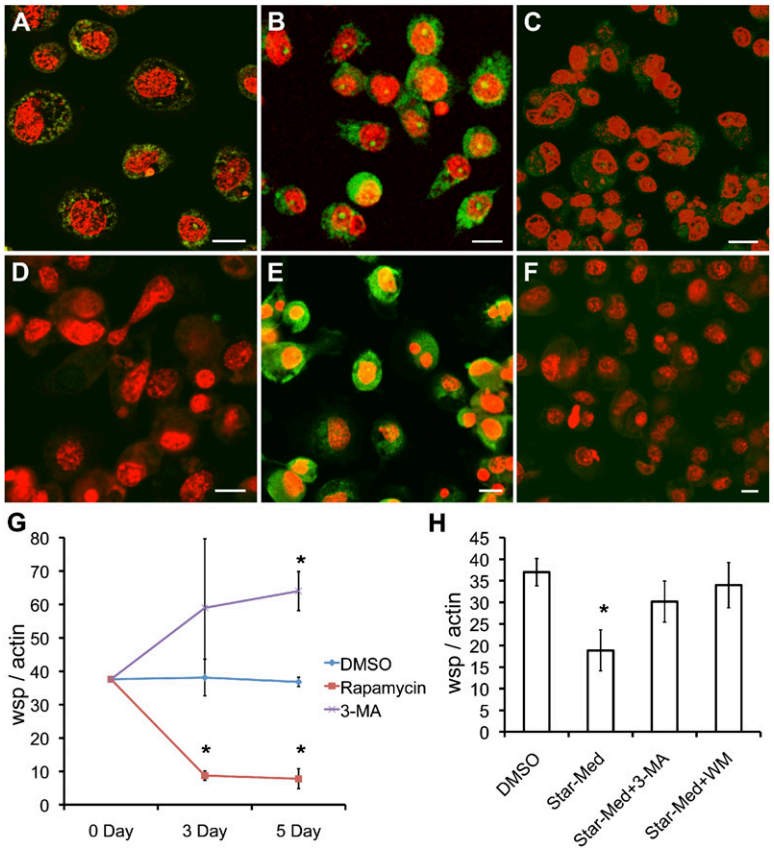

Fig. 5. Regulation of autophagy controls ATG8a expression and *Wolbachia* load in *A. albopictus* C6/36 cells. (*A–F*) Detection of ATG8a (green) in C6/36 (wAlbB) cells (*A–C*) and uninfected C6/36 cells (*D–F*) during the treatment. (*A* and *D*) Control (DMSO-treated) cells. (*B* and *E*) Rapamycin-induced cells display up-regulated ATG8a signals. (*C* and *F*) 3-MA–treated cells show suppressed expression of ATG8a. (Scale bars: 5 μm). (*G* and *H*) qPCR analysis of *Wolbachia* (WSP) and host actin gene copies in mosquito cells after treatment. (G) Ratio of *wsp*:*actin* in the C6/36 (wAlbB) cells treated with rapamycin, 3-MA, or DMSO (control). (*H*) Ratio of *wsp*:*actin* in the C6/36 (wAlbB) cells exposed to starvation (Star-Med) and treated with autophagy inhibitors Wortmannin (WM) or 3-MA. $^*P < 0.001$.

Chemical inducers of autophagy as potential antiwolbachial therapeutics. To test whether drugs that induce the activation of autophagy could lead to a reduction in *Wolbachia* populations in vivo, we treated gerbils infected with *B. malayi* with rapamycin and spermidine. Rapamycin and spermidine extend the lifespan of yeast, flies, and worms and have beneficial effects on the health of rodents (16, 17). Rapamycin slows tumorigenesis and extends lifespan in mice (17, 18), and spermidine leads to enhanced resistance to oxidative stress and decreased cell death (17, 19). In the first experiment gerbils were infected with L3 larvae and divided into three groups ($n = 3$ per group). The first group acted as a vehicle control and received 50 μL DMSO (20%)/EtOH (10%, vol/vol) in PBS by s.c. injection; the second group was injected s.c. daily for 14 d with 50 μL rapamycin [5 mg/kg in DMSO (20%)/EtOH (10%) (vol/vol)] in PBS. The third group received 30 mM spermidine in drinking water, which was changed daily, also for 14 d. Worms (L4 larvae) were collected after 14 d, and *Wolbachia* loads were analyzed by qPCR. In

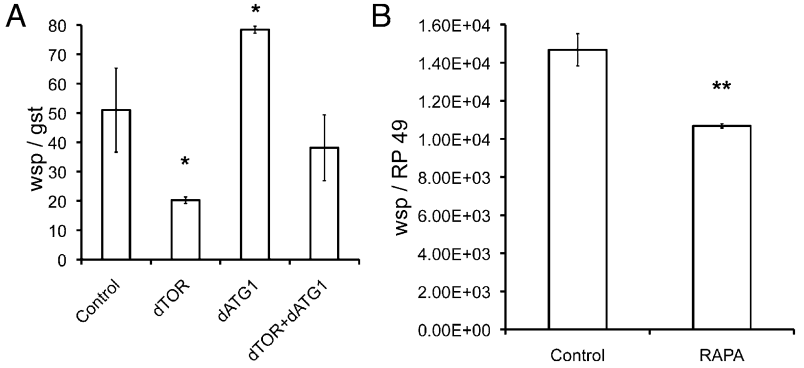

Fig. 6. Autophagy activation controls *Wolbachia* populations in *Drosophila*. (*A*) Effects of siRNA treatment on *w*MelPop populations in *Drosophila* cells (PC15). Ratio of *wsp*:*RP49* in PC15 (*w*MelPop) cells. (*B*) Reduction of the *wsp*:*RP49* ratio in *D. melanogaster* (w1118) naturally infected with *w*MelPop and treated with rapamycin (RAPA).

a second experiment gerbils ($n = 4$ per group) were treated as in the first experiment but for a period of 35 d; then *Wolbachia* loads of adult worms were analyzed by qPCR. *Wolbachia* loads in parasites treated with rapamycin or spermidine for 14 d were reduced by 30.7% and 47.3%, respectively, in L4 larvae as compared with the untreated control (Fig. 7). In adult females, treatment with rapamycin or spermidine reduced *Wolbachia* loads by 66–68% for both groups as compared with the control. These results provide a proof of concept that drug-induced activation of autophagy is as effective as antibiotic therapy in reducing *Wolbachia* populations in vivo and identify a bactericidal mode of action that can be exploited in the discovery and development of antifilarial treatments.

Discussion

Here we show that autophagy is a key regulator of *Wolbachia* populations in diverse host–symbiont relationships that range from mutualism to pathogenicity. Infection and expansion of *Wolbachia* populations activate the autophagy pathway, acting as a conserved immune recognition process across a wide range of invertebrate hosts. The genetic manipulation of the TOR–Atg1 signaling pathway or pharmacological activation or suppression of autophagy regulates *Wolbachia* loads in all host organisms and cells investigated.

The activation of autophagy through initiation and elongation steps was associated with *Wolbachia* infection dynamics and was up-regulated during periods of rapid bacterial growth and population expansion. The cellular distribution of the clustering of the major autophagosomal protein marker ATG8a was associated closely with *Wolbachia* distribution in nematode and insects cells. We show that activation of autophagy by *Wolbachia* is a process common to all three *Wolbachia*–host associations studied: (*i*) *Wolbachia* from *B. malayi* (*w*Bm), which has a mutualistic association with the host filarial nematode; (*ii*) *w*AlbB from the mosquito *Aedes albopictus,* a commensal/parasitic strain that induces cytoplasmic incompatibility; and (*iii*) *w*MelPop, which has a pathogenic effect on *Drosophila*, decreasing the lifespan of the host.

Recognition and activation of autophagy by *Wolbachia* in filarial nematodes demonstrates that, even when a host has become entirely dependent on *Wolbachia* for growth, development, and survival, *Wolbachia* still is recognized as a foreign invader and must circumvent the host intracellular defense system to survive. This phenomenon is shared with other mutualistic bacterial host symbioses (20, 21). As in other host–symbiont relationships, there must be a balance among (*i*) accommodating the symbiont to provide sufficient essential factors that serve the mutualistic association, (*ii*) regulating the symbiont population to avoid unnecessary fitness costs and pathogenicity; and (*iii*) retaining an intact host defense against other related pathogens.

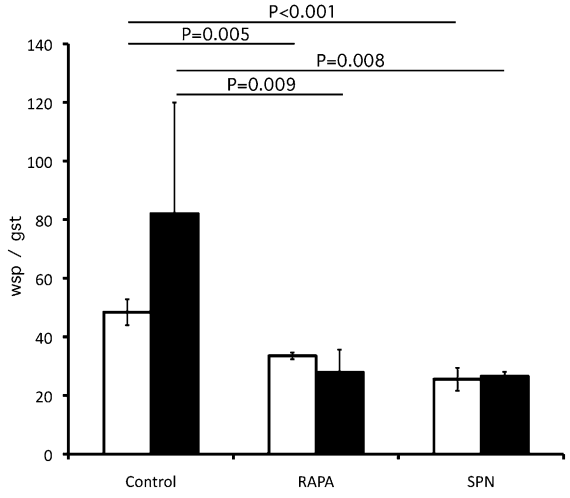

Fig. 7. qPCR analysis of *Wolbachia* number in *B. malayi* after in vivo treatment with rapamycin (RAPA) or spermidine (SPN) and in controls. Reductions of *Wolbachia* were seen in worms treated with inducers of autophagy. White bars indicate *wsp*:*gst* ratio in L4 larvae (treated for 14 d), black bars indicate *wsp*:*gst* ratio in adult females treated for 35 d.

Activation of nematode autophagy can increase the lifespan of *C. elegans* and protection from bacterial infection (22), illustrating the dual key roles played by this process in cell homeostasis and host defense. The filarial nematodes that host *Wolbachia* are renowned for their longevity of 10–15 y (23). Removal of *Wolbachia* with antibiotics such as doxycycline leads to a rapid blockage in embryogenesis and larval development that is associated with a *Wolbachia*-mediated prevention of cell apoptosis, probably through the provision of essential factors necessary for the most metabolically demanding periods of the nematode's development (10). However, this process does not appear to account for the more long-term consequences on adult worm survival, because apoptosis is confined to embryonic and larval somatic cells and adult female germline cells but does not occur in most adult somatic cells (10). After *Wolbachia* depletion with antibiotics, it takes 1–2 y before the adult worms die prematurely (23). It is intriguing to speculate that the activation of autophagy by *Wolbachia* may contribute to this extended lifespan of filarial nematodes and that the depletion of *Wolbachia* sentences the adult worms to a shorter lifespan and one more typical of an adult nematode.

To survive and serve as an essential mutualist, nematode *Wolbachia* must have developed a mechanism to evade autophagy-mediated removal from the cell. Other Ricketsiales, such as *Anaplasma phagocytophilum*, subvert the autophagy system to grow and replicate in early autophagosomes but prevent their maturation to late autophagosomes and fusion with lysosomes (24). Activation of autophagy with rapamycin favors *A. phagocytophilum* infection and growth, and the inhibition of autophagy with 3-MA arrests their growth (24). This effect is in stark contrast to our observations with *Wolbachia*, in which activation of autophagy leads to the elimination of bacteria and its inhibition promotes population expansion, highlighting important differences in the mechanisms by which these closely related bacteria avoid autophagy-mediated destruction. Our results show that induction of autophagy through TOR-Atg1 results in an increase in the number of lysosomes, that *Wolbachia*-containing vacuoles can fuse with lysosomes, leading to their elimination, and that the inhibition of autophagy and lysosomal activity by 3-MA increases the number of *Wolbachia* in host organisms and cells. The mechanism by which *Wolbachia* populations maintain their levels may depend on a fine balance between the rate of population growth and the rate of elimination by autophagy. One process that might contribute to maintaining this balance is the possible modification or mimicry of key autophagy proteins by the bacteria that block or delay autophagosomal maturation. Our observation of the localization of ATG8a antibody reactivity to components within the bacterial matrix and membranes may be one example of such modification or mimicry by which *Wolbachia* masks or subverts host ATG8a function. We are exploring this possibility with further experimental approaches.

Transcriptional analysis of two other *Wolbachia* symbioses—a feminizing association in the woodlouse, *Armadillidium vulgare*, and obligate symbiosis in the parasitoid wasp, *Asobara tabida*—provide further evidence for regulation of the autophagy pathway by *Wolbachia.* In the isopod atg7 and atg12 were underexpressed in infected ovaries, and autophagy genes were down-regulated in the wasp association, suggesting widespread regulation of autophagy by *Wolbachia* is required for bacterial survival (25, 26).

Autophagy is not the only host-defense mechanism that can be activated by *Wolbachia*. Natural and experimental infections of *Drosophila* and mosquitoes with the overreplicating and life-shortening *w*MelPop strain can induce up-regulation of host immune responses and inhibit microbial infection with viruses, protozoa, and helminth parasites (27–30). Nevertheless, not all *Wolbachia*–host associations lead to activation of host immunity, and among the strains that do not activate host immunity are natural strains infecting *Drosophila* and *Aedes aegypti* (31, 32). The induction of host defense and protection from microbial infection therefore is strain dependent and appears to be restricted to strains that have a high replication rate and widespread tissue tropisms (29, 31, 33). Alternately, it has been suggested that the metabolic demands of such overreplicating bacteria may prevent microbial infection and transmission through competition for host cell resources (27).

Although the mechanism by which *Wolbachia* protect host insects from microbial infection remains to be fully resolved, our result suggests that autophagy activation and manipulation is a mechanism that might contribute to this phenomenon. The enhanced activation of autophagy by rapidly replicating bacteria such as *w*Bm during larval development and in adult worm populations and induced by *w*MelPop in *Drosophila* also may influence the successful infection and transmission of viruses. For example, the requirement of arboviruses (Dengue and Chikungunya) for an intact host autophagy system and their use of autophagosomes for successful replication and transmission (34, 35) may be blocked by *Wolbachia*-mediated manipulation of autophagosomal maturation, a hypothesis we are testing currently.

Finally, the use of antibiotics such as doxycycline to target *Wolbachia* elimination from filarial nematodes has emerged as a promising approach to the treatment and control of onchocerciasis and lymphatic filariasis (23). Antiwolbachial therapy is more effective than existing standard antifilarial drugs because of the permanent sterilization of adult worms and long-term macrofilaricidal effects. However, widespread mass administration of doxycycline is compromised by the relatively lengthy course of treatment (4 wk) and the exclusion of pregnant women and children <9 y of age. These barriers stimulated the formation of the Anti-*Wolbachia* (A·WOL) consortium (http://www.a-wol.net) to search for drugs active against *Wolbachia* that overcome these restrictions. Our observation that activation of nematode autophagy with drugs and small molecules leads to reductions of *Wolbachia* populations similar in magnitude to those achieved with gold-standard antibiotics such as doxycycline, both in vitro and in vivo in animal models, provides important proof of concept of a bactericidal mode of action that could be exploited for the discovery and development of drugs against filarial diseases. This proof of concept can stimulate the search for drugs that preferentially activate host nematode autophagy as an alternative approach to the elimination of this essential symbiont.

In conclusion, we have described how the regulation of *Wolbachia* populations is under the control of host autophagy and show that, to ensure their survival, the bacteria must manipulate or modulate this process through mechanisms that are distinct from those adopted by closely related bacteria. All *Wolbachia*/host associations studied, ranging from mutualism through to pathogenicity, display similarities in the activation of autophagy, which is associated particularly with overreplicating strains and periods of rapid replication and population expansion. In filarial nematodes, which host a mutualistic association with *Wolbachia,* the activation of host nematode autophagy provides a bactericidal mode of action to target *Wolbachia* for the development of chemotherapeutic agents against filarial diseases and in insects may represent an alternative host-defense mechanism to account for *Wolbachia*-mediated protection against viruses and other microbial pathogens.

Materials and Methods

Parasite Material. *B. malayi* was maintained in the peritoneal cavity of gerbils (*Meriones unguiculatus*). Parasites originally were obtained from TRS Labs, and the life cycle was maintained in house at the Liverpool School of Tropical Medicine. L3 larvae were collected from *Ae. aegypti* mosquitoes. Microfilaria, L4 larvae, and adult worms were collected from the peritoneal cavities using preheated (37 °C) standard culture medium (RPMI-1640 supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, 2.5 mg/mL amphotericin B, and 25 mM Hepes) (GIBCO). Individual worms were frozen at −80 °C for future extraction of protein/RNA/DNA. The remaining worms were cultivated in 24-well plates with either rapamycin (Sigma) at a final concentration of 5 μM for 5 d or siRNA for 7 d (37 °C, 5% CO_2). Adults were incubated individually with 10 worms per experimental group; L3 and L4 larvae with 8–10 worms per group, and microfilaria with 10,000 worms per experimental group. To treat worms with specific siRNA (45 mg/mL), freshly prepared siRNA molecules were dissolved in 0.5 mL nonsupplemented RPMI-1640 (without FBS), and worms were incubated at 37 °C, 5% CO_2 for 2 h (36). Then the medium was replaced with culture medium (supplemented with heat-inactivated FBS) containing 45 mg/mL siRNA. Worms were cultivated for 7 d before collection. After chemical or siRNA treatment, all worms were washed individually with PBS and stored for future analysis.

To obtain *Wolbachia*-depleted parasites, gerbils infected with L3 larvae were treated with tetracycline administered in drinking water (2.5 mg/mL final concentration) for 14 d (for L4 larvae). Gerbils with adult worm infections were treated for 6 wk. Worms were collected 2 wk following treatment, as described above, washed, and frozen for future analysis.

For in vivo treatment, gerbils (n = 3 or 4 animals per group) infected with L3 larvae were treated with (*i*) rapamycin injected s.c. in a concentration of 5 mg/kg every day for 14 d for analysis of L4 larvae or for 35 d to collect adults. (*ii*) Spermidine was delivered in drinking water (30 mM final concentration) daily for 14 for L4 larvae and 35 d adult worms. (*iii*) Control animals received vehicle solution (DMSO 20%/EtOH 10% in PBS) by s.c. following the regime used in the rapamycin group. Worms were collected as described above, washed, and frozen for future analysis.

All animal experiments were carried out in strict accordance with the Animals Scientific (Procedures Act) 1986 (UK) under a license granted by the Home Office (London). Experimental procedures were reviewed and approved by the Animal Welfare Committee, Liverpool School of Tropical Medicine and the Home Office (London).

***Drosophila* Maintenance.** *D. melanogaster* (w1118) naturally infected with *w*MelPop and *D. melanogaster* (w1118 NI) were maintained at 25 °C and a 12-h dark/light regime. Agar-yeast standard food was changed every 20 d (37).

To study the effect of rapamycin on the *Wolbachia* loads in *Drosophila*, 6-d-old females were placed overnight to lay new eggs in vials containing standard food (as the control) or food supplemented with rapamycin (5 μM). Then adult flies were removed from the vials. The new generation was collected from vials on day 5 and frozen for future analysis of *Wolbachia* population by qPCR.

Mosquitoes and *Drosophila* Cell Lines. Mosquito cell line C6/36 (NI), originally uninfected with *Wolbachia*, was established from *Ae. albopictus*. The cell line C6/36 (*w*AlbB) was infected with *w*AlbB derived from Aa23 (*Ae. albopictus*) at the Liverpool School of Tropical Medicine and was cultivated successfully for 4 y in the laboratory (38, 39). The cell lines were cultured routinely in 25-cm^2 plastic culture flasks at 26 °C in 5 mL of Leibovitz-15 medium consisting 10% of heat-inactivated FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, and 2 mM L-glutamine. Cells were transferred into a new flask every 4–5 d.

One day before the experiments, cells were transferred to a 96-well plate at 10,000 cells per well. On the next day the medium and nonattached cells were removed, and fresh medium with compounds was added. Rapamycin (5 μM) and 3-MA (100 mM) were used to treat C6/36 cells for 3 and 5 d, respectively. For the starvation experiment, cells were cultivated in medium without FBS for 2 h; then the medium was replaced with standard medium. This procedure was repeated every day during the 5-d experiment. To suppress autophagy in the starved cells, medium was supplemented with 3-MA (100 mM) or Wortmannin (10 μM). At the end of the experiment cells were washed twice with PBS, and DNA was extracted using a Promega DNA-extraction kit following the manufacturer's instructions.

Drosophila PC15 (*w*MelPop) cells were derived from naturally infected *D. melanogaster* (w1118) females. The cell line was cultured routinely in 25-cm^2 plastic culture flasks at 26 °C in 5 mL of Schneider's insect medium consisting of 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 mg/mL

streptomycin. Cells were transferred into a new flask routinely once every 10 d. One day before the experiments, cells were transferred to a 96-well plate at 10,000 cells per well. On the next day the medium and nonattached cells were removed, medium without FBS and containing 20 mg siRNA was added in the wells, and cells were cultivated for 2 h. Then the modified medium was replaced with standard medium containing 20 mg siRNA. This procedure was repeated three times during the 7-d period of cell cultivation. At the end of the experiment (on day 7) cells were washed twice with PBS, and DNA was extracted as described above.

Production of RNA for dsRNA and siRNA. Total RNA was extracted from *B. malayi* or *D. melanogaster* adult females by a TRIzol-based method (40). Purified RNA was treated with 1 U DNase I (Epicentre) at 37 °C for 30 min followed by inactivation by EDTA. Treated RNA (5 μg) was used as a template for cDNA synthesis performed by SuperScript III (Invitrogen). The cDNA template was amplified by PCR using specific primers containing the T7 promoter sequence (Table S1), and the product was used as a template for T7 RNA polymerase to synthesize the dsRNA by the HiScribe T7 in vitro transcription kit (New England BioLabs). Quality and integrity of dsRNA was checked by standard agarose gel electrophoresis. siRNA (18–25 bp) corresponding to the specific target was produced by digesting transcribed dsRNA with the ShortCut RNase III kit (New England BioLabs) following the manufacturer's instructions. The siRNA was quantified by comparison on agarose gel to siRNA standard (New England BioLabs). RNA of the green fluorescent protein (*gfp*) gene was used as a control for siRNA treatment. The RNA was extracted from *Drosophila* flies containing *gfp*-gene insertion using the methods and procedures used for the production of experimental siRNA.

Gene Expression. Total RNA was extracted from 10,000 microfilaria, five L3 larvae, five L4 (14-d old) larvae, five male or female adults, or individual female *Drosophila*. Then cDNA synthesis was performed as described above. Specific primers for detection of *atg8a* gene expression level were designed by tehePrimerPrimier 4.0 program using cDNA of *atg8a B. malayi* or *D. melanogaster* as templates (Table S1). All amplifications and fluorescence quantifications were performed by a Bio-Rad Chromo 4 real-time PCR Detector (Bio-Rad). A ΔΔCt-based method was used to analyze *atg8* levels and the *gst* gene of *B. malayi* or the RP49 gene of *Drosophila* for normalization (10, 41, 42). All comparisons were replicated with at least three biological repeats with three technical replicates for each repeat.

Western Blot. *B. malayi* worms (microfilaria, L4 larvae, and adults) were collected from treated and untreated gerbils. L3 larvae were obtained by dissection of infected mosquitoes. All samples were washed three times in PBS and lysed with 50 μL of Tissue Extraction Reagent (Invitrogen). The concentration of the proteins was estimated by bicinchoninic acid assay (Invitrogen) following the manufacturer's instructions. Lysates of worms were mixed with LDS sample buffer (NuPAGE; Invitrogen), boiled, and run in 12% PAGE. Protein was transferred to nitrocellulose membranes and used in the Western blot as previously described (43). Western blot detection of ATG8a was performed using anti-ATG8a (LC3-II) antibody (Invitrogen and New England BioLabs). Western blot was performed using three independent protein samples analyzed in parallel.

DNA and qPCR. DNA was extracted from worms, mosquito or *Drosophila* cells, and *Drosophila* flies by using the QIAGenes Expression Kit (QIAGEN) following the manufacturer's instructions. *Wolbachia* numbers were quantified by qPCR using a single-copy gene: *wsp* (for *Wolbachia*) as previously described (9). To estimate the dynamics of the bacterial populations in *B. malayi* microfilaria, we calculated the ratio of single-copy genes *wsp* and *gst* (*B. malayi*) (9, 10); to estimate *Wolbachia* loads in the cells, we calculated the ratio of *wsp*:*actin* for C6/36 cells and *wsp*:*RP 49* for *Drosophila* to standardize the data (39).

Microscopy. *B. malayi* adult females were fixed using 4% formaldehyde in PBS with 0.05% Triton-X100 (PBST) for 20 min for confocal microscopy analysis of ATG8a protein localization. During fixation, worms were cut to improve distribution of the fixative. Samples then were washed three times in PBST and treated with RNase A (100 mg/mL) overnight at 4 °C (10). The following day, samples were washed in PBS and blocked with 5% BSA for 15 min and incubated overnight at 4 °C with anti-Atg8a (LC3-II) antibody (Invitrogen) diluted 1:200. Secondary antibody labeled with FITC was used at 1:500. After incubation with antibodies samples were costained with propidium iodide for 20 min to visualize DNA (host nuclei and *Wolbachia*) and were viewed with an LSM 5 Pascal confocal microscope (Zeiss).

For TEM, worms were fixed with 2.5% glutaraldehyde for 2 h. During the fixation worms were cut into ∼5-mm pieces. After fixation, samples were washed three times in PBS and postfixed by 4% OsO_4 for 1 h. Samples then were washed and dehydrated using a series of ethanol concentrations (50–100%) with a final wash of acetone. Samples were embedded in plastic (Agar 100) and prepared for sectioning. Ultrathin sections were contrasted with uranyl acetate (1%) and lead citrate and then were analyzed under the Tecnai G2 Spirit BioTWIN TEM by the TEM unit, University of Liverpool (Liverpool, UK).

For immuno-TEM worms were fixed by 4% paraformaldehyde dissolved in PBS for 4 h at 4 °C. During fixation, worms were cut. After fixation, samples were washed in PBS (three times on ice) and dehydrated in a series of ethanol concentrations (50–100%) on ice. Dehydrated samples were embedded in Lowicryl Gold plastic resin. Ultrathin sections were blocked by 5% BSA and incubated with primary anti-Atg8a (LC3-II) antibody (Invitrogen) diluted 1:200 in 1% BSA overnight at 4 °C. On the next day, sections were washed three times in PBS and incubated with secondary antibody labeled with 10-nm gold particles. Sections then were washed with water and contrasted by uranyl acetate (1%) and lead citrate and then were analyzed under the Tecnai G2 Spirit BioTWIN TEM by the TEM unit, University of Liverpool (Liverpool, UK).

C6/36 cell lines were grown on glass overnight and then were fixed by 4% formaldehyde in PBST for 20 min for confocal microscopy analysis of ATG8a protein localization. After fixation, cells were washed three times in PBS and processed as described above. Stained cells were investigated under an LSM 5 Pascal confocal microscope (Zeiss).

Statistical Analysis. Differences between means were analyzed using one-way ANOVA with Dunnett's multiple comparison tests as appropriate. For in vitro experiments, means were obtained from 8–10 biological replicates for qPCR and from three biological replicates for qRT-PCR. For in vivo experiments, means were obtained from three or four biological replicates. Analysis of each biological replicate was performed in triplicate. A Dunn–Šidák adjustment was made for multiple comparisons, using a normal P value of $P = 0.006$ for individual tests to provide an overall significance (α) level of 0.05. All analyses were performed using the PASW Statistics 17 statistical computer program (IBM).

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