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The fungal pathogen *Cryptococcus neoformans* manipulates macrophage phagosome maturation

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Summary

Phagocytosis by cells of the innate immune system, such as macrophages, and the subsequent successful maturation of the phagosome, is key for the clearance of pathogens. The fungal pathogen *Cryptococcus neoformans* is known to overcome killing by host phagocytes and both replicate within these cells and also escape via a non-lytic process termed vomocytosis. Here we demonstrate that, during intracellular growth, cryptococci modify phagolysosome maturation. Live cryptococci, but not heat-killed pathogens or inert targets, induce the premature removal of the early phagosome markers Rab5 and Rab11. In addition, significant acidification of the phagosome, calcium flux and protease activity is hindered, thus rendering the phagosome permissive for cryptococcal proliferation. Interestingly, several attenuated cryptococcal mutants retain this ability to subvert phagosomal maturation, suggesting that hitherto unidentified pathogen mechanisms regulate this process.

Introduction

The phagocytosis of microbes and subsequent killing of these ingested particles by phagocytic cells is a vital component of the innate immune system. However, many microbial pathogens have evolved a variety of ways to avoid the intracellular killing mechanisms of host phagocytes (Smith and May, 2013). One example of such an organism is the fungal pathogen *Cryptococcus neoformans*. This opportunistic yeast is responsible for an estimated 1 million infections and approximately 600 000 deaths per annum (Park *et al*., 2009). A dramatic rise in the incidence of cryptococcosis over the last century coincides with an increasing number of immunocompromised individuals, especially in sub-Saharan Africa, the epicentre of the acquired immune deficiency syndrome pandemic (Park *et al*., 2009). The disease, cryptococcosis, is thought to start with the inhalation of infectious particles (desiccated yeast or spores) followed by survival and proliferation within the lung, dormancy within the host, reactivation, dissemination and infection of the central nervous system and subarachnoid space (Chen *et al*., 2014). The interaction of *Cryptococcus* with macrophages is thought to be crucial in determining disease progression (Diamond *et al*., 1972; Diamond and Bennett, 1973; Vecchiarelli *et al*., 1994; Shao *et al*., 2005) and correlates with disease outcome in humans (Alanio *et al*., 2011; Mansour *et al*., 2011; Sabiiti *et al*., 2014). Furthermore, macrophages have been shown to potentially exacerbate infection and aid dissemination of this yeast (Santangelo *et al*., 2004; Kechichian *et al*., 2007).

During the successful clearance of microbes by phagocytes, the microbe is phagocytosed and finds itself within a membrane compartment, termed the phagosome, created by the invaginated plasma membrane of the host cell (Flannagan *et al*., 2012). The phagosome must then undergo a series of maturation steps to become increasingly inhospitable to the cargo, eventually resulting in degradation of the microbe. This is achieved by multiple fusion (delivery) and fission (recycling) events with early endosomes, late endosomes and lysosomes. These events create a dynamic phagosome membrane that evolves into a fully matured phagolysosome marking the end of phagosome maturation (Kinchen and Ravichandran, 2008; Smith and May, 2013).

The disruption of phagosome maturation is a tactic adopted by many microbial pathogens such as *Mycobacterium tuberculosis* (Via *et al*., 1997; Malik *et al*., 2003), *Legionella pneumophila* (Horwitz, 1983; Coers *et al*., 1999) and *Histoplasma capsulatum* (Strasser *et al*., 1999). *C. neoformans* is well known to survive within, replicate (Feldmesser *et al*., 2001) and escape from (Alvarez and Casadevall, 2006; Ma *et al*., 2006) the phagosome. This observation suggests that the fungus is either able to withstand the anti-microbial onslaught of the mature phagolysosome or is able to manipulate the host

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environment to reduce its anti-microbial activity. Previous studies have demonstrated the cryptococcal phagosome can be co-localized with maturation markers such as major histocompatibility complex II (MHC II) and CD63 (Artavanis-Tsakonas *et al*., 2006) and the lysosomal-associated membrane protein 1 (LAMP1) (Levitz *et al*., 1999; Johnston and May, 2010; Qin *et al*., 2011), suggesting that the phagosome is at least partially mature. However, the cryptococcal phagosome is known to become permeabilized (Cox *et al*., 2001; Tucker and Casadevall, 2002; Chayakulkeeree *et al*., 2011) and it remains unclear whether this compartment retains a normal level of antimicrobial activity.

In this study we have investigated several markers of phagosome maturation. First, we looked for the presence of the Rab GTPases Rab5, Rab7, Rab9 and Rab11. Rab5 is a well-known marker of early endosomes and phagosomes, which is recruited rapidly to new phagosomes and activated by its guanine exchange factor. Once active, Rab5 is able to recruit a host of effectors, including early endosome marker 1 (EEA1) (via hVPS34) and Rab7, which is considered a late phagosome marker thought to play an integral role in the fusion of the phagosome with lysosomes. Also present on early phagosomes is Rab11, which is located on recycling endosomes, aiding the recovery of phagocytic receptors back to the plasma membrane. Finally, Rab9 is a marker of endoplasmic reticulum-derived membranes and often associated with vesicles of the late endocytic pathway (Kinchen and Ravichandran, 2008). We then monitored the levels of acidification and cathepsin activity within *Cryptococcus*-containing phagosomes. Acidification is often used as a hallmark for fully matured phagosomes (Kinchen and Ravichandran, 2008). The cathepsins are a family of degradative enzymes that accrue to high levels within mature phagosomes in order to aid digestion of the phagosomal cargo. Cathepsin L is most active in the latest stages of phagosome maturation in macrophages (Claus *et al*., 1998; Lennon-Dumenil *et al*., 2002). Lastly, the flux of calcium within the *Cryptococcus*-containing phagosome was explored as calcium is a key second messenger in phagocytic cells, required for some forms of phagocytosis and also for the progression of phagosome maturation (Nunes and Demaurex, 2010).

Together, the data we present demonstrate that *C. neoformans* subtly alters the maturation of the phagosome it resides within in order to generate a compartment that is amenable to cryptococcal replication.

Results

Acquisition of Rab GTPases is altered on Cryptococcus*-containing phagosomes*

Rab GTPases are a family of proteins that perform critical functions in coordinating vesicle maturation (Barr, 2013). Thus, we examined the distribution of Rab family GTPases on maturing cryptococcal-containing phagosomes. To determine which Rab GTPases are recruited to *Cryptococcus*-containing phagosomes, we infected J774 murine macrophages with live *C. neoformans* H99, heat-killed H99 or latex beads and at time points between 5 min and 2 h post-infection immunolabelled for the presence of Rab5, 7, 9 and 11 (Fig. 1A–D).

Phagosomes containing live cryptococci showed a remarkably different pattern of Rab acquisition than those containing either heat-killed cryptococci or inert latex beads (Fig. 1). At 15 min post-phagocytosis, both heat-killed cryptococci and latex beads had recruited the early endocytic markers Rab5 and Rab11 strongly, but these GTPases were missing from phagosomes with live cryptococci (Fig. 1A and B). In contrast, by 2 h post-phagocytosis, all particles showed similar levels of Rab5, 7 and 11, but Rab9 levels were markedly higher on phagosomes containing live cryptococci (Fig. 1C). Subsequent examination of earlier events revealed that Rab5 was recruited strongly to all targets at 5 min post-phagocytosis, but subsequently lost much more rapidly from phagosomes containing live cryptococci than from those containing killed yeast (Fig. 1E). A similar pattern was confirmed with another strain of *C. neoformans*, the serotype D strain B3501 (Fig. S1). Example images of H99-containing phagosomes with immunolabelling for Rab GTPases are provided in Fig. S2.

Live cryptococci are able to block acidification of the phagosome

Normal phagosome maturation culminates in an acidic phagolysosome containing high levels of hydrolytic enzymes. Thus, we asked whether subtle changes to the phagosome, orchestrated by cryptococci, may lead to a modification of later stages of maturation. To determine if the *Cryptococcus*-containing phagosome reaches a typical acidic pH (as low as 4.3–4.5 for J774 cells) (Chen, 2002), we used the acidotropic dye LysoTracker Red to monitor pH during live imaging of the macrophage–*Cryptococcus* interaction. Over the 18-h infection of J774 macrophages with live H99, very few cryptococcal-containing phagosomes became acidic (Fig. 2A and E and Movie S1). In contrast, heat-killed or UV-killed H99-containing phagosomes always became highly acidic (acquired LysoTracker positive signal) over the course of the 18-h experiment, typically within 1 h of engulfment (Fig. 2A and E and Movie S2). These findings were confirmed in human monocyte-derived macrophages (HMDMs) (Fig. 2B) and with another strain of *C. neoformans*, with a high rate of vomocytosis (Johnston and May, 2010), ATCC 90112 (Fig. S3).

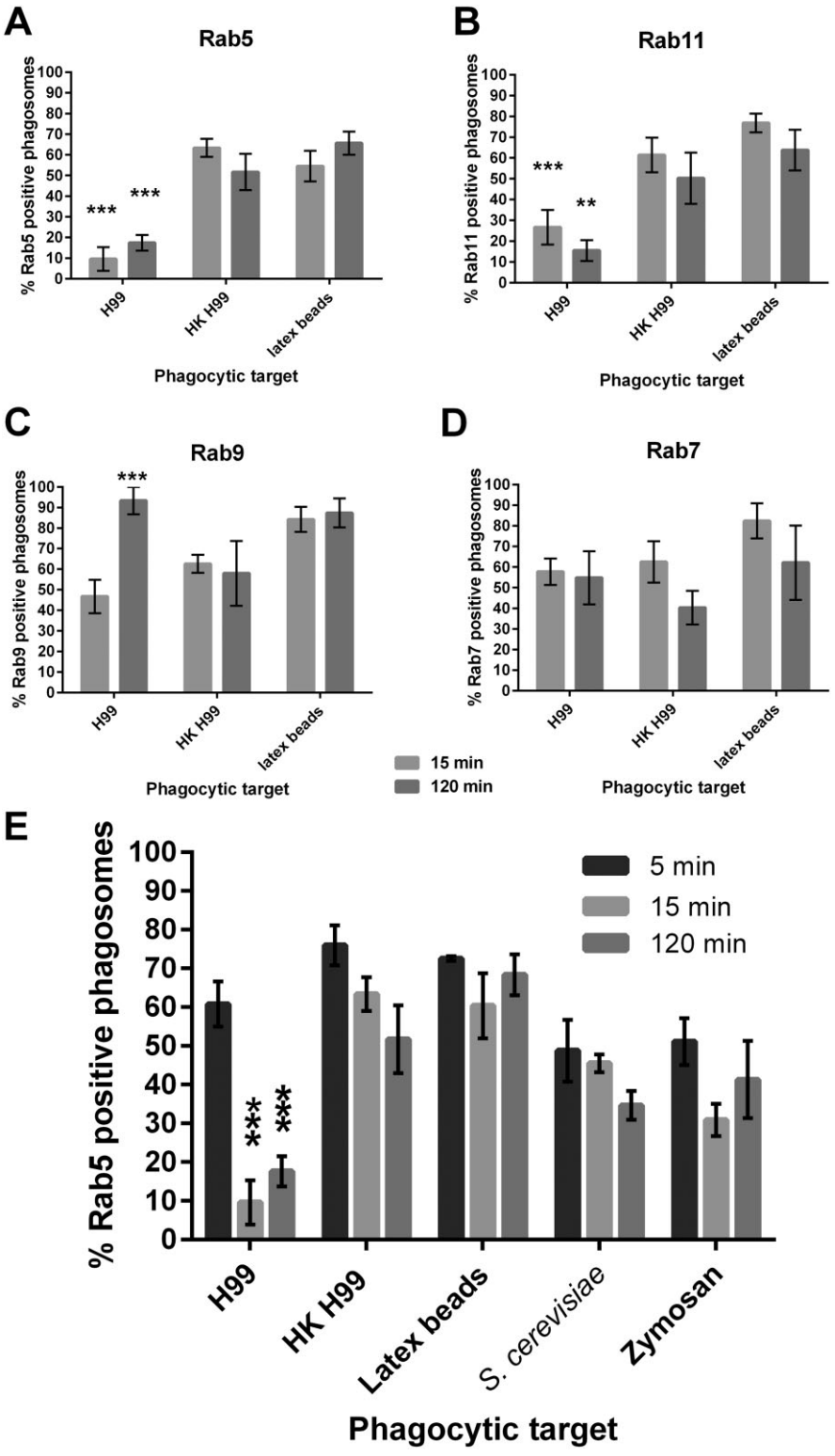

Fig. 1. Acquisition of Rab GTPases onto the *Cryptococcus*-containing phagosome. At 15 min and 2 h, Rab5 (A), Rab11 (B), Rab9 (C) and Rab7 (D) recruitment to phagosomes containing live cryptococci was monitored. Recruitment at 5 min is comparable between phagosomes containing live *C. neoformans* H99, heat-killed H99 and a variety of inert targets (E). All data were collected from immunofluorescence analysis of J774 phagocytosed particles. All bars represent data collected from observing 100–664 phagosomes for each target at each time point over three to six biological repeats, mean ± SEM. Data presented for H99 and HK H99 at 15 and 120 min are replicated in A and E. ***$P < 0.001$, **$P < 0.01$ Fisher's exact test.

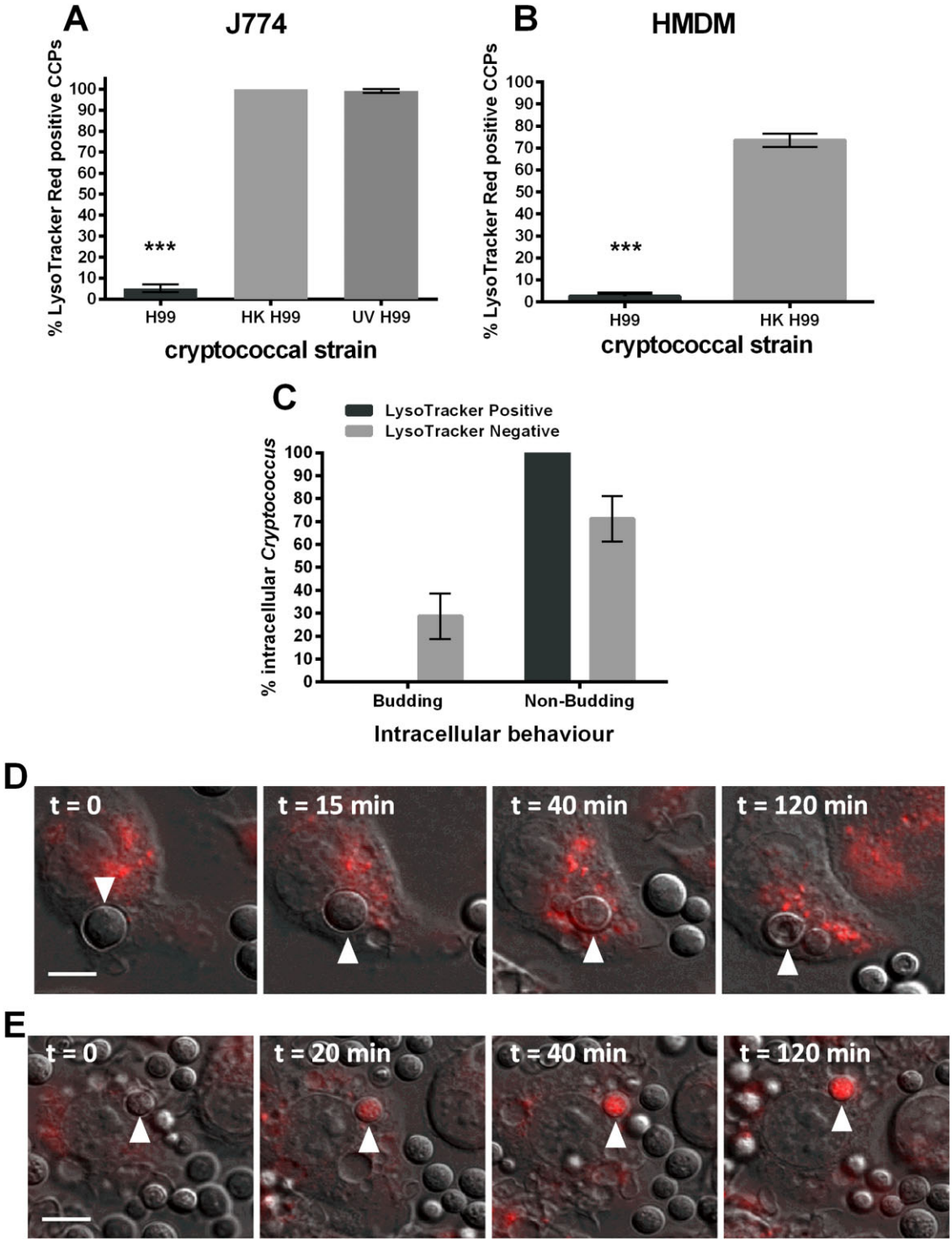

Fig. 2. Acidification of the *Cryptococcus*-containing phagosome. Phagosomes containing live *C. neoformans* H99 do not acquire the acidification reporter LysoTracker Red in either J774 cells (A) or HMDMs (B). Phagosomes containing either heat-killed (HK H99) or UV-killed (UV H99) cryptococci mature normally and become acidic in both cell types. Only H99 yeasts residing in non-acidified phagosomes are capable of budding in HMDMs (C). Panels D and E represent still images taken from time lapse microscopy experiments with J774 cells and a 60× objective (Movie S1, Movie S2) at the indicated times post–phagocytosis; displayed is the merged DIC and red fluorescence (LysoTracker Red) images. Live H99 (D) resides in a LysoTracker negative phagosome, whereas heat-killed H99 rapidly acquire LysoTracker (E). CCPs, cryptococcal-containing phagosomes. Scale bars represent 5 μm. Graphs A, B and C display mean ± SEM of 95–202 phagosomes monitored over 18 h for each target across three to five biological repeats. Significant figures for A and B come from a Fisher's exact test of the raw data. ***$P < 0.001$.

Because most phagosomes containing live cryptococci do not acidify, we wondered whether the rare instances in which acidification was seen might reflect variation in the yeast population within the inoculum. Reanalysis of the time lapse movies demonstrated that budding or vomocytosis activity of the intracellular cryptococci was restricted exclusively to yeast resident in non-acidified (LysoTracker negative) phagosomes (Fig. 2C). Thus, the rare acidification events (< 10%) that are observed in the infections performed with live H99 probably represent dead cryptococci in the inoculum, in line with the number of non-viable cryptococci that we typically detect in overnight cultures (Fig. S4).

Interestingly, cryptococci within non-acidified phagosomes of HMDMs were seen to be mostly (71.27%) in a quiescent, non-budding state (Fig. 2C). Thus, even non-acidified phagosomes reduce replication of cryptococci to rates below that seen in 'stress-free' environments such as YPD broth or serum-free Dulbecco's modified Eagle's medium (Fig. S5).

Cryptococcus-*containing phagosomes do not harbour cathepsin activity*

We exploited the fluorescent reporter dye MagicRed, which is activated after cleavage by cathepsin L (and to a lesser extent cathepsin B) to monitor cathepsin activity within phagosomes containing cryptococci. As with LysoTracker, we saw very little evidence of cathepsin activity in phagosomes containing live cryptococci (Fig. 3A and C and Movie S3). In rare cases when cathepsin activity was detectable, these cryptococci failed to bud or vomocytose. Conversely, heat-killed H99-containing phagosomes accumulate active cathepsin L (Fig. 3A and D and Movie S4). These findings were also confirmed with *C. neoformans* strain ATCC 90112 (Fig. S6). Interestingly, time-resolved monitoring of MagicRed demonstrated that there is a relatively short (~ 30 min) 'window' within which phagosomes acquire cathepsins; phagosomes that are negative for MagicRed during this 'window' remained negative throughout the remaining 17.5 h of imaging (Fig. 3B).

Cryptococcus neoformans *alters phagosomal calcium levels*

We investigated the presence of calcium in *Cryptococcus*-containing phagosomes using cell-permeable Oregon Green® BAPTA-1 and measuring resulting fluorescence after binding Ca^{2+} ions. Phagosomes containing live cryptococci display a reduced level of intraphagosomal calcium in relation to cytosolic levels (Fig. 4). Conversely, phagosomes containing heat-killed H99 gradually accrue a level of calcium greater than that of the cytoplasm. Our data suggest that live *C. neoformans* manipulate not only GTPase recruitment, acidification and cathepsin activity but also calcium transport into the phagosome it resides within.

A variety of cryptococcal mutants are still able to block acidification

To begin to decipher the possible mechanism *C. neoformans* employs to alter the maturation of the phagosome, we monitored the behaviour of several mutant strains known to be attenuated in animal models of disease. These mutant strains included a knock out of the phospholipase B gene (Δ*plb1*), a tri-functional enzyme required for full virulence in mouse models and implicated in permeabilization of the phagosome (Cox *et al*., 2001; Chayakulkeeree *et al*., 2011), as well as its associated Sec14 secretion system (Chayakulkeeree *et al*., 2011). We also tested mutants lacking functional urease expression (Δ*ure1* and Δ*ure7*) (Cox *et al*., 2000; Singh *et al*., 2013) as urease is thought to play an important role in intracellular parasitism (Rutherford, 2014). Lastly, we studied the acapsular mutant Δ*cap67* and its parental strain, B3501 (Jacobson *et al*., 1982), as the production of capsule is considered one of the most important virulence determinants of cryptococci with a key role in protecting the pathogen from the host attack (Vecchiarelli *et al*., 2013).

Interestingly, none of these mutants showed any alteration in their ability to prevent acidification of the phagosome (Fig. 5). Thus, even attenuated cryptococcal mutants retain the ability to modify host phagosome maturation, suggesting the existence of hitherto unidentified virulence mechanisms in this pathogen.

Discussion

The relationship between macrophages and *Cryptococcus* species is known to be a complex one. Cryptococci not only are able to survive and replicate within the macrophage phagosome but also escape the phagocyte by a non-lytic expulsion process termed vomocytosis (Alvarez and Casadevall, 2006; Ma *et al*., 2006). Previous research, by our group and others, has indicated that the cryptococcal-containing phagosome acquires several markers of 'normal' maturation. However, by examining very early events post-phagocytosis, we now show that cryptococcal-containing phagosomes display altered acquisition of the Rab family of small GTPases, in particular Rab5 and Rab11, within minutes of uptake. Such behaviour is not seen with heat-killed *Cryptococcus*, UV-killed *Cryptococcus*, latex beads, zymosan particles or *Saccharomyces cerevisiae*, strongly implicating the existence of a pathogen-driven

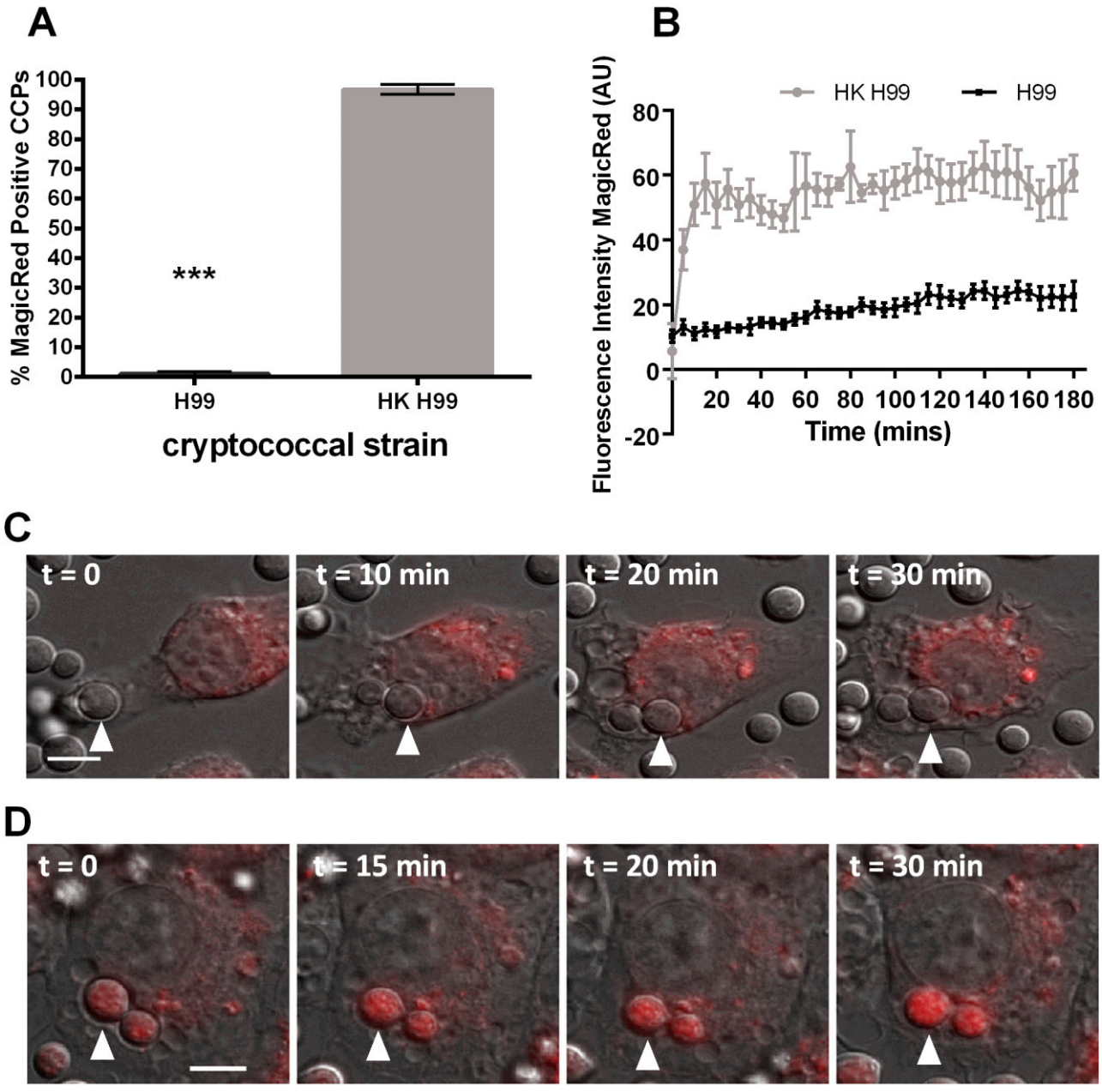

Fig. 3. Cathepsin activity within the *Cryptococcus*-containing phagosome. Cathepsin L activity was negligible in phagosomes containing live *C. neoformans* H99 (A). Graph represents mean ± SEM of 396 H99-containing phagosomes and 193 HK H99-containing phagosomes across $n = 5$. For analysis a Fisher's exact test was completed on the raw data.
B. Time lapse imaging reveals that cathepsin L activity either develops rapidly (within 30 min post-phagocytosis) or is never acquired. Graph represents mean fluorescent intensities from respective regions of interest ± SEM after background subtracting the mean intensity of three extracellular cryptococci selected from the same field of view ($n = 5$ phagosomes).
C, D. Example images from time-lapse movies showing lack of cathepsin activity in live H99-containing phagosome (C) and a cathepsin L active (MagicRed positive) HK H99-containing phagosome (D). Scale bars represent 5 μm. CCPs, cryptococcal-containing phagosomes.
***$P < 0.001$.

mechanism to disrupt phagosome maturation. Mechanisms that other pathogens use to manipulate Rab GTPases varied but include effector proteins such as SopB of *Salmonella*, which is able to reduce the levels of negatively charged PI(4,5)P_2 and phosphatidylserine on the phagosome, resulting in dissociation of Rab proteins and therefore a delay in phagosome fusion (Hernandez *et al*., 2004; Bakowski *et al*., 2010). Similarly, *M. tuberculosis* manipulates phagosome maturation (Fratti *et al*., 2001; Purdy *et al*., 2005) via at least two mechanisms: the

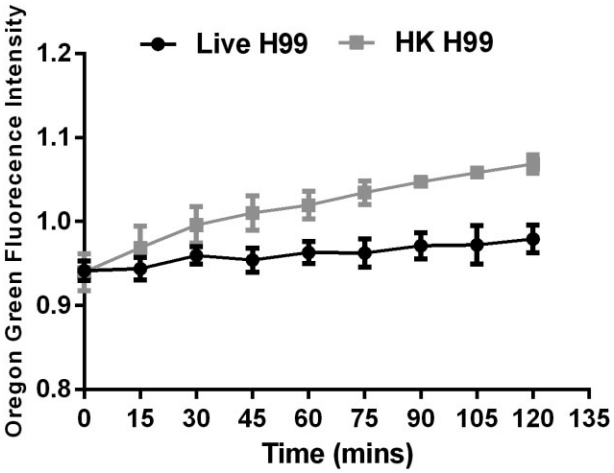

Fig. 4. Calcium levels within the phagosome are altered by live *C. neoformans*. Phagosomes containing live cryptococci do not accumulate calcium. J774 macrophages were pre-loaded with Oregon Green BAPTA-1 1 h before infection with live *C. neoformans* H99 or heat-killed H99 (HK H99). Fluorescent intensity data from each cryptococcal-containing phagosome were normalized to a randomly selected region of cytoplasm within the same cell. Data displayed are fluorescent intensity relative to cytoplasm, sampled every 15 min over the course of a time lapse experiment, mean ± SEM ($n < 35$ phagosomes) across three biological repeats.

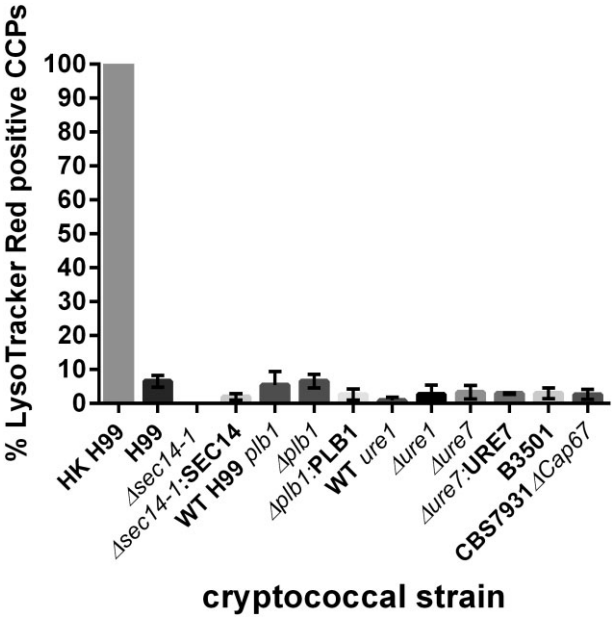

Fig. 5. Several mutants of *C. neoformans* are able to alter phagosome acidification. A variety of *C. neoformans* mutants are still able to modify the acidification of the phagosome in J774 cells. J774 macrophages infected with cryptococci were observed with live cell imaging for 18 h. CCPs, cryptococcal-containing phagosomes. Graph displays mean ± SEM of 100–176 phagosomes for each target from at least three biological repeats.

dephosphorylation of Rab5-guanosine triphosphate (GTP) and Rab7-GTP, resulting in inactivation of both critical GTPases (Sun *et al*., 2010) and via Lipoarabinomannan modification of the host calcium/calmodulin, EEA1, phosphatidylinositol 3-kinase hVPS34 and PI3P production pathway (Vergne *et al*., 2003). Lastly, both *Listeria monocytogenes* and the lesser known intracellular pathogen *Tropheryma whipplei* disrupt Rab5 activity via a glyceraldehyde-3-phosphate dehydrogenase, which induces Rab5a-specific adenosine diphosphate ribosylation and blocks the guanosine diphosphate/GTP exchange activity (Alvarez-Dominguez *et al*., 2008). In the case of *T. whipplei*, this leads to a chimeric phagosome displaying both Rab5 and Rab7 on its surface (Mottola *et al*., 2014). To date, it is unknown whether intracellular fungal pathogens have analogous routes to manipulate host endocytic trafficking, but recent advances in high-throughput genetics in cryptococci make this an exciting and promising avenue for future exploration.

The acidification of the cryptococcal-containing phagosome has been previously examined via ratiometric imaging (Levitz *et al*., 1999). This approach indicated an average phagosomal pH of 5, which was described as acidic. However, the macrophage phagosome is capable of reaching pH 4.3 (Chen, 2002), and our data suggest an alternative theory that the cryptococcal-containing phagosome actually does not fully mature but may reach only an intermediate pH that is insufficient for full antimicrobial activity. This modification of maturation is driven only by the live pathogen, indicating the existence of a currently unknown host manipulation pathway in this organism that is independent of major virulence factors such as capsule, urease expression or phospholipase B production.

How *C. neoformans* is able to alter the acidification of the phagosome is currently unclear. The cryptococcal-containing phagosome has previously been shown to permeabilize soon after phagocytosis (Tucker and Casadevall, 2002; Chayakulkeeree *et al*., 2011). This permeabilization could allow for dilution of phagosomal content and increase in the luminal pH throughout phagosome biogenesis. This would mean that markers of the phagosomal membrane would still be present (as seen in many other studies), but that the antimicrobial activity of the lumen would not be maximal. An alternative, or additional, mechanism could be avoidance of fusion with lysosomes. A lack of cryptococcal killing by human alveolar macrophages in a previous study was hypothesized to be due to a lack of phagosome–lysosome fusion (Vecchiarelli *et al*., 1994). A similar lack of acidification has been seen in microglia cells (specialized phagocytes of the central nervous system), in which only 35% of phagosomes underwent full acidification (Orsi *et al*., 2009).

Cryptococci are not the only fungal pathogens capable of manipulating host phagosomal biology. *Candida glabrata* has also been shown to modify the pH of the macrophage phagosome, possibly via mannosyltransferases (Kasper *et al*., 2014). *Candida albicans* rapidly undergoes filamentation within phagosomes, growing and eventually lysing the host cell (Lorenz *et al*., 2004), in a way that is pH dependent and requires the amino acid permease Stp2p (Vylkova and Lorenz, 2014). *C. albicans* phagosomes show only minimal acquisition of late-stage markers such as lysobisphosphatidic acid and vATPase and actively remove LAMP1 and cathepsin D from the phagosome (Fernandez-Arenas *et al*., 2009). *C. albicans* also expresses catalase, which plays an important role in virulence and hydrogen peroxide resistance (Enjalbert *et al*., 2007). *C. albicans* has very recently been discovered to be capable of non-lytic escape, in a similar way to *Cryptococcus*, although how this expulsion is achieved is as yet unknown (Bain *et al*., 2012). *Candida krusei* is also capable of modifying phagosome maturation. *C. krusei*-containing phagosomes are devoid of LAMP1 and are also seen to be less acidic compared with phagosomes containing heat-killed yeast (Garcia-Rodas *et al*., 2011).

Our findings therefore suggest that elaborate manipulation of phagosomal maturation is not restricted to the *Candida* genus but may represent a widespread approach for virulence in fungal pathogens. Indeed such a model is supported by the observation that another fungal pathogen, *H. capsulatum*, also survives within macrophages by modifying the phagosome and keeping it at pH 6.5 by blocking acidification (Strasser *et al*., 1999). Interestingly, *Histoplasma* actively retains a slightly acidic phagosomal pH even when phagosomal acidification is blocked by the vATPase inhibitor Bafilomycin (Strasser *et al*., 1999), suggesting that the pathogen itself creates the slightly acidic phagocytic lumen rather than allowing partial vATPase activity. Whether such mechanisms exist for *C. neoformans* is unknown, but it is striking that this pathogen also resides in a phagosomal compartment with an intermediate pH.

We also report unusual calcium dynamics in phagosomes containing live cryptococci. Although the precise roles of calcium in phagosome maturation are still debated, some pathogens have already been recognized to alter maturation via calcium signalling pathways. These include the lipoarabinomannan (LAM) toxin of *M. tuberculosis* that blocks the recruitment of calmodulin and EEA1 to the phagosome (Vergne *et al*., 2003) and LPG of the protozoan intracellular parasite *Leishmania donovani*, which depletes periphagosomal filamentous actin in a calcium-dependent manner (Holm *et al*., 2001; Tejle *et al*., 2002). Thus, live *C. neoformans* might also be using the relationship between calcium and phagosome maturation to create a phagosome amenable to survival and replication.

In summary, using a combination of immunofluorescence and live cell microscopy we show that *C. neoformans* modifies the phagosome it resides within. Understanding the route by which it does this, may offer opportunities to improve the control of this pathogen in patients with impaired adaptive immunity.

Experimental procedures

Cryptococcal strains, growth conditions and opsonization

All reagents were acquired from Sigma unless otherwise stated. Strains used were *C. neoformans* var. *grubii* serotype A strain H99 and mutants in this genetic background: WT H99 *URE1*, *ure1*Δ (Cox *et al*., 2000), *ure7*Δ, *ure7*Δ:*URE7* (Singh *et al*., 2013) WT H99 *PLB1*, *plb1*Δ, *plb1*Δ:*PLB1* (Cox *et al*., 2001), *sec14-1*Δ, *sec14-1*Δ:*SEC14-1* (Chayakulkeeree *et al*., 2011) as well as the serotype D strain B3501 and its acapsular derivative CBS7931 *cap67*Δ (Jacobson *et al*., 1982) along with the wild-type serotype A strain ATCC 90112. Cryptococcal strains were grown for 18 h overnight in yeast peptone dextrose (YPD) medium (2% glucose, 1% peptone and 1% yeast extract) with shaking (240 r.p.m.) at 25°C. Yeasts from overnight cultures were collected by centrifugation at 6500 r.p.m. for 2 min and washed in triplicate with 1× phosphate buffered saline (PBS). Yeasts were then counted and their concentration adjusted to 10^7 yeast ml^{-1}. Where necessary yeasts were heat killed at 55°C for 30 min or UV killed with 1 J UV in a UVILink CL-508G cross-linker (UVITec). Yeasts were then opsonized with 10 $\mu g\ ml^{-1}$ mouse immunoglobulin G (IgG) 18B7 (a monoclonal antibody to glucuronoxylomannan, a component of the cryptococcal capsule, kindly provided by Arturo Casadevall) for 1 h at room temperature. When latex beads or zymosan were used, these were resuspended to the required concentration in PBS before use in infection assays.

Macrophage cell line culture

Murine macrophage-like cells J774A.1 were maintained in DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 $\mu g\ ml^{-1}$ streptomycin and 100 U ml^{-1} penicillin. The cell line was incubated in a humidified environment at 37°C with 5% CO_2 and used between 5 and 15 passages after thawing. J7741.A cells (1×10^5 per well) were seeded in 24-well plates (with 13 mm in diameter glass coverslips for immunofluorescence experiments) or onto glass bottom 96-well plates at 0.25×10^5 per well, 18 h before infection.

HMDMs isolation and differentiation

HMDMs were isolated from healthy volunteers. Blood was diluted at least 1:3 in sterile PBS before layering to Ficoll plaque (GE Healthcare). This was then centrifuged for 30 min at 400× g with no braking. White blood cells were then collected from the Ficoll interface and diluted in an excess PBS. Cells were then centrifuged for 10 min at 300× g with braking and the resulting pellet resuspended in an excess of PBS. Cells were then centrifuged

for 10 min at 200× g and resuspended in PBS twice. HMDMs were then resuspended in RPMI 1640 with 10% FBS and counted. HMDMs were adjusted to 3×10^5 cells ml^{-1} and seeded into 48-well tissue culture plates. After 1 h non-adherent cells were removed and RPMI 1640 with 10% FBS and 50 ng ml^{-1} granulocyte-macrophage colony-stimulating factor was added for differentiation of monocytes to macrophages. Media were replaced every 3 days. Differentiated HMDMs were used between 7 and 10 days after isolation.

Infection of macrophages with Cryptococcus

J774 cells were activated with 150 ng ml^{-1} PMA for 1 h in DMEM without FBS prior to infection with cryptococci. For calcium flux experiments, 10 μM of Oregon Green 488 BAPTA-1 acetoxymethyl (Molecular Probes, Life Technologies), a reporter that fluoresces upon binding of Ca^{2+}, was also added to the media during this 1 h PMA activation.

HMDMs were activated 24 h prior to infection with LPS 1 μg ml^{-1} and interferon-γ 1000 U ml^{-1} (Immuno Tools) and then incubated in serum-free RPMI 1 h before infection. Macrophages were then infected with opsonized *Cryptococcus*, 3 μm latex beads, *S. cerevisiae* or Zymosan A at a multiplicity of infection of 10:1 for immunofluorescence experiments or 5:1 for live imaging experiments. At the time of infection, serum-free RPMI or DMEM (for HMDMs or J774s, respectively) containing either 50 nM LysoTracker Red DND-99 (Invitrogen, molecular probes), an acidotropic dye for the visualization of acidic cellular compartments, or 5 μM MR-$(FR)_2$ MagicRed (Immunochemistry Technologies) for fluorescent detection of the lysosomal cysteine protease cathepsin L, were added to the macrophages. Cells were then taken for live imaging or incubated in a humidified environment at 37°C with 5% CO_2 for the stated time before fixation.

Labelling and imaging of fixed cells

At the stated times post-infection, cells on coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then treated with 50 nM NH_4Cl for 10 min and permeabilized in 0.1% Triton X-100 for 4 min. Coverslips were then blocked with 5% goat serum for 1 h and then washed in PBS before being treated with 0.5 μg ml^{-1} primary antibody (monoclonal rabbit anti-Rab5, Rab7, Rab9 or Rab11) (Cell Signaling) with 2.5 μg ml^{-1} human IgG for 30 min. After PBS washing, coverslips were then treated with 2 μg ml^{-1} secondary goat anti-rabbit IgG-FITC (Sigma) with 10 μg ml^{-1} human IgG for 30 min. Coverslips were visualized with a Nikon Eclipse T*i*-S microscope, Plan Apo 60×/1.40 NA oil DIC objective (Nikon) and captured with QICAM Fast1394 camera (QImaging). All image analysis was performed with NIS Elements AR software (Nikon).

Live imaging

Time lapse images were captured on a Nikon TE2000 enclosed in a temperature controlled and humidified environmental chamber (Okolabs) with 5% CO_2 at 37°C, with Digital Sight DS-Qi1MC camera (Nikon), Plan Apo Ph1 20× objective (Nikon) or Plan Apo DIC 60×/1.40 NA oil objective (Nikon), using NIS elements AR software (Nikon). Images were captured every 5 min for 18 h.

Statistics

Statistical analysis of data was completed using GraphPad Prism version 6.04 (GraphPad Software Inc.). Categorical data were analysed for statistical significance with a two-tailed Fisher's exact test. All data are presented as mean ± standard error of the mean from at least three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

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Conflict of interest

The authors have no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Rab5 acquisition is also altered on other strains of *Cr. neoformans*. Acquisition of Rab5 to phagosomes containing *Cr. neoformans* B3501 or heat-killed B3501 at 5, 15 and 120 min post-infection. All data were collected from immunofluorescence analysis of J774 phagocytosed particles. All bars represent data collected from three to six biological repeats, mean ± SEM. ***$P < 0.001$, **$P < 0.01$ Fisher's exact test.

Fig. S2. Immunolabelling of Rab GTPases in the H99-containing phagosome. Images are given for live H99-containing phagosomes after 15 min inside J774 macrophages. Negative and positive examples are shown for labelling of each Rab GTPase; Rab5, Rab7, Rab9 and Rab11. Images were captured after labelling with anti-rabbit IgG-FITC. Scale bars represent 5 μm.

Fig. S3. Acidification of the ATCC 90112-containing phagosome. Phagosomes containing live *Cr. neoformans* ATCC 90112 do not acquire the acidification reporter LysoTracker Red in human monocyte-derived macrophages (HMDMs). The majority of phagosomes containing heat-killed cryptococci (HK ATCC 90112) mature normally and become highly acidic. Graph displays mean ± SEM of 202 phagosomes (ATCC 90112) and 95 phagosomes (HK ATCC 90112) monitored over 18 h for each target across four biological repeats. Significance figure from Fisher's exact test of the raw data. ***$P < 0.001$.

Fig. S4. Proportion of viable cryptococci in overnight cultures. Cryptococci were grown overnight (18 h) in YPD broth, washed three times with PBS and total number of cryptococci counted via haemocytometer. Suspensions were then serially diluted, plated on YPD agar, incubated for 48 h and the numbers of CFU counted for each strain. Percentage viable cryptococci were calculated as the proportion of CFU yeasts in total yeasts from haemocytometer counts. Data presented are mean ± SEM from three biological repeats.

Fig. S5. Proportion of *C. neoformans* H99 population which undergo budding. Graph presents data on the percentage of

cryptococci found budding when grown in either YPD broth or serum-free DMEM (SF DMEM). Yeasts were collected after overnight culture in YPD broth, washed with PBS and their concentration adjusted (as would be done for infection experiments). Multi-well plates containing either YPD or SF DMEM were then inoculated with H99. Yeasts were then observed with live cell imaging for 18 h to assess the number of cryptococci that replicate by budding. Data are mean ± SEM from three biological repeats.

Fig. S6. *C. neoformans* ATCC 90112 modifies the cathepsin activity of J774 phagosomes. Cathepsin L activity was also negligible in phagosomes containing live *C. neoformans* ATCC90112. Similarly to H99, very few live ATCC 90112-containing phagosomes acquire the MagicRed cathepsin reporter. Graph represents mean ± SEM of $n = 4$. For analysis a Fisher's exact test was completed on the raw data. CCPs, cryptococcal-containing phagosomes. $^{***}P < 0.001$.

Movie S1. Example lack of acidification of the live *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualization of infection of J774 macrophages with live *C. neoformans* H99. LysoTracker Red does not accumulate in phagosomes containing live H99. Media contain cell-permeable LysoTracker Red, displayed in red in this DIC and fluorescent channel merge. Each frame represents 5 min real time.

Movie S2. Example acidification of the heat-killed *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualization of infection of J774 macrophages with heat-killed *C. neoformans* H99. LysoTracker Red signal accumulates rapidly and intensely in phagosomes containing heat-killed H99. Media contain cell-permeable LysoTracker Red, displayed in red in this DIC and fluorescent channel merge. Each frame represents 5 min real time.

Movie S3. Example lack of cathepsin L activity of the live *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualization of infection of J774 macrophages with live *C. neoformans* H99. MagicRed signal does not accumulate in phagosomes containing live H99, indicating a lack of cathepsin L activity. Media contain cell-permeable MagicRed, displayed in red in this DIC and fluorescent channel merge. Each frame represents 5 min real time.

Movie S4. Example of rapid acquisition of cathepsin L activity to the heat-killed *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualization of infection of J774 macrophages with heat-killed *C. neoformans* H99. Strong MagicRed signal is seen, indicating significant acquisition of active cathepsin L to phagosomes containing heat-killed H99. Media contain cell-permeable MagicRed, displayed in red in this DIC and fluorescent channel merge. Each frame represents 5 min real time.