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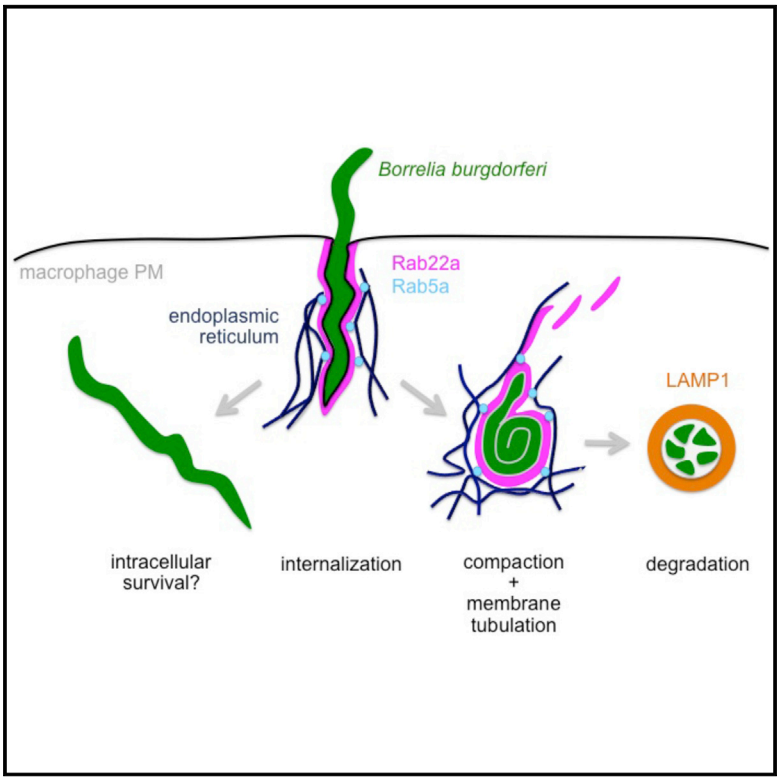
ER-Coordinated Activities of Rab22a and Rab5a Drive Phagosomal Compaction and Intracellular Processing of *Borrelia burgdorferi* by Macrophages

Graphical Abstract



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In Brief

Borrelia burgdorferi is the causative agent of Lyme disease. Naj and Linder show that borreliae internalized by macrophages are compacted in Rab22a-positive phagosomes, which is driven by membrane tubulation, controlled by Rab22a and Rab5a, and coordinated by the ER. Rab22a and Rab5a thus regulate the intracellular survival of spirochetes.

Highlights

- Rab22a is an early regulator of borreliae phagocytosis, preceding Rab5a
- Compaction of borreliae in phagosomes is driven by membrane tubulation
- The ER coordinates Rab22a and Rab5a activities
- Rab22a and Rab5a regulate intracellular survival of borreliae

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ER-Coordinated Activities of Rab22a and Rab5a Drive Phagosomal Compaction and Intracellular Processing of *Borrelia burgdorferi* by Macrophages

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SUMMARY

***Borrelia burgdorferi* is the causative agent of Lyme disease, a multisystemic disorder affecting the skin, joints, and nervous system. Macrophages and dendritic cells counteract *Borrelia* dissemination through internalization and degradation of spirochetes. We now show that *Borrelia* internalization by primary human macrophages involves uptake and compaction into Rab22a-positive phagosomes that are in close contact with Rab5a-positive vesicles. Compaction of borreliae involves membrane extrusion from phagosomes, is driven by Rab22a and Rab5a activity, and is coordinated by ER tubules forming contact sites of Rab22a phagosomes with Rab5a vesicles. Importantly, Rab22a and Rab5a depletion leads to reduced localization to lysosomes and to increased intracellular survival of spirochetes. These data show that Rab22a- and Rab5a-driven phagosomal uptake is a crucial step in the vesicular cascade that leads to elimination of spirochetes by macrophages. Rab22a and Rab5a thus present as potential molecular targets for the modulation of intracellular processing of borreliae in human immune cells.**

INTRODUCTION

Borrelia burgdorferi is the causative agent of Lyme disease, a multisystemic disorder affecting skin, nervous system, and joints (Margos et al., 2011). Humans are inadvertent hosts for the spirochete and can be infected by *Borrelia*-bearing *Ixodes* ticks feeding on blood (Dolan et al., 1998). Infection with borreliae can be indicated by an *erythema migrans*, a skin lesion that occurs in ~50% of all cases (Biesiada et al., 2010). This lesion is enriched in macrophages and dendritic cells (Salazar et al., 2003), which are the first immune cells to encounter borreliae in the human body. Accordingly, successful uptake and elimination of spirochetes by these cells is decisive for the outcome of a respective infection (Berende et al., 2010; Montgomery et al., 2007; Olson et al., 2007).

Phagocytic uptake of captured borreliae involves a variety of signaling pathways. In particular, the molecular axis involving Toll-like receptor 2 (TLR2) and myeloid differentiation factor 88 (MyD88) has emerged as a major signaling pathway that regulates internalization of spirochetes (Cervantes et al., 2011; Hirschfeld et al., 1999; Shin et al., 2009). Accordingly, TLR2-knockout mice show increased spirochete loads during early infection (Wang et al., 2004). However, also TLR2-independent pathways, either involving urokinase receptor (Hovius et al., 2009) or an interaction between complement receptor 3 (CR3) and CD14 (Hawley et al., 2012), play a role in this process. Phagocytosed borreliae were shown to colocalize with TLR2 and TLR8 in human monocytes (Cervantes et al., 2011) and to be processed in lysosomes of human dendritic cells (Filgueira et al., 1996) or macrophages (Montgomery and Malawista, 1996). However, the specific molecular pathways that regulate trafficking and maturation of borreliae-containing phagosomes are largely unknown.

In this study, we investigated the potential influence of several RabGTPases on the uptake and intracellular processing of *B. burgdorferi* by primary human macrophages. RabGTPases are major regulators of vesicular trafficking in both endo- and exocytic pathways (Hutagalung and Novick, 2011; Schwartz et al., 2007; Stenmark, 2009). They act as molecular switches (Stenmark et al., 1994) that mediate the binding of vesicles to target organelles (Zerial and McBride, 2001). The more than 70 members of this molecular family influence multiple aspects of intracellular transport (Hutagalung and Novick, 2011; Stenmark, 2009), notably maturation and trafficking of pathogen-containing phagosomes. Accordingly, Rab5 has been shown to be important in phagocytosis of *Staphylococcus aureus* (Hagiwara et al., 2014) and *Yersinia pseudotuberculosis* by COS cells (Sarantis et al., 2012) or of *Helicobacter pylori* by human macrophages (Borlace et al., 2011), whereas Rab9 and Rab14 were identified as factors involved in the maturation of *Salmonella-enterica*-containing vesicles (Kuijl et al., 2013). However, the mechanisms that allow spatiotemporal coordination of different RabGTPases during pathogen uptake are unclear.

One such mechanism might be contact of phagosomes with the ER. The influence of the ER on phagocytic processes, including uptake of pathogens such as *Salmonella typhimurium* (Gagnon et al., 2002), has been demonstrated (Touret et al., 2005a), with the current consensus being that the contribution of ER-derived membrane to phagosomes is minimal

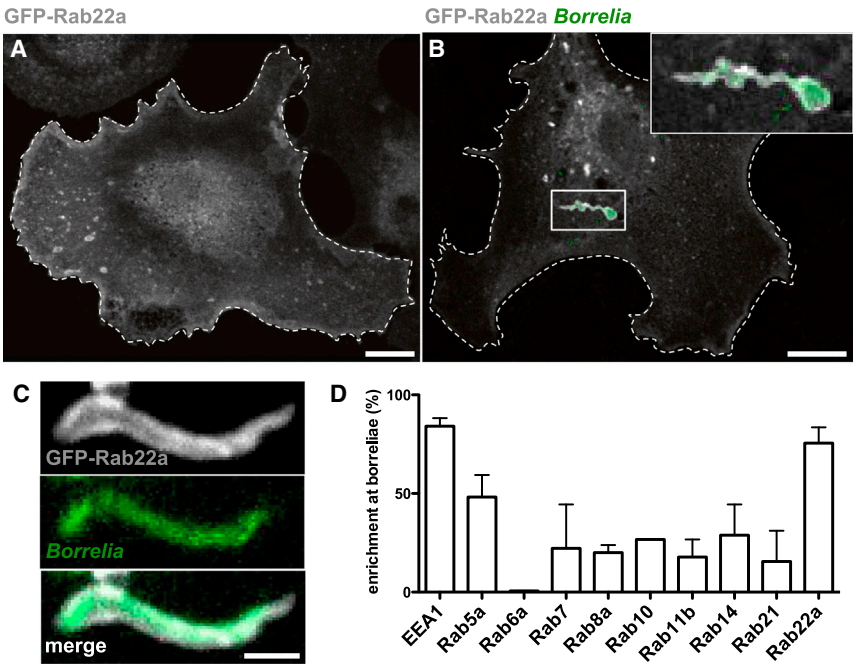


Figure 1. Internalized Borreliae Are Present in a Compartment Positive for EEA1, Rab5a, and Rab22a

(A and B) Confocal micrographs of primary macrophages expressing GFP-Rab22a. Macrophage in (B) was coincubated with borreliae stained with anti-*Borrelia burgdorferi sensu stricto* antibody (green). Dashed lines indicate cell circumference. The scale bars represent 10 μm. White box in (B) indicates area of detail image.

(C) Example for colocalization screen of host proteins with borreliae. GFP-Rab22a (white) and *Borrelia* stained with Hoechst 33342 (green) is shown. The scale bar represents 2 μm.

(D) Statistical evaluation of colocalization screen. The number of internalized borreliae was set to 100%. Percentages of borreliae positive for indicated proteins are given as means ± SEM. For each value, 3 × 15 borreliae internalized by macrophages from three different donors were evaluated.

(Campbell-Valois et al., 2012; Rogers and Foster, 2007; Touret et al., 2005b). Still, ER-localized proteins were shown to influence phagosomal maturation in HeLa cells and mouse neutrophils (Hatsuzawa et al., 2006, 2009; Nunes et al., 2012), and both Rab5-positive early endosomes (Friedman et al., 2013; Rowland et al., 2014) as well as Rab7- (Friedman et al., 2013; Rowland et al., 2014) or LAMP-1-positive (Alpy et al., 2013) late endosomes in COS-7 and HeLa cells have been shown to be in close contact with the ER (Rowland et al., 2014).

In this study, we show that intracellular processing of *Borrelia burgdorferi* by primary human macrophages is regulated by Rab22a and Rab5a. Internalization of borreliae proceeds via uptake and compaction into Rab22a-positive phagosomes that are contacted by Rab5a vesicles. This process depends on the activities of both RabGTPases, involves extrusion of membrane from the phagosome surface, and is facilitated by contact of both Rab22 phagosomes and Rab5 vesicles with ER tubules. Importantly, we show that Rab22a and Rab5a activity is crucial for development of *Borrelia*-containing phagosomes into degradative lysosomes and for elimination of spirochetes by human macrophages.

RESULTS

Internalized Borreliae Are Positive for Rab5a, Rab22a, and EEA1

To screen for regulators of *B. burgdorferi* processing, primary human macrophages were transfected with constructs encoding GFP or GL (green lantern) fusions of RabGTPases or of the RabGTPase effector EEA1 (Simonsen et al., 1998; GFP-EEA1-CT). Macrophages expressing respective fusion proteins, which were mostly present in dot- or ring-like accumulations reminiscent of vesicles (Figure 1A) or at the Golgi (not shown), were coincubated with borreliae for 1 hr. Specimens were fixed and stained with anti-*B. burgdorferi* antibody (Figure 1B) or with Hoechst 33342 to visualize DNA (Figure 1C). Intracellular borreliae were analyzed for enrichment of expression constructs (Figure 1D). Pronounced enrichment was observed for EGFP-EEA1-CT, GL-Rab5a, and GFP-Rab22a at internalized borreliae; moderate enrichment (10%–30%) for fusions of Rab7, Rab8a, Rab10, Rab11b, Rab14, and Rab21; and no enrichment for GFP-Rab6a or for EGFP alone (Figures 1D and S1A). (Note: overexpression levels of Rab22a and Rab5a were calculated as 108.0% and 130.7%, as determined by western blot analysis corrected for transfection efficiency; Figures S1M and S1N.) Importantly, enrichment of EEA1, Rab5a, and Rab22a at intracellular borreliae was further confirmed by staining of endogenous proteins (Figures S1D–S1L).

Phagocytic Uptake of Borreliae Is Coupled with Their Compaction in Phagosomes

To analyze the process of EEA1, Rab5a, and Rab22a enrichment at borreliae in more detail, respective cells were analyzed by live-cell imaging. Internalization of borreliae was accompanied by their uptake into a vesicular/tubular compartment positive for EGFP-EEA1-CT, GL-Rab5a, or GFP-Rab22a (Figures 2A–2C; Movies S1, S2, and S3), typical for phagosomes/early endosomes (Kauppi et al., 2002; Simonsen et al., 1998). Notably, both EGFP-EEA1-CT and GFP-Rab22a were present as continuous "coats" around the surface of borreliae-containing phagosomes (Figures 2A and 2B), whereas GL-Rab5a was enriched in discrete dots at the surface of this compartment (Figure 2C).

Strikingly, we observed progressive compaction of elongated spirochetes into globular forms during uptake into phagosomes (Figures 2A and 2B; Movies S1 and S2). These phagosomes sometimes fused, and several borreliae could thus be present within a single vesicle (Figure 2C; Movie S3). Moreover, compaction of elongated phagosomes into globular vesicles was accompanied by loss of membrane material (Figure 2D; Movie

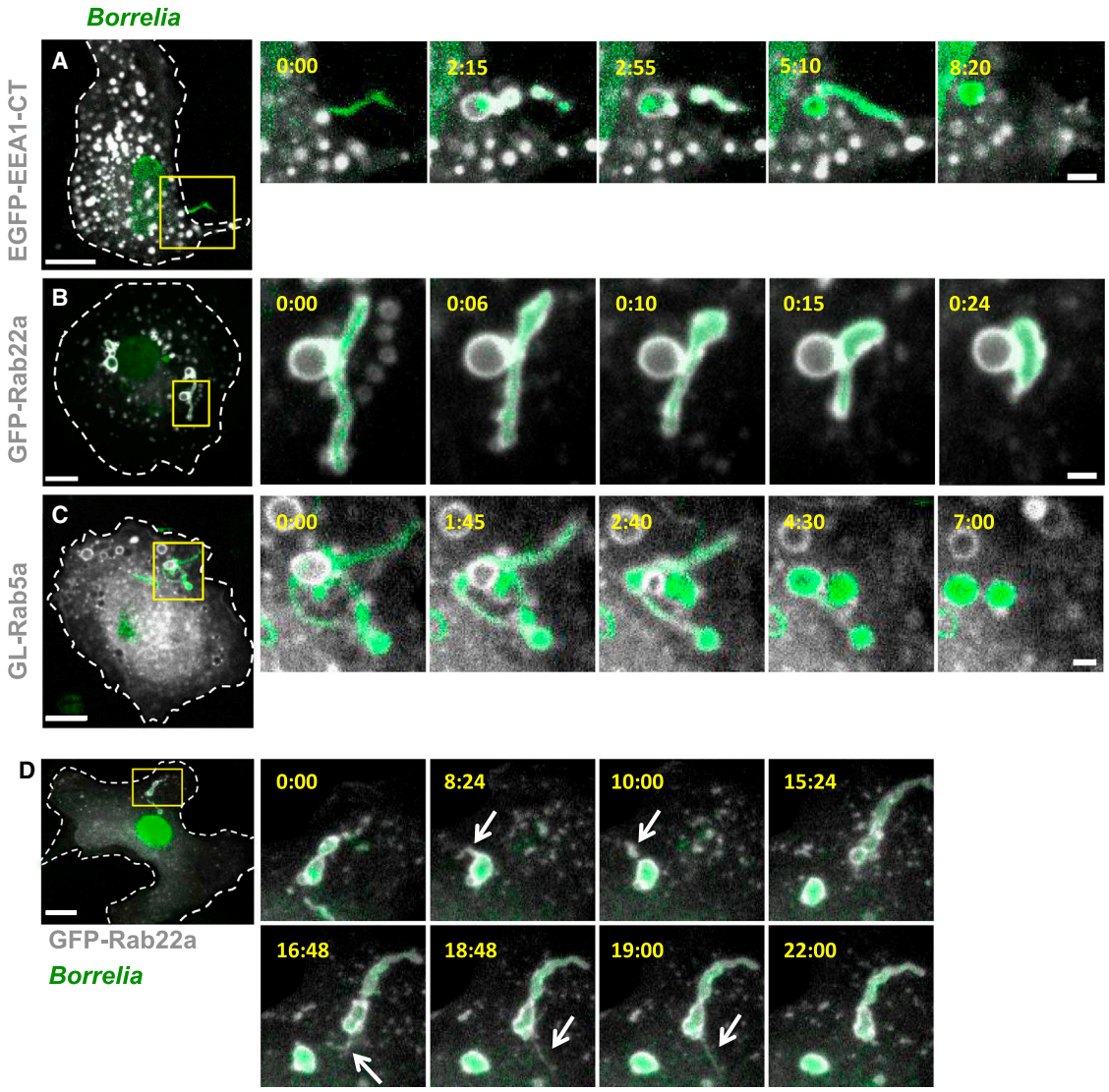


Figure 2. Internalized Borreliae Are Compacted in Phagosomes Positive for EEA1, Rab5a, and Rab22a

Confocal micrographs of macrophages expressing EGFP-EEA1 (A), GFP-Rab22a (B and D), or GL-Rab5a (C), coincubated with borreliae. Expression constructs are shown in white, with borreliae stained by Hoechst 33342 (green). (D) Compaction of borreliae-containing phagosomes is accompanied by extrusion of membrane tubules (white arrows). Still images are taken from Movies S1, S2, and S3. Dashed lines indicate cell circumference. Yellow boxes indicate area of detail images. Time since start of the experiments is indicated in minutes. The scale bars in large images represent 10 μm and in gallery images 2 μm.

S4), indicating that compaction of borreliae is probably due to reduction of the phagosomal surface. Consistently, borreliae not positive for Rab5a or Rab22a, in both fixed and live specimens, were also not compacted (not shown). This subset (~1%–5% of internalized borreliae) retained their elongated morphology and appeared to be present in the cytoplasm without a phagosomal membrane. Further experiments using *B. afzelii*, *B. garinii*, and *B. spielmanii* (not shown) revealed phagosomal compaction also for these *Borrelia* species. Moreover, also heat-killed (56°C; 30 min) *B. burgdorferi* cells were compacted to a similar degree as their viable counterparts (Figure S2A). Collectively, these observations indicated that internalization of borreliae proceeds via uptake into RabGTPase-enriched phagosomes and is accompanied by compaction of borreliae and a reduction of the phagosome surface.

Rab22a and Rab5a Regulate Phagosomal Compaction of Borreliae

To explore the potential roles of Rab22a and Rab5a on phagosomal compaction of borreliae, macrophages expressing respective dominant active (DA) or dominant negative (DN) fusion proteins were coincubated for 1 hr with borreliae, and specimens were fixed and stained. Spirochetes were scored into groups, according to morphology. Expression of GL-Rab5a DN led to a 100% increase of "elongated" borreliae, accompanied by a respective, although not statistically significant, decrease in

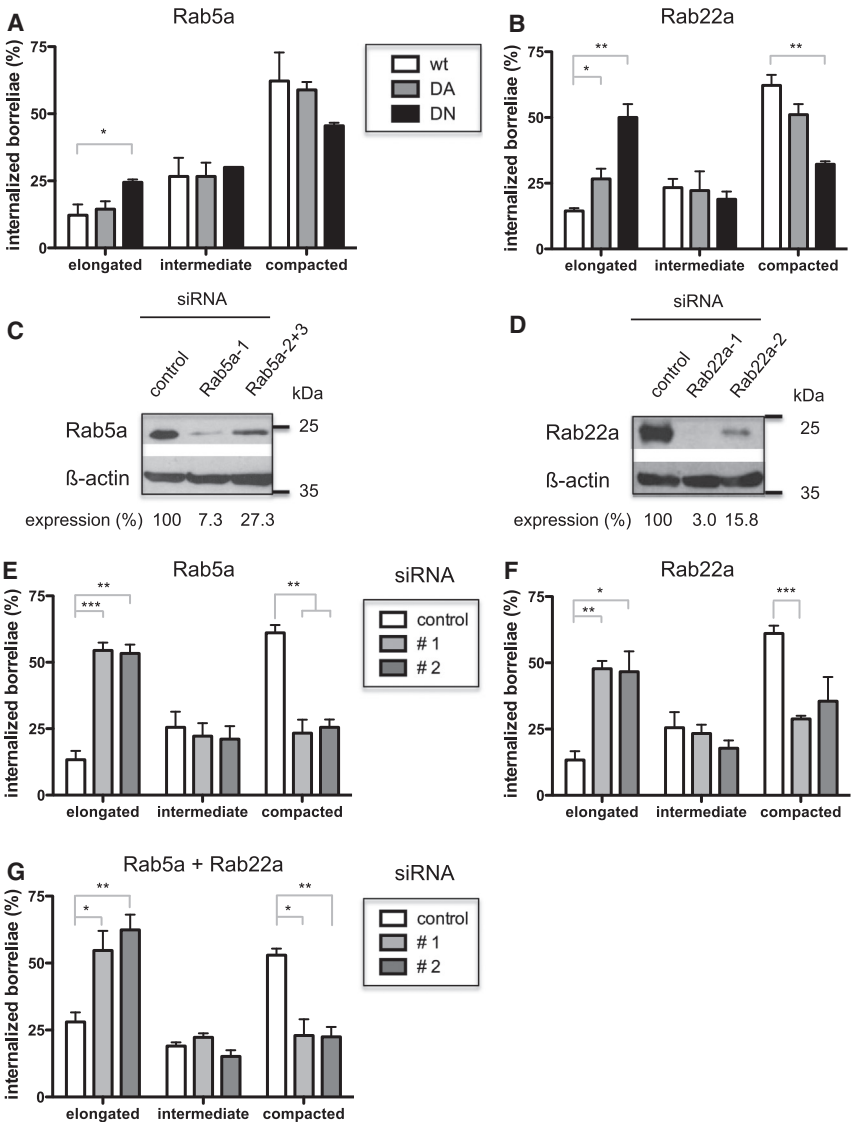


Figure 3. Rab5a and Rab22a Drive Phagosomal Compaction of Borreliae

(A and B) Statistical evaluation of phenotypes of internalized borreliae in macrophages expressing Rab5a (A) or Rab22a (B) wild-type (wt), dominant active (DA), or dominant negative (DN) constructs. The percentage of internalized borreliae showing elongated, intermediate, or compacted phenotypes is indicated. Values are given as means ± SEM. For each value, 3 × 30 borreliae internalized by macrophages from three different donors were evaluated. Single and double asterisks indicate $p < 0.0424$ and $p < 0.0024$, respectively.

(C and D) Western blots of cell lysates from macrophages treated with siRNA against Rab5a (C) or Rab22a (D) and developed with antibodies against respective RabGTPase (upper blots) or β-actin as loading control (lower blots). For each RabGTPase, two independent siRNAs were used (with Rab5a no. 2 being a combination of two siRNAs), with siRNA for firefly luciferase as control. Normalized expression levels and molecular weight in kDa are indicated.

(E–G) Statistical evaluation of phenotypes of internalized borreliae in macrophages treated with Rab5a- or Rab22a-specific siRNA (E and F), combinations thereof (G), or controls. The percentage of internalized borreliae showing respective phenotypes is indicated. Values are given as means ± SEM. For each value, 3 × 30 borreliae internalized by macrophages from three different donors were evaluated.

Single, double, and triple asterisks indicate $p < 0.0301$, $p < 0.0070$, and $p < 0.0008$, respectively.

the number of "compacted" borreliae (Figure 3A). Expression of GL-Rab5a DA did not result in pronounced changes in phagosomal compaction. In contrast, expression of GFP-Rab22a DA or DN led to pronounced changes in the number of compacted borreliae, corresponding to increases for elongated forms of 85% and 246% and to decreases for compacted forms of 18% and 48%, respectively (Figure 3B).

We next established siRNA-mediated knockdown of both RabGTPases, using each time two previously validated siRNAs (Wiesner et al., 2013). After 3 days, macrophages with established knockdown of Rab5a (Figure 3C) or Rab22a (Figure 3D) were coincubated with borreliae for 1 hr, and internalized borreliae were scored into groups. We observed elevated levels of elongated borreliae in macrophages depleted for Rab5a (308% and 300%; Figure 3E) or Rab22a (258% and 250%; Figure 3F). This increase was accompanied by a respective decrease in the level of compacted borreliae (Rab5a siRNA: decreases of 62% and 58%; Figure 3E; Rab22a siRNA: decreases of 53% and 42%; Figure 3F). To clarify whether Rab5a and Rab22a work in the same pathway, this experiment was also performed using macrophages treated with combinations of Rab5a- and Rab22a-specific siRNA. Combined knockdown of both RabGTPases led to an increase of elongated borreliae (Figure 3G), accompanied by a decrease of compacted forms (Figure 3G), comparable to single knockdown of Rab22a (~50% increase of elongated and ~25% decrease of compacted forms; see Figures 3A and 3B). Importantly, also phagosomes containing heat-killed borreliae acquired EGFP-EEA1-CT (Figures S2B–S2D), GFP-Rab22a (Figures S2E–S2G), and GL-Rab5a (Figures S2H–S2J), whereas siRNA-mediated knockdown of Rab5a (Figure S2K) or Rab22a (Figure S2L) led to similar reduction of borreliae compaction, compared to controls.

Collectively, these data show that Rab5a and Rab22a are important regulators of *Borrelia* uptake into phagosomes and that the activity of both proteins is necessary to drive phagosomal compaction of borreliae. The absence of an additive effect for combined knockdown further indicates that both proteins act in the same pathway. Moreover, use of heat-killed borreliae shows that compaction does not require

mobility or viability of spirochetes but is driven by the phagocytosing macrophage.

Coordination of Rab22a and Rab5a Activity Is Facilitated by the ER

To investigate the spatiotemporal coordination of Rab5a and Rab22a, macrophages co-expressing GFP-Rab22a and mRFP-Rab5a were coincubated with borreliae and imaged by time-lapse video microscopy. Strikingly, an accumulation of GFP-Rab22a around borreliae was observed already at initial stages of spirochete uptake (Figure 4A). Typically (>90%), GFP-Rab22a accumulated as a continuous coat around the internalized parts of *Borrelia* cells, leading to the presence of GFP-Rab22a at the entire surface of the phagosome upon completion of internalization (Figures 2B, 2D, and 4A; Movie S5). In contrast, mRFP-Rab5a was present at dot-like, motile vesicles, which accumulated in regions of *Borrelia* uptake and contacted borreliae-/GFP-Rab22a-positive phagosomes (Figure 4A; Movie S5). Interestingly, these contacts persisted for several minutes and were mostly observed at sites of curvature of *Borrelia*-containing phagosomes (Figure 4A). Moreover, contact with Rab5a vesicles was often associated with sites of membrane tubule extension from the surface of Rab22a-positive phagosomes (Figure 4B; Movie S6). These observations indicated that Rab5a and Rab22a are present at discrete vesicular entities during internalization of borreliae. Moreover, contact between these compartments was often associated with the extrusion of membrane tubules, leading to a reduction of the phagosome surface.

Recent research indicates that early endosomes are in close contact with the ER (Eden et al., 2010; Rowland et al., 2014). We thus performed live-cell imaging of macrophages co-expressing the ER marker Sec61β, fused to mCherry (Rowland et al., 2014), together with GFP-Rab22a, and incubated them with borreliae (Figure 5A). mCherry-Sec61β expression revealed an extensive network of ER tubules throughout the macrophage cytoplasm, particularly in the central parts of cells. Strikingly, this network was specifically extended toward sites of *Borrelia* uptake. Sec61β-positive ER tubules were typically in close contact with borreliae-/GFP-Rab22a-positive phagosomes (Figures 5A and 5A'; Movie S7) and tightly enwrapped this compartment (Figure 5A''; Movie S7). Co-expression of GL-Rab5a and mCherry-Sec61β revealed that also Rab5a-positive vesicles were in constant close contact with the ER network (Figure 5B; Movie S8). Collectively, these observations show that ER tubules contact both borreliae-containing Rab22a-positive phagosomes and also Rab5a vesicles, facilitating contact between these compartments.

To investigate whether ER tubules are required for phagosomal compaction of borreliae, we generated a DN mutant of atlastin3, a GTPase involved in ER tubulation. This mutant contains a point mutation (Y192C) in the GTPase domain, leading to disruption of the ER tubule network (Kornak et al., 2014) (Figure 6A). In contrast to WT GFP-atlastin3, which decorated mCherry-Sec61β-positive ER tubules in control macrophages (Figures 6B–6D), GFP-atlastin3-Y192C led to a strong reduction of ER tubules, especially in the cell periphery (Figures 6E–6G). Macrophages expressing either WT GFP-atlastin-3 or GFP-atlastin-3-Y192C were incubated with borreliae, and the morphology of internalized spirochetes was quantified. Surprisingly, the values for elongated, intermediate, and compacted borreliae did not differ significantly between the two groups (Figure 6H). To explore the possibility whether the few remaining peripheral ER tubules were sufficient to regulate borreliae compaction, we again prepared specimens of GFP-atlastin-3-Y192C expressing macrophages, coincubated with borreliae, and stained specimens with anti-GFP antibody to enhance the atlastin signal. Indeed, we found ER tubules contacting and enwrapping internalized, CFP-Rab22-positive borreliae, comparable to WT cells. These data show that ER tubules facilitate contact between Rab22a and Rab5a compartments during borreliae compaction and that this process is highly efficient and can be performed even by a strongly reduced ER tubule network. Similar results were obtained using heat-killed borreliae (Figure S4A), again pointing to the macrophage providing the driving force for borreliae compaction.

Rab22a and Rab5a Target Borreliae to Degradative Lysosomes

Western blots of lysates from macrophages, prepared 1 hr and 2 hr after internalization of borreliae and developed using a polyclonal anti-*B. burgdorferi* antibody, showed that (1) a prominent *Borrelia* signal can be detected even after 1 hr of internalization and (2) is diminished in the 2-hr lysate (Figure 7A). Transient survival of borreliae in macrophages is thus likely (see also Figure 7P), but most of the internalized spirochetes appear to be quickly degraded subsequently to their uptake into phagosomes.

Analyzing the further processing of internalized borreliae, we found enrichment of endogenous LAMP1, a lysosome marker (Chen et al., 1988), around compacted borreliae (Figures 7B–7D), whereas intermediate or elongated forms mostly showed no such enrichment (Figures 7E–7G). To determine whether this LAMP1-positive compartment was also degradative, macrophages were coincubated with borreliae in the presence of GFP-coupled, dequenched BSA (DQ-BSA), a reporter of proteolysis (Vázquez and Colombo, 2009). In cells incubated with DQ-BSA, compacted borreliae mostly colocalized with DQ-BSA fluorescence signals (Figures 7H–7J), in contrast to intermediate or elongated forms (Figures 7K–7M). These findings indicated that borreliae compacted in phagosomes are subsequently targeted to a degradative lysosomal compartment.

To test whether Rab5a and Rab22a are involved in this process, macrophages were treated with respective siRNA and the number of internalized borreliae showing LAMP1 or DQ-BSA enrichment was quantified. In all cases, we found pronounced reduction of LAMP1 accumulations around internalized borreliae, corresponding to relative decreases of 21% and 42% in Rab5a-depleted cells and to relative decreases of 41% and 40% in Rab22a-depleted cells (Figure 7N). Similar values were gained for the DQ-BSA analysis, corresponding to relative decreases of 15% and 32% in Rab5a knockdown cells and to relative decreases of 39% and 45% in Rab22a knockdown cells (Figure 7O). We conclude from these data that Rab5a and Rab22a are important regulators of borreliae-containing phagosomes that ultimately enable their maturation into a degradative lysosomal compartment.

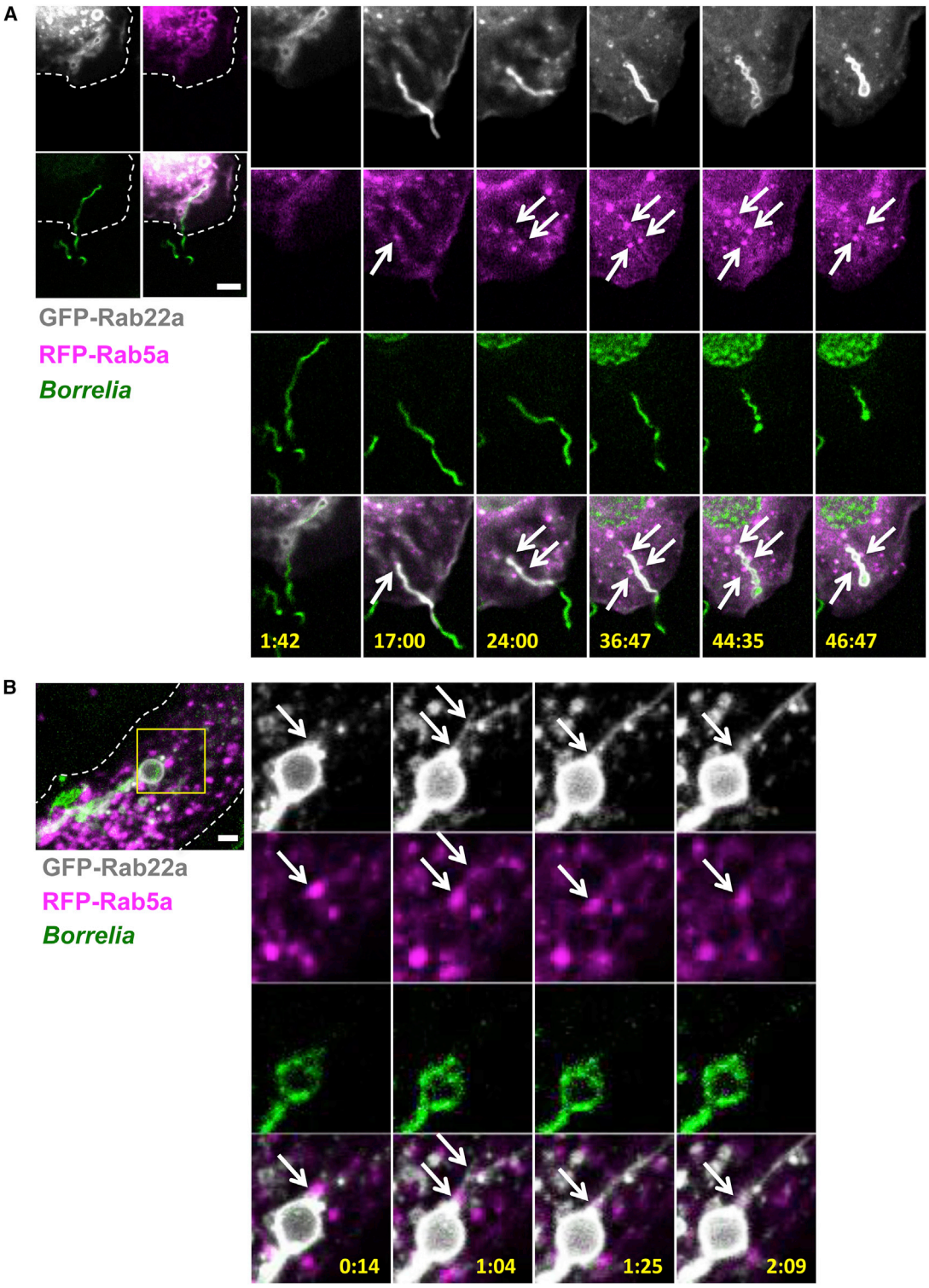


Figure 4. Rab22a-Positive Phagosomes and Rab5a-Positive Vesicles Cooperate in Compaction of Borreliae

Confocal micrographs of macrophages expressing GFP-Rab22a (white; A and B) and m-RFP-Rab5a (magenta; B), coincubated with borreliae stained by Hoechst 33342 (green). GFP-Rab22a is present as a phagosomal coat during early stages of *Borrelia* uptake (A) and is contacted by mRFP-Rab5a-positive vesicles (white arrows; A). (B) mRFP-Rab5a-positive vesicles contact *Borrelia*-containing phagosomes at sites of membrane tubule extrusion. Still images are taken from Movies S4, S5, and S6. Dashed lines indicate cell circumference. Yellow boxes indicate area of detail images. Time since start of the experiments is indicated in min.

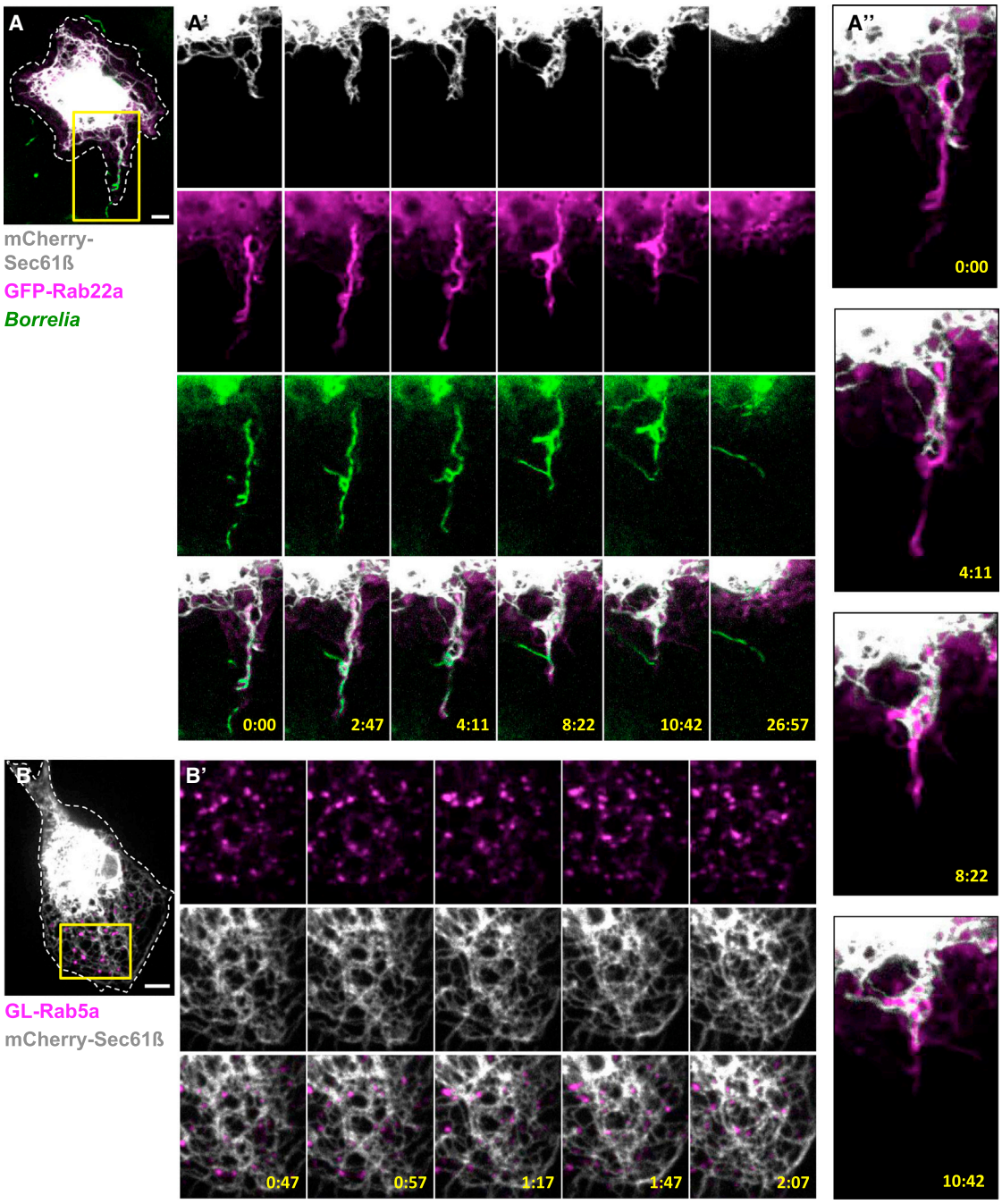


Figure 5. The ER Is in Close Contact with Rab22a-Coated Borreliae and Rab5a-Positive Vesicles

(A) Confocal micrographs of macrophage expressing ER-marker mCherry-Sec61β (white) and RFP-Rab22a (magenta) coincubated with borreliae stained by Hoechst 33342 (green). Note protrusion of ER tubules into *Borrelia*-capturing cell extension and close contact of ER with the *Borrelia*-surrounding coat of RFP-Rab22a.

(B) Confocal micrographs of macrophage expressing mCherry-Sec61β (white) and GL-Rab5a (magenta). Note close and persistent contact of GL-Rab5a-positive vesicles with ER tubules. Still images are from Movies S7 and S8. Dashed lines indicate cell circumference. Yellow boxes indicate area of detail images. Time since start of the experiments is indicated in min.

Considering the involvement of Rab5a and Rab22a in the progression of borreliae-containing phagosomes to lysosomes, we asked whether compaction of borreliae could also be due to acidification within maturing phagosomes. To test this, macrophages were coincubated with borreliae in the presence of the V-ATPase inhibitor bafilomycin (Bowman et al., 1988).

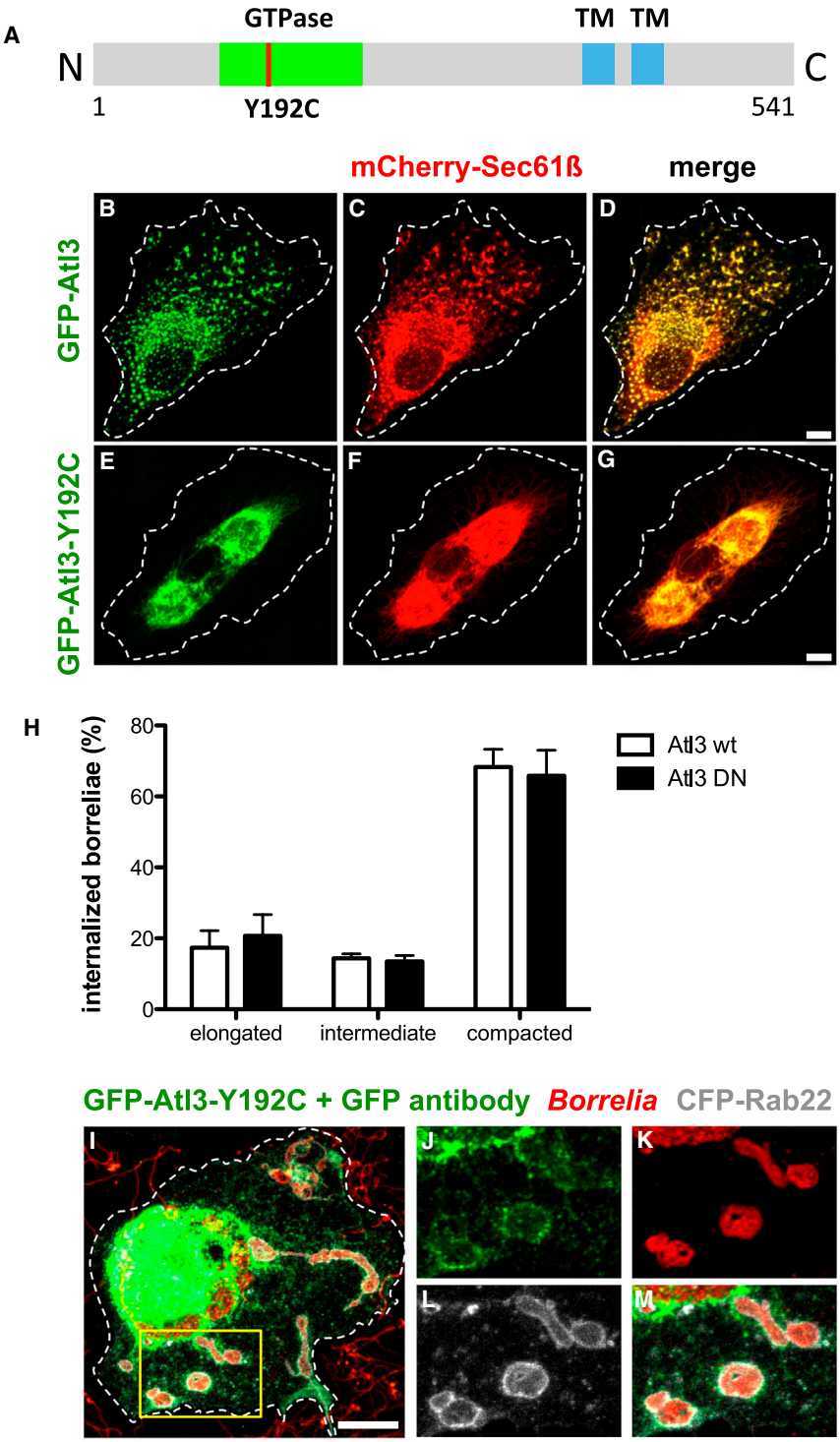


Figure 6. ER Tubules Efficiently Regulate Phagosomal Compaction of Borreliae

(A) Domain structure of atlastins, GTPase, and transmembrane (TM) domains and number of N- and C-terminal residues are indicated. Tyrosine residue at position 192, mutated to cysteine in the GTPase-inactive mutant (Y192C), is indicated in red.

(B–G) Confocal micrographs of macrophages expressing mCherry-Sec61β (red; C and F) together with GFP-atlastin-3 (green; B) or the GFP-atlastin-3-Y192C mutant (green; E), with merges in (D) and (G). Dashed white lines indicate cell borders. The scale bars represent 10 μm.

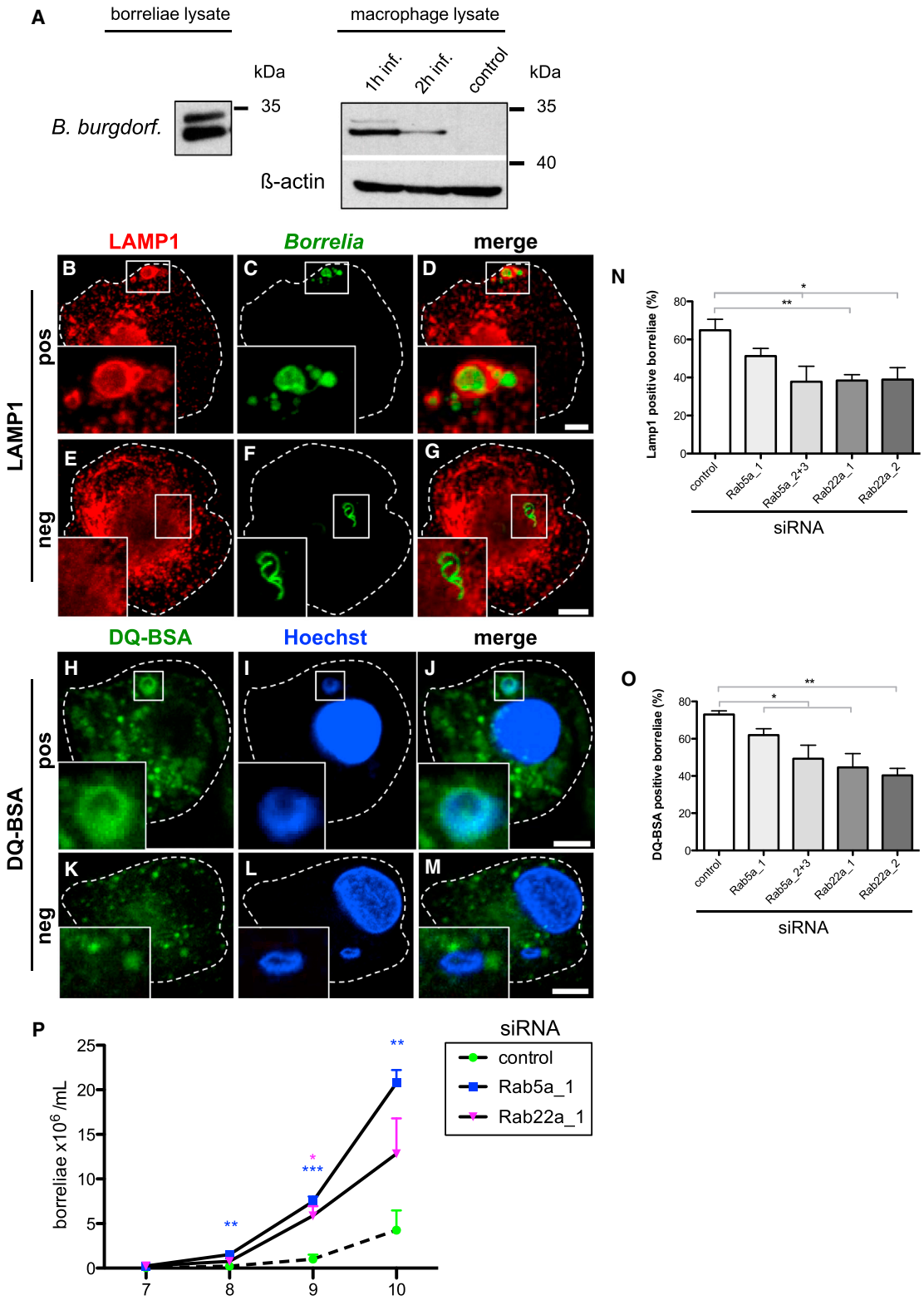
(H) Statistical evaluation of morphology of internalized borreliae in cells expressing wild-type atlastin3 or the Y192C mutant.

(I–M) Confocal micrograph (I) of macrophage expressing GFP-atlastin-3-Y192C and stained with anti-GFP antibody (green; J), co-expressing CFP-Rab22 (white; L), and coincubated with borreliae stained with anti-*B. burgdorferi* antibody (red; K). Yellow box in (I) indicates detail region in (J)–(M). Dashed white line indicates cell border. The scale bar represents 10 μm.

Morphological evaluation of borreliae showed no difference between spirochetes internalized by bafilomycin-treated cells, compared to controls (Figures S3A–S3C). This was corroborated by incubation of borreliae in acidic medium (pH 4.0), which did not alter the elongated morphology of spirochetes (Movie S9). We conclude that compaction of borreliae is not due to acidification within phagosomes.

Depletion of Rab22a or Rab5a Leads to Enhanced Intracellular Survival of Borreliae

The data so far show that Rab5a and Rab22a are important regulators of *Borrelia* uptake and targeting to lysosomes. To test the role of both RabGTPases in the regulation of intracellular processing of spirochetes, macrophages were treated with respective siRNAs, or with luciferase siRNA as control; incubated for 3 days to establish knockdown of the respective RabGTPase; and infected with borreliae at a MOI of 100. After 1 hr of incubation, resulting in internalization of spirochetes, macrophages were washed and incubated for 1 hr with medium containing gentamicin and kanamycin to kill extracellular bacteria. Samples were again washed extensively and lysed by mechanical disruption. Lysate aliquots were added to *B. burgdorferi* culture medium, and the number of re-cultured borreliae was determined for 10 consecutive days. Pronounced differences in bacteria numbers were found in re-cultures from macrophages treated with Rab5a- or Rab22a-specific siRNA, compared to controls (Figure 7P). This was particularly apparent at days 9 and 10, with borreliae densities from cultures of Rab5a-depleted cells increased 7.4-fold and



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4.9-fold and in Rab22a-depleted cells 5.8-fold and 3.0-fold, compared to controls. Similar results were gained in re-cultures from macrophages overexpressing GL-Rab5a or GFP-Rab22a (Figure S4G), indicating that upsetting the balance of Rab5a or Rab22a activity by either depletion or overexpression results in impaired intracellular processing of borreliae. Collectively, these data indicate that the number of borreliae that survive intracellularly is significantly increased in macrophages with altered levels of Rab5a or Rab22a. Both RabGTPase thus emerge as important regulators of intracellular survival of borreliae.

DISCUSSION

In this study, we identify Rab22a and Rab5a as important regulators of uptake and intracellular processing of *Borrelia burgdorferi* by primary human macrophages. We find that borreliae are internalized through uptake into phagosomes that are enriched in Rab22a and EEA1 and are subsequently contacted by Rab5a-positive vesicles. Rab5 is recognized as an early regulator of the RabGTPase cascade during phagocytosis (Fairn and Grinstein, 2012). The early enrichment of Rab22a at internalized borreliae, preceding Rab5a, is thus surprising. However, a similar succession has been shown for Rab20 at phagosomes containing IgG-opsonized beads in macrophages. In this system, Rab20 recruits Rabex-5, a GEF for Rab5, leading to subsequent enrichment of Rab5 (Pei et al., 2014). Our data now indicate that also Rab22a can be upstream of Rab5 during phagocytosis.

These data do not rule out involvement of other RabGTPases. Indeed, we found less pronounced (<30%), but robust, association of internalized borreliae with Rab7, Rab8a, Rab10, Rab11b, Rab14, and Rab21. This indicates the involvement of other RabGTPase-controlled pathways (Schwartz et al., 2007) on intracellular processing of borreliae, most probably including lysosomal degradation (Rab7), endosomal recycling (Rab11b, Rab14, and Rab21), and biosynthesis pathways (Rab8a). The only tested Rab protein without detectable localization to internalized borreliae was Rab6a, rendering a respective role for Rab6a and its main function in endosome to trans-Golgi trafficking (Schwartz et al., 2007) unlikely.

Our findings are in line with the general involvement of Rab5a and Rab22a in early steps of endocytosis and phagosome maturation (Schwartz et al., 2007). Specifically, Rab5 has been shown to be involved in intracellular uptake of pathogens, including phagocytosis of *Staphylococcus aureus* (Hagiwara et al., 2014) or of *Yersinia pseudotuberculosis* by COS-7 cells (Sarantis et al., 2012) or, together with EEA1, of *Helicobacter pylori* by primary human macrophages (Borlace et al., 2011). The importance of RabGTPases for phagocytosis and elimination of bacteria is also illustrated by the fact that a variety of pathogens have developed mechanisms that target RabGTPase signaling to modulate their uptake or intracellular processing (Stein et al., 2012). For example, p40 protein of *Listeria monocytogenes* causes Rab5-specific ADP ribosylation, thus inhibiting Rab5-dependent phagosome-endosome fusion (Alvarez-Dominguez et al., 2008), whereas VipD from *Legionella pneumophila* binds active Rab5 and Rab22 on endosomes and blocks endosome fusion through catalyzing the removal of PI(3)P (Gaspar and Machner, 2014; Ku et al., 2012). In the current study, we observed that a small subset of phagocytosed borreliae (<5%) acquire Rab22a or Rab5a only transiently. This could be indicative of endolysosomal avoidance, as described for other pathogens such as *Legionella* (Gaspar and Machner, 2014; Molofsky et al., 2005), although *Borrelia* does not possess a type III secretion system (Tilly et al., 2008).

The observed compaction of borreliae in phagosomes is in good agreement with earlier reports of compacted intracellular spirochetes, using electron microscopy (Montgomery and Malawista, 1996) or live-cell imaging (Cervantes et al., 2011). Using mutant constructs and siRNA-mediated knockdown, we could show that Rab5a and Rab22a are important regulators of the compaction process. We found similar values of compaction for both live and heat-killed borreliae in all treatments. Compaction is therefore driven by the macrophage, requiring neither motility nor viability of borreliae, and is most likely a response of the immune cell to the elongated morphology of spirochetes. Interestingly, compaction of borreliae was accompanied by membrane loss from the phagosomal surface. This indicates a reduction of the phagosomal surface, which is probably involved in this effect. This is in line with the finding that compaction is not based on acidification within phagosomes, as shown by incubation of borreliae at pH 4.0 or treatment of macrophages with the V-ATPase inhibitor bafilomycin. This seems to be consistent with the early onset of compaction during borreliae internalization (Figure 2). Interestingly, a mechanism for membrane tubule formation involving microtubules and associated motors has been

Figure 7. Rab22a and Rab5a Regulate Maturation of Borreliae-Containing Phagosomes and Intracellular Survival of Borreliae

(A) Western blot of lysates from macrophages with internalized borreliae. Borreliae are detected by using polyclonal anti-*B. burgdorferi* antibody, β-actin staining as loading control. Left: control blot of borreliae lysate is shown. Right: blot of lysates from macrophages prepared 1 hr or 2 hr after internalization of borreliae or of uninfected control cells is shown. Molecular weight is indicated on right.

(B–M) Confocal micrographs of macrophages with internalized borreliae (green), stained with LAMP1 antibody (red; B–G) or incubated with DQ-BSA (green; H–M). Borreliae were stained with anti-OspC antibody (B–G) or by staining DNA using Hoechst 33342 (H–M). (B–D and H–J) Examples of borreliae localizing to LAMP1- (B–D) or DQ-BSA- (H–J) positive compartments are shown. (E–G and K–M) Examples of absent or unspecific accumulation of LAMP1 (D–F) or DQ-BSA (J–L) signals surrounding borreliae are shown. The scale bars represent 10 μm.

(N and O) Statistical evaluation of borreliae localized to LAMP1-positive (N) or DQ-BSA-positive (O) compartments in macrophages treated with Rab5a- or Rab22a-specific siRNA or luciferase siRNA as control. Values are given as means ± SEM. For each value, 3 × 30 borreliae internalized by macrophages from three different donors were evaluated. Single and double asterisks indicate $p < 0.0467$ and $p < 0.0036$, respectively.

(P) Statistical evaluation of intracellular survival of borreliae in macrophages treated with Rab5a- or Rab22a-specific siRNA or control siRNA. Graphs show growth curves of borreliae re-cultured from respective macrophage cell lysates. Number of re-cultured borreliae in growth medium (10^6/ml) is indicated on y axis; days after start of the experiment are indicated on x axis.

Single, double, and triple asterisks indicate $p < 0.0149$, $p < 0.0072$, and $p < 0.0010$, respectively.

reported for fusion of Rab5-positive endosomes in MDCK cells (Skjeldal et al., 2012) and also for Rab7-positive dextran-containing phagosomes of RAW 264.7 macrophages (Harrison et al., 2003), the latter also involving PI3K signaling. However, we could not detect an influence of microtubule integrity or PI3K activity on membrane tubule formation from phagosomes or on compaction of borreliae (Figures S3D and S3E).

Combined knockdown of Rab22a and Rab5a indicated that both GTPases work in the same pathway during borreliae processing. As RabGTPase activity is thought to be restricted to the compartment where they are located (Zerial and McBride, 2001), this is in good agreement with the enrichment of Rab22a and Rab5a at internalized borreliae. However, each isoform is present on a different vesicular entity: Rab22a as a coat on borreliae-containing phagosomes and Rab5a on vesicles that contact phagosomes. As previous reports showed that Rab5-positive endosomes are closely associated with the ER (Friedman et al., 2013; Rowland et al., 2014), we investigated localization of the ER in respect to internalized borreliae. Strikingly, overexpression of the ER marker Sec61β (Deshaies et al., 1991; Friedman et al., 2013) showed that ER tubules closely enwrap nascent Rab22a-positive, borreliae-containing phagosomes. This contact persists beyond completion of uptake, and the whole phagosomal complex is moved toward the cell interior. Consistent with earlier reports (Friedman et al., 2013; Rowland et al., 2014), the majority (>90%) of Rab5-positive vesicles in a macrophage cell are in constant and dynamic contact with the ER. Combined with the observations that (1) protrusion of membrane tubules during phagosomal compaction happens preferentially at sites of Rab5a vesicle contact with Rab22a phagosome coats (Figure 4B) and (2) Rab22a membrane tubules extend along the ER network (not shown), the ER apparently acts as a hub for the coordination of Rab22a and Rab5a activity. Interestingly, ER contact sites have recently been shown to act as preferential sites of fission of Rab5-positive endosomes in COS7 cells (Rowland et al., 2014). Notably, in COS7 cells, Rab5-positive endosomes themselves were cleaved, whereas in macrophages, Rab5a endosomes define the sites of membrane protrusion from borreliae-containing phagosomes. Still, in both cases, the ER is involved in the reshaping of endosomal membranes.

To test whether ER tubules are required for phagosomal compaction of borreliae, the number of peripheral ER tubules was decreased by expression of an atlastin-3 mutant construct. Atlastin GTPases mediate membrane tethering through trans-oligomerization between molecules of adjacent membranes (Orso et al., 2009; Pendin et al., 2011), with atlastin-2 and -3 being localized to the ER in most tissues (Rismanchi et al., 2008). Accordingly, atlastin proteins lacking GTPase activity, such as the Y192C mutant used here, lead to strong reduction of ER tubulation (Kornak et al., 2014), whereas leaving secretory traffic unaffected (Rismanchi et al., 2008). As expected, macrophages expressing atlastin-3-Y192C showed strongly reduced numbers of ER tubules. Surprisingly, however, phagosomal compaction of borreliae was not affected. A more-detailed analysis, enhancing the ER-based signals, revealed that the few remaining ER tubules still contact and enwrap internalized borreliae, comparable to the situation in wild-type cells. These data show that ER-coordinated compaction of borreliae is a highly efficient process that can be performed even by a strongly reduced ER tubule network. The contribution of the ER to endocytic processes is an emerging field. Our data contribute to this debate by underlining the importance and apparent efficiency of ER-based processes during phagocytosis.

Uptake of bacteria into phagosomes indicates their eventual processing in mature phagolysosomes (Fairn and Grinstein, 2012). Indeed, the majority of internalized borreliae localized to a LAMP-1-positive compartment that also exhibited proteolytic activity, as indicated by DQ-BSA signals. This is in line with earlier reports showing localization of internalized borreliae at lysosomes (Hechemy et al., 1992; Montgomery and Malawista, 1996). Considering that also extra-lysosomal degradation of borreliae has been proposed (Rittig et al., 1994), we explored this possibility by using the autophagosome marker GFP-LC3 (Huang et al., 2009). However, we were unable to detect GFP-LC3 localization at internalized borreliae (not shown). Importantly, knockdown of Rab22a and Rab5a led to a 40%–50% reduction of borreliae localization to lysosomes, indicating that both RabGTPases are important for targeting of internalized borreliae to this compartment, most probably through the described uptake into phagosomes, followed by maturation into phagolysosomes.

Moreover, we also observed a subset of elongated borreliae in the cytoplasm of macrophages, which were not compacted in phagosomes. Similar findings of elongated borreliae in the cytoplasm were reported for human dendritic cells (Filgueira et al., 1996), leukocytes and macrophages (Ionescu et al., 1997), and Vero cells (Hechemy et al., 1992). Combined, these data indicate a potential phagolysosomal avoidance of this spirochete subset. We therefore explored the possibility that avoidance of Rab22a-/Rab5a-induced phagosome uptake could lead to increased persistence of borreliae in macrophages. Indeed, re-culturing experiments using borreliae-infected macrophages treated with respective siRNA led to ~4-fold increased levels of re-cultured borreliae, compared to controls. Similar survival rates were found in macrophages overexpressing either Rab5a or Rab22a, pointing to a delicate balance of RabGTPase activity being necessary for efficient intracellular processing of borreliae. Comparable results have been reported earlier, as for example, Rab22a-controlled recycling of transferrin receptor is impaired upon either knockdown of Rab22a or overexpression of EGFP-Rab22a in CHO cells (Magadán et al., 2006). Collectively, these results indicated that phagolysosomal avoidance or escape is a potential mechanism for *Borrelia* survival in human immune cells and could play a role in the observed clinical persistence of borreliae (Tilly et al., 2008). Our data indicate that Rab22a and Rab5a could be promising molecular targets to modify this process.

Collectively, we show that internalization of *Borrelia burgdorferi* by primary human macrophages proceeds through uptake into Rab22a-positive phagosomes. This involves extrusion of membrane tubules at sites of phagosome contact with Rab5a-positive endosomes, leading to compaction of borreliae. This process is facilitated by a close spatial relationship with the ER. Importantly, uptake of borreliae into phagosomes is critical for their further intracellular processing. In consequence,

interference with Rab22a or Rab5a activity results in increased intracellular survival of spirochetes, pointing to a potential strategy of intracellular persistence for borreliae.

These results reveal a novel role for Rab22a as an early and important player in the phagocytic RabGTPase cascade in macrophages. They also show how the activity of different RabGTPases that are present at discrete vesicular entities can be coordinated by the ER to regulate phagosomal reshaping. Moreover, our finding that ER tubules facilitate these processes in a highly efficient manner adds new information to the current debate on the role of the ER in endocytic processes. Finally, this study also demonstrates the usefulness of the unique spirochete morphology to detect and analyze in high detail organelle interactions during the phagocytic process that might not be discernible using smaller or more globular bacteria, thus pointing to *B. burgdorferi* as a valuable model system for the study of phagocytosis.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Wild-type (WT) *B. burgdorferi* B31 ATCC 35210 strain (provided by Peter Kraiczy, Universitäts-Klinikum Frankfurt) was cultivated as described (Naj et al., 2013). *Escherichia coli* strain DH5α was used for cloning and DNA plasmid amplification.

Eukaryotic Cell Culture

Human peripheral blood monocytes were isolated from buffy coats (provided by Frank Bentzien, UKE) and differentiated into macrophages, as described (Naj et al., 2013).

Plasmid Constructs and Mutagenesis

For information about plasmids, see respective section in Supplemental Information. GFP-atlastin3-Y192C was cloned by introduction of a point mutation in GFP-atlastin3 (QuikChange; Stratagene) using following primers: F-primer: 5′- AGCTCTTCACAGAATGCGGTCGTCTGGCAAT -3′ and R-primer: 5′- ATT GCCAGACGACCGCATTCTGTGAAGAGCT-3′.

Cell Transfection and siRNA Experiments

Macrophages were transiently transfected using the Neon Transfection System (Life Technologies) with these settings: pulse Voltage 1,000 V; pulse width 40 ms; and pulse number 2. Cells were transfected at a ratio of 10^5 cells to 0.5 μg DNA. siRNA-treated cells were transfected twice at 0 hr and 48 hr and evaluated after a further incubation of 24 hr. For siRNA sequences, see Supplemental Information.

Phagocytosis Assays

Macrophages were infected with borreliae at a MOI of 100:1. Cells were pretreated for 2 hr with inhibitors or DMSO (Sigma-Aldrich) and infected in the presence of inhibitors at the following concentrations: 1 μM nocodazole (Sigma-Aldrich); 200 nM bafilomycin (Sigma-Aldrich); and 100 nM wortmannin (AppliChem). After 20 min, non-adherent borreliae were removed by washing and cells were further incubated with monocyte cultivation medium at 37°C. Cells were washed at indicated time points and fixed with 4% methanol-free formaldehyde in PBS (Thermo Scientific).

Antibodies and Staining Reagents

For details on antibodies and staining reagents, see Supplemental Information.

Immunoblot Analysis

Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane with the iBlot dry-blot system (Life Technologies) with program P2. Membranes were rinsed in PBS and incubated for 30 min in blocking solution, containing 5% milk powder in TBS and 0.01% Tween 20 (TBST). Dilutions of primary antibodies were prepared in 5% milk powder in TBST, and the membrane was incubated overnight at 4°C. After three washing steps in TBST, the membrane was incubated at room temperature for 1 hr in dilutions of goat anti-mouse or anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (Dianova). Enhanced chemiluminescence (SuperSignal West Femto or SuperSignal West Pico kit; Pierce; Thermo Fisher Scientific) was used, and chemiluminescence signals were collected on X-ray film (Fujifilm) and developed automatically (Agfa Curix 60; Agfa HealthCare).

Immunofluorescence Microscopy

Fixed samples were permeabilized in 0.5% Triton X-100/PBS, washed, blocked with 1% BSA, 5% goat serum, and 5% human serum in PBS and incubated with the primary antibody solution followed by incubation with the secondary antibody solution, optionally supplemented with fluorescence-labeled phalloidin. Coverslips were mounted in Mowiol 4-88 (Carl Roth), containing p-phenylenediamine (Sigma-Aldrich). For detection of extracellular bacteria, samples fixed with 4% methanol-free formaldehyde (Thermo Scientific) in PBS were blocked and incubated with *B.-burgdorferi*-specific antibody without permeabilization, followed by incubation with a secondary antibody labeled with Alexa 488. After washing three times with PBS, samples were permeabilized and extracellular and intracellular borreliae were stained with the primary antibody, followed by incubation with the secondary antibody labeled with Alexa Fluor 588. Thus, intracellular bacteria were stained only in red whereas extracellular bacteria were labeled in green and red. Microscopy was performed with a Leica TCS SP2 confocal laser scanning microscope (with a Leica TCS SP2 AOBS confocal point scanner) or with a Leica TCS SP5 (with a Leica TCS SP5 AOBS confocal point scanner), both equipped with an oil-immersion HCX PL APO 63× NA 1.4 λ_{blue} objective.

Live-Cell Imaging

Live-cell imaging was performed with an Improvision spinning-disk microscope system and Volocity 4.3.1 software (PerkinElmer), based on an Axiovert 200M stand with a Plan-Apochromat 63× NA 1.4 oil-immersion objective lens (Zeiss), a CSU-22 spinning disk unit (Yokogawa), an EM-CCD camera (C9200-50; Hamamatsu Photonics), and an environmental chamber (Solent Scientific). Videos were corrected for photobleaching. Macrophages were seeded on 18-mm glass coverslips (Paul Marienfeld) at a density of 2×10^5 cells. Coverslips were assembled in a Chamlide magnetic chamber dish (Live Cell Instrument). Macrophages were incubated with *B. burgdorferi* prestained for 1 hr with Hoechst 33342 (A0741; AppliChem) at a MOI of 100.

Intracellular Survival Assay

Macrophages were treated with siRNA or selected for overexpression of constructs by the LNGFR MACSelect Cell Selection kit (Miltenyi Biotec). Cells were infected with borreliae at a MOI of 100:1 for siRNA-treated cells or 500:1 for construct-expressing cells in RPMI. After incubation at 37°C for 1 hr, cells were washed five times with DPBS and 1.5 ml warm RPMI was pipetted on the cells, supplemented with 100 μg/ml kanamycin and gentamicin to kill extracellular bacteria. Samples were incubated for 1 hr with antibiotics at 37°C and washed ten times with DPBS. One hundred microliters DPBS was pipetted on the samples and transferred to 3 ml BSK-H medium containing 6% rabbit serum. No bacteria growth was observed in control samples. Two hundred microliters DPBS was added to samples, which were lysed mechanically using a cell scraper, transferred in a 1.5-ml reaction tube, and centrifuged at 12,000 rpm for 8 min. The pellet was resuspended in the supernatant, and 100 μl were transferred to a 15-ml reaction tube containing 3 ml BSK-H medium (Sigma-Aldrich) with 6% rabbit serum (Bio&Sell) and cultivated under microaerophilic conditions at 33°C and 1% CO_2. The number of borreliae was determined from day 7 to day 10 in a C-Chip Neubauer improved hemocytometer (Digital Bio) by dark-field microscopy with a Standard WL Upright Microscope, equipped with a central field stop, a Neofluar 16× numerical aperture (NA) 0.40/Ph2 objective lens, and two KF × 10/18 mm oculars (Zeiss).

DQ-BSA Assays

1×10^5 macrophages were seeded on a 35-mm dish within the area of the 12-mm glass coverslip in the center of the dish (GWSt-3512; Willco Wells). Cells were preincubated for 30 min at 37°C with 12.5 μg/ml DQ Green BSA

(DQ-BSA) (Life Technologies) in macrophage cultivation medium. *B. burgdorferi* were prestained for 1 hr with Hoechst 33342 (A0741; AppliChem) and resuspended in RPMI with 12.5 μg/ml DQ-BSA, centrifuged for 3 min at 1,020 rpm onto the cells at a MOI 100:1, and incubated for 1 hr at 37°C. Samples were washed three times with DPBS, incubated for 1 hr with 12.5 μg/ml DQ-BSA in macrophage cultivation medium at 37°C, and fixed for 10 min using 4% methanol-free formaldehyde in PBS.

Statistical Analysis

Statistical evaluation of data sets was performed using two-tailed Student's t test in GraphPad Prism 5 (GraphPad Software).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, three tables, and nine movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.027.

AUTHOR CONTRIBUTIONS

X.N. designed and performed experiments. S.L. designed experiments and wrote the manuscript.

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