Title of thesis: Modulation of host cell signalling by effectors of *Mycobacterium tuberculosis* and *Helicobacter pylori*.

*Mycobacterium tuberculosis* replicates in host macrophages to cause tuberculosis. We observed that the virulence associated secreted protein ESAT-6 plays a key role in miR-155 induction and its subsequent effects on Bach1 and SHIP1 repression. We observed that miR-155 upregulation is directly linked to the attenuation of Bach1 and SHIP1. Bach1 is a transcriptional repressor of HO-1, whereas SHIP1 inhibits the activation of the serine/threonine kinase AKT. We hypothesize that ESAT-6 induced miR-155 induction leads to repression of Bach1, which augments the expression of HO-1, a documented activator of the *M. tuberculosis* dormancy regulon. SHIP1 repression facilitates AKT activation, which is required for *M. tuberculosis* survival. Thus, our results offer new insights into the role of miRNAs in modulation of the host innate immune response by *M. tuberculosis* for its own benefit.

Mannose-capped lipoarabinomannans (ManLAMs) are members of the family of *Mycobacterium tuberculosis* effectors that regulate signalling in host immune cells. Here we use an unbiased approach to understand how ManLAM regulates macrophage cell signalling. We demonstrate that the ability of ManLAM to attenuate host cell apoptosis, rests partially on its ability to induce expression of the anti-apoptotic Bcl2 family member A1 in macrophages.

Autophagy is an intracellular catabolic process that is required to maintain cellular homeostasis. Pathogen-elicited host cell autophagy may favour containment of infection or may help in bacterial survival. Pathogens have developed the ability to modulate host autophagy. The secreted antigen HP0175 is a peptidyl prolyl cis,trans isomerase of *Helicobacter pylori*. Here we show how it executes autophagy in gastric epithelial cells. Autophagy is dependent on the unfolded protein response (UPR) that activates the expression of PERK. This is accompanied by phosphorylation of eIF-2α and transcriptional activation of ATF4 and CHOP. Knockdown of UPR-related genes inhibits the conversion of LC3I to LC3II, a marker of autophagy. The autophagy-inducing ability of *H. pylori* is compromised when cells are infected with an isogenic *hp0175* mutant. Autophagy precedes apoptosis. Increased apoptosis of gastric epithelial cells is known to be linked to *H. pylori*-mediated gastric inflammation and carcinogenesis. This study demonstrates how HP0175 links UPR-dependent autophagy and apoptosis during *H. pylori* infection.
Identification of a novel role of ESAT-6-dependent miR-155 induction during infection of macrophages with *Mycobacterium tuberculosis*

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**Summary**

*Mycobacterium tuberculosis* (M.tb.) replicates in host macrophages to cause tuberculosis. We have investigated the role of miRNAs in M.tb.-infected murine RAW264.7 cells and bone marrow-derived macrophages (BMDMs), focusing on miR-155, the most highly upregulated miRNA. We observed that miR-155 upregulation is directly linked to the attenuation of expression of BTB and CNC homology 1 (Bach1) and SH2-containing inositol 5′-phosphatase (SHIP1). Bach1 is a transcriptional repressor of haem oxygenase-1 (HO-1), whereas SHIP1 inhibits the activation of the serine/threonine kinase AKT. We hypothesize that M.tb.-induced miR-155 induction leads to repression of Bach1, which augments the expression of HO-1, a documented activator of the M.tb. dormancy regulon. SHIP1 repression facilitates AKT activation, which is required for M.tb. survival. In addition, M.tb.-induced miR-155 inhibits expression of cyclooxygenase-2 (Cox-2) and interleukin-6 (Il-6), two modulators of the innate immune response. Importantly, we observed that the virulence-associated secreted protein ESAT-6 plays a key role in miR-155 induction and its subsequent effects on Bach1 and SHIP1 repression. Inhibition of miR-155 hindered survival of M.tb. in RAW264.7 and in murine BMDMs. Thus, our results offer new insights into the role of miRNAs in modulation of the host innate immune response by M.tb. for its own benefit.

**Introduction**

MicroRNAs (miRNAs) are short 22 bp regulatory RNAs that post-transcriptionally control target mRNA expression or cause translational repression. MiRNAs usually bind to mRNA targets in their 3′-untranslated regions (3′-UTRs). Based on sequence complementarity, this binding leads to translational repression and/or degradation of the mRNA (Baek et al., 2008; Selbach et al., 2008). Among a host of processes, miRNAs play vital roles in development, cancer, neuronal cell fate and immune function in diseased states such as rheumatoid arthritis (Johnston et al., 2005; Rodriguez et al., 2009; Liu et al., 2009; Hou et al., 2009; Lee and Dutta, 2009). Innate immunity constitutes the first line of defence against pathogens. An important branch of innate immune signalling is triggered by ligation of the Toll-like receptors (TLRs) followed by intracellular assembly of signalling scaffolds dependent on adaptor proteins such as MyD88 and TRIF, that activate protein kinases eventually leading to the activation of key transcription factors such as NF-κB (Moresco et al., 2011). Bacterial lipopolysaccharide (LPS)-treated monocytes, macrophages and dendritic cells have been used as models to explore the role of miRNAs in TLR signalling (O’Connell et al., 2007; Tili et al., 2007; Ceppi et al., 2009; Liu et al., 2009; Lu et al., 2009; McCoy et al., 2010; Martinez-Nunez et al., 2011; Zhang et al., 2011). A trio of miRNAs comprising miR-155, miR-146a and miR-21 are now regarded as...
central miRNAs, which regulate inflammatory pathways in myeloid cells (Quinn and O’Neill, 2011). miR-146a plays a role in endotoxin tolerance by virtue of its ability to knock-down IRAK1 and TRAF6 (Taganov et al., 2006). In mature human dendritic cells, miR-155 targets TAB2 to downregulate inflammatory cytokine production (Ceppi et al., 2009). MI-21 dampens the expression of PDCD4 thereby upregulating IL-10 and negatively regulating the pro-inflammatory response (Sheedy et al., 2010). In addition, miRNAs such as let-7 family members and miR-132 (Androulidaki et al., 2009; Lagos et al., 2010) have also recently been recognized as important regulators of innate immune signalling.

Subtle differences in miRNA expression profiles are likely to have a significant influence on the outcome of an encounter between the host and pathogen. Other than the available literature on the role of TLR ligands in miRNA production in cells of the innate immune system, there is only sketchy knowledge on the role of miRNAs in guiding the outcome of host-pathogen interactions. Tuberculosis (TB) caused by Mycobacterium tuberculosis (M.tb.) remains a global health problem. The outcome of M.tb. infection depends on the balance between innate immune mechanisms designed to eliminate the pathogen, and the pathogen’s ability to skew the innate immune responses in a direction favouring its survival in the host. The mechanisms exploited by virulent M.tb. to tilt the balance of the innate immune response in its favour remain incompletely understood. miRNAs by virtue of their ability to fine tune innate immune signalling, stand out as candidate regulators of M.tb.-induced signalling. In this context, a recent study has investigated the global miRNA response of macrophages infected with M. avium ssp. hominissuis infection (Sharbati et al., 2011). In this study, we have explored the role of miRNAs in influencing macrophage signalling in response to M.tb. infection. Our results demonstrate that miR-155 is the principal miRNA produced by murine macrophages in response to M.tb. infection. MiR-155 induction is dependent on early secreted antigenic target 6 kDa protein (ESAT-6) and is lower in M. bovis BCG (which lacks the esat-6-encoding genomic region) compared with M.tb. H37Rv. We describe the unexpected finding that miR-155 induction benefits the pathogen, and helps in its survival in the early stages of infection in an in vitro model. miR-155 attenuates the SH2-containing inositol 5'-phosphatase (SHIP1) and the transcriptional repressor BTB and CNC homology 1 (Bach1). We hypothesize that SHIP1 attenuation is associated with activation of the serine/threonine kinase AKT, while Bach1 repression upregulates haem oxygenase 1 (HO-1). At the same time, suppression of miR-155 expression with anti-miR-155 demonstrates an attenuating role of miR-155 in the production of cyclooxygenase-2 (Cox-2) and interleukin-6 (Il-6). Most importantly, we demonstrate that inhibition of miR-155 attenuates the ability of M.tb. to survive in macrophages, supporting the contention that miR-155 induction likely offers a survival advantage to M.tb. within its host.

Results

miRNA induction in M.tb.-infected RAW264.7

In order to identify miRNAs that are differentially expressed in M.tb.-infected macrophages, we performed a global analysis using Taqman Low Density Arrays (TLDA; Applied Biosystems, Foster City, CA). MiR-155 was identified as the most highly upregulated miRNA. TLDA analysis (performed in technical replicates) showed a 27-fold induction of miR-155 24 h after infection. In addition, TLDA analysis showed 1.4- to 2-fold induction (24 h post infection) of miR-146a and miR-21, two miRNAs reported to be part of the triumvirate of miRNAs (along with miR-155), which are induced upon challenge of cells of the innate immune system with pathogen-derived effectors. Northern analysis confirmed the induction of these two miRNAs (Fig. S1). miR-155 is the focus of our studies in the present communication. miR-155 is rapidly induced in a dose (moi)- and time-dependent manner, and persists even up to 24 h post infection in RAW264.7 (Fig. 1A).

Recent reports have suggested that lack of miR-155 induction correlates with the ability of a pathogen to suppress the host immune response. MiR-155 induction by the avirulent Francisella novicida but not the virulent F. tularensis is reportedly linked to pro-inflammatory cytokine production (Cremer et al., 2009). The apparent conflict between these findings and our own observations clearly reflecting high induction of miR-155 in cells infected with a pathogenic mycobacterium, prompted us to investigate the consequences of miR-155 expression in M.tb.-infected macrophages in greater detail.

ESAT-6 is linked to miR-155 induction by M.tb.

Previous studies (Rajaram et al., 2011; Schulte et al., 2011) have suggested that sensing of surface-exposed lipoglycans triggers the early miRNA response to bacterial pathogens. We chose to test the involvement of other players in the early miRNA response by examining the effect of the esxt1-encoded ESAT-6. Using a mouse 96-well miRNA array (SA Biosciences), we observed that miR-155 was the only miRNA to be induced by ESAT-6. Northern analysis showed that ESAT-6 upregulated miR-155 in a dose- (data not shown) and time-dependent manner (Fig. 1B), qRT-PCR analysis also confirmed that ESAT-6 induces the induction of miR-155 (Fig. S2).

Further, ESAT-6 also induced miR-155 in primary bone marrow-derived macrophages (BMDMs) (Fig. 1C).
order to test the hypothesis that ESAT-6 is a player in miR-155 induction by M.tb., we compared miR-155 induction between H37Rv and the Δesat-6 mutant, or between M. bovis BCG and M. bovis BCG-2F9 (which harbours the RD1 locus of M.tb.; henceforth referred to as BCG:2F9). Induction of miR-155 was attenuated in RAW26.47 cells (Fig. 1D, Fig. S3) or in BMDMs (Fig. 1E) infected with H37Rv:Δesat-6 compared with H37Rv. In addition, miR-155 induction was higher in RAW264.7 (Figs 1F and S4) or in BMDMs (Fig. 1G) infected with the strain 2F9 compared with M. bovis BCG. These results argued in favour of a role of ESAT-6 in miR-155 induction by M.tb. in macrophages.

**miR-155-dependent downregulation of SHIP1 in M.tb.-infected macrophages**

In an effort to directly analyse the role of miR-155 in the M.tb.-induced immune response, we assessed the expression of selected miR-155 targets. The first target chosen was the Src homology 2-containing inositol 5′-phosphatase 1, SHIP1, a validated, direct target of miR-155. SHIP1 is expressed in haemopoietic cells and hydrolyses the 5′-phosphate of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5-P3)) to negatively regulate the activation of the serine/threonine kinase AKT (Krystal, 2000). It fine tunes lipid phosphate-dependent signalling events and acts in concert with other negative regulators of TLR signalling to promote endotoxin tolerance (Rauh et al., 2004). Deletion of SHIP1 phenocopies many of the effects of miR-155 knockout in mice. Based on reports that AKT is required for the survival of M.tb. in macrophages (Kuijl et al., 2007; Kumar et al., 2010), we contended that miR-155-dependent regulation of SHIP1 could play a role in the survival of M.tb. in macrophages. To begin with, we tested the expression of SHIP1 in M.tb.-treated RAW264.7. A downregulation of SHIP1 was observed in M.tb.-infected macrophages (Fig. 2A) in a dose-dependent manner. Concomitant with a decrease in SHIP1, an increase in AKT phosphorylation was observed in the treated macrophages (Fig. S5). The efficacy of the miR-155 inhibitor was confirmed by analysing attenuation of the luciferase activity in RAW264.7 cells transfected with either control or miR-155 inhibitor along with the SHIP1 3′-UTR construct (Fig. S6A). The status of SHIP1 was then tested in cells transfected with either control or miR-155 inhibitor compared with M. bovis BCG. These results argued in favour of a role of ESAT-6 in miR-155 induction by M.tb. in macrophages.

*Fig. 1. M.tb. or ESAT-6 induces expression of miR-155 in RAW264.7 and bone marrow derived macrophages (BMDMs). (A) Cells were infected with M.tb. H37Rv at different moi for 4 h, followed by removal of bacteria and incubation in complete medium for different periods of time (as indicated). Bacteria were washed as described in Experimental procedures and RNA was isolated. °P-labelled probe specific for miR-155 was used for Northern blotting. As a loading control, the blot was reprobed with a probe specific for U6 RNA. (B) RAW264.7 cells and (C) BMDMs were left untreated (−) or treated with recombinant ESAT-6 (5 μg ml⁻¹) for different periods of time (B) or for 6 h (C). RNA was isolated and Northern blotting was performed using DIG-labelled probe specific for miR-155. RAW264.7 (D and F) or BMDMs (E and G) were left uninfected or infected with M.tb. H37Rv or H37Rv:Δesat-6 (D and E), or M. bovis BCG or BCG:2F9 (F and G), at a moi of 5 followed by isolation of RNA and Northern blotting as described in (B). Data are representative of the results obtained in three (A and B) or two independent experiments (C–G).*

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Control inhibitor (Fig. S6B). We hypothesize that at least within 24 h post infection, the miR-155-dependent SHIP1 downregulation favours activation of AKT, thereby exerting a pro-survival effect on macrophages, which likely benefits the pathogen.

miR-155-dependent downregulation of Bach1 in M. tb.-infected macrophages

Haem oxygenase-1 (HO-1) encoded by the *hmox1* gene is a stress-responsive gene that responds to oxidative stress by catalysing the degradation of haem to form biliverdin, carbon monoxide (CO) and free iron (Otterbein et al., 2003). The induction of *hmox1* is regulated by two upstream enhancers, E1 and E2 (Alam, 1994; Alam et al., 1995) that contain Maf recognition elements (MAREs) (Kataoka et al., 2001). The MARE can be bound by heterodimeric basic leucine zipper (bZip) factors including Nrf2, BTB and CNC homology 1 (Bach1), Maf and AP-1 families. Bach1 is a basic region leucine zipper transcriptional regulator. It forms heterodimers with the small Maf proteins and the resulting Bach1 heterodimers repress MARE-dependent transcription. With increasing haem levels, the repressor activity of Bach1 is lost, shifting the balance towards activation of *hmox1*. It is well documented that there is a rise in *hmox1* expression upon infection of macrophages with M.tb. (Kumar et al., 2008; Shiloh et al., 2008). We reasoned that since Bach1 is a target of miR-155 (Xu et al., 2010), the elevated miR-155 expression could be involved in repression of Bach1 leading to increased *hmox1* expression. In order to test this hypothesis, we determined the levels of Bach1 in M.tb.-infected macrophages. Bach1 levels were decreased in M.tb.-infected macrophages compared with uninfected macrophages (Fig. 3A). This effect was partially reversed in cells treated with miR-155 inhibitor (Fig. 3B). The effect of the miR-155 inhibitor could also be demonstrated in BMDMs infected with M.tb. (Fig. 3C). HO-1 levels were expected to decrease after M.tb. infection in miR-155 inhibitor treated cells. The repression of HO-1 levels in miR-155 inhibitor treated cells infected with M.tb. could be demonstrated in RAW264.7 and BMDMs (Fig. 3 D and E).

The role of ESAT-6 in miR-155-dependent regulation of SHIP1 in RAW264.7

In order to test the role of ESAT-6 in regulation of SHIP1 levels in RAW264.7, we analysed the status of SHIP1 in cells treated with ESAT-6. ESAT-6 downregulated the expression of SHIP1 in a time-dependent manner (Fig. 4A). Concomitantly, there was upregulation of p-AKT (Fig. 4B). In order to further investigate the role of ESAT-6 in the context of the intact bacterium, RAW264.7 was infected with *M. bovis* BCG or BCG:2F9. The downregulation of SHIP1 was observed in BCG:2F9-infected but not in BCG-infected cells (Fig. 4C), arguing in favour of a role of ESAT-6 in downregulating SHIP1 levels in infected cells.

The role of ESAT-6 in miR-155-dependent regulation of Bach1 in RAW264.7

Bach1 was downregulated in ESAT-treated RAW264.7 (Fig. 5A) and increased upon inhibition of miR-155 (Fig. 5B). These results supported a role of ESAT-6 in miR-155-dependent Bach1 regulation in RAW264.7. At the same time, HO-1 levels were upregulated in ESAT-6 treated cells (Fig. 5C). Bach1 levels decreased in BCG-infected RAW264.7 compared with uninfected cells. However, there was a more pronounced decrease in Bach1 in RAW264.7 cells infected with BCG:2F9 expressing ESAT-6, compared with BCG-infected cells (Fig. 5D). In harmony with the decrease in Bach1, HO-1 levels increased in M.tb.-infected RAW264.7 (Fig. S7). The induction of HO-1 was lower in BCG compared with M.tb., whereas it was higher in BCG:2F9 compared with BCG (Fig. S7). Further, in miR-155 inhibitor-treated cells, ESAT-6 treatment showed higher Bach1 levels compared with cells treated with control inhibitor (Fig. S8A). Concomitantly, there was a decrease in *hmox1* (Fig. S8B) and

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Taken together, these observations support the contention that ESAT-6 plays a role in augmenting HO-1 expression by inhibiting Bach1 expression in a miR-155-dependent manner.

miR-155 regulates the production of cyclooxygenase-2 in M.tb.-infected macrophages

The balance between the production of the eicosanoids prostaglandin E2 (PGE2) and lipoxin A4 has been reported to determine the cellular fate of macrophages infected with M.tb. M.tb. infection elicits the expression of prostaglandin E synthase (PGES) [or cyclooxygenase-2 (COX-2)] in macrophages (Rand et al., 2009). COX-2 activity leading to generation of PGE2 limits the production of anti-inflammatory lipoxin A4 (LXA4) and prevents necrosis of the infected macrophage (Chen et al., 2008). Infection of prostaglandin PGES−/− macrophages in vitro with M.tb. H37Rv results in higher bacterial burden compared with the wild-type macrophages (Chen et al., 2008). Considering the important role of COX-2 in mycobacterial infection, we asked the question whether miR-155 influences the expression of Cox-2 after infection. Transfection of RAW264.7 macrophages with miR-155 inhibitor augmented the expression of Cox-2 after infection with M.tb. (Fig. 6A). This result suggests that miR-155-mediated inhibition of Cox-2 could favour the survival of M.tb. in macrophages.

miR-155 regulates the production of IL-6 in M.tb.-infected macrophages

We next wanted to test the global role of miR-155 production in M.tb.-infected macrophages through direct or
indirect effects by analysing the regulation of selected cytokines or chemokines in the absence of presence of miR-155 inhibitor. We tested the expression of Tnf-α, Tgf-β, Il-12 p40, Il-6, Ccl5 (RANTES) and Ccl2 (MCP-1) by qRT-PCR. Out of these, Il-6 was found to be differentially regulated in the absence or presence of miR-155 inhibitor. Transfection of RAW264.7 macrophages with miR-155 inhibitor augmented the expression of Il-6 (Fig. 6B). This was evident at the level of the IL-6 protein released into the supernatant as well (Fig. S9). IL-6 plays a key role in the granuloma maintenance response to mycobacterial trehalose dimycolate (Welsh et al., 2008).

In addition, there is evidence that virulent M.tb. strains elicit reduced levels of pro-inflammatory cytokines (including TNF-α and IL-6) and RNI from macrophages compared with less virulent ones. This phenomenon has been described for the hypervirulent HN878 strain of M. tuberculosis and the hypervirulent mce1 mutant of M.tb. (Shimono et al., 2003; Manca et al., 2004; Reed et al., 2004). The dampening of Il-6 induction could therefore be a response favouring the pathogen.

miR-155 expression is required for growth of M.tb. in macrophages

Considering the fact that miR-155 modulated important immune function regulatory molecules of macrophages infected with M.tb., we asked the question whether miR-155 expression affects the growth of M.tb. in macrophages. In order to test the role of miR-155, RAW264.7 or BMDMs were transfected with either control inhibitor or miR-155 inhibitor, followed by infection with M.tb., cells were lysed after infection and viable counts (cfu) were determined by plating out the bacteria. Significant differences were not observed at 0 h post infection (Fig. 7A)

Fig. 6. Inhibition of miR-155 enhances the expression of Cox-2 and Il-6 in M.tb.-infected RAW264.7 and hinders the survival of M.tb. in macrophages. RAW264.7 cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155). After 20 h, transfected cells were either left uninfected or infected with M.tb. H37Rv at a moi of 5. RNA was isolated and expression of Cox-2 (A) or Il-6 (B) was assessed by qRT-PCR using Gapdh expression for normalization. Relative expression is shown with respect to the expression in uninfected cells. Data are shown as mean ± SD of three separate determinations. Similar results were obtained in at least three independent experiments. *P < 0.05, **P < 0.01.
and B). Relative to control inhibitor-transfected cells, there was significant reduction in bacterial colony-forming units (cfu) in miR-155 inhibitor-transfected RAW264.7 cells 24 h post infection (Fig. 7B). In similar experiments performed with BMDMs, colony-forming units were significantly reduced in cells transfected with miR-155 inhibitor compared with cells transfected with control RNA at 24 and 48 h post infection (Fig. 7D and E).

**Discussion**

The mechanisms exploited by virulent M.tb. to tilt the balance of the innate immune response in its favour remain incompletely understood. The role of miRNAs in controlling the outcome of an encounter between host and pathogen has been investigated in a limited manner in the context of intracellular pathogens. With this in view, we undertook an analysis of the miRNAs regulated when M.tb. infects murine RAW264.7 cells. Our results show that a core set of miRNAs (miR-155, miR-146a and miR-21) previously identified to act as first-line innate immune effectors is upregulated following challenge of macrophages with M.tb. Considering the fact that miR-155 was the most highly regulated, we focused on this particular miRNA in the present study. Bacterial components such as surface lipoglycans likely have a role in miR-155 induction as described by Rajaram et al. (2011). However, we show for the first time that initial sensing of an intracellular pathogen is not confined to sensing of surface-exposed lipoglycans. The induction of miR-155 depends at least in part on the presence of the secreted antigen, ESAT-6. Using exogenous ESAT-6, M.tb. or an esat-6 knockout strain, *M. bovis* BCG or BCG expressing ESAT-6, we provide evidence that miR-155 induction requires ESAT-6. Mir-155 transcription is dependent on NF-κB, while exogenous ESAT-6 inhibits TLR/MyD88-dependent NF-κB activation (Pathak et al., 2007). This would appear contradictory. However, it is pertinent to point out that miR-155 induction is dependent on several other transcription factors such as AP-1 and SMAD4 (Kong et al., 2008; Yin et al., 2008). In addition, regulation could also be at the post-transcriptional level (Ruggiero et al., 2009). Therefore, the overall effect of ESAT-6 could be dependent on its ability to influence these pathways as well.

Rajaram et al. (2011) have suggested that miR-155 induction is low in M.tb.-infected human macrophages, 6 h post infection. Our results show markedly increased levels of miR-155 24 h post infection. More work is...
required to understand the similarities and differences in the response of human and murine macrophages with regard to miRNA production. It is also pertinent to note that stimulation of PBMCs from active tuberculosis patients with purified protein derivative (PPD) leads to activation of miR-155 (Wu et al., 2012), suggesting a global role of miR-155 in the immune response to tuberculosis that deserves further investigation.

A link between miR-155 and the innate immune response is well established through experiments, showing increased expression following stimulation of macrophages or monocytes with LPS or lipoprotein, or in the splenocytes of mice infected withSalmonella enteritidis-derived LPS. (Taganov et al., 2006; O’Connell et al., 2007; Tili et al., 2007). We demonstrate that the induction of miR-155 correlates directly with suppression of its targets SHIP1 and Bach1. Based on the knowledge that SHIP1 attenuates AKT activation, we hypothesize that miR-155-dependent inhibition of SHIP1 favours AKT activation, which is required for survival of M.tb. in macrophages. Shiloh et al. (2008) have hypothesized HO-1-induced CO production supports long-lasting activation of the dormancy regulon. We rationalize that a link exists between miR-155 induction, HO-1 expression and activation of the dormancy region, based on our observation that miR-155 downregulates the hmoxt transcriptional repressor, Bach1.

In addition to its effects on SHIP1 and Bach1, miR-155 negatively regulates two important immune regulators Cox-2 and Il-6 probably through indirect effects involving pathways, which are yet to be understood. Considering that PGES−/− mice harbour higher burdens of M.tb., suppression of Cox-2 by miR-155 appears to be yet another miR-155-dependent mechanism favouring the survival of the pathogen. In addition, miR-155 inhibits the expression of the pro-inflammatory mediator Il-6. Again, this is unlike the case of the non-pathogenic F. novicida, which induces higher levels of Il-6 in miR-155 expressing cells compared with miR-155-negative cells, supporting a pro-inflammatory role of miR-155 in Francisella infection. Our results also differ from the report of Kurowska-Stolarska et al. (2011) who show that miR-155 enhances Il-6 production from M-CSF-differentiated human macrophages. Our findings suggest that in the context of M.tb. infection in murine macrophages, miR-155 exerts the opposite effect.

Mir-155 has been reported to oscillate after TNF-α stimulation. These oscillations could result in positive or negative effects on target proteins which are important mediators of NF-κB signalling, such as IKKα, or cellular death pathways, such as FADD. Mir-155 expression is regulated by multiple transcription factors. It is therefore important to elucidate in detail the mechanism and the temporal pattern of miR-155 induction by M.tb. in order to have comprehensive knowledge about its role in infection. In addition, the effects of miR-155 cannot be viewed in isolation. A host of miRNAs most likely engage in orchestrated feedback loops to guide the fates of the pathogen and its host. These are subjects of ongoing investigations.

The central finding of the present study is that expression of miR-155, occurring early following infection of murine macrophages with M.tb., sets off a chain of events that favours establishment of infection. Work with other pathogens has shown that miR-155 expression is an early event in the innate immune response, and is set off by cell surface-exposed lipopigycans. Our results uncover hitherto unappreciated roles of miR-155 in dampening the innate immune response and describe for the first time that a secreted protein, ESAT-6, functions as a direct effector of miR-155 production.

Experimental procedures

Antibodies and reagents

Beta-actin antibody, EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] and 1-methylimidazole were obtained from Sigma Chemical Co., St. Louis, MO. UltraHyb, miRNA isolation kit and RNA molecular weight markers were from Ambion. Antibodies specific for SHIP1 (Cat. No. 2728) and phosphorylated AKT (Cat. No. 9271) were from Cell Signaling Technology, Beverly, MA. Antibodies specific for AKT (sc-8312), Bach1 (sc-27121) and tubulin (sc-23948) were from Santa Cruz Biotechnology; anti-HO1 antibody was from Enzo Life Sciences, Ann Arbor; MI (ADI-OSA-150-D). DIG-labelled LNA-DNA mixed oligonucleotide probes against mature murine miRNA-155 and U6 RNA were obtained from Exiqon. M-CSF was a product of Prospec Protein Specialists, East Brunswick, NJ.

Cell culture and transfection

The murine macrophage cell line RAW264.7 was obtained from Dr Dipshikha Chakravortty, Indian Institute of Science, Bangalore and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine and penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO2. BMDMs were prepared from C57BL/6 mice and cultured for 7–8 days in the presence of M-CSF to allow them to mature (Boone et al., 2004; Gómez-Muñoz et al., 2004).

Transfections were carried out using Lipofectamine 2000 (Invitrogen). Briefly, cells were seeded 24 h before transfection in 12-well plates at a density of 3 x 10^5 cells per well and the mmu-miR-155 inhibitor (Ambion, AM17010 assay ID 13058) or miRNA inhibitor negative control (Ambion, AM 17000) was transfected at a final concentration of 150 nM. For confirmation of the downregulation of miR-155, co-transfections of SHIP1 3′-UTR construct (gift from Prof. David Baltimore, California Institute of Technology, USA), β-galactosidase expressing construct and miRNA-155 inhibitor (or miRNA inhibitor negative control) were carried out. Cells were lysed after 48 h of transfection and luciferase activity was measured using a luciferase assay kit (Promega) according to the manufacturer’s protocol. Luciferase

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activity was normalized by measuring beta-galactosidase activity using beta-galactosidase assay kit (Promega).

**Bacterial strains**

*Mycobacterium tuberculosis* strain H37Rv, and the Δesat-6 mutant (H37RvΔesat-6) (both from the laboratory of Professor David Sherman, SBRI, Seattle) were grown in Middlebrook 7H9 broth (Difco) supplemented with 10% ADC (Becton Dickinson). Recombinant cosmids vector RD1-2F9 harbouring the RD1 locus of *M. tuberculosis* (Brodin et al., 2005) was a gift from Professor Stewart Cole of the École Polytechnique Fédérale de Lausanne (EPFL), Switzerland. *M. bovis* BCG (Copenhagen) was transformed with cosmids 2F9 as described by Chatterjee et al. (2011). *M. bovis* BCG Copenhagen and BCG-2F9 were grown in Middlebrook 7H9 (Difco) supplemented with 10% OADC (Becton Dickinson) and 40 mM sodium pyruvate. Briefly, 50 μg ml⁻¹ hygromycin was used for culturing H37RvΔesat-6, while 50 μg ml⁻¹ hygromycin and 100 μg ml⁻¹ ampicillin were used for culturing BCG-2F9.

**Infection**

Bacteria were grown up to mid-log phase and washed thoroughly prior to infection. Bacterial clumps were removed by passing the washed suspension through a 27-gauge syringe. Infections were carried out for 4 h at the indicated multiplicity of infection (m.o.i.). Cells were washed and treated with gentamicin for 2 h in order to remove adhered bacteria. Incubations in DMEM containing 10% FBS were carried out for 20 h post infection (unless otherwise stated) or as indicated.

**Bacterial cfu determinations**

For the quantification of mycobacterial growth within macrophages cells were lysed after infections and colony-forming units (cfu) were counted after plating serial dilutions on Middlebrook 7H11 agar supplemented with OADC, using standard procedures.

**Western blotting**

Cells were washed with cold phosphate-buffered saline and lysed in lysis buffer (Cell Signaling Technology). Proteins were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 1% Tween 20 (TBST) for 1 h at room temperature, washed and subsequently incubated overnight at 4°C with primary antibodies in TBST supplemented with 5% (w/v) bovine serum albumin or non-fat dry milk. Following three washes of 5 min each with TBST, the blots were incubated with horse radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology or Cell Signaling Technology, Beverly, MA) followed by development with chemiluminescence detection reagent according to the manufacturer’s protocol (Cell Signalling Technology).

**RNA extraction**

RAW264.7 cells (6 × 10⁴ to 8 × 10⁵) were lysed in Qiazol lysis reagent (Qiagen). RNA was isolated using the mirVana miRNA isolation kit (Ambion). In some instances, the RT-qPCR-Grade miRNA isolation kit (SA Biosciences, Part No. 1033A) was used for isolating miRNA.

**Northern blotting**

The non-radioactive Northern blotting of miRNA was carried out as described by Kim et al. (2010). Briefly, total (or small RNA) was separated on 8 M urea-15% polyacrylamide gels and transferred to positively charged nylon membranes (Roche Applied Science). RNA was cross-linked by incubating the membrane in EDC cross-linking solution for 90 min at 60°C. For DIG-labelled LNA probes, pre-hybridization was carried out at 37°C in ULTRAhyb hybridization buffer (Ambion) for 1 h, followed by hybridization at 37°C for 16 h with slow rotation using 0.25 nM DIG-labelled LNA probe for miR-155 (Exiqon, Product No. 39471-15) (Pall and Hamilton, 2008). After washing the membranes, anti-DIG-AP Fab fragment (Roche Applied Science) was used for detection. After stripping the membranes, reprobing was carried out using DIG-labelled U6 probe (Exiqon, Product No. 99002-15).

Radioactive probes were prepared by phosphorylating 10 pmol of each DNA probe (Table S1) with 20 pmol of [γ-³²P]-ATP and T4 polynucleotide kinase (Fermentas) followed by purification using Quick Spin Columns (Roche Applied Science). Pre-hybridization was performed in 6× SSC, 10× Denhardt’s solution and 0.2% SDS for 1 h at 65°C, followed by hybridization in 6× SSC, 5× Denhardt’s solution, 0.2% SDS containing 4 × 10⁶ cpm 5'-end labelled probe for 16 h at 37°C with slow rotation.

**Real-time quantification of RNA**

Global analysis of miRNA expression was performed using Taqman Low Density Array (TLDA) in technical replicates at Life Technologies, Gurgaon, India. Two independent experiments were carried out for this purpose. For the quantification of miR-155, cDNA was synthesized using the Taqman MicroRNA reverse transcription kit (Applied Biosystems) and specific reverse transcription primers for mmu-miR 155 and housekeeping small RNA snoRNA142 (Applied Biosystems). Real-time PCR was carried out using the Taqman universal PCR master mix (Applied Biosystems) in a 7500 Real-time PCR system (Applied Biosystems). The relative expression of the target gene normalized to the endogenous reference gene was quantified using the comparative C. method. Alternatively, cDNA was synthesized from miRNAs using the RT-miRNA First Strand Kit (SA Biosciences, Cat. No. MA-03) and miRNAs were assayed in 96-well plate format (SA Biosciences, Cat. No. MAM-001-C2) according to the manufacturer’s instructions.

For quantification of gene expression, cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas) and SYBR Green-based real-time PCR was carried out using the primers provided in Table S2.

**Expression and purification of His-ESAT6**

*Escherichia coli* BL21 (DE3) transformed with pET 23b* carrying the ESAT6 gene was grown in LB medium containing 100 μg ml⁻¹ ampicillin. IPTG was added at a concentration of 0.25 mM when...
the A600 reached 0.6–0.7, and the cells were harvested after 4 h by centrifugation at 4°C. The cell pellet from 50 ml bacterial culture was resuspended in 3 ml Bugbuster HT (Novagen) supplemented with 0.5 mM EDTA and 100 mM Pefabloc (Roche Applied Science) and incubated on a shaking platform for 20 min at room temperature, followed by centrifugation at 16 000 g for 20 min at 4°C. The soluble fraction containing ESAT6 was loaded on a Ni2+-NTA- agarse column equilibrated in 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl (buffer A). After washing the column with 50 mM imidazole in buffer A, ESAT6 was eluted with 250 mM imidazole in buffer A. The purified protein was stored at –70°C.

IL-6 analysis

IL-6 was measured in the supernatants of infected cells using the IL-6 ELISA kit from Ray Biotech. All measurements were done at least in duplicate.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. Data have been expressed as means ± SD. Student’s t-test was used when comparing two groups. Differences in values were considered significant for P<0.05.

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References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression of miR-21 and miR-146a in RAW264.7 infected with M.tb. H37Rv. RAW264.7 cells were left uninfected or infected with M.tb. H37Rv at a moi of 5 for 24 h. Bacteria were washed as described under ‘Methods’ and RNA was isolated. The presence of the control inhibitor is set at 100.

Fig. S2. Expression of miR-155 in RAW264.7 treated with ESAT-6. RAW264.7 cells were left untreated (−) or treated (+) with ESAT-6 (5 μg/ml) for 6 h. Expression of miR-155 was measured by qRT-PCR. Data were normalized with respect to snoRNA142. Data represent mean ± SD of triplicates. *P < 0.05. Similar results were obtained in two independent experiments.

Fig. S3. Expression of miR-155 in RAW264.7 infected with M. tuberculosis H37Rv or ∆esat-6. RAW264.7 cells were left uninfected or infected with the indicated strains at a moi of 5. Expression of miR-155 was measured by qRT-PCR performed in triplicate. Data were normalized with respect to snoRNA142.

Data are expressed relative to the expression of miR-155 in H37Rv set at 100. P < 0.05, 3 determinations.

Fig. S4. Expression of miR-155 in RAW264.7 infected with M. bovis BCG or BCG:2F9. RAW264.7 cells were left uninfected or infected with M. bovis BCG or BCG:2F9 at a moi of 5. Expression of miR-155 was measured by qRT-PCR performed in triplicate. Data were normalized with respect to snoRNA142. Relative miR-155 expression is shown with respect to the expression in uninfected cells (considered to be 1). Data represent mean ± SD of triplicates. *P < 0.05. Similar results were obtained in two independent experiments.

Fig. S5. M.tb.-induced phosphorylation of Akt. RAW264.7 cells were infected with M.tb. at different moi. Cells were lysed and immunoblotted with phospho-AKT antibody. The blot was reprobed with AKT antibody. The blot is representative of two independent experiments.

Fig. S6. 3′-SHIP1-UTR luciferase reporter assay and SHIP1 regulation in infected BMDMs. (A) Shown is an analysis of expression of luciferase reporter in the presence of control (filled bar) or miR-155 inhibitor (open bar). Luciferase expression in the absence of the control inhibitor is set at 100. (B) Cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155) and then infected with M.tb. Cell lysates were immunoblotted with SHIP1 antibody, and blots were reprobed with beta actin antibody. The fold change in SHIP1 was calculated with respect to infected cells transfected with control inhibitor. Data are representative of the results obtained in at least two independent experiments.

Fig. S7. HO-1 expression in infected RAW264.7. Cells were left uninfected or infected with the indicated strains at a moi of 5 for 20 h. Expression of HO-1 was analysed in the lysates by Western blotting. Data represent the results obtained in two independent experiments.

Fig. S8. Bach1, hmox1 and HO-1 expression in RAW264.7 cells transfected with miR-155 inhibitor and treated with ESAT-6. RAW264.7 cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155). After 20 h, transfected cells were treated with ESAT-6 (5 μg/ml) for 6 h. RNA was isolated and expression of Bach1 (A) or hmox1 (B) was assessed by qRT-PCR using Gapdh expression for normalization. Data are shown as mean ± SD of three separate determinations. *P < 0.01. (C) Cells were transfected with control inhibitor (ctrl) or miR-155 inhibitor (miR-155). Transfected cells were treated with ESAT-6 for 6 h followed by immunoblotting with HO-1 antibody. Blots were reprobed with actin antibody. The fold change in HO-1 expression is shown with respect to cells transfected with control inhibitor (ctrl).

Fig. S9. Role of miR-155 in IL-6 release by M.tb.-infected RAW264.7. RAW264.7 cells were transfected with either control inhibitor (ctrl) or miR-155 inhibitor (miR-155). Transfected cells were infected with M.tb. H37Rv and IL-6 was measured in the supernatant 20 h post infection. *P < 0.001.

Table S1. Probes for Northern blotting.
Table S2. Primers for qRT-PCR.

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The secreted antigen, HP0175, of *Helicobacter pylori* links the unfolded protein response (UPR) to autophagy in gastric epithelial cells

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**Summary**

Autophagy is an intracellular catabolic process that is required to maintain cellular homeostasis. Pathogen-elicited host cell autophagy may favour containment of infection or may help in bacterial survival. Pathogens have developed the ability to modulate host autophagy. The secreted antigen HP0175, a peptidyl prolyl cis,trans isomerase of *Helicobacter pylori*, has moonlighting functions with reference to host cells. Here we show that it executes autophagy in gastric epithelial cells. Autophagy is dependent on the unfolded protein response (UPR) that activates the expression of PKR-like ER kinase (PERK). This is accompanied by phosphorylation of eukaryotic initiation factor 2α (eIF-2α) and transcriptional activation of ATF4 and CHOP. Knockdown of UPR-related genes inhibits the conversion of LC3I to LC3II, a marker of autophagy. The autophagy-inducing ability of *H. pylori* is compromised when cells are infected with an isogenic *hp0175* mutant. Autophagy precedes apoptosis. Silencing of BECLIN1 augments cleavage of caspase 3 as well as apoptosis. Increased apoptosis of gastric epithelial cells is known to be linked to *H. pylori*-mediated gastric inflammation and carcinogenesis. To the best of our knowledge, this study provides the first demonstration of how HP0175 endowed with moonlighting functions links UPR-dependent autophagy and apoptosis during *H. pylori* infection.

**Introduction**

*Helicobacter pylori* infects the human population worldwide. It is a Gram-negative, flagellated, microaerophilic bacterium that colonizes the gastric mucosa (Suerbaum and Michetti, 2002). *H. pylori* causes a spectrum of disorders ranging from gastritis and gastroduodenal ulcers to gastric carcinomas and lymphomas (Amieva et al., 2002; Peek and Blaser, 2002). Colonization of the stomach involves an interplay between host immune mechanisms geared for elimination of the pathogen and bacterial effectors that act to protect the niche of the bacterium enabling it to multiply and survive. Current evidence suggests that *H. pylori* can invade epithelial cells, macrophages and dendritic cells (Petersen and Krogfelt, 2003; Terebiznik et al., 2006; Ito et al., 2008; Wang et al., 2009, 2010). The ability of a subpopulation of the bacteria to do so likely allows *H. pylori* to survive within the host even under antibiotic pressure. Autophagy is presently acknowledged to play a key role in the response of host cells challenged with either extracellular or intracellular bacterial pathogens. Apoptosis and autophagy are two processes that are intimately linked to the survival or elimination of the pathogen and to the extent of the damage exerted on the gastric epithelium.

Autophagy is a process that is involved in maintaining cellular homeostasis under conditions of stress such as under starvation or in pathological conditions (Cuervo, 2004; Klionsky, 2005; Yorimitsu and Klionsky, 2005; Yuan et al., 2011). Dysfunctional cellular components are removed through autophagy by fusion of autophagosomes with the lysosomal machinery. The breakdown of long-lived proteins also ensures cell survival during starvation. Environmental stresses such as oxidative stress, accumulation of misfolded proteins and irradiation trigger autophagy (Mizushima, 2005; Yorimitsu and Klionsky, 2005). Autophagy is initiated under starvation by the dissociation of mTORC1 from Unc-51-like kinase (ULK1)-ATG13, which controls the early steps of autophagosome formation (Mizushima, 2010). The class 3 phosphatidylinositol-3-kinase 3, vacuolar protein sorting 34 (Vps34), BECLIN1 and a number of other
proteins form a complex that is required for nucleation and assembly of the double-membrane phagophore. Two ubiquitin-like conjugation systems ATG12–ATG16 (Suzuki et al., 2001; Hanada et al., 2007) and ATG8 (or MAP1LC3B)–phosphatidylethanolamine are recruited to the phagophore that elongates to form the autophagosome containing encapsulated cytoplasmic material (Mizushima et al., 1998; 2011). Subsequently, another set of proteins mediates the fusion of autophagosomes with lysosomes (Tumbarello et al., 2012), and a set of lysosomal hydrolases digests the contents of the phagolysosome under acidic conditions (Kroemer et al., 2010). The dynamics of these macromolecular complexes regulate the process of autophagy in response to stress (Kroemer et al., 2010; Yang and Klionsky, 2010).

In recent years, it has been established that autophagy is associated with the immune response of host cells challenged by pathogens (Ma et al., 2013). Autophagy facilitates capture of invading microbes and their delivery to a degradative pathway culminating in their removal. For example, the clearance of Pseudomonas aeruginosa by alveolar macrophages requires autophagy (Yuan et al., 2011). On the other hand, viruses and bacteria are also known to take refuge in autophagosomes where they survive and replicate (Dorn et al., 2002; Nakagawa et al., 2004; Campoy and Colombo, 2009; Deretic and Levine, 2009; Deretic, 2011). In summary, pathogens have evolved to either evade or to exploit autophagy as a means of facilitating their own survival, whereas autophagy is a necessary response of the host that enables it to successfully eradicate the pathogen. In this context, the role of autophagy in H. pylori-caused gastric epithelial cell pathology and disease remains poorly understood.

The unfolded protein response (UPR) has been linked with autophagy as well as apoptosis (Walter and Ron, 2011). The presence of misfolded proteins in the endoplasmic reticulum triggers a signal transduction pathway termed the UPR (Hetz, 2012). The UPR is activated by the ER stress sensors PKR-like ER kinase (PERK) also known as eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3), IRE-1 and activating transcription factor 6 (ATF6) (Ron and Walter, 2007; B’Chir et al., 2013). PERK is a kinase that phosphorylates eukaryotic initiation factor 2α (eIF-2α) to inhibit the initiation of translation. However, a subset of transcripts is translated under these conditions. This includes the transcription factor ATF4. The second arm of the UPR involves the splicing and activation of the transcription factor XBP1 mediated by the endonuclease activity of IRE-1. The third arm involves the release of the transcription factor ATF6 from the membrane by proteolytic cleavage.

H. pylori has been reported to induce autophagy in phagocytic cells or in gastric epithelial cells (Tererbiznik et al., 2009; Tang et al., 2012). Immune-modulating functions have been attributed to individual bacterial factors. The vacuolating cytotoxin VacA is so far the only known virulence factor that has been demonstrated to regulate autophagy in H. pylori-infected gastric epithelial cells (Raju et al., 2012; Tang et al., 2012). The low-density lipoprotein receptor-related protein-1 functions as the VacA receptor for toxin-induced autophagy that precedes apoptosis in AZ-521 cells (Yahiro et al., 2012). However, the mechanism of VacA-induced autophagy remains unclear. There is evidence suggesting that autophagy protects against H. pylori infection. Patients with polymorphisms in ATG16L1 associated with Crohn’s disease are more susceptible to H. pylori infection (Raju et al., 2012). It has also been suggested that by inducing the microRNA miR30b, H. pylori is able to evade autophagy. miR30b targets the autophagy pathway proteins BECLIN1 and ATG12 (Tang et al., 2012). In spite of this recent body of work linking autophagy with the pathogenesis of H. pylori infection, there has been no investigation detailing whether there is any crosstalk between autophagy and processes of cell death such as apoptosis.

We have established the moonlighting functions of the secreted antigen HP0175, a peptidyl prolyl cis,trans isomerase. Our previous work has shown that it induces apoptosis in gastric epithelial cells (Basak et al., 2005). Inhibition of apoptosis in AGS cells associated with disruption of the hp0175 gene clearly showed that HP0175 is one of the proteins regulating apoptosis in these cells. The findings suggested that factors other than VacA are involved in regulation of apoptosis in gastric epithelial cells. Here we show that H. pylori-mediated autophagy also depends on HP0175. HP0175-dependent transcriptional up-regulation of MAP1LC3B, ULK1, BECLIN1 and ATG5 is linked with the UPR and depends on PERK and the transcription factors ATF4 and CHOP. The UPR and the genes of the canonical autophagy pathway are involved in H. pylori-mediated autophagy. To the best of our knowledge, this study provides the first demonstration of a link between the UPR and autophagy during H. pylori infection. We further demonstrate that inhibition of autophagy enhances apoptosis of H. pylori-treated gastric epithelial cells, strengthening the view that there is cross-talk between autophagy and apoptosis. This study also adds to the increasing knowledge of the moonlighting functions of bacterial proteins (Kundu, 2013) and their likely link with bacterial pathogenesis. Our investigations also suggest that in addition to VacA, H. pylori-mediated autophagy and apoptosis in gastric epithelial cells depends on other factors such as the presence of HP0175.
Results

HP0175 regulates expression of autophagy pathway-linked genes in AGS

We have previously established that the secreted antigen HP0175 is an inducer of apoptosis in the gastric epithelial cell line AGS (Basak et al., 2005). Considering that cross-talk between apoptosis and autophagy is documented (Shimizu et al., 2004; Huang et al., 2013), we asked the question whether HP0175 could regulate autophagy in AGS cells. In order to explore whether HP0175 regulates autophagy through transcriptional regulation of autophagy-associated genes, we analysed the expression of an array of autophagy pathway genes in cells challenged with HP0175. Interestingly, HP0175 induced transcription as well as expression of a number of proteins (MAP1LC3B, ULK1, ATG5 and BECLIN1) associated with the autophagy pathway (Fig. 1). Each of the quantitative real-time polymerase chain reaction (qRT-PCR) assays was validated by analysis of the melting curves (Fig. S1). Moreover, HP0175 was also able to activate the MAP1LC3B and BECLIN1 promoters (Fig. S2). These results argued in favour of a likely role of HP0175 in regulating autophagy. VacA is known to induce autophagy in epithelial cells. In order to understand the independent roles of HP0175 and VacA, in H. pylori-induced autophagy, a KO mutant of vacA (VacA-KO) was generated. Vacuolating activity of this isogenic mutant was severely compromised in comparison with the wild-type (WT) H. pylori (Fig. 2A), confirming the inactivation of vacA. Challenge of AGS cells with H. pylori resulted in significant induction of MAP1LC3B, ULK1, ATG5 and BECLIN1 (Fig. 2B–E). This induction of autophagy-associated genes was not apparent in cells treated with HP0175-KO (Fig. 2B–E), although this mutant still showed functional vacuolating activity (Fig. 2A). However, in the absence of vacA, autophagy-associated genes were still significantly up-regulated in treated versus untreated cells (Fig. 2B–E). These results suggested that HP0175-induced up-regulation of autophagy-associated genes occurred independent of functional VacA.

HP0175 plays a role in the conversion of LC3I to LC3II in AGS cells

The mammalian homologue of yeast Atg8, LC3 (or MAP1LC3B), exists as a cytosolic 18 kDa polypeptide. This is cleaved in its C-terminal region to generate LC3I

Fig. 1. HP0175 up-regulates genes linked to autophagy. AGS cells were left untreated (U) or treated with HP0175 (1 μg ml⁻¹) for different periods of time and RNA was isolated. Transcription of MAP1LC3B (A), ULK1 (B), ATG5 (C) and BECLIN1 (D) was analysed by qRT-PCR. Gene expression was normalized with respect to GAPDH. Results represent means ± SD of three determinations. Comparisons have been made against the untreated samples (U). *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant. Expression of ULK1 (E), ATG5 (F), Beclin1 (G) and PERK (G) was analysed by immunoblotting. Blots were reprobed with tubulin antibody. The intensity of each band was quantitated by densitometric scanning and the relative intensity of each band with respect to the corresponding loading control was determined. Data were then normalized with the untreated control.
that is lipidated on its C-terminal glycine to yield a membrane-bound form, LC3II by the E1/E2-like enzymes that are homologues of Atg7 and Atg3 (Kabeya et al., 2000; Tanida et al., 2004). LC3II formation is used as a marker for early autophagosome formation (Klionsky et al., 2012). Time-dependent conversion of LC3I to LC3II was observed in HP0175-treated AGS cells (Fig. 3A). Conversion of LC3I to LC3II was further augmented when phagosomal degradation was inhibited by treating cells with bafilomycin A (Fig. 3B), suggesting that the observed difference in the first instance was not due to a block in autophagic flux. However, at a longer time point (16 h), autophagy appeared to be compromised. This was in harmony with our earlier observations that at longer periods of incubation, HP0175 induces apoptosis (Basak et al., 2005). The role of HP0175 in the induction of autophagy was further confirmed by comparing the ability of the WT strain and its isogenic hp0175 mutant (KO) to induce the conversion of LC3I to LC3II. LC3II formation was compromised in cells treated with the KO strain both in the absence (Fig. 3C and D) and in the presence of bafilomycin (Fig. S3A). The absence of VacA was also

Fig. 2. H. pylori-mediated induction of autophagy-linked genes depends on HP0175. 
A. AGS cells were left untreated (U) or treated with soluble extract of H. pylori (WT) or its isogenic hp0175 mutant (KO) or VacA mutant (VacA-KO) and vacuolating activity was measured by neutral red uptake assay. Data represent means ± SD (n = 3). Comparisons were made as indicated. ***P < 0.001; NS, not significant. 
B–E. AGS cells were infected with H. pylori (WT or KO) or VacA-KO at an MOI of 50 or 100 for 6 h. Transcription of MAP1LC3B (B), ULK1 (C), ATG5 (D) and BECLIN1 (E) was analysed by qRT-PCR as described in Fig. 1. Results represent means ± SD of three determinations. NS, not significant; **P < 0.01; ***P < 0.001. In all cases, comparison was made with untreated cells (U).
Fig. 3. HP0175 stimulates autophagy. AGS cells were treated with HP0175 (1 μg ml⁻¹) for different periods of time in the absence (A) or in the presence of bafilomycin (10 nM) (B) or with H. pylori (WT or KO) at an MOI of 50 for different periods of time (C). Cells were lysed, lysates were separated on sodium dodecyl sulphate gels and immunoblotted with LC3 antibody followed by reprobing with β-actin antibody. The blot shown is representative of three independent experiments. The last lane in (B) is a positive control showing LC3II conversion in starved cells kept in EBSS medium for 6 h to induce autophagy.

D. LC3III was quantitated by densitometric scanning and the relative intensity of each band with respect to the corresponding β-actin band was determined. Data were normalized against the uninfected control (time 0). Data represent means ± SD (n = 3). Comparisons were made as indicated. **P < 0.01; ***P < 0.001. AGS cells were left untreated (E) or treated with HP0175 for 5 h (F), washed and stained with LC3 antibody followed by Alexa-546-conjugated secondary antibody (red). The number of cells showing more than five puncta were counted and expressed as percentage of total number (> 200) of cells (G). Results represent means ± SD of three determinations. *P < 0.05; **P < 0.01.

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associated with decreased conversion of LC3I to LC3II (Fig. S3B), suggesting that both proteins likely have independent roles in regulating autophagy. To further confirm the role of HP0175 as an inducer of autophagy, HP0175-treated AGS cells were immunostained for LC3B. The stain was evenly distributed in untreated AGS cells (Fig. 3E), whereas in HP0175-treated cells, clear punctate structures were found (Fig. 3F), suggesting the activation and localization of LC3 in autophagosomes. Cells having more than five puncta were counted and a significant increase was found in HP0175-treated cells compared with untreated cells (Fig. 3G). Evidence of HP0175-triggered autophagic flux was further corroborated by analysing the fluorescence of mRFP-GFP-LC3B, a tool developed to analyse trafficking to autolysosomes (Ni et al., 2011). This method is based on the selective loss of green fluorescence but retention of red fluorescence in acidic compartments. In the presence of bafilomycin, that is, when autophagic flux was blocked, both green and red punctate structures were visible in cells treated with HP0175 (Fig. S4). Merging showed yellow punctate structures. In the absence of bafilomycin, merging showed an increase in proportion of red punctate structures, typical of autophagic flux (Fig. S4). In order to confirm the role of HP0175 in autophagy, AGS cells were treated with H. pylori WT, KO or VacA-KO for 4 h at MOI 50 followed by immunostaining with LC3 antibody. The absence of either HP0175 or VacA led to significant decrease in the formation of punctuated cells compared with that observed in the case of cells treated with WT bacteria (Fig. S3C and D). In a recent paper, Irving et al. (2014) showed that outer membrane vesicles (OMVs) from a H. pylori vacA mutant induced autophagosome formation in AGS cells to an extent similar to that observed using WT H. pylori or rapamycin. OMV of the WT contain VacA (Rici et al., 2005). However, the similar extent of autophagosome formation suggested redundancy for VacA in H. pylori OMV-induced autophagy. It is evident from this study that VacA is probably not the only factor associated with H. pylori-mediated autophagy induction in epithelial cells.

Involvement of BECLIN1, ATG5 and ULK1 in H. pylori-driven autophagy

In the canonical pathway of autophagy, ULK1, BECLIN1, Vps34 and ATG14L integrate upstream signals and are involved in the nucleation process. Autophagosome formation and elongation requires the conjugation of ATG12 to ATG5 mediated by ATG7 and ATG10 and subsequent interaction with ATG16 (Hanada et al., 2007; Mizushima et al., 2011). The ATG12–ATG5–ATG16 complex binds to the membrane and dissociates upon completion of the autophagosome. Knockdown of BECLIN1 or ATG5 or ULK1 attenuated the conversion of LC3I to LC3II in AGS cells infected with H. pylori (Fig. 4). This confirmed a role of the canonical pathway in H. pylori-driven autophagy in AGS cells.

Induction of genes associated with the UPR in gastric epithelial cells by H. pylori and HP0175

The UPR is activated by the ER stress sensors PERK also known as EIF2AK3, IRE-1 and ATF6 (Rouschop et al., 2013). PERK is a kinase that phosphorylates eIF-2α to inhibit the initiation of translation. However, a subset of transcripts is translated even under these conditions. This includes the transcription factor ATF4. Transcriptional up-regulation of several genes of the autophagy pathway depends on transcription factors that are induced as part of the UPR, including ATF4 and CHOP (Rouschop et al., 2013). Along with the genes associated with canonical autophagy, we observed up-regulation of the transcription of UPR-linked genes PERK, ATF4 and CHOP in cells challenged with HP0175 (Fig. 5A–C). A concomitant increase in the expression of PERK, CHOP and ATF4 was also observed at the level of the proteins in cells treated with HP0175 (Figs 1G, 5B and C, respectively). This suggested a link between the UPR and HP0175-mediated autophagy. In order to confirm the role of HP0175 in induction of the UPR in H. pylori-treated AGS cells, the expression of the above genes was tested in cells treated with the WT or KO strain. WT H. pylori but not the KO strain induced expression of these UPR-associated genes (Fig. 5D–F). Taken together, these results suggested that HP0175 plays an important role in UPR-linked autophagy in H. pylori-treated gastric epithelial cells.

Role of PERK in H. pylori-mediated autophagy

As an immediate response, UPR is known to activate PERK (Hetz, 2012). We observed time-dependent activation of PERK when AGS cells were treated with HP0175 (Fig. 6A). Activation of PERK was found to be dependent on HP0175 since phosphorylation of PERK was attenuated in the cells infected with hp0175 KO (Fig. 6B). In order to determine the role of PERK in H. pylori-induced autophagy in AGS cells, we compared LC3I to LC3II conversion in cells after PERK silencing. Silencing of PERK (Fig. 6C) led to diminished LC3I to LC3II conversion (Fig. 6D), suggesting a role of PERK in H. pylori-induced autophagy. PERK-dependent eIF-2α phosphorylation precedes activation of downstream genes such as ATF4. Four hours after challenge of cells with H. pylori, there was a greater than sixfold increase in intensity of the phosphorylated form of eIF-2α (Fig. 6E). This increase was not evident when cells were challenged with hp0175.
KO (Fig. 6F) corroborating the role of HP0175 in the induction of UPR-associated signalling pathway. To further investigate the role of eIF-2α phosphorylation on autophagy, AGS cells were transfected with either empty vector or a construct defective in phosphorylation of eIF-2α [eIF-2α (S51A)] prior to challenge with H. pylori. Overexpression of eIF-2α (S51A) inhibited H. pylori-induced LC3II formation (Fig. 6G). Collectively, these data suggest a likely role of the UPR in H. pylori-induced autophagy.

**Role of ATF4 and CHOP in regulating the expression of autophagy-linked genes during H. pylori infection of AGS**

Considering that ATF4 and CHOP are transcriptionally activated in H. pylori or HP0175-treated AGS cells, we tested the role of these transcription factors in regulating the expression of autophagy-associated genes during H. pylori infection. qRT-PCR showed that ATF4 knockdown (Fig. 7A and B) attenuated the expression of MAP1LC3B, ULK1, CHOP and ATG5 (Fig. 7C–F), whereas CHOP knockdown (Fig. 7G and H) attenuated the expression of ATG5 (Fig. 7I). These results confirmed that both ATF4 and CHOP are critical for the expression of key players in canonical autophagy.

**Crosstalk between autophagy and apoptosis in H. pylori-challenged cells**

The link between autophagy and cell death has been debated for some time, and a cause-and-effect relationship has not been unequivocally established. It has been suggested that treatment-induced damage to organelles such as in anti-cancer therapy could trigger autophagy as a protective mechanism. Caspase-independent cell death may follow the induction of autophagy. Considering that HP0175 plays a role in autophagy and apoptosis, we explored the role of autophagy in regulating apoptosis and the activation of the executioner caspase, caspase 3, in H. pylori-challenged cells. Knockdown of BECLIN1, a player in H. pylori-driven autophagy of AGS cells, was associated with increased formation of cleaved caspase 3, an indicator of apoptotic cell death (Fig. 8A and B). In addition, Annexin V fluos staining of these cells was enhanced, further confirming that the cells were undergoing apoptotic cell death (Fig. 8C). These results suggested that inhibition of autophagy leads to apoptotic cell death in H. pylori-challenged AGS cells.
Fig. 5. HP0175-mediated induction of PERK, ATF4 and CHOP. AGS cells were left untreated (U) or treated with HP0175 (1 μg ml$^{-1}$) (A–C) or infected with H. pylori (WT or KO) (D–F) at different MOIs for 6 h. Transcription of PERK, CHOP or ATF4 was quantified by qRT-PCR as described in Fig. 1. Results represent means ± SD of three determinations. The expression of CHOP and ATF4 was quantitated by immunoblotting (B and C) after treatment with HP0175.

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Discussion

In recent times it has become increasingly evident that H. pylori can invade, survive and multiply in both epithelial cells as well as in professional phagocytes (Semino-Mora et al., 2003; Dubois and Borden, 2007; Necchi et al., 2007; Ozbek et al., 2010). H. pylori enters the gastric adenocarcinoma cell line AGS (Amieva et al., 2002; Kwok et al., 2002). This makes it likely that autophagy could facilitate either elimination of the bacterium by the host or survival of the bacterium within the host. It is therefore of importance to understand how H. pylori regulates autophagy in gastric epithelial cells.

Several H. pylori factors have been recognized as having immunomodulatory properties. Our recent studies have brought to light the moonlighting functions of the secreted antigen, HP0175. We have established that it modulates host cell signaling pathways both in gastric epithelial cells (Basak et al., 2005; Basu et al., 2008) as well as in cells of the monocytic lineage (Amedei et al., 2014). HP0175 induces apoptosis in gastric epithelial cells (Basak et al., 2005). In view of the recently documented reports of crosstalk between autophagy and apoptosis in epithelial cells (Tanaka et al., 2012), we investigated whether HP0175 elicits autophagy in the gastric cell line, AGS. Our present findings bring to light the autophagy-inducing ability of HP0175. The following observations supported HP0175-induced autophagy in AGS cells: (i) increased amounts of LC3II formation seen in immunoblots; (ii) increased formation of LC3 puncta observed by immunofluorescence; and (iii) increased autophagic flux demonstrated by analysis of trafficking of mRFP-GFP-LC3 by fluorescence microscopy.}

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Transcriptional up-regulation of autophagy-associated genes was distinctly different from that of VacA. We demonstrate that *H. pylori* induces transcriptional activation of genes of the canonical autophagy pathway, including MAP1LC3B, ULK1, ATG5 and BECLIN1, with HP0175 playing a role in this process.

VacA-induced autophagy in AZ-521 cells has been reported to occur in an ATG5-dependent, BECLIN1-independent manner (Yahiro *et al.*, 2012). On the other hand, Tang *et al.* (2012) have reported that *H. pylori*-induced autophagy in AGS cells involves BECLIN1 in line with the present findings. These reports suggest that the results of individual studies require careful consideration and further investigation about possible strain- and cell line-specific effects.

The UPR has been linked to autophagy in organisms ranging from *Drosophila* (Nagy *et al.*, 2013) to mammals (Kouroku *et al.*, 2007). PERK is one of the protein kinases that is activated by misfolded proteins in the endoplasmic reticulum (Harding *et al.*, 1999). It triggers phosphorylation on serine 51 of the α subunit of eIF-2α (Wek *et al.*, 2006). Phosphorylation of eIF-2α results in global decrease in translation, with the exception of a subset of mRNAs. This includes a set of bZIP transcription factors such as ATF4 and CHOP that bind to C/EBP–ATF response element sequences of a subset of genes. Here we show for the first time that *H. pylori*-induced autophagy is linked to the UPR. In support of this, we demonstrate that *H. pylori* enhances the expression of PERK and also activates the phosphorylation of PERK.
an HP0175-dependent manner. This is accompanied by phosphorylation of eIF-2α. Downstream of eIF-2α phosphorylation, we observed transcriptional activation of ATF4 and CHOP. The concerted activation of this set of genes linked to the UPR provided the motivation for testing the role of PERK, ATF4 and CHOP in regulating H. pylori-induced autophagy. Knockdown of PERK inhibited the conversion of LC3 to LC3II. Furthermore, transfection of cells with a phosphorylation-defective mutant of eIF-2α also inhibited the conversion of LC3 to LC3II. These findings suggested that the UPR is linked to H. pylori-mediated autophagy. Based on the report of B’Chir et al. (2013) linking ATF4 and CHOP to the transcriptional induction of a set of autophagy genes in response to amino acid starvation, we tested whether these transcription factors were regulating the expression of some of the genes of the canonical autophagy pathway during infection of AGS cells by H. pylori. We observed that knockdown of ATF4 inhibited the transcriptional induction of MAP1LC3B, ULK1, CHOP and ATG5, whereas CHOP inhibited the transcriptional activation of ATG5. ATF4 binding elements have been identified on the MAP1LC3B, ULK1 and CHOP promoter (Rouschop et al., 2010; Pike et al., 2013), whereas CHOP has been shown to bind to the ATG5 promoter (Rouschop et al., 2010). The effect of ATF4 silencing on ATG5 is likely due to its inhibitory effect on CHOP expression. The transcription factors ATF4 and CHOP therefore play a critical role in the activation of genes of the canonical autophagy pathway, thereby regulating H. pylori-induced autophagy. A model of H. pylori-induced autophagy involving the UPR is given in Fig. 9.

We next attempted to decipher whether there is any crosstalk between autophagy and apoptosis. Recent reports have linked autophagy to prosurvival effects. Pharmacological inhibitors of autophagy have been reported to enhance cell death in endothelial cells (Choi et al., 2009). Tanaka et al. (2012) have shown that inhibition of autophagy by silencing of LC3B promotes hypoxia-induced cell death in epithelial cells. It is evident from our studies that HP0175-induced autophagy (as assessed by the conversion of LC3I to LC3II) occurs at early time points of infection (up to 6 h) and gradually diminishes thereafter (Fig. 3). Further, our previous studies have shown that H. pylori-induced apoptosis occurs at later time points (peaking between 18 and 24 h after infection) (Basak et al., 2005). It has also been reported that VacA, one of the inducers of autophagy in AGS cells, prevents maturation of the autolysosome at longer times of exposure (Raju et al., 2012). Taken together, it appears that autophagy and apoptosis occur in temporally distinct phases, with autophagy preceding apoptosis. In this study we observed that silencing of BECLIN1 increases apoptosis, suggesting a link between the two processes.

**Fig. 8. Helicobacter pylori induced apoptosis is regulated by BECLIN1.**

A. AGS cells were transfected without (C) or with (si) BECLIN1 siRNA for 48 h and left untreated (U) or infected with H. pylori at an MOI of 25 or 50 for 16 h. Cells were lysed and immunoblotted with antibody against cleaved caspase 3. The blot was reprobed with β-actin antibody. This blot is representative of the results obtained in three separate experiments. For each of the three experiments, the relative intensity of each band with respect to the corresponding loading control was determined by densitometric scanning. Data were then normalized against the untreated control. Quantification of the results is shown in panel B. Results shown are means ± SD (n = 3).

C. AGS cells transfected without or with BECLIN1 siRNA were infected with H. pylori at different MOIs for 16 h. Cells were washed and apoptosis was quantified by staining with Annexin V followed by flow cytometry. Results shown are means ± SD (n = 3).

*P < 0.05; **P < 0.01.

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By analysing *H. pylori* replication in AGS cells and in gastric tissues, Tang et al. have suggested that autophagy is a host–beneficial process that restricts *H. pylori* replication. *H. pylori*-induced miR30b targets ATG12 and BECLIN1 to inhibit autophagy and facilitate *H. pylori* replication. We hypothesize that *H. pylori* induces autophagy at the early stages of infection and at lower MOIs. Autophagy is subsequently tuned down by regulatory processes including the synthesis of miRNAs, allowing apoptosis to take over. It is open to question whether autophagy or apoptosis is beneficial for the host or for the bacteria. Chu et al. (2010) have reported that autophagic vesicles induced by *H. pylori* are the sites of replication. Apoptosis of infected epithelial cells could help in maintaining cellular homeostasis by removing damaged cells. The UPR-induced autophagy reportedly regulates the hypoxia-induced tumour microenvironment (Rouschop et al., 2010). What is evident from the present studies is that HP0175 regulates the balance between autophagy and apoptosis in *H. pylori*-infected epithelial cells. It drives PERK-mediated transcriptional activation of key autophagy-linked genes.

Epithelial cell turnover events likely dictate the outcome of *H. pylori* infection, which manifests in pathology ranging from ulcers to gastric adenocarcinoma. Atrophic gastritis and gastric dysplasia are associated with accelerated apoptosis in gastric epithelium after *H. pylori* infec-

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**Antigen HP0175 of *H. pylori* links UPR to autophagy**

**Fig. 9.** Schematic diagram of HP0175-mediated induction of autophagy in gastric epithelial cells. HP0175-mediated induction of PERK leads to phosphorylation of eIF-2α which in turn activates transcription of ATF4 and CHOP. These transcription factors finally induce a number of autophagy-associated genes leading to autophagy.

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**Experimental procedures**

**Reagents**

Antibodies against LC3B, ATF4, ATG5, BECLIN1, cleaved caspase3 (Asp 175), p-EIF-2α, EIF-2α and ULK1 were purchased from Cell Signaling Technology. Antibodies against CHOP, p-PERK, tubulin and TBP as well as CHOP and ATF4 siRNAs were purchased from Santa Cruz Biotechnology. CHOP, p-PERK, tubulin and TBP as well as CHOP and ATF4 siRNAs were purchased from Santa Cruz Biotechnology. BECLIN1, ULK1 and PERK siRNA were purchased from Cell Signaling Technology. β-Actin antibody was obtained from Sigma. LC3B antibody from MBL was used for immunostaining. Bafilomycin A was purchased from EMD Biosciences. *H. pylori* selectable supplement Dent was from Oxoid. Isovitalex, brain–heart infusion (BHI) agar, BHI broth and blood agar were obtained from Difco, BBL. Annexin fluos was from Roche Applied Science. The eIF-2α (S51A) plasmid was a gift from Dr Kasper Rouschop, Maastricht University, The Netherlands.

**Cell culture**

The human gastric epithelial cell line AGS was obtained from National Centre of Cell Science, Pune and maintained in Ham’s F-12 (Invitrogen) medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin in a humidified atmosphere containing 5% CO₂.

**Bacterial growth and infection**

*Helicobacter pylori* 26695 or its isogenic hp0175 KO mutant (Basak et al., 2005) (*H. pylori* KO) or vacA KO (VacA-KO) (this paper) was grown in BHI agar containing 10% heat-inactivated FCS, Isovitalex and Dent under microaerophilic conditions at 37°C. Kanamycin (20 μg ml⁻¹) was added for the growth of both the KO mutants. AGS cells were incubated at the indicated mul-
tplicities of infection (MOIs) for different periods of time, washed and used for further experiments.

**Generation of the Vac A KO mutant**

The gene encompassing vacA was amplified by PCR using genomic DNA from *H. pylori* strain 26695 as template and primers 5′-ATGGTACCCGCTTTCACAAACGGTGATC 3′ (primer 1) and 5′-ATGGATCCGAAATTATGGCATTAGCTG 3′ (primer 2) and cloned between the Knpl and BamHI in pBluescript SK+. A Psil site was created 800 base pairs (bp) downstream from the N terminus of vacA by overlap extension PCR using primers 1, 2, 3 (5′-CAACGCGCTCTGGCAGGATTATCGCTAGT 3′) and 4 (5′-ACTAGGCGATAATGCCTGCAAGACGGCGCTTG 3′). The initial rounds of PCR were carried out using the primer pairs 1, 3 and 2, 4. The products of each PCR were purified and used as templates for the second round of PCR using primers 1 and 2.

The vacA gene was disrupted by introducing the kanamycin resistance cassette (aphA gene) excised from pUC4k at the Psil site within vacA. The identity of the resultant clone was verified by sequencing. The plasmid was then electroporated into competent *H. pylori* and the transformants were plated on BHI agar plate containing 10% FCS, Isovitalex, *H. pylori*-selective supplement Dent and 20 μg ml⁻¹ kanamycin under microaerophilic conditions at 37°C. The kanamycin-resistant colonies were selected and disruption of vacA by insertion of the kanamycin resistance cassette was confirmed by PCR using genomic DNA from the kanamycin-resistant vacA mutant (VacA-KO) as template. The loss of functional vacuolating activity was confirmed by neutral red dye uptake assay.

**Expression and purification of HP0175**

N-terminal His tagged HP0175 was purified by Ni²⁺ affinity chromatography as described earlier (Basak et al., 2005). For all experiments, HP0175 was used at a concentration of 1 μg ml⁻¹ unless otherwise stated.

**Transfections**

Transfections of AGS cells with siRNAs were performed using an Amaxa Nucleofector system (Lonza). Briefly, 10⁶ cells were electroporated with different siRNAs (150 nM). Transfected cells were grown for 48–72 h prior to use. Transfections with plasmid were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were grown for 24 h prior to use.

**Western blotting**

After infections or treatment with HP0175, cells were washed with ice-cold phosphate buffered saline (PBS) and lysed at 4°C in lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na₂ ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis (β-amino ethyl ether) tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₂VO₃, 1 μg ml⁻¹ leupeptin and protease inhibitor cocktail (Roche Applied Science)] for 15 min. For the analysis of cleaved caspase 3, cells were lysed in CHAPS buffer containing 50 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 6.5, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol and protease inhibitor cocktail using three freeze-thaw cycles. For the analysis of CHOP, samples were lysed in high salt extraction buffer, containing 25 mM HEPES, pH 7.4, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% NP-40 and protease inhibitor cocktail for 10 min at room temperature. The supernatant (soluble extract) was filter sterilized and used for vacuolation assay. There were 10⁶ AGS cells plated in microtitre plates in antibiotic-free Roswell Park Memorial Institute medium supplemented with 10% FBS. After overnight incubation, the medium was replaced by fresh medium containing 10 mM ammonium chloride and fivefold diluted *H. pylori* soluble extract. Neutral red uptake assay was performed as described earlier (Cover et al., 1991). After overnight incubation, the medium was replaced with 100 μl of staining solution containing 0.05% neutral red in 0.9% saline per well and incubated for 5 min. Cells were then washed three times rigorously with 0.9% saline. Neutral red was extracted from cells using acidified alcohol (70% ethanol in water containing 0.4% HCl) and absorbance was measured at 540 nm. In each case, OD₅₄₀ of wells containing cells incubated with medium without soluble extract was subtracted from experimental wells to yield net OD₅₄₀.

**Cloning of the BECLIN1 and MAP1LC3B promoters in pGL3 and assay of promoter activity**

The BECLIN1 (~644 to +197) and MAP1LC3B promoter (~965 to +118) were amplified from genomic DNA of AGS cells using primers given in Table S1. The resulting PCR products were cloned between the Knpl and HindIII sites of the vector pGL3 basic (Promega) harbouring the firefly luciferase gene. These promoter constructs were co-transfected into AGS cells along with β-galactosidase expressing construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were treated 24 h after transfection, lysed and luciferase activity was measured using the luciferase assay kit (Promega) according to the manufacturer’s protocol. Luciferase activity was normalized by measuring β-galactosidase activity using the β-galactosidase assay kit (Promega).

**Annexin V fluores staining and flow cytometry**

Cells after treatment were washed twice with PBS and stained with Annexin V fluores for 10 min at room temperature in the dark...
according to the manufacturer's instruction. Cells were analysed by flow cytometry (FACS Calibur; Becton Dickinson) using an excitation of 488 nm green fluorescence was collected between 505 and 545 nm. There were 10,000 cells analysed per sample. Data analysis was performed with Cell Quest software (Becton Dickinson).

RNA isolation and qRT-PCR

Total RNA was isolated from 10^6 cells using the RNeasy kit (Qiagen) according to the manufacturer's protocol. For qRT-PCR, cDNA was synthesized from 2 μg of total RNA using the Revert Aid First Strand synthesis kit (Thermo Scientific, Fermentas) according to the manufacturer's protocol. SYBR green based real-time PCR was carried out using primers described in Table S2 and Mesagreen Master Mix (Eurogentec). The relative expression of target gene was normalized to the reference gene GAPDH using the comparative Ct method.

Fluorescence microscopy

For immunostaining, AGS cells (1 × 10^5) were grown on cover slips placed in wells of a 24-well plate with each well containing 500 μl medium. After treatment, cells were prefixed by adding 500 μl of 10% neutral buffered formalin solution and incubated for 2 min. The medium was then replaced with 500 μl of 10% formalin solution and incubated for 20 min at room temperature followed by permeabilization with 0.05% TritonX-100 (v/v) in PBS for 15 min. Fixed cells were blocked in 2% BSA (w/v) at 4°C overnight, washed extensively with PBS and finally stained in PBS for 30 min, incubated with primary antibody (1:1000) at 4°C overnight, washed extensively with PBS and finally stained with Alexa fluor 546-conjugated goat-anti rabbit antibody (1:750). After washing with PBS, the coverslips were mounted on glass slides using Prolong gold and slow fade gold antifade reagent (Molecular Probes, Invitrogen) and observed using a Zeiss microscope.

Autophagic flux was detected using a fluorescence microscope. By monitoring the distribution and alteration of mRFP-GFP-LC3B (Molecular Probes, Invitrogen) and observed using a Zeiss microscope.

Acknowledgements

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References


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Autophagic flux was analysed by fluorescence microscopy by monitoring the distribution and alteration of mRFP-GFP-LC3B fluorescent signals. AGS cells were transfected with pTf3c plasmid (Addgene) expressing both mRFP and eGFP for 24 h followed by treatment with HP0175 (1 μg ml^-1) for 5 h. Localization of RFP-GFP was detected using a fluorescence microscope.


Supporting information

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

Fig. S1. Derivative melting curves of autophagy linked genes. MAP1LC3B (A), ULK1 (B), ATG5 (C), BECLIN1 (D).

Fig. S2. HP0175 activates promoters of genes linked to the autophagy pathway. AGS cells were transfected with promoter constructs of BECLIN1 and MAP1LC3B for 24 h. Cells were left untreated (U) or treated with HP0175 (1 μg ml−1) for different periods of time. Cells were lysed followed by measurement of luciferase activity.

Fig. S3. HP0175 induces autophagy in AGS cells. Cells were infected with H. pylori (WT or KO or VacA-KO) (A and B) in the presence of bafilomycin at MOI 50 for different periods of time. Cells were lysed and immunoblotted with LC3 antibody followed by Alexa-546-conjugated secondary antibody (red). Formation of punctated lysosome was visualized. The number of cells showing more than five puncta were counted and expressed as percentage of total number (> 200) of cells (D).

Fig. S4. HP0175-mediated induction of autophagic flux. AGS cells were transfected with ptk8c plasmid (expressing eGFP-mRFP-LC3) for 24 h. Cells were either left untreated (A) or treated with HP0175 in the presence (B) or absence (C) of bafilomycin. Cells were then fixed with 10% formalin solution followed by fluorescence microscopy. Green, red and yellow dots per cell shows GFP-RFP−, GFP-RFP+ and GFP-RFP+ puncta, respectively. Scale bar represents 50 μm.

Table S1. Sequences of primers used for cloning of promoter constructs.

Table S2. Sequences of primers used for qRT-PCR.