# ATP-Dependent Lon Protease Contributes to *Helicobacter pylori*-Induced Gastric Carcinogenesis

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## Abstract

*Helicobacter pylori* infection is the strongest risk factor for development of gastric cancer. Host cellular stress responses, including inflammatory and immune responses, have been reported highly linked to *H. pylori*-induced carcinogenesis. However, whether mitochondrial regulation and metabolic reprogramming, which are potently associated with various cancers, play a role in *H. pylori*-induced gastric carcinogenesis is largely unknown. Here we revealed that Lon protease (Lonp1), which is a key inductive of mitochondrial unfolded protein response (UPR$^{mt}$) and is required to maintain the mitochondrial quality, was greatly induced in *H. pylori* infected gastric epithelial cells. Importantly, we uncovered that knockdown of Lonp1 expression significantly diminished the metabolic switch to glycolysis and gastric cell proliferation associated with low multiplicity of *H. pylori* infection. In addition, Lonp1 overexpression in gastric epithelial cells also promoted glycolytic switch and cell overgrowth, suggesting *H. pylori* effect is Lonp1 dependent. We further demonstrated that *H. pylori* induced Lonp1 expression and cell overgrowth, at least partially, via HIF-1α regulation. Collectively, our results concluded the relevance of Lonp1 for cell proliferation and identified Lonp1 as a key regulator of metabolic reprogramming in *H. pylori*-induced gastric carcinogenesis.

## Introduction

*Helicobacter pylori* infection is a major cause of chronic gastritis and is the strongest risk factor of gastric cancer [1]. *H. pylori* has also been defined as a class I carcinogen by the international Agency for Research on Cancer (IARC) [2,3]. It is well known that *H. pylori* influences gastric cell proliferation, apoptosis, and cancer cell transformation via production of multiple virulence factors, including CagA, peptidoglycans, VacA, adhesins and outer membrane proteins (OMPs) [4,5]. However, growing evidence indicated that host chronic gastric stresses also play important roles in *H. pylori*-induced gastric adenocarcinoma [6]. For example, a few regulators of immune response, inflammation and hypoxia underlying carcinogenesis, like NF-κB and HIF-1α, are activated by *H. pylori* infection [7,8]. Beside

that, however, the molecular mechanism(s) by which *H. pylori*-induced stress responses in epithelial cells contribute to carcinogenesis is still not fully characterized.

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Lonp1 is a conserved serine peptidase and an important responsive marker for UPR$^{mt}$, which mounts protective effects to compensate stress-associated mitochondrial injury by inducing the expression of mitochondrial enzymes, chaperons, as well as proteases, to maintain mitochondrial proteostasis and function [9]. Lonp1 plays an essential role in clearance of misfolded or damaged proteins and maintains mitochondrial function and cell viability under diverse physiological conditions, including oxidative stress, endoplasmic reticulum (ER) stress, and hypoxia [10,11]. In addition, Lonp1 also supports mitochondrial DNA (mtDNA) stability and mitochondrial electron transport chain integrity [12,13]. Importantly, recent studies indicated that Lonp1 up-regulation in melanoma cells results in robust changes in mitochondrial complexes, leading to impaired mitochondrial respiration and metabolic switch to glycolysis, which are the major features of tumorigenesis [14]. As a consequence, the proliferation rate and viability of melanoma cells, as well as cervical cancer cells, were significantly increased regarding Lonp1 induction [14,15].

In this study, we performed integrative analysis of published microarray data of *H. pylori* infection and uncovered the *Lonp1*, as well as other UPR$^{mt}$ genes, were significantly induced in gastric epithelial cells. We further demonstrated that Lonp1 plays an important role in metabolic switch toward glycolysis and gastric epithelial cell proliferation in response to low multiplicity of *H. pylori* infection.

## Results

### Differentially Expressed Genes in H. pylori-Infected Mouse Gastric Epithelial Cells

To assess the influence of *H. pylori* infection on the transcriptional profile of gastric epithelial cells, we analyzed microarray data that was downloaded from NCBI-GEO website (GSE10262). In that study, Gordon and his colleagues treated mouse gastric stem-like cells with chronic atrophic gastritis (ChAG)- and cancer-associated *H. pylori* strains to examine the transcriptional alterations during the transition from ChAG to gastric adenocarcinoma [16]. In order to obtain a broader range of differentially expressed genes for statistic modeling, we set the cut-off as fold change (FC) >1.5 and false discovery rate (FDR) <0.05, which was a little different from the criteria (FC >2 and FDR <0.05) in Gordon's work [16]. Eventually, 2507 (1574 up- and 933 down-regulated) genes and 1483 (956 up- and 527 down-regulated) genes were identified in both Kx1 (ChAG-associated) and Kx2 (cancer-associated) *H. pylori* infection models, respectively (Figure 1, A–B). We further found that 73% of up-regulated genes and 82% of down-regulated genes in Kx2 model were consistently differentially expressed in Kx1 model (Figure 1, A–B).

Interestingly, the gene ontology (GO) enrichment analysis of overlapping genes that were differentially expressed in both Kx1 and Kx2 models revealed that GO terms related to phosphorylation regulation, cell growth, stress response, and RNA processing were significantly enriched in up-regulated genes, while GO terms related to transport regulation, cell death, macromolecular complex organization, lipid metabolism and oxidation regulation were significantly enriched in down-regulated genes (Figure S1, A–B). In addition to biological process, we also found that cellular compartment GO terms related to nuclear lumen, endosome, lysosome, proteasome complex, and V-type ATPase complex were significantly enriched in up-regulated genes (Figure S2C). On the other hand, cellular compartment GO terms related to mitochondrion, ER, Golgi apparatus, and cytoskeleton were significantly enriched in down-regulated genes (Figure 1C). The down-regulated mitochondrial genes included those ones encoding components of electron transport chain (ETC), tricarboxylic acid cycle (TCA), as well as beta-oxidation (Figure S2).

We also performed GO enrichment analysis of unique genes that were only differentially expressed in Kx2 models. However, those very few significantly enriched GO terms were all included in Kx1-Kx2-overlapping gene enrichment analysis (data not shown), indicating that cancer-associated *H. pylori* (Kx2) infection did not cause additional changes of biological processes or cellular compartments as compared with ChAG-associated *H. pylori* (Kx1) infection. Thus, we speculated that *H. pylori*-induced carcinogenesis is due to the cumulative effects of chronic stress response in gastric cells.

### H. pylori Infection Up-Regulates UPR$^{mt}$ Genes in Mouse Gastric Epithelial Cells

Impaired expression of genes associated with mitochondrial activity or organelle architecture has been shown to trigger mitochondrial-to-nuclear signaling and increase UPR$^{mt}$ gene expression, including mitochondrial enzymes, chaperones, proteases, as well as transcription factors, to maintain protein quality and mitochondrial function [9,17]. To assess whether down-regulated functional mitochondrial genes (Figure S2) induces UPR$^{mt}$ signaling, we examined the expression levels of UPR$^{mt}$ genes [18]. Surprisingly, most UPR$^{mt}$ genes were significantly were induced by *H. pylori* infection in mouse gastric cells. Except *Hsp90ab1* (encodes HSP90) that was significantly decreased, *Ddit3* (encodes transcriptional factor CHOP), *Cebpb* (encodes transcriptional factor C/EBP), *Hmox1* (encodes heat shock protein HSP32), *Lonp1*, *Hspa9* (encodes mitochondrial HSP70), *Clpp* (encodes subunit of protease Clpp), and *DNAJA3* (encodes mitochondrial HSP40) were significantly elevated by *H. pylori* infection in both Kx1 and Kx2 models (Figure 1D). Thus, our results strongly indicated that UPR$^{mt}$ signaling was induced in mouse gastric epithelial cells upon *H. pylori* infection.

### H. pylori Infection Significantly Induces Lonp1 Expression in Human Gastric Epithelial Cells

Mitochondrial proteases are important UPR$^{mt}$ indicators and are essential for mitochondrial maintenance and cell survival in response to exogenous stresses [18]. We thus measured the expression of mitochondrial protease genes in the context of *H. pylori* infection. *Yme1l1* (encodes YME1L1) and *Oma1* (encodes OMA1) were not significantly affected in both Kx1 and Kx2 infection models. *Htra2* (encodes HTRA2 peptidase) was only significantly induced in Kx1 but not Kx2 model (Figure 2A). Only *Lonp1* and *Clpp* were significantly increased in both infection models. Noted that, *Lonp1* was with the greatest induction, 9.9 and 7.6 folds in Kx1 and Kx2 infection models, respectively (Figure 2A). Thus, we speculated that, as an important regulator of mitochondrial protection and cell proliferation [14,15], Lonp1 induction might contribute to *H. pylori*-induced gastric carcinogenesis.

Since UPR$^{mt}$ regulation and *H. pylori*-associated gastric responses are similar across species [18,19], we next confirmed whether Lonp1 is induced by *H. pylori* infection in human gastric epithelial cells in a similar manner. Confluent MKN28 cells were infected by *H. pylori* strain NCTC 11637 (MOI = 50) for 0, 1, 4, 12 and 24 hours. We found that *Lonp1* mRNA level was increased by *H. pylori* infection by
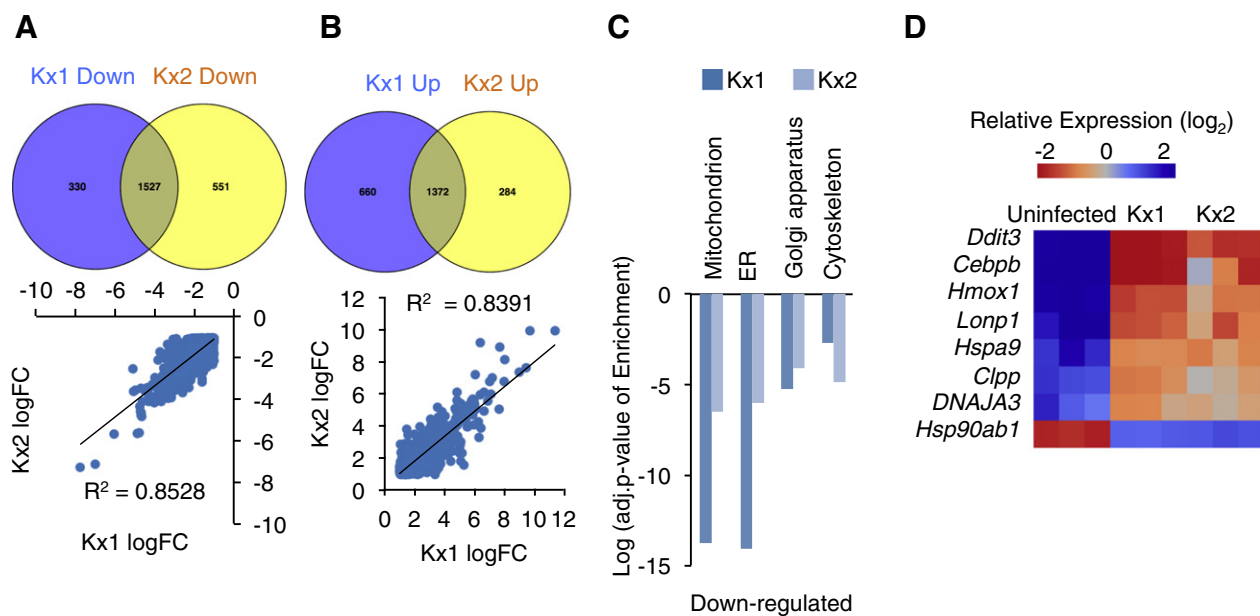
**Figure 1.** Differentially expressed genes in gastric cells in *H. pylori* infected mouse gastric epithelial cells. (A-B) Significantly down- and up-regulated genes (fold change >1.5 and FDR < 0.05) were identified in Kx1 and Kx2 *H. pylori* infection models. Venn diagram analysis (up) and Pearon's correlation analysis (down) of significant down- (A) and up-regulated (B) genes. "LogFC" means the value of log₂ fold-change for each gene in Kx1 or Kx2 infection model. (C) Significantly enriched (EASE score < 0.05) GO terms regarding cellular compartment were identified in overlapping down-regulated genes. (D) Heatmap showing the significantly deregulated UPR^mt genes in both Kx1 and Kx2 *H. pylori* infection models.

1.9, 4.2, 8.1 and 12.6 folds compared to uninfected cells ([Figure 2](#)*B*). MKN28 cells were then infected with different MOIs of NCTC 11637 *H. pylori* for 12 hours and *Lonp1* mRNA level was increased in a dose-dependent manner ([Figure 2](#)*C*). Importantly, in addition to *Lonp1*, other UPR^mt genes, including transcriptional factors, enzymes, and chaperons, were also induced by *H. pylori* infection (MOI = 50, 12 hours) (Figure S3). We further treated MKN28 cells with different strains of *H. pylori* (MOI = 50, 12 hours), 26695, J99, BCM-300, as well as BCS 100. Consistently, similar to NCTC 11637, cag⁺ *H. pylori* strains, 26695, J99, and BCM-300, potently induced *Lonp1* mRNA expression ([Figure 2](#)*D*). However, cag⁻ *H. pylori* strain BCS 100 failed to increase *Lonp1* mRNA level ([Figure 2](#)*D*), suggesting that *cag*-encoding virulence factors, including CagA, are essential for *Lonp1* induction. We next assessed Lonp1 protein level change and found that *H. pylori* infection significantly elevated Lopn1 protein levels, indicated by western blot, in MKN28 cells in a time-dependent manner ([Figure 2](#)*E*). Taken together, our results indicated that *H. pylori* infection increases Lonp1 expression in human gastric epithelial cells at both mRNA and protein levels.

### Lonp1 Regulates Mitochondrial Function in Response to H. pylori Infection

Previous studies have reported that *H. pylori* infection impairs host cell mitochondrial function via virulence factor production, including CagA and VacA [20]. We also accessed the mitochondrial regulation in response to *H. pylori* infection by measuring mitochondrial mass. Strikingly, low multiplicity of *H. pylori* infection resulted in a great increase in mitochondrial mass, including mitochondrial density and mitochondrial DNA level ([Figure 3](#), *B–C*). Lonp1 has been reported to restore mitochondrial function under diverse stresses [14,21]. To examine whether Lonp1 is required for

mitochondrial mass increase in response to *H. pylori* infection, we knocked down Lonp1 expression in MKN28 cells using lentivrius-induced RNAi (sh-Lonp1). Compared to sh-GFP, Lonp1 expression was dramatically decreased at both mRNA and protein levels in sh-Lonp1 cells ([Figures 3](#)*A* and S5). Interestingly, Lonp1 deficiency significantly, but partially, diminished *H. pylori*-associated increase of mitochondrial intensity and mitochondrial DNA level compared to control (sh-GFP) ([Figure 3](#), *B–C*).

Respiration rate and ATP production are two major features of mitochondrial activity. We next studied whether Lonp1 regulates mitochondrial respiration in response to *H. pylori* infection. However, even though high multiplicity of *H. pylori* infection has been shown to impair mitochondrial respiration, including oxygen consumption and electron transport [22], low multiplicity of *H. pylori* infection failed to suppress basal or maximal oxygen consumption rate in control MKN28 cells (sh-GFP) ([Figure 3](#)*D*). In Lonp1-deficient cells oxygen consumption was slight decreased without *H. pylori* infection ([Figure 3](#)*D*). Surprisingly, both basal and maximal oxygen consumption rate in Lonp1-deficient cells were further significantly decreased by 24 hours of low multiplicity of *H. pylori* infection ([Figure 3](#)*D*), indicating that Lonp1 induction is essential for mitochondrial respiration maintenance in response to *H. pylori* infection. We next clarified Lonp1 roles in mitochondrial respiration regulation by overexpressing Lonp1 in MKN28 cells and found that, however, maximal oxygen consumption rate in MKN28 cells was also significantly suppressed by Lonp1 overexpression ([Figure 3](#)*F*).

We also measured ATP production in gastric cells regarding *H. pylori* infection. Slightly different from mitochondrial respiration, ATP production in MKN28 cells was elevated by 24 hours of low multiplicity of *H. pylori* infection (MOI = 25, 50, 100), but decreased
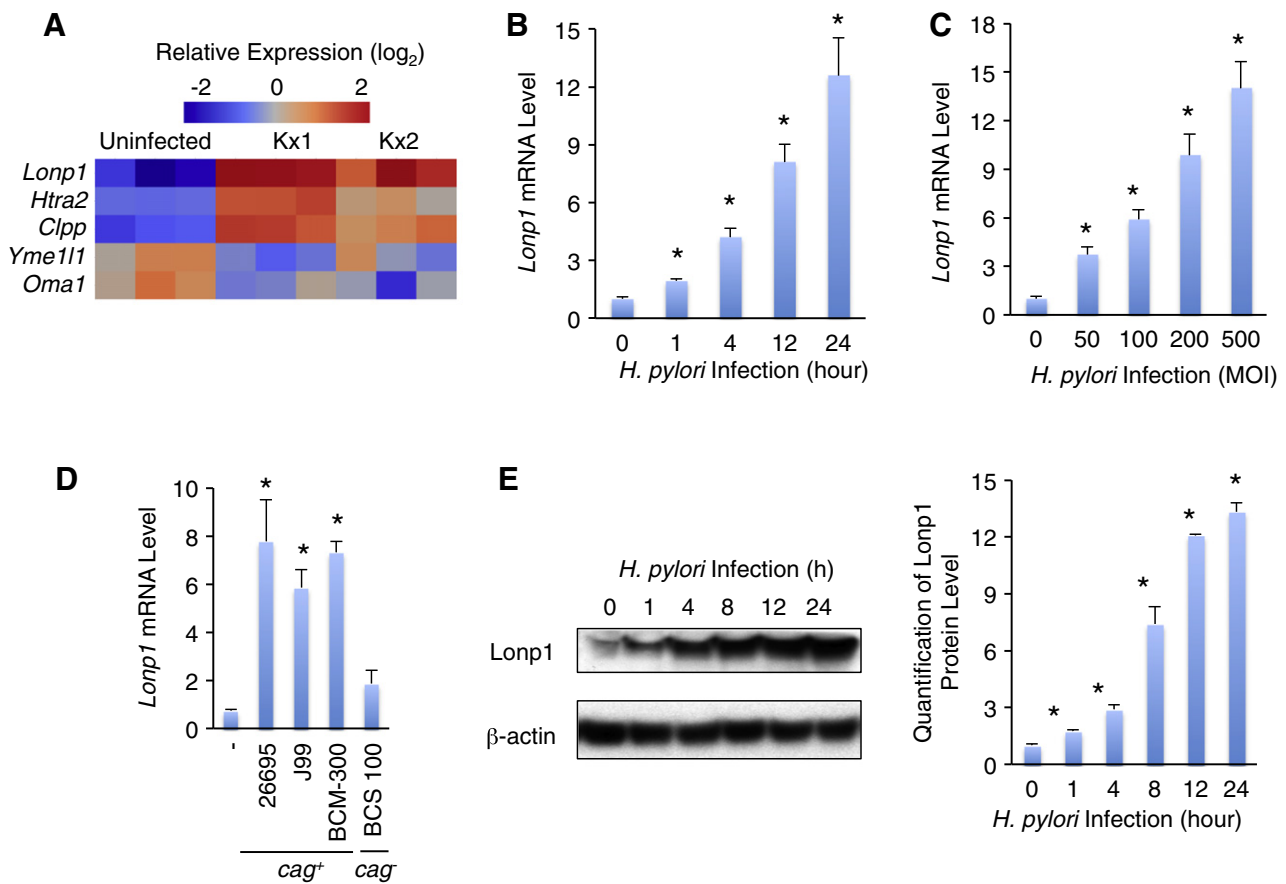
**Figure 2.** Lonp1 expression is induced by *H. pylori* infection in human gastric epithelial cells. (A) Heatmap showing the expression pattern of mitochondrial proteases in Kx1 and Kx2 *H. pylori* infection models. (B) *Lonp1* mRNA levels in MKN28 gastric cells were indicated by qPCR in response to *H. pylori* infection (MOI = 50, NCTC 11637) at different time points. (C) *Lonp1* mRNA levels in MKN28 gastric cells were induced by different MOIs of *H. pylori* NCTC 11637 for 4 hours. (D) *Lonp1* mRNA levels in MKN28 gastric cells were induced by different *H. pylori* strains (MOI = 50, 26695, J99, BCM-300, and BCS 100) for 12 hours. (E) Lonp1 proteins that were indicated by western blot (left) and quantified (right) in MKN28 gastric cells were elevated by *H. pylori* infection (MOI = 50, NCTC 11637) at different time points. Data represent the mean ± SEM from three separate experiments. * $P < .05$.

by high multiplicity of *H. pylori* infection (MOI = 500) (Figure S4A). Interestingly, Lonp1 deficiency mildly decreased ATP levels without *H. pylori* infection, however, significantly abolished ATP production induced by low multiplicity of *H. pylori* infection in MKN28 cells (Figure 3E). In addition, Lonp1 overexpression elevated ATP production by ~1.5-fold without *H. pylori* infection (Figure 3F). Collectively, our results indicated that Lonp1 plays a critical role in homeostatic regulation of mitochondrial activity in response to *H. pylori* infection.

### Lonp1 Contributes to H. pylori-Induced Metabolic Switch to Glycolysis

A metabolic switch towards glycolysis over mitochondrial respiration was observed in most solid tumor cells [23]. Our gene expression analysis in muscle gastric cells also indicated that major components involved in glycolysis were significantly increased in *H. pylori* infection models (Figure S2, right). We therefore hypothesized that *H. pylori* infection promotes metabolic glycolytic switch to influence gastric carcinogenesis. To address this hypothesis, we measured both glucose incorporation and lactate production, which are indicative of glycolysis [24], in MKN28 gastric epithelial

cells. Low multiplicity of *H. pylori* infection for 24 hours dramatically promoted glucose incorporation and lactate production in sh-GFP MKN28 cells (Figure 4, A–B). Endogenous Lonp1 deficiency in MKN28 cells somehow slightly elevated glucose incorporation and lactate production without *H. pylori* infection (Figure 4, A–B). Strikingly, both glucose incorporation and lactate production associated with *H. pylori* infection were potently diminished in Lonp1-deficient cells (Figure 4, A–B), suggesting Lonp1 is essential for *H. pylori*-induced glycolytic shift. In contrast, Lonp1 overexpression in MKN28 cells significantly promoted both glucose incorporation and lactate production without *H. pylori* infection (Figure 4, C–D). Taken together, our results indicated that Lonp1 is required to enhance glycolytic switch in response to *H. pylori* infection.

### Lonp1 is Required for H. pylori-Induced Gastric Cell Proliferation

Metabolic switch toward glycolysis is highly associated with tumorigenesis/cell overproliferation [23]. We next examined whether Lonp1 contributes to gastric cell overgrowth in response to *H. pylori* infection. Previous studies indicated that low MOI (<100) of *H. pylori* promotes, whereas high MOI (>400) suppresses, cell proliferation [25–27]. To obtain an optimal gastric cell proliferative
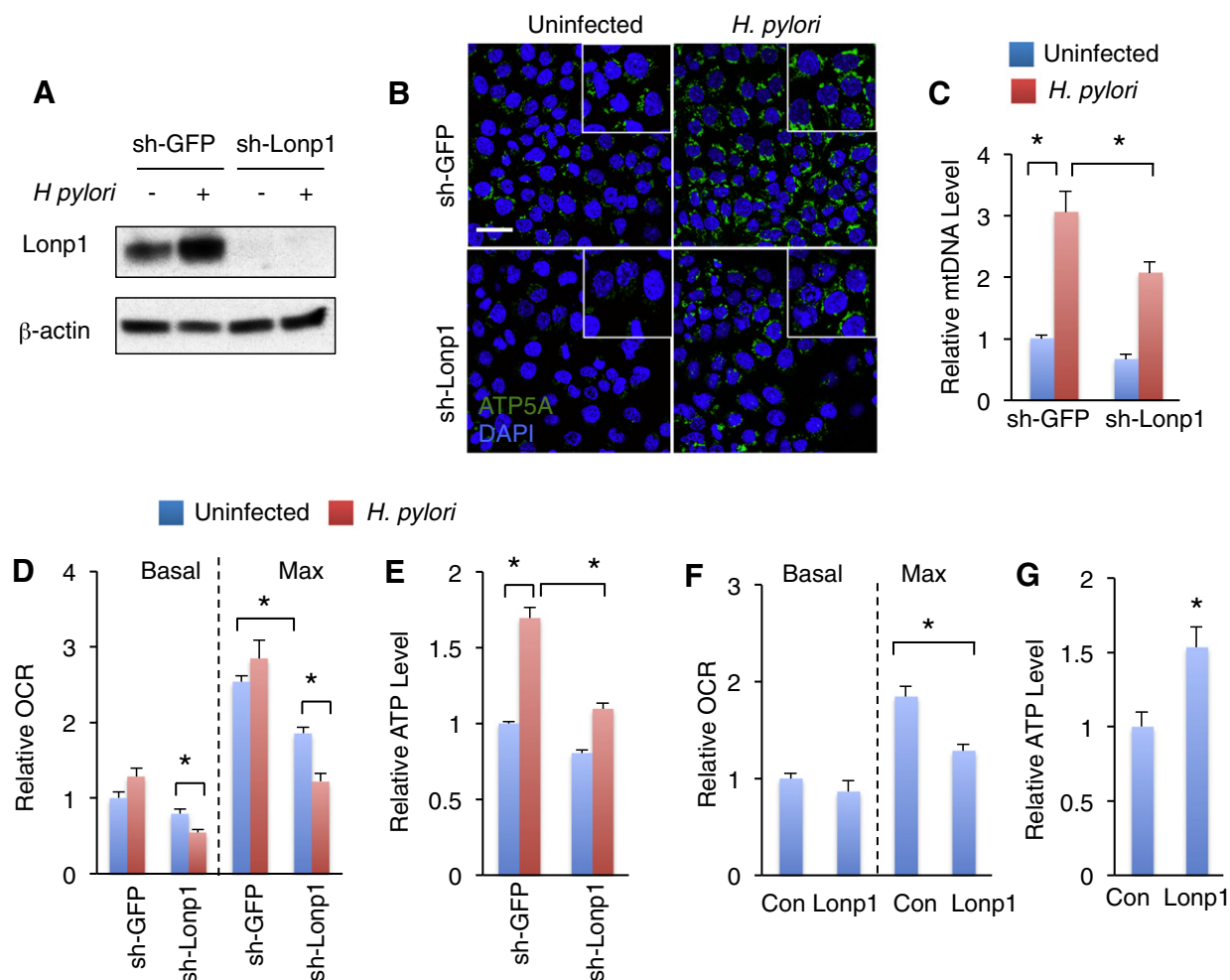
**Figure 3.** Induction of Lonp1 maintains mitochondrial function in response to low multiplicity of *H. pylori* infection. (A) Western blot indicating Lonp1 protein levels in MKN28 cells in response to low multiplicity of *H. pylori* infection (MOI = 50, NCTC 11637) and lentivrius-induced RNAi. (B-C) Mitochondrial mass, which was indicated by immunostaining of ATP5A (B), and mtDNA level (C) in MKN28 cells under low multiplicity of *H. pylori* infection for 24 hours (MOI = 50, NCTC 11637). (D-E) Basal and maximal mitochondrial oxygen rates (D) and cellular ATP levels (E) in control (sh-GFP) and Lonp1-deficient (sh-Lonp1) MKN28 cells in response to 24 hours of low multiplicity of *H. pylori* infection were measured. (F-G) Basal and maximal mitochondrial oxygen rates (F) and cellular ATP levels (G) in control (Con) and Lonp1-overexpressing (Lonp1) MKN28 cells. Data represent the mean ± SEM from three separate experiments. * $P < .05$.

condition for *H. pylori* infection, we tested different MOIs of *H. pylori* using a 3-dimensional model system that provides interactions between cells, growth factors, and an extracellular matrix. Consistent with previous reports, we found that MOI = 50 of *H. pylori*, which potently promotes glycolysis, manifested a maximal proliferative effect on MKN28 gastric epithelial cells (Figure S4B). In order to examine whether Lonp1 is important for *H. pylori*-induced gastric cell proliferation, we measured proliferation rate in Lonp1-deficient cells in the context of low multiplicity of *H. pylori* infection (MOI = 50). Strikingly, Lonp1 deficiency significantly diminished the increase in both cell growth and proliferation rate associated with *H. pylori* infection (Figure 5A, C–D). On the other hand, we tested whether elevated Lonp1 expression is sufficient to promote gastric epithelial cell proliferation. Consistent with the previous studies in cervical cancer cells and melanoma cells [14,15], overexpression of Lonp1 significantly increased cell growth and proliferation rate of

gastric epithelial cells without *H. pylori* infection (Figure 5B, E–F). Thus, our results demonstrated that low multiplicity of *H. pylori* infection results in gastric cell proliferation via Lonp1 function.

### HIF-1α Regulates Lonp1 Expression in Response to H. pylori Infection

We next assessed the molecular mechanism(s) by which Lonp1 expression is induced by *H. pylori* infection. HIF-1α has been shown to directly target Lonp1 promoter and regulate Lonp1 expression in HeLa cells under hypoxia conditions [28]. The activity and protein level of HIF-1α are increased in gastric epithelial cells upon *H. pylori* infection [7]. In addition, mRNA profiling from *H. pylori* infected mouse gastric cells also indicated that well-known HIF-1α target genes [29] were up-regulated in both Kx1 and Kx2 models (Figure 6A). Thus, we speculated that HIF-1α might regulate Lonp1 expression upon *H. pylori* infection. To address this
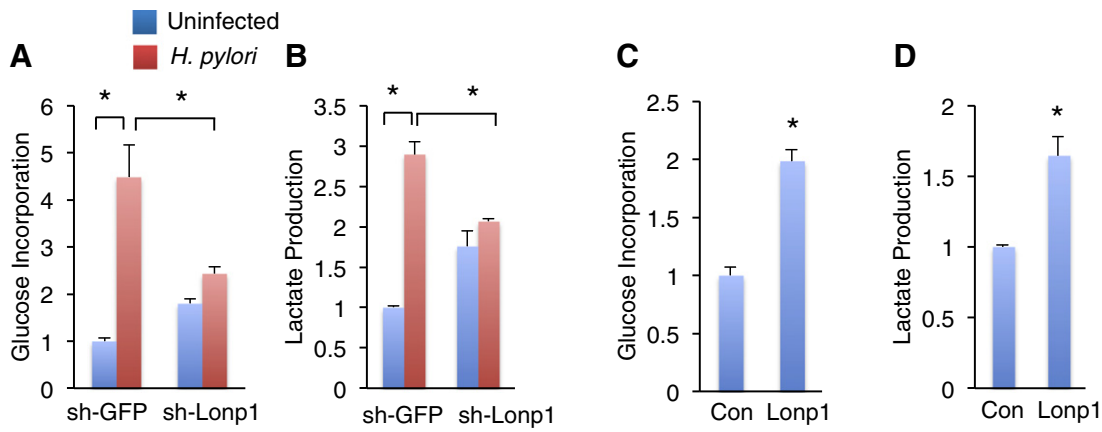
**Figure 4.** Low multiplicity of *H. pylori* infection results in glycolytic switch via Lonp1 induction. (A-B) Glucose incorporation (A) and lactate production (B) in control (sh-GFP) and Lonp1-deficient (sh-Lonp1) MKN28 cells in response to low multiplicity of *H. pylori* infection (MOI = 50, NCTC 11637) for 24 hours were indicated. (C-D) Glucose incorporation (C) and lactate production (D) in control (Con) and Lonp1-overexpressing (Lonp1) MKN28 cells were measured. Data represent the mean ± SEM from three separate experiments. * $P < .05$.

hypothesis, we first examined the HIF-1α expression in response to *H. pylori* infection and found that low multiplicity of *H. pylori* infection elevated HIF-1α protein level at 1 hour and in a time-dependent manner (Figure 6B). However, in contract to protein level, low multiplicity of *H. pylori* infection failed to elevated *HIF1a* mRNA level at early time points (1 hour and 4 hours) (Figure 6C),
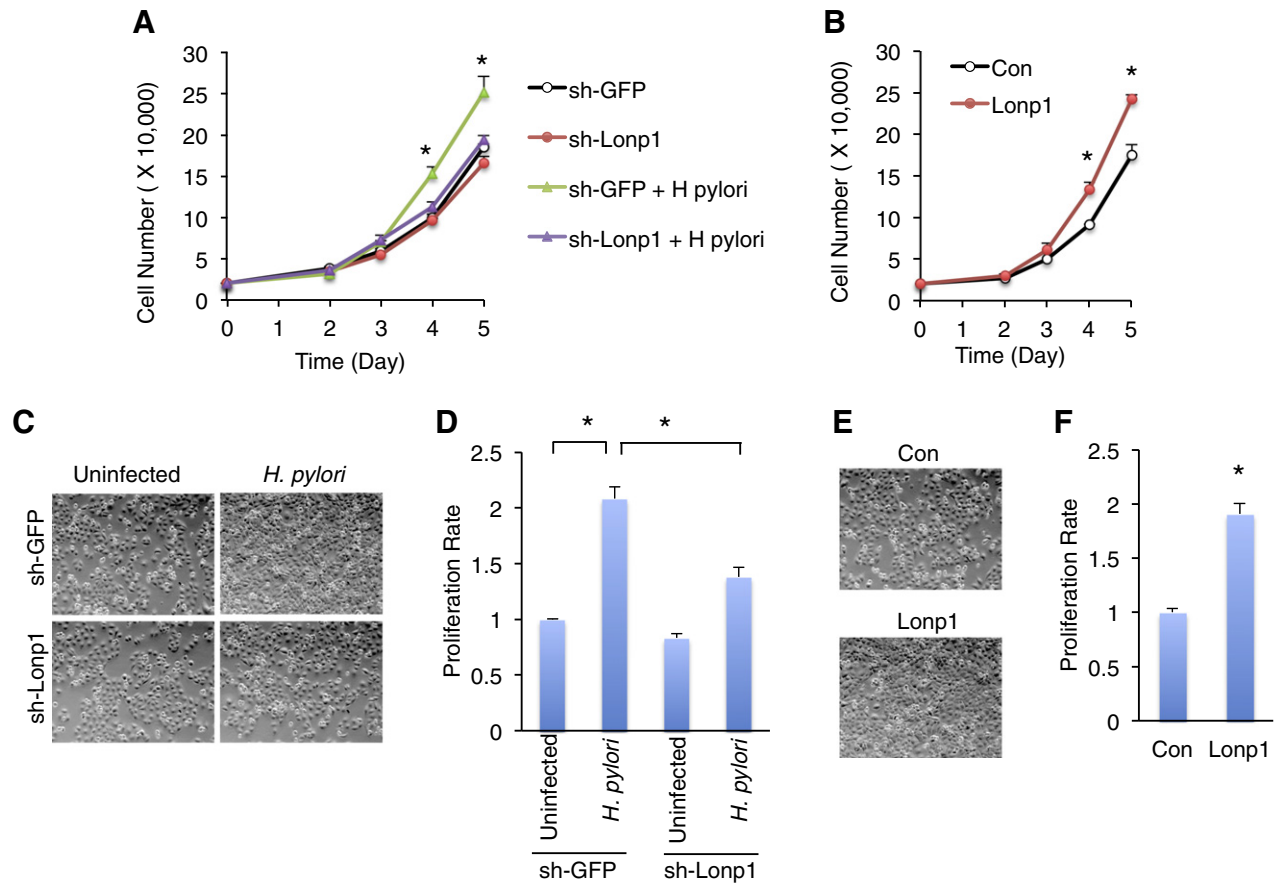


**Figure 5.** Lonp1 is required for low multiplicity of *H. pylori*-induced gastric cell proliferation. (A-B) Cell growth curves of control (sh-GFP) and Lonp1-deficient (sh-Lonp1) MKN28 cells in response to low multiplicity of *H. pylori* infection (MOI = 50, NCTC 11637) (A) and Lonp1-overexpressing MKN28 cells (B). (C-D) Images (C) and proliferation rates indicated by MTT assays (D) of control (sh-GFP) and Lonp1-deficient (sh-Lonp1) MKN28 cells in response to low multiplicity of *H. pylori* infection at day 5 (MOI = 50, NCTC 11637). (E-F) Images (E) and proliferation rates indicated by MTT assays (F) of control (Con) and Lonp1-overexpressing (Lonp1) MKN28 cells. Each group was compared with sh-GFP (A) or Con (B) at each time point in growth curve experiment. Data represent the mean ± SEM from three separate experiments. * $P < .05$.
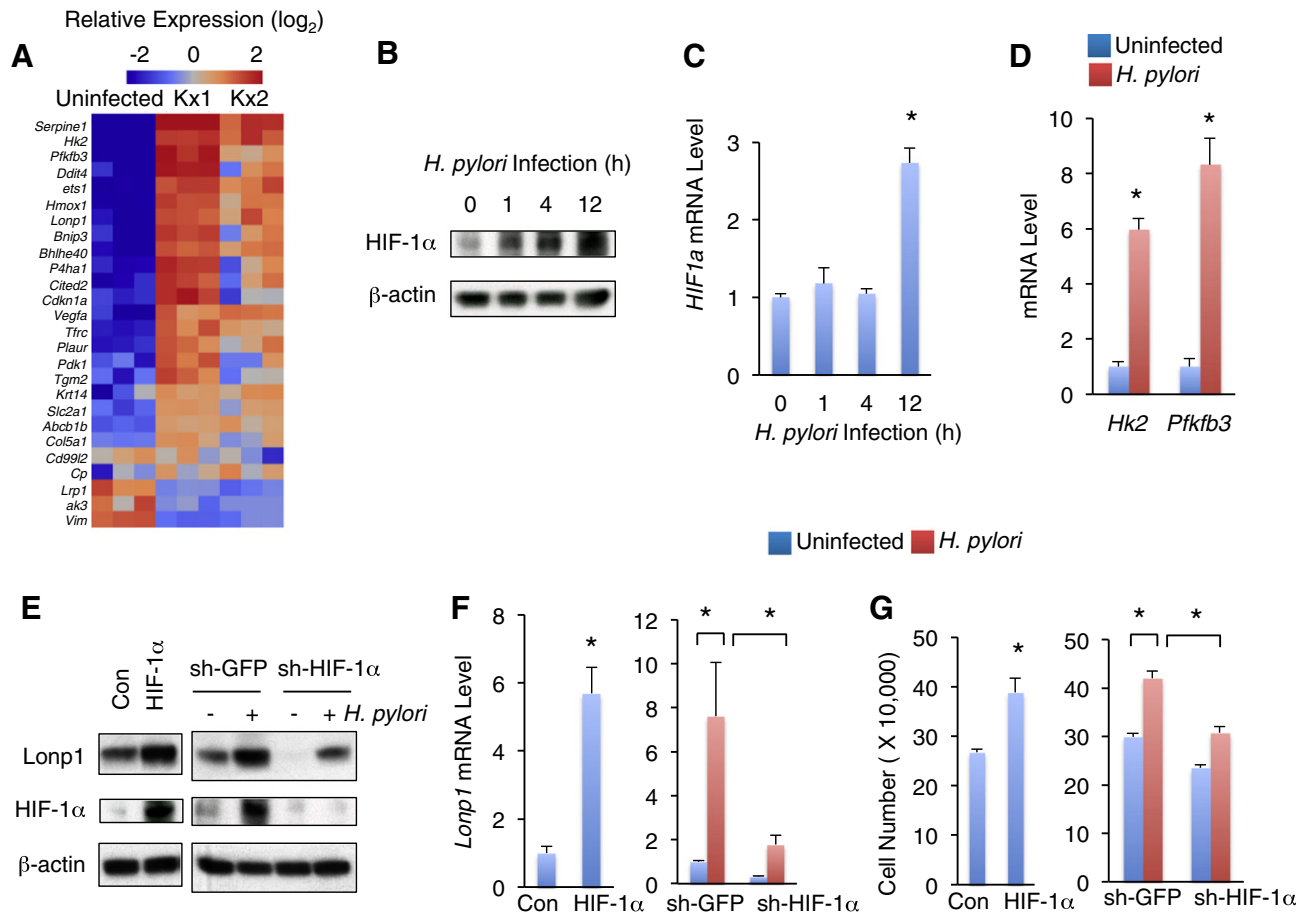
**Figure 6.** HIF-1α contributes to low MOI of *H. pylori*-induced Lonp1 induction and gastric cell proliferation. (A) Heatmap showing differential expression of known HIF-1α targets in Kx1 and Kx2 *H. pylori* infection models. (B-C) HIF-1α protein levels indicated by western blot (B) and *HIF1a* mRNA levels indicated by qPCR (C) in MKN28 gastric cells in response to low multiplicity of *H. pylori* infection at different time points (MOI = 50, NCTC 11637). (D) *Hk2* and *Pfkfb3*, two HIF-1α targets, in MKN28 cells were transcriptionally elevated by low multiplicity of *H. pylori* infection (MOI = 50, NCTC 11637) for 12 hours. (E-F) Lonp1 protein levels (E) and mRNA levels (F) in HIF-1α-overexpressing MKN 28 cells (left) or HIF-1α-knockdown MKN28 cells in response to low multiplicity of *H. pylori* infection for 12 hours (MOI = 50, NCTC 11637) (right). (G) Cell growth of control and HIF-1α-overexpressing MKN28 gastric cells (left) or HIF-1α-knockdown cells in response to low multiplicity of *H. pylori* infection (right). Data represent the mean ± SEM from three separate experiments. * *P* < .05.

suggesting that *H. pylori* infection elevates HIF-1α expression, at least, via post-transcriptional regulation. Consistent to HIF-1α protein level, two HIF-1α target genes, *Hk2* and *Pfkfb3*, were dramatically up-regulated in MKN28 gastric cells in response to *H. pylori* infection (Figure 6D).

Then we clarified the HIF-1α role in Lonp1 transcriptional regulation in gastric epithelial cells by overexpressing HIF-1α. Interestingly, lenti-viral-induced HIF-1α overexpression significantly induced Lonp1 expression in MKN28 cells at both mRNA and protein levels (Figure 6, E–F, left). To confirm whether HIF-1α directly regulates Lonp1 transcription in gastric cells, we performed luciferase report assay by transfecting luciferase reporter plasmids that contained *Lonp1* promoter regions into HIF-1α overexpressing or control MKN28 gastric cells. We found that HIF-1α dramatically enhanced *Lonp1* promoter activity (Figure S6), suggesting HIF-1α directly regulates Lonp1 transcription. Similar to Lonp1 proliferative roles, HIF-1α overexpression also promoted MKN28 gastric epithelial cell growth (Figure 6G, left).

We therefore assessed whether HIF-1α is essential for Lonp1 expression and gastric epithelial cell proliferation in response to *H. pylori* infection by knocking down HIF-1α expression (Figure 6E). Strikingly, *H. pylori*-induced Lonp1 expression at both mRNA and protein levels were dramatically, but not completely, diminished in HIF-1α knockdown cells (Figure 6, E–F). In addition, HIF-1α knockdown also significantly alleviated *H. pylori*-induced gastric cell proliferation (Figure 6G, right). Taken together, our results demonstrated that HIF-1α is required for Lonp1 expression and cell proliferation in response to *H. pylori* infection.

## Discussion

Differential gene expression has been shown to link to various biological processes, including aging, stem cell proliferation, metabolism and tumorigenesis, across species [30–33]. Using bioinformatics method to unify the representation of gene-related biological processes and molecular regulation will help us systemically understand molecular modulation under diverse physiological

conditions. The mechanism how *H. pylori* infection results in gastric carcinogenesis is still not fully understood. Thus, in this study, to get a comprehensive understanding of *H. pylori*-induced molecular change regarding gastric carcinogenesis, we analyzed transcriptional prolife in a *H. pylori*-infection gastric cancer model. Even though the dataset has been published and Gordon and his colleagues have hypothesized that some unique genes, which were induced only in cancer-associated (Kx2) *H. pylori*-infected mouse gastric cells, might contribute to gastric carcinogenesis [16], we surprisingly found that most of deregulated genes in Kx2 infected cells were also included in deregulated genes in ChAG-associated (Kx1) infected cells (Figure 1, A–B). Our GO enrichment analysis further indicated that no additional GO terms were significantly enriched in Kx2-associated gene expression compared to Kx1, suggesting cancer-associated *H. pylori* infection failed to cause additional changes in biological process or cellular compartment as compared with ChAG-associated *H. pylori*. Thus, we hypothesized that *H. pylori*-induced gastric carcinogenesis relies on the cumulative effects of chronic stress response in gastric cells. Then we focused on the overlapping deregulated genes in both Kx1 and Kx2 models and uncovered that genes associated to UPR$^{mt}$, glycolysis, as well as HIF-1α targets, were significantly increased in mouse gastric epithelial cells. The similar regulation was further confirmed in human gastric cells in response to *H. pylori* infection and then shed the light on our molecular mechanism investigation.

*H. pylori* infection has been shown to cause mitochondrial dysfunction or perturbation in gastric epithelial cells in a dose-dependent manner [34,35]. Although severe mitochondrial dysfunction is detrimental, the salutary effects of mild mitochondrial perturbation have been reported in multiple organisms [17,36–38]. Consistent with this notion, we observed that low MOI of *H. pylori* promotes, but high MOI of *H. pylori* suppresses, gastric cell proliferation, suggesting the homeostatic interaction of mitochondrial injury and compensatory protection effects. Growing evidence suggested that UPR$^{mt}$ signaling networks, which induce expression of chaperons and proteases to maintain mitochondrial quality and function, could be a contributing factor. For example, mitochondrial heat shock proteins, HSP60/70/90, and protease, Clpp & Lonp1, have been implicated in cancer cell protection from stresses [9,14,39–42]. The compensatory UPR$^{mt}$ signaling might provide novel insights into the molecular mechanisms of gastric cell overproliferation associated with low multiplicity of *H. pylori* infection. In this study, we first characterized that mitochondrial protease Lonp1 is induced by *H. pylori* infection in gastric epithelial cells in a dose- and time-dependent manner. Furthermore, consistent with previous reports of Lonp1 role in mitochondrial maintenance and glycolysis switch [14,15], we demonstrated that Lonp1 is essential for mitochondrial restoration, metabolic shift to glycolysis and gastric cell over-proliferation associated with low multiplicity of *H. pylori* infection (Figure 7). Our findings manifested the novel molecular mechanisms by which *H. pylori* infection promotes gastric carcinogenesis and offered potential therapeutic opportunities for *H. pylori*-induced gastric cancer by targeting Lonp1.

UPR$^{mt}$ signaling as well as Lonp1 function have been shown to contribute to delicate modulation of mitochondrial activity and hemostatic transition between oxidative and glycolytic metabolism [14,17]. In this study, we have found that both overexpression and knockdown of Lonp1 promote glycolytic switch, including glucose incorporation and lactate production, in gastric epithelial cells
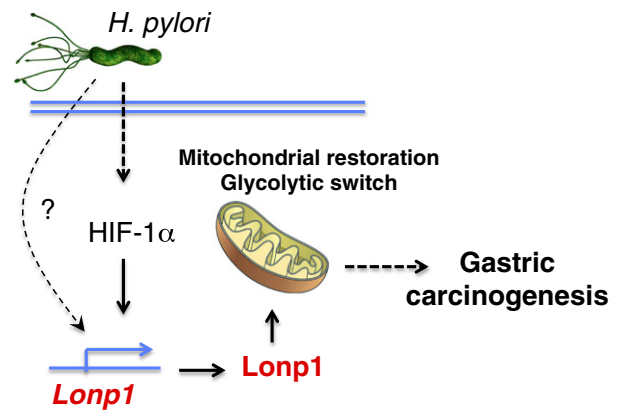


**Figure 7.** Schematic of molecular mechanism of Lonp1 induction and gastric carcinogenesis in response to *H. pylori* infection. Low multiplicity of *H. pylori* infection increases Lonp1 expression in gastric cells via HIF-1α regulation and other unknown mechanisms. Subsequently, excess Lonp1 results in mitochondrial restoration and metabolic switched toward glycolysis and contribute to gastric cell overproliferation and gastric carcinogenesis.

(Figure 4). The similar regulation has been coincidently observed in López-Otín's work [14]. Their results demonstrated that Lonp1 overexpression triggers metabolic reprogramming and promotes cellular glycolytic switch via gene expression regulation. Meanwhile, mitochondrial dysfunction caused by genetic or pharmaceutical manipulation results in a compensatory increase in glycolysis to maintain energy homeostasis [43–45]. We've exhibited that Lonp1 knockdown decreased mitochondrial mass and oxygen consumption in gastric cells, thus we speculated that the glycolytic increase in Lonp1 knockdown cells could be an obligate consequence of the mitochondrial dysfunction.

HIF-1α is an important hypoxia responsive factor and is found to enhance Lonp1 expression in HeLa cells [28]. *H. pylori* infection also stabilizes HIF-1α and increases its activity in gastric cells [7]. In this study, we confirmed that HIF-1α enhances Lonp1 expression in response to *H. pylori* infection, as HIF-1α overexpression potently increased Lonp1 expression, whereas HIF-1α knockdown significantly diminished Lonp1 induction associated with *H. pylori* infection. However, we observed that knockdown of HIF-1α failed to completely abolish Lonp1 induction in response to *H. pylori* infection, suggesting other unknown regulators are also involved in Lonp1 induction. UPR$^{mt}$ transcriptional factor CHOP/C/EBPβ is not likely to regulate Lonp1 expression, as its cognate binding elements are absent in *Lonp1* promoter region [46]. Interestingly, Lonp1 expression is robustly induced by PERK signaling in response to ER stress [47]. Since transmission of cell stress from ER to mitochondria is largely unknown in *H. pylori*-induced gastric carcinogenesis, it is worth studying how PERK is involved in Lonp1 induction in response to *H. pylori* infection in our future study.

In addition to *Lonp1*, other UPR$^{mt}$ responsive genes were also significantly induced in *H. pylori* infected gastric epithelial cells (Figures 1D and S3), indicating these genes and their associated biological processes might be involved in *H. pylori*-induced gastric carcinogenesis. *Didt3* and *Cebpb* encode CHOP and C/EBPβ, respectively, to form hetero-dimers and activate transcription of UPR$^{mt}$ responsive genes [46]. *Hmox1* encodes a heme oxygenase and exhibits anti-oxidation effects [48]. *Hspa9* and *DNAJA3* have been shown to encode HSP70 and

HSP40, respectively, and *Clpp* encodes a mitochondrial protease to maintain mitochondrial proteostasis and activity [9]. Thus, in the future we will characterize the roles of each of these UPR$^{mt}$ responsive genes in *H. pylori*-induced gastric cell proliferation and uncover more potential mechanism(s) for gastric carcinogenesis.

## Material and Methods

### Bioinformatics Analysis of Microarray Data

Microarray data was obtained from NCBI-GEO (http://www.ncbi.nlm.nih.gov/geo/) (GSE10262). Expression levels of probes were calculated using RMA normalization and mapped in CDF version Affymetrix Mouse 430_2. Genes with fold change > 1.5, false discovery rate (FDR) < 0.05 were considered as significant. All comparisons were made between infected and uninfected. The significantly up- and down-regulated genes were uploaded separately to DAVID Bioinformatics Resource (https://david.ncifcrf.gov). The mouse genome 430 2.0 was used as a background for the GO analysis. The GO terms with EASE score < 0.05 were selected for interpretation [49].

### Cell Culture, Bacteria Culture and Reagents

MKN28 human gastric epithelial cell line (JCRB Cell Bank, JCRB0253) was a kind gift from Cyril Benes. MKN28 cells were cultured in growth medium, RPMI-1640 medium containing 10% FBS (Invitrogen) and 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C with 100% humidity and 5% $CO_2$. *H. pylori* strains NCTC 11637 (ATCC 43504), 26695 (ATCC 700392), J99 (ATCC 700824), BCM-300 (ATCC BAA-1606) and BCS 100 (ATCC BAA-945) were obtained from ATCC and were grown in *Brucella* broth with 5% FBS at 37°C. For *H. pylori* infection assays, bacteria cells were harvested by centrifugation at 8000 rpm for 5 minutes and added to gastric cells at multiplicity of infection (MOI) of bacteria to cell from 25:1 to 500:1. Lonp1 polyclonal antibody (HPA002192), β-actin monoclonal antibody (A5441), and pLKO.1-puromysin shRNA lentiviral vectors for *Lonp1* (TRCN0000291803) and *HIF-1α* (TRCN0000003810) were purchased from Sigma. ATP5A monoclonal antibody used for tracking mitochondria (ab14748) was obtained from Abcam. HIF-1α antibody was purchased from Novus Biologicals (NB100-105). Lentiviral overexpression vector pLJM1-EGFP (19319) and pcDNA3 vector containing full-length HIF-1α (18949) were obtained from Addgene. pCMV6-XL4 vector containing full-length Lonp1 (SC117111) was purchased from Origene.

### Lentivirus-Induced RNAi and Overexpression

HIF-1α and Lonp1 were subcloned from commercial vectors containing full-length cDNAs into lentiviral pLJM1-EGFP vector. pLKO.1-puromysin vectors containing shRNAs and pLJM1-EGFP-puromysin overexpression vectors were used for knocking down and overexpressing target genes (Lonp1 and HIF-1α), respectively. Lentivrius packaging was performed as previously described [50]. For lentivrius transduction, MKN28 cells at 50% confluence were incubated with packed viruses in growth medium containing 8 μg/mL Polybrene (Sigma) overnight. Infected cells were cultured for 48 hours and then selected with 3 μg/mL puromycin for 72 hours to generate stable cells. Knockdown efficiency and overexpression was further confirmed with western blot.

### Real-Time Quantitative PCR

Cells were washed three times with PBS and immediately homogenized in Trizol (Invitrogen) and processed through alcohol precipitation according to the manufacturer's instructions. RNAs were dissolved in nucleas-free water (Ambion) and were quantified using a NanoDrop spectrophotometer. cDNA was synthesized from 1 μg of total RNA using iScript cDNA Synthesis Kit (BioRad). qPCR was performed in 20 μL reaction that contains 10 μL iQ™ SYBR Green Supermix (2X), 250 nM forward or reverse primer and 10 ng cDNA. The following program was run in CFX96 Real-time PCR system (Bio-Rad):

| Polymerase activation and DNA denaturation at 95°C | Amplification | | | Melt curve analysis 55-95°C 0.5°C increment |
| | Denaturation at 95 °C | Annealing/extension + plate read at 60°C | Cycles | |
| --- | --- | --- | --- | --- |
| 3 min | 15 s | 30 s | 40 | 3 s/step |

Relative mRNA level (target gene mRNA/*Gapdh* mRNA) was calculated by a comparative Ct method, using the following equation: target gene mRNA/*Gapdh* mRNA = $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{target\ gene} - Ct_{Gapdh}$. The following primers for each gene with 90% to 110% efficiencies were used:

*Lonp1*-forward: CCTGACTGCAGAGATCGTGA
*Lonp1*-reverse: CCCATGTCGCTCAGGTAGAT
*Ddit3*-forward: CAGAACCAGCAGAGGTCACA
*Ddit3*-reverse: AGCTGTGCCACTTTCCTTTC
*Cebpb*-forward: CTCGCAGGTCAAGAGCAAG
*Cebpb*-reverse: GACAGCTGCTCCACCTTCTT
*Hmox1*-forward: CTCAAACCTCCAAAAGCC
*Hmox1*-reverse: TCAAAAACCACCCCAACCC
*Hspa9*-forward: GGACTATCGCTCCATGCCAA
*Hspa9*-reverse: CTTTACTTGGGGCTCTGCCA
*Clpp*-forward: CTCATTCCCATCGTGGTGGA
*Clpp*-reverse: GATAACAAGGCTGGCAACGC
*DNAJA3*-forward: GGTGTCAGCCTTACAGGAAGAT
*DNAJA3*-reverse: ACCCCCTTTGCAGCTTGATT
*Htra2*-forward: AGAGTTTCTGCATCGTGGGG
*Htra2*-reverse: AGACCAGCCCTGGGACTC
*Hsp90ab1*-forward: ATGGAAGAGAGCAAGGCAAA
*Hsp90ab1*-reverse: GCAGCAAGGTGAAGACACAA
*Hif1a*-forward: CTGACCCTGCACTCAATCAA
*Hif1a*-reverse: TCCATCGGAAGGACTAGGTG
*Hk2*-forward: ACCCACATTCGAGTTGGAAG
*Hk2*-reverse: CAGTGGGTTGTCATGAGTGG
*Pfkfb3*-forward: GACAAATGCGACAGGGACTT
*Pfkfb3*-reverse: TAGTACACGATGCGGCTCTG
*Gapdh*-forward: CAGCCTCAAGATCATCAGCA
*Gapdh*-reverse: TGTGGTCATGAGTCCTTCCA

### Luciferase Assay

*LONP1* promoter sequences were amplified from genomic DNA of MKN28 cells by PCR using the following primers with MluI and BglII restriction sites, digested, and ligated into pGL3-Basic luciferase report vector (Promega), which encodes firefly luciferase. HIF-1α stable or control cells were transfected with firefly luciferase reporter plasmid (200 ng) and control reporter plasmid pAct-Renilla (40 ng), which encodes Renilla luciferase, using Effectene (Qiagen). The ratio of firefly to Renilla luciferase activity was determined using the Dual-Luciferase Assay System (Promega).

−435/−415-Forward:  GCTCTTACGCGTGGGAGTCGC
TGCACAATCCGA

−338/−318-Forward:  GCTCTTACGCGTCTCCGCCTG
AATCTTGAGCAT

+1/+22-Forward:  ATGCTCTTACGCGTAGCTCCCTGAAG
CGGCTGTTTC

+71/+92-Reverse:  TTACTTAGATCTTCATTTCCGCT
CGCCGCGAAAC

### Western Blot

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate) containing protease inhibitor and phosphatase inhibitor cocktails (ThermoFisher). 40 μg total proteins were separated by SDS-PAGE gel electrophoresis and transferred to PVDF membranes. Total protein levels were assessed by western blot using primary antibodies and HRP-conjugated secondary (1:3000) antibodies.

### Immunostaining

Cells were planted and infected with or without *H. pylori* in glass chamber slides and were washed three times with PBS. Then cells were fixed with 4% paraformaldehyde in PBS for 30 minutes and washed with PBS containing 0.2% Triton-X (PBST). After being blocked with PBST containing 1% BSA, cells were immunostained with monoclonal anti-ATP5A antibody (1:500) overnight at 4°C. Slides were washed and incubated with AlexaFluor 488-conjugated secondary antibody (1:500, Invitrogen) and DAPI (1:5000) for 1 hour at room temperature and mounted for confocal imaging.

### mtDNA Analysis

Similar to qPCR, mtDNA was also qualified using iQ™ SYBR Green Supermix with CFX96 Real-time PCR system (Bio-Rad). Total DNA was used as a template and relative mtDNA copy numbers were assessed after mitochondrial gene *Nd1* normalization by the single-copy nuclear gene *Pk*. Relative mtDNA copy number (mtDNA amount/nDNA amount) was calculated by a comparative Ct method, using the following equation: mtDNA/nDNA=$2^{-\Delta Ct}$, where $\Delta Ct = Ct_{mitochondrial} - Ct_{nuclear}$. The primers with 90% to 110% efficiencies are listed like below.

*Nd1*-forward: CCCTAAAACCCGCCACATCT
*Nd1*-reverse: GAGCGATGGTGAGAGCTAAGGT
*Pk*-forward: AGCCCAAATGGCCTTGAA
*Pk*-reverse: AGAGACAGAATGCCAGTGAGC

### Lactate, Glucose and ATP Measurements

Cells seeded in triplicated in 24-well plates were infected with or without *H. pylori* for 24 hours. After that, the cells were washed and lysed with passive lysis buffer (Promega). The lysates were centrifuged at 12,000 rpm at 4°C for 5min, and supernatants were collected. The protein concentration of the supernatant was measured with Bradford kit (Sigma). The ATP level of supernatant was determined using the ATP determination kit (Invitrogen) according to the instructions in a SpectraMax plate reader (Molecular Devices) and normalized to protein levels. The media were collected and the content of lactate was determined using lactate assay kit (Sigma) and normalized to cell protein levels. As for glucose incorporation, the cells were infected with or without *H. pylori* for 24 hours in growth medium containing 1 μCi/mL 2-deoxy-D-[$^3$H] glucose (PerkinElmer). Then, the cells were washed and lysed in 0.4 mL 1% Na-dodecyl sulfate. Intracellular [$^3$H] glucose

content was determined in 4 mL scintillant using a Beckman scintillation counter and normalized to cell protein level.

### Mitochondrial Oxygen Consumption Analysis

After *H. pylori* infection for 24 hours, mitochondrial oxygen consumption in intact cells was measured using a Seahorse Bioscience extracellular flux analyzer according to manufacturer's instructions. Briefly, after 15 minutes equilibration, three measurements of 3 minutes were performed, separated by 3 minutes of mixing. Maximal membrane potential was assessed by adding 1 μM oligomycin, and uncoupled mitochondrial respiration was induced with 1 μM CCCP. Both 1 μM rotenone and antimycin were used to stop the mitochondrial oxygen consumption.

### Cell Proliferation Assay

Tissue culture plates were coated with Matrigel™ Matrix (BD) containing growth factors at 37°C for 1 hour. Then, 20,000 cells were planted per well on Matrigel™ matrix and 12 hours later were infected with or without *H. pylori*. After trypsinization, cells were re-suspended in an equal volume of growth medium containing 0.05% trypan blue. The number of live cells in triplicated wells that excluded trypan blue was counted for 5 days to generate cellular growth curve. MTT assay was also used for measuring cell proliferation rate. Briefly, after 5-day cell growth with or without *H. pylori* infection, cells were incubated with 1 mg/mL MTT (Roche) in growth medium for 2 hours at 37°C and then with extraction buffer (20% SDS, 50% dimethylformamide) overnight at 37°C. Absorbance was measured at 590 nm in SpectraMax plate reader and the proliferation rate was normalized to control cells.

### Statistical Analysis

All experimental results are indicated as mean ± error bars represent SEM. Unpaired Student's t test and one-way ANOVA followed by post-hoc test were performed to assess the differences. $P < .05$ was considered statistically significant and shown with asterisks.

### Author Contributions

MH. P. and K. W. conceived and design the experiments. B. L., MG. W., NY. H. and X. H. performed most of the biochemical and cell proliferation experiments. GQ. J. and XP. Q. performed immunostaining and confocal imaging. XF. Z., Y. L. and K. L. performed cloning and *H. pylori* culture. W. S. performed bioinformatics analysis of microarray data. MH. P. and K. W. discussed the results and wrote the manuscript.

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