Bacterial CagA protein compromises tumor suppressor mechanisms in gastric epithelial cells

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Approximately half of the world's population is infected with the stomach pathogen *Helicobacter pylori*. Infection with *H. pylori* is the main risk factor for distal gastric cancer. Bacterial virulence factors, such as the oncoprotein CagA, augment cancer risk. Yet despite high infection rates, only a fraction of *H. pylori*–infected individuals develop gastric cancer. This raises the question of defining the specific host and bacterial factors responsible for gastric tumorigenesis. To investigate the tumorigenic determinants, we analyzed gastric tissues from human subjects and animals infected with *H. pylori* bacteria harboring different CagA status. For laboratory studies, well-defined *H. pylori* strain B128 and its cancerogenic derivative strain 7.13, as well as various bacterial isogenic mutants were employed. We found that *H. pylori* compromises key tumor suppressor mechanisms: the host stress and apoptotic responses. Our studies showed that CagA induces phosphorylation of XIAP E3 ubiquitin ligase, which enhances ubiquitination and proteasomal degradation of the host proapoptotic factor Siva1. This process is mediated by the PI3K/Akt pathway. Inhibition of Siva1 by *H. pylori* increases survival of human cells with damaged DNA. It occurs in a strain-specific manner and is associated with the ability to induce gastric tumor.

Introduction

Interaction between human cells and microbial agents significantly alters the intracellular homeostasis and causes various diseases, including cancer. *Helicobacter pylori (H. pylori)* is the main risk factor for gastric cancer, with almost 90% of new cases of noncardia gastric cancers attributed to this bacterium (1). More than half of the world's population is infected with *H. pylori* (2). However, even though *H. pylori* infection is very common, only a small fraction of infected individuals develop gastric cancer, suggesting complexity of tumorigenic interactions between bacteria and human cells.

Among bacterial factors associated with gastric carcinogenesis is the *cag* pathogenicity island (*cag* PAI) that encodes CagA and other components of a type IV secretion system (T4SS) (3, 4). Using the T4SS, bacteria inject the CagA protein into human gastric cells, where it is phosphorylated by host kinases causing alterations of multiple signaling pathways (3–5). Several studies pointed out the oncogenic function of CagA (3, 5, 6). *H. pylori* also causes strong cellular oxidative and genotoxic stresses, including induction of double strand breaks in DNA (7–9). Little is currently known about how these cellular stresses are resolved.

Siva1 protein is one of the important factors regulating cellular stress responses. It is a proapoptotic protein that is activated by both extrinsic and intrinsic apoptosis signaling pathways (10, 11). Cellular stresses cause upregulation of Siva1 protein, resulting in induction of apoptosis (12, 13). Siva1 has also apoptosis-independent functions in normal cells (14). p53 protein has several binding sites in the promoter of the *Siva1* gene and directly induces its transcription (12). However, the role of Siva1 remains controversial, as recent studies suggested that Siva1 has oncogenic properties. It was found that Siva1 facilitates non–small cell lung cancer in vivo (15). In addition, Siva1 can inhibit p14ARF and p53 tumor suppressors in certain conditions (16–18). The present study aimed to investigate the regulation of the cellular stress response and Siva1 protein in *H. pylori*–infected cells.

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Results

Siva1 protein is downregulated by H. pylori. Given the important role played by Siva1 in the regulation of key cellular processes, we investigated this protein in *H. pylori*–infected cells. We started our analyses exploring expression of Siva1 protein in *H. pylori*–infected mice. Mice were challenged with *H. pylori* strain PMSS1 (n = 8), which successfully colonizes the murine stomach (19). Control animals (n = 8) received Brucella broth. Following successful infection, gastric tissues were collected and analyzed for Siva1 protein using immunohistochemistry and Western blotting (Figure 1, A and B). In control animals Siva1 protein was primarily expressed in chief cells at the base of the oxyntic glands (Figure 1A). Pit epithelial cells also showed some staining. In the antrum, Siva1 expression was limited to the foveolar cells and, to a lesser extent, to epithelial cells located at the base of the gastric antral glands (Figure 1A). Some mucosal mesenchymal cells also showed staining. Comparing expression of Siva1 protein in infected and

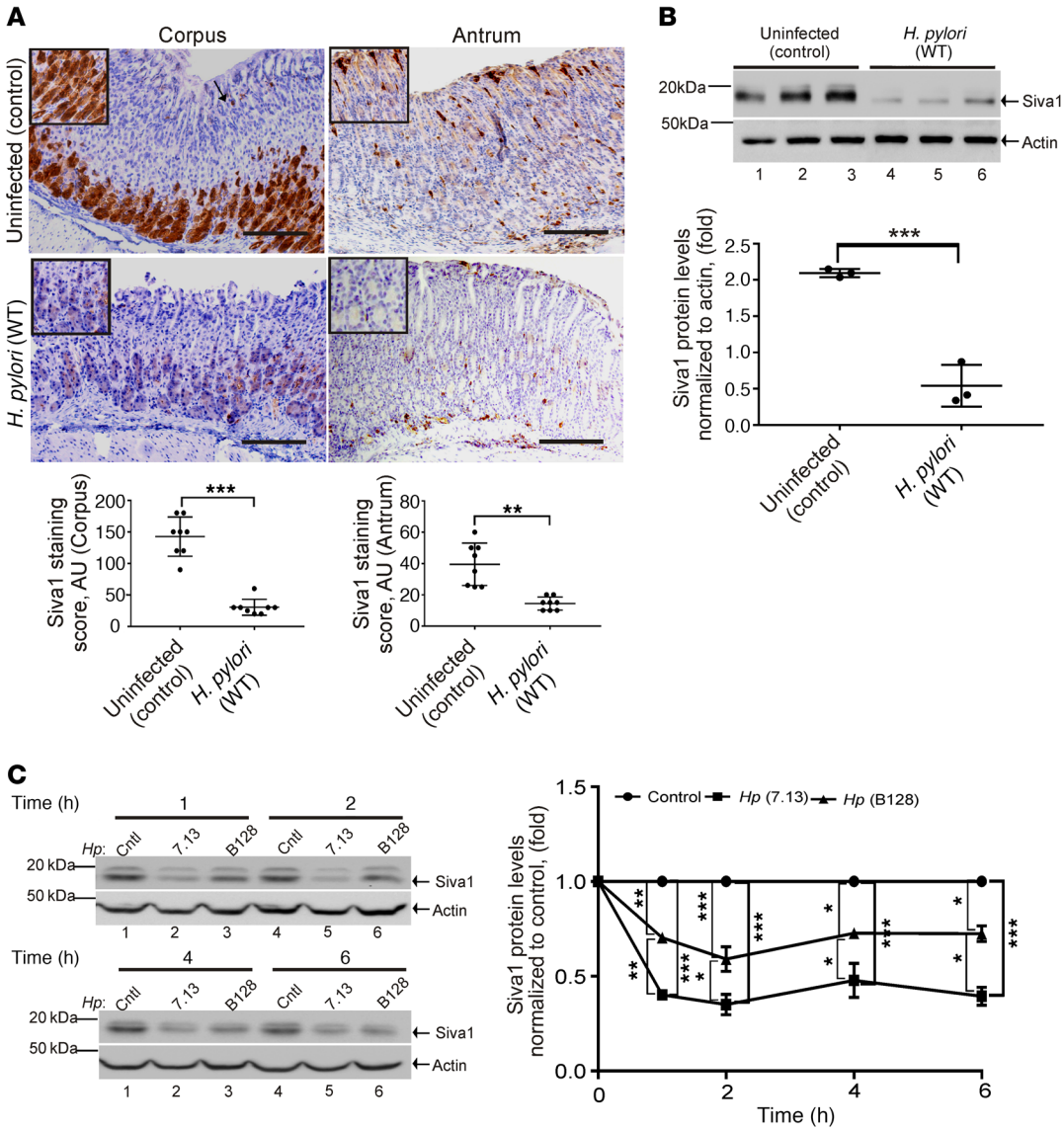

Figure 1. *H. pylori* infection leads to downregulation of Siva1. (**A**) Representative IHC staining for Siva1 protein in the corpus and antrum of uninfected and infected mice. Mice were infected with *H. pylori* strain PMSS1 for 8 weeks. Scale bars: 50 μm. Insets show magnified views, ×40. Histograms show IHC scores for Siva1 protein expression (n = 8/group). (**B**) Western blot analysis of Siva1 protein expression in gastric tissues collected from control and infected mice. Bottom panel shows densitometric analysis (n = 3/group). (**C**) Western blot analyses of Siva1 protein after coculture of AGS cells with *H. pylori* strains 7.13 and B128 for the indicated time. The graph panel shows quantification of Siva1 protein by densitometry, normalized to actin (n = 3). Expression of Siva1 protein at zero time point was arbitrarily set at 1. Data in **A** and **B** were calculated using unpaired 2-tailed t test; data in **C** were calculated using 1-way ANOVA followed by Tukey's multiple comparison test. Data are displayed as mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. For additional clarity, **A** and **B** are also shown in Figure 8B and Supplemental Figure 8B.

control animals, we found that infection with *H. pylori* leads to significant downregulation of Siva1 protein in the gastric mucosa compared with uninfected controls (Figure 1, A and B).

To analyze Siva1 protein in a more controlled environment, we carried out additional experiments in vitro. We took advantage of previously characterized *H. pylori* clinical isolate B128 and its oncogenic derivative 7.13 (20). The latter strain strongly activates cellular oncogenes, resulting in induction of premalignant and malignant gastric lesions in different rodent models (20, 21). AGS and SNU1 gastric epithelial cells were cocultured with *H. pylori* strains 7.13 and B128 for the indicated time and analyzed for expression of Siva1 protein. Similar to infected animals, protein levels of Siva1 were significantly downregulated after coculture with both *H. pylori* strains (Figure 1C and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI130015DS1). Notably, tumorigenic strain 7.13 was more potent in downregulation of Siva1 than its parental strain B128. Siva1 downregulation was also observed with another *H. pylori* clinical isolate J166 (Supplemental Figure 1B).

Siva1 protein regulates apoptosis in gastric epithelial cells infected with H. pylori. Previous studies have demonstrated that *H. pylori* infection causes apoptosis in infected patients (22, 23). Since Siva1 functions as a proapoptotic protein, we next asked whether Siva1 regulates the apoptosis response in *H. pylori*–infected cells.

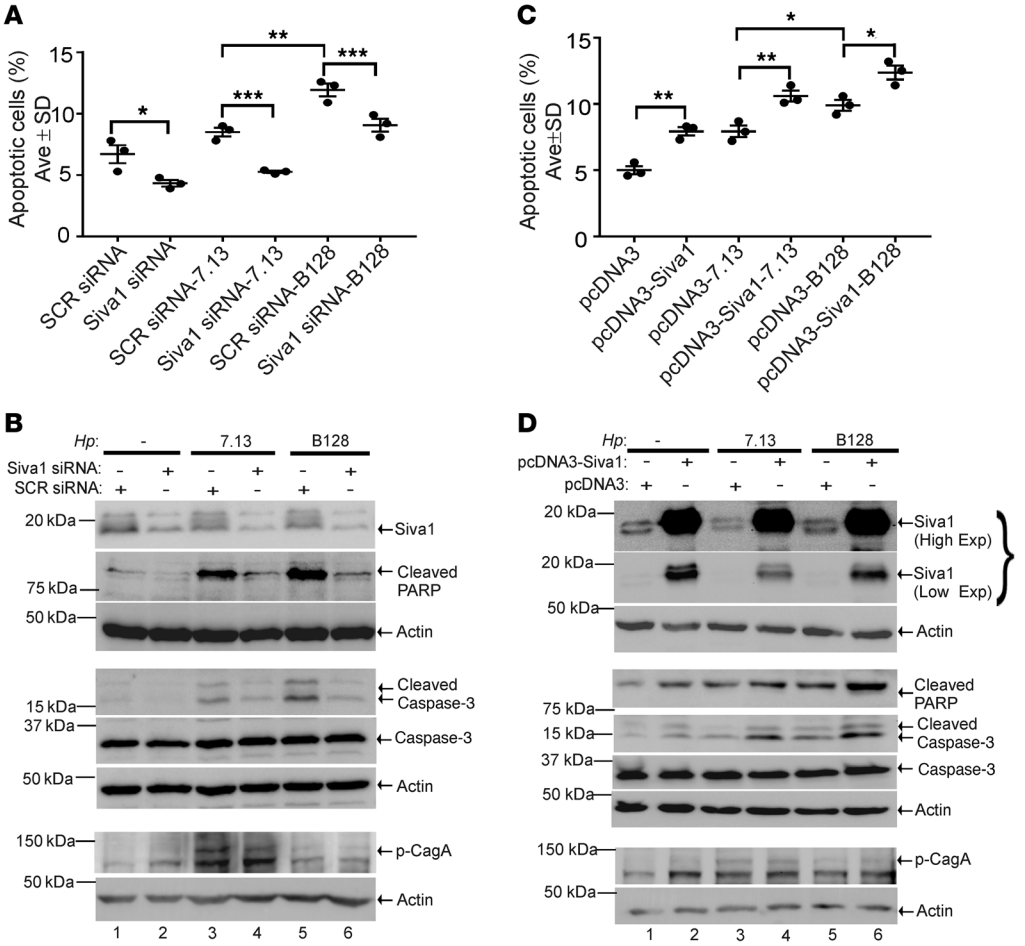

Figure 2. Siva1 protein regulates the apoptotic response in *H. pylori*–infected cells. (**A**) Flow cytometric analysis of apoptosis using Annexin V staining in AGS cells transfected with Siva1 siRNA or scrambled siRNA and then either left uninfected or cocultured with *H. pylori* strains 7.13 or B128 for 18 hours. The graph panel shows the percentage of apoptotic cells (n = 3). (**B**) The same as **A**, but Western blotting was used to analyze cleaved PARP1 and caspase-3 proteins in AGS cells. Cells transfected with scrambled siRNA were used as a control. (**C**) Flow cytometric analysis of apoptosis using Annexin V staining in AGS cells transfected with pcDNA3-FLAG-Siva1 expression plasmid or empty pcDNA3 vector and then either left uninfected or cocultured with *H. pylori* strains 7.13 or B128 for 18 hours. The graph panel shows the percentage of apoptotic cells. (**D**) The same as **C**, but Western blotting was used to analyze cleaved PARP1 and caspase-3 proteins in AGS cells. Cells transfected with empty vector (pcDNA3) were used as a control. Phosphorylation of CagA was analyzed after coculture of AGS cells with the indicated *H. pylori* strains for 2 hours. Statistical significance was calculated using 1-way ANOVA followed by Tukey's multiple comparison test. Data are displayed as mean ± SE and are representative of 3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Representative flow cytometry scatter plots are presented in Supplemental Figure 2. Flow cytometry and Western blot analyses were performed using the same transfected cells. Apoptosis in control cells was caused by transfection. Brackets indicate low and high exposures of Western blot images.

To answer this question, AGS cells, in which endogenous Siva1 protein was downregulated with specific siRNA, were cocultured with *H. pylori* strains 7.13 or B128 for 18 hours. Apoptosis was then measured by the Annexin V assay using flow cytometry (Figure 2A and Supplemental Figure 2A). Cleavage of PARP1 and caspase-3 proteins was also assessed in the same cellular lysates (Figure 2B). Our analyses showed that downregulation of Siva1 protein significantly decreases Annexin V staining, PARP1, and caspase-3 cleavage (Figure 2, A and B). Notably, *H. pylori* strain 7.13 delivered more CagA protein into host cells than strain B128, as indicated by a stronger tyrosine phosphorylation of CagA protein in cells infected with strain 7.13 (Figure 2, B and D). To validate our data, we conducted rescue experiments in which Siva1 was targeted with siRNA directed against the 3′UTR, followed by the reconstitution of Siva1 protein (Supplemental Figure 3A). We also stably expressed shRNA against Siva1 (Supplemental Figure 3B). These experiments further confirmed that downregulation of Siva1 consistently inhibits apoptosis induced by *H. pylori*.

In complementary experiments, gastric cells were transiently or stably transfected with Siva1-expressing or control plasmids, cocultured with *H. pylori* strains 7.13 or B128 for 18 hours, and then apoptosis was analyzed as discussed above. We found that enforced expression of Siva1 strongly augments apoptosis in infected cells (Figure 2, C and D, Supplemental Figure 2B, and Supplemental Figure 3C).

We also analyzed apoptosis and Siva1 protein using, respectively, TUNEL assay and immunohistochemistry in infected mice described above. Expression of Siva1 protein was found to closely correlate with TUNEL positivity (R^2 = 0.77–0.84; $P < 0.01$) (Supplemental Figure 4, A and B).

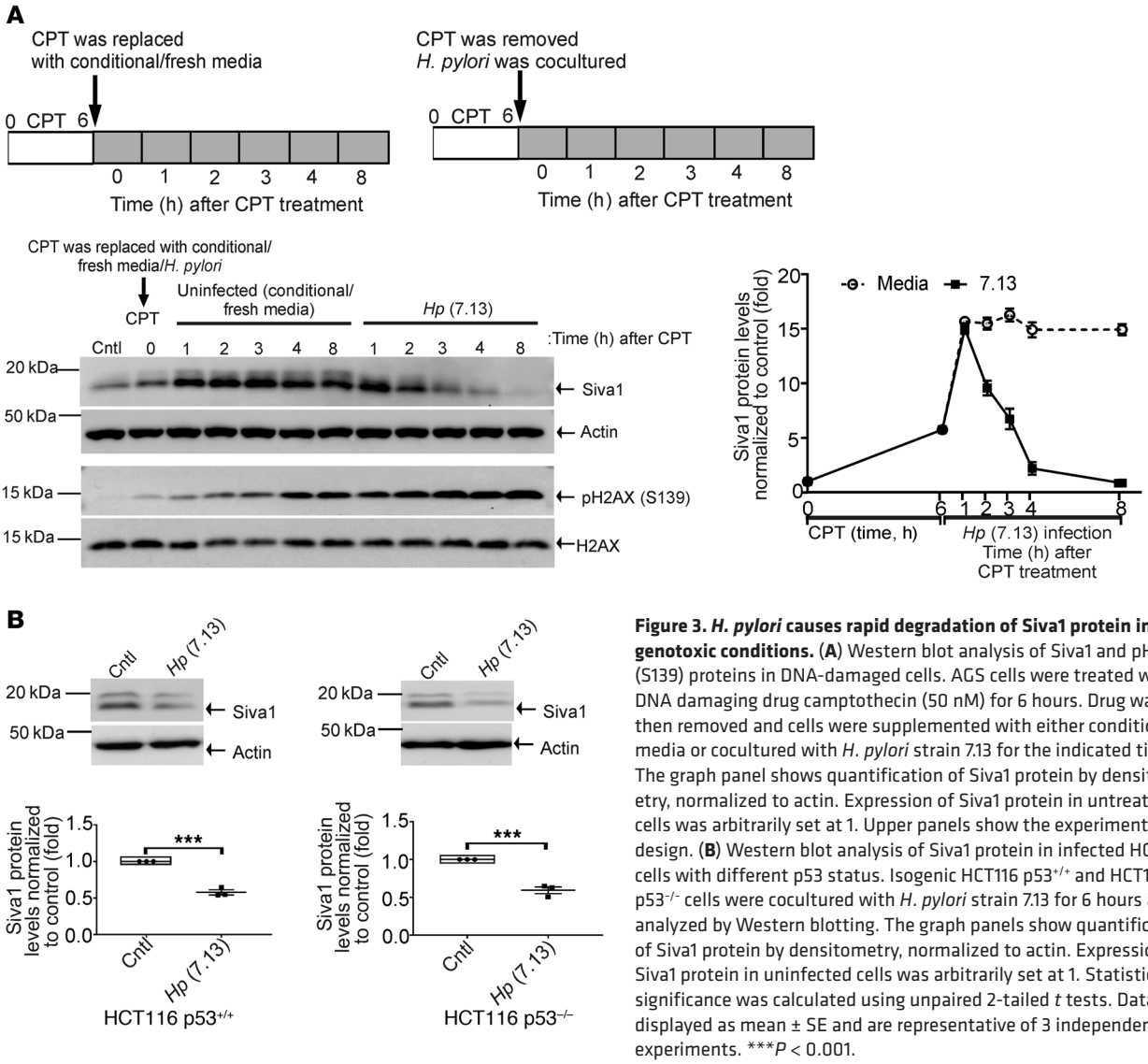

Figure 3. *H. pylori* causes rapid degradation of Siva1 protein in genotoxic conditions. (**A**) Western blot analysis of Siva1 and pH2AX-(S139) proteins in DNA-damaged cells. AGS cells were treated with DNA damaging drug camptothecin (50 nM) for 6 hours. Drug was then removed and cells were supplemented with either conditional media or cocultured with *H. pylori* strain 7.13 for the indicated time. The graph panel shows quantification of Siva1 protein by densitometry, normalized to actin. Expression of Siva1 protein in untreated cells was arbitrarily set at 1. Upper panels show the experimental design. (**B**) Western blot analysis of Siva1 protein in infected HCT116 cells with different p53 status. Isogenic HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells were cocultured with *H. pylori* strain 7.13 for 6 hours and analyzed by Western blotting. The graph panels show quantification of Siva1 protein by densitometry, normalized to actin. Expression of Siva1 protein in uninfected cells was arbitrarily set at 1. Statistical significance was calculated using unpaired 2-tailed *t* tests. Data are displayed as mean ± SE and are representative of 3 independent experiments. ***$P < 0.001$.

Taken together, our studies show that Siva1 protein is an important determining factor in the apoptotic response to infection.

H. pylori inhibits Siva1 under DNA damage conditions. It was previously demonstrated that *H. pylori* infection causes different types of DNA damage, including oxidative modifications and single and double strand breaks (7, 9, 24). To investigate how the DNA damage response is regulated in infected cells, AGS cells were treated with the DNA-damaging drug camptothecin (50 nM), which is known to induce Siva1 protein (12). Six hours after treatment, drug was removed and cells were cultured in the presence of either conditioned media or *H. pylori* strain 7.13 for the indicated time (Figure 3A). As expected, treatment with camptothecin induced Siva1 protein and caused DNA damage, as indicated by an increased phosphorylation of histone H2AX (pH2AX S139). In contrast to control uninfected cells in which Siva1 was upregulated, levels of Siva1 protein were rapidly decreased in infected cells even in the presence of strong DNA damage (Figure 3A and Supplemental Figure 5). Since Siva1 is a direct transcriptional target of p53 protein (12), which is strongly induced by DNA damage, we investigated the regulation of Siva1 by p53 in infected cells using previously described isogenic cell lines HCT116-$p53^{+/+}$ and HCT116-$p53^{-/-}$ (25). We found that *H. pylori* efficiently inhibits Siva1 in p53-null cells, suggesting that Siva1 is downregulated in a p53-independent manner (Figure 3B). Combined, our data implicate *H. pylori* in compromising the DNA damage response by inhibiting Siva1 protein.

H. pylori enhances ubiquitination and proteasomal degradation of Siva1 protein. The ubiquitin-proteasomal and autophagy-lysosomal pathways are crucial for protein homeostasis (26, 27). Both systems are altered in *H. pylori*–infected cells (6, 28). To define the mechanism by which *H. pylori* regulates Siva1, AGS cells were cocultured with *H. pylori* strain 7.13 or B128, treated with autophagy inhibitor bafilomycin A (100 nM) or proteasomal inhibitor MG132 (20μM) for 4 hours, and analyzed for Siva1 protein. The concentrations of inhibitors were selected based on previous reports showing no adverse effects on *H. pylori* activities (6, 28). Our analyses found that MG132, but not bafilomycin A, significantly inhibits downregulation of

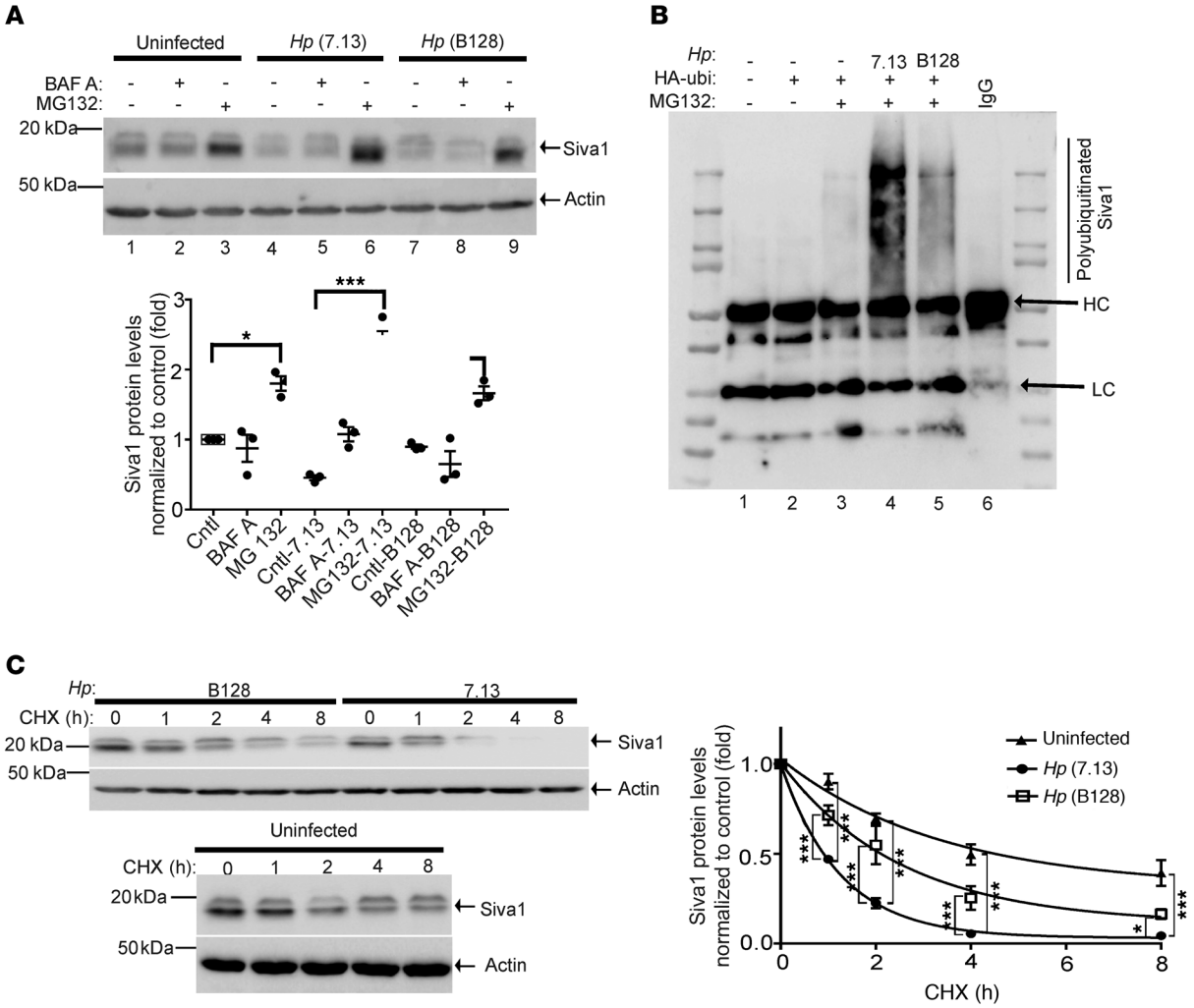

Figure 4. *H. pylori* infection induces ubiquitination and proteasomal degradation of Siva1 protein. (**A**) Western blot analysis of Siva1 protein in AGS cells cocultured with the indicated *H. pylori* strains and treated with MG132 (20 μM) or bafilomycin A (100 nM). The graph panel shows quantification of Siva1 protein by densitometry, normalized to actin. Expression of Siva1 protein in control uninfected cells was arbitrarily set at 1. (**B**) Western blot analysis of Siva1 protein ubiquitination in AGS cells transfected with ubiquitin expressing plasmid and treated as indicated at the top of the panel. Ubiquitination of Siva1 was analyzed with HA tag antibody after Siva1 protein immunoprecipitation. Immunoprecipitation with normal mouse IgG was used as a control (lane 6). (**C**) Analyses of Siva1 protein stability in infected cells. Stability of Siva1 protein was determined in AGS cells cocultured with the indicated *H. pylori* strains using the cycloheximide chase method (see Methods section). Levels of Siva1 protein were analyzed at the indicated time points. The graph panel shows quantification of Siva1 protein by densitometry, normalized to actin. Protein expression of Siva1 at zero time point was arbitrarily set at 1. Statistical significance was calculated using 1-way ANOVA followed by Tukey's multiple comparison test. Data are displayed as mean ± SE and are representative of 3 independent experiments. $^*P < 0.05$; $^{***}P < 0.001$.

Siva1 protein, suggesting that it is degraded in the proteasomes in infected cells (Figure 4A).

To further explore this possibility, we analyzed ubiquitination of Siva1 protein. AGS cells were transfected with a plasmid expressing HA-tagged ubiquitin, cocultured with *H. pylori* strains 7.13 or B128 for 1 hour, and treated with proteasomal inhibitor MG132 (20 μM) for an additional 8 hours. Siva1 was then immunoprecipitated with Siva1 antibody and analyzed by Western blotting with anti-HA antibody (Figure 4B). Our results showed that *H. pylori* significantly increases ubiquitination of Siva1 protein, and strain 7.13 is a stronger inducer of Siva1 ubiquitination than its counterpart strain B128 (Figure 4B).

To define how *H. pylori* affects the stability of Siva1 protein, cells were cocultured with the aforementioned *H. pylori* strains and incubated with cycloheximide (150 μM), which inhibits de novo protein synthesis. Levels of Siva1 protein were then analyzed at the indicated time intervals by Western blotting (Figure 4C). Consistent with data on the ubiquitination shown above, we found decreased stability of Siva1 protein in *H. pylori*–infected cells. The half-lives of Siva1 protein were estimated to be 48 and 105 minutes for infections with strains 7.13 and B128, respectively (Figure 4C). Collectively, our results demonstrate that *H. pylori* is able to induce proteasomal degradation of Siva1.

H. pylori activates XIAP E3 ubiquitin ligase. To identify E3 protein ligase(s) responsible for polyubiquitination of Siva1 protein in infected cells, we first analyzed Cbl-b and XIAP proteins that were previously reported to regulate Siva1 (18, 29). Cbl-b and XIAP E3 ligases were downregulated with siRNAs in AGS cells, which were

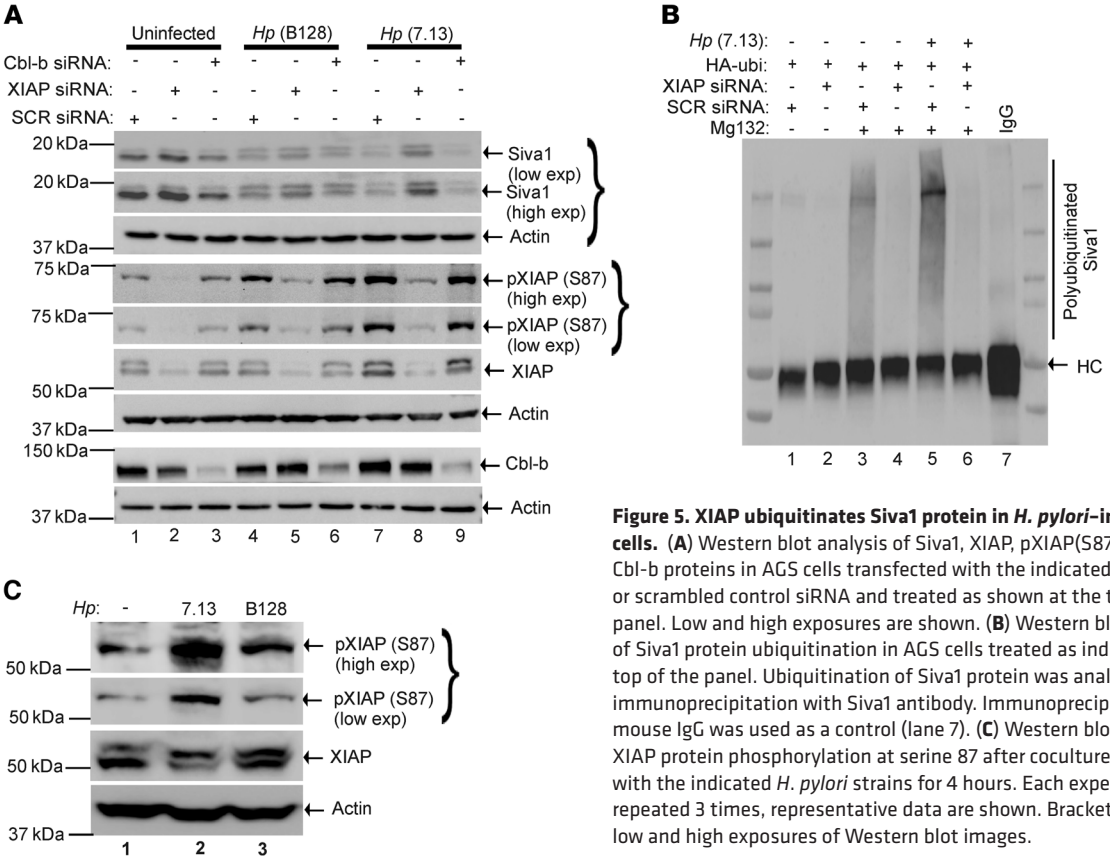

Figure 5. XIAP ubiquitinates Siva1 protein in *H. pylori*–infected cells. (**A**) Western blot analysis of Siva1, XIAP, pXIAP(S87), and Cbl-b proteins in AGS cells transfected with the indicated siRNA or scrambled control siRNA and treated as shown at the top of the panel. Low and high exposures are shown. (**B**) Western blot analysis of Siva1 protein ubiquitination in AGS cells treated as indicated at the top of the panel. Ubiquitination of Siva1 protein was analyzed after its immunoprecipitation with Siva1 antibody. Immunoprecipitation with mouse IgG was used as a control (lane 7). (**C**) Western blot analysis of XIAP protein phosphorylation at serine 87 after coculture of AGS cells with the indicated *H. pylori* strains for 4 hours. Each experiment was repeated 3 times, representative data are shown. Brackets indicate low and high exposures of Western blot images.

then cocultured with *H. pylori* and analyzed for expression of Siva1 protein. We found that inhibition of XIAP, but not Cbl-b, alleviates the inhibitory effect of *H. pylori* and leads to a marked upregulation of Siva1 protein (Figure 5A). This effect was especially robust in cells cocultured with *H. pylori* strain 7.13, which is more potent in inhibition of Siva1 than strain B128 (Figure 5A).

We validated our findings using rescue experiments in which XIAP was targeted with siRNA directed against the 3′UTR, followed by the XIAP reconstitution (Supplemental Figure 6A). These results were confirmed with siRNA and shRNA that target different regions of the XIAP transcript (Supplemental Figure 6, B and C). In addition, we found that overexpression of XIAP enhances degradation of Siva1 protein in infected cells (Supplemental Figure 7A).

To further investigate whether XIAP is required for degradation of Siva1, AGS cells were cotransfected with vector expressing HA-ubiquitin and XIAP siRNA, cocultured with *H. pylori* strain 7.13, and treated with proteasomal inhibitor MG132. Then, ubiquitination of Siva1 protein was assessed as described above. We found that downregulation of XIAP significantly inhibits polyubiquitination of Siva1 in infected cells (Figure 5B). These data were confirmed using additional siRNA, which targets a different region of the XIAP transcript (Supplemental Figure 7B).

We next asked whether *H. pylori* affects expression of XIAP protein. Comparison of infected and noninfected cells showed no significant changes in XIAP protein levels (Figure 5C). On the contrary, *H. pylori* strongly increased phosphorylation of XIAP protein at serine 87 in different cell lines. This effect was the most noticeable in cells infected with strain 7.13 (Figure 5C and Supplemental Figure 7C).

H. pylori activates the PI3K/Akt pathway to phosphorylate XIAP. Our characterization of XIAP as a regulator of Siva1 in infected cells led us to another important question: What are the mechanisms responsible for the XIAP activation? To answer this question, we performed a series of experiments using a panel of chemical inhibitors of various proteins that were previously reported to be activated by *H. pylori*: GSK-3β, MEK1/2, SRC, FAK, PKC, cAbl, Akt, PI3K, SHP2, and NF-κB. AGS cells were pretreated with each inhibitor (10 μM) for 1 hour, cocultured with *H. pylori* strain 7.13 for 4 hours, and analyzed by Western blotting with Siva1 antibody. Among tested compounds, only inhibitors of Akt and PI3K prevented degradation of Siva1 protein (Figure 6A). To confirm these findings and minimize off-target effects, PI3K was downregulated with siRNA. Cells were then cocultured with *H. pylori* strain 7.13 and analyzed for expression of the indicated proteins (Figure 6B). This set of experiments was repeated with siRNA against Akt1/2 (Figure 6C). Similar to chemical inhibitors, downregulation of PI3K or Akt inhibited phosphorylation of XIAP(S87) and prevented degradation of Siva1 protein. Given that PI3K is an upstream regulator of Akt, we determined activity of Akt protein by analyzing its phosphorylation at Thr308. We found upregulation of pAkt by *H. pylori* in AGS and SNU1 cells (Supplemental Figure 8A) Notably, strain 7.13 was a stronger activator of Akt than strain B128. Thus, Akt kinase is responsible for activation of XIAP protein.

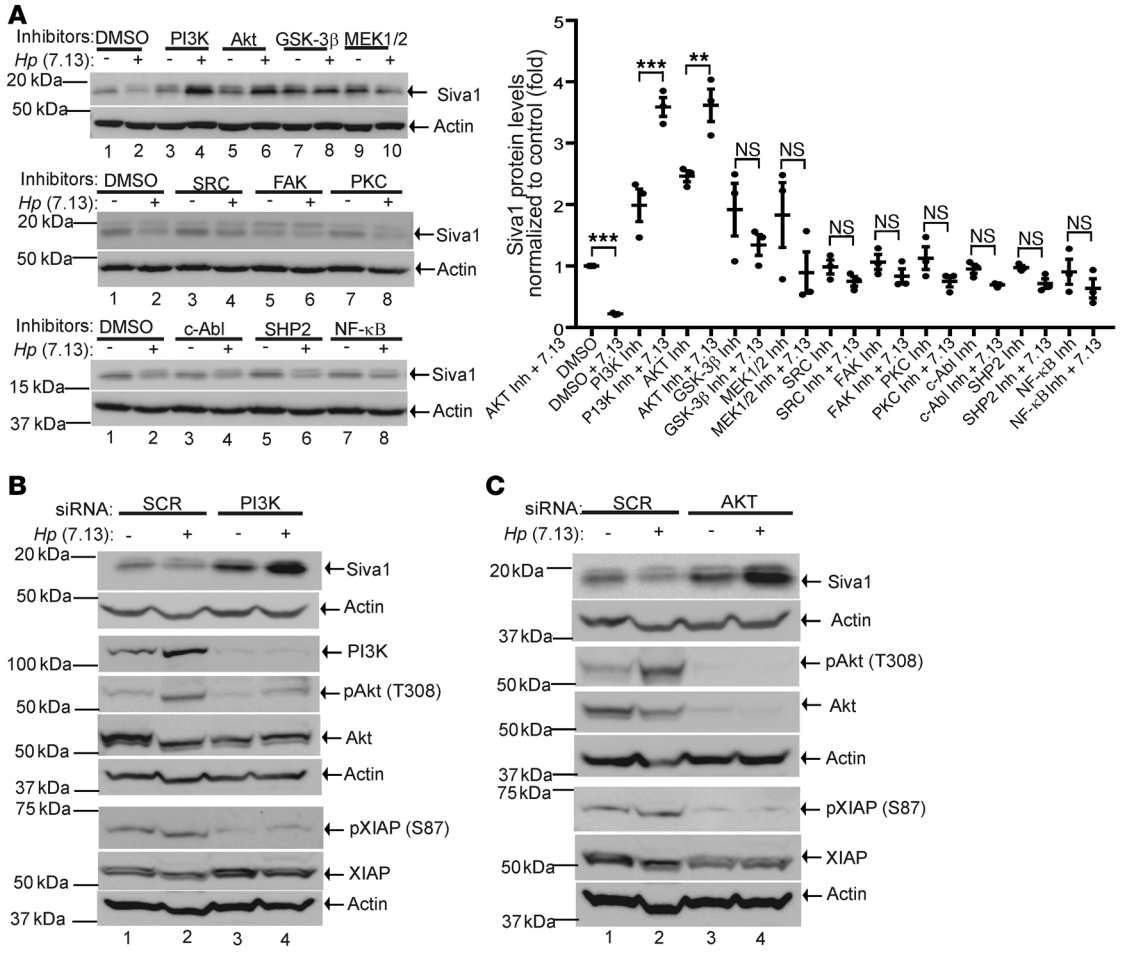

Figure 6. Activation of the PI3K/Akt pathway by *H. pylori* augments phosphorylation of XIAP leading to degradation of Siva1. (**A**) Western blot analysis of Siva1 protein in AGS cells treated with chemical inhibitors (final concentration 10 μM) for the indicated enzymes and cocultured with *H. pylori* strain 7.13. The graph panel shows quantification of Siva1 protein by densitometry, normalized to actin ($n = 3$). Expression of Siva1 protein in control cells treated with vehicle (DMSO) was arbitrarily set at 1. (**B**) Western blot analysis of Siva1 and XIAP proteins in AGS cells transfected with PI3K siRNA or scrambled siRNA and then either left uninfected or cocultured with *H. pylori* strain 7.13 for 4 hours ($n = 3$). (**C**) The same as **B**, but siRNA against Akt protein was used ($n = 3$). Statistical significance was calculated using unpaired 2-tailed *t* tests and *P* value corrected by Bonferroni's multiple comparison adjustment. Data are displayed as mean ± SE and are representative of 3 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

Bacterial virulence factor CagA regulates Siva1 protein. Bacterial proteins encoded by the *cag PAI* significantly affect host-microbe interactions (30). Many molecular alterations caused by *H. pylori* infection are associated with bacterial CagA protein that is injected into host cells through the T4SS (3). To investigate whether CagA is involved in regulation of Siva1 protein, Siva1 expression plasmid (Siva1-pcDNA3) was cotransfected with an increasing amount of CagA expression plasmid (CagA-pSP65SRα) in AGS cells (28). GFP-expression plasmid (pEGFP-N1) was used to normalize transfection efficiency. We found that ectopic expression of CagA increases phosphorylation of XIAP protein and downregulation of Siva1 in a dose-dependent manner (Figure 7A). In the complementary experiments, the effect of ectopically expressed CagA was analyzed in AGS cells, in which Akt protein was downregulated with siRNA. Consistent with our results shown in Figure 6, downregulation of Akt significantly inhibited phosphorylation of XIAP and degradation of Siva1 protein induced by CagA (Figure 7B). To further investigate the role of CagA, expression of XIAP and Siva1 proteins was analyzed in AGS cells cocultured with WT *H. pylori* strain J166 and its *cagA*$^-$ and *cagE*$^-$ isogenic mutants, which are deficient in delivery of CagA (31). We also compared how WT bacteria and *cagA*$^-$ mutant affect Siva1 protein in cells undergoing DNA damage (Supplemental Figure 5). Our analyses showed that mutants have a reduced ability to inhibit Siva1 and phosphorylate XIAP protein, implicating CagA in regulation of Siva1 (Figure 7C).

To validate our findings in vivo, C57BL/6 mice were infected with *H. pylori* strain PMSS1 or its *cagE*$^-$ isogenic mutant and analyzed for expression of XIAP protein by immunohistochemistry (IHC). Gastric tissues were also analyzed by Western blotting in 9 specimens (3 animals per group). XIAP protein was found to be primarily expressed in chief cells at the base of the oxyntic glands and gastric foveolar epithelial cells in the corpus of uninfected animals ($n = 8$). In contrast to XIAP, uninfected animals showed a weak staining for phosphorylated XIAP protein in the gastric corpus and antrum. Infection with WT *H. pylori* or *cagE*$^-$ mutant had a minimal effect on the expression pattern of XIAP protein (Figure 8A). At the same time, WT *H. pylori* strain, but not *cagE*$^-$ mutant, caused a robust increase in phosphorylation of XIAP protein in

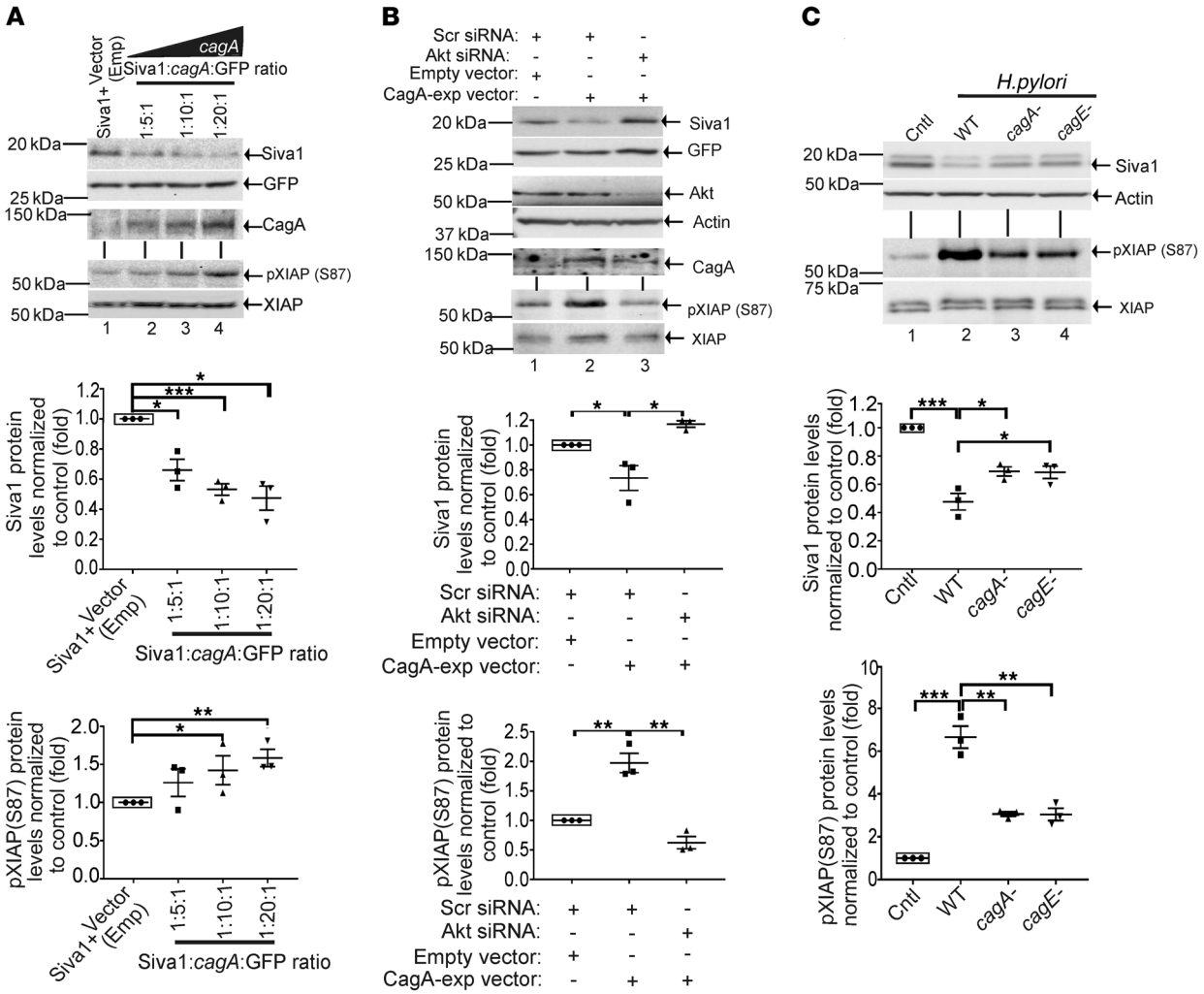

Figure 7. CagA protein induces phosphorylation of XIAP protein and degradation of Siva1. (**A**) Western blot analysis of Siva1 and pXIAP(S87) proteins in AGS cells cotransfected with Siva1, CagA, and GFP expression plasmids at the indicated ratios for 24 hours. GFP was used to normalize transfection efficiency. pXIAP(S87) protein levels were normalized to XIAP protein expression. (**B**) Western blot analysis of Siva1 and pXIAP(S87) proteins in AGS cells cotransfected with CagA expression vector (CagA-pSP65SRα) or empty vector (pSP65SRα) and Akt siRNA or scrambled siRNA as shown at the top of the panel. β-actin and GFP were used to normalize protein loading and transfection efficiency, respectively. (**C**) Western blot analysis of Siva1 and pXIAP(S87) proteins in AGS cells cocultured with WT *H. pylori* strain J166 or its *cagA*⁻ or *cagE*⁻ isogenic mutants. The graph panels show quantification of Siva1 and pXIAP(S87) proteins by densitometry, normalized to actin and XIAP, respectively. Expression of Siva1 and pXIAP(S87) proteins in control cells was arbitrarily set at 1. Data were analyzed using 2-tailed Student's *t* test. Data are displayed as mean ± SE and are representative of 3 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

both the antrum and corpus, which was accompanied by downregulation of Siva1 protein (Figure 8, A and B, and Supplemental Figure 8B).

To further solidify our data, we analyzed expression of Siva1 and XIAP proteins in the stomach of human individuals infected with *cagA*⁺ ($n = 13$) and *cagA*⁻ ($n = 13$) *H. pylori* bacteria as well as uninfected human subjects ($n = 6$). Immunohistochemical staining of antral biopsies revealed that Siva1 and XIAP proteins are primarily expressed in foveolar epithelial cells and epithelial cells of the pyloric glands in uninfected individuals (Figure 9). Infection with *cagA*⁺ bacteria was associated with significant increase in XIAP protein phosphorylation at S87 and downregulation of Siva1, compared with uninfected individuals or subjects infected with *cagA*⁻ bacteria (Figure 9). At the same time, expression of XIAP protein did not significantly change, showing a similarity with infected mice discussed above (Figure 8, A and B). Combined, our results show that CagA protein is involved in regulation of Siva1 and XIAP proteins.

Discussion

Our studies provide the first evidence that *H. pylori* inhibits cell death by negatively regulating Siva1 protein, suggesting that this microbial pathogen can actively modulate the intracellular stress environment. We found that *H. pylori* activates the E3 ubiquitin ligase XIAP, which increases ubiquitination of Siva1 protein, resulting in its proteasomal degradation. Another interesting finding is that *H. pylori* strains varied in the ability to inhibit Siva1, pointing out the role of bacterial CagA protein. We found that infection of mice with WT *H. pylori* strongly induces pXIAP and inhibits Siva1, whereas *cagE*⁻ isogenic mutant, which is defi-

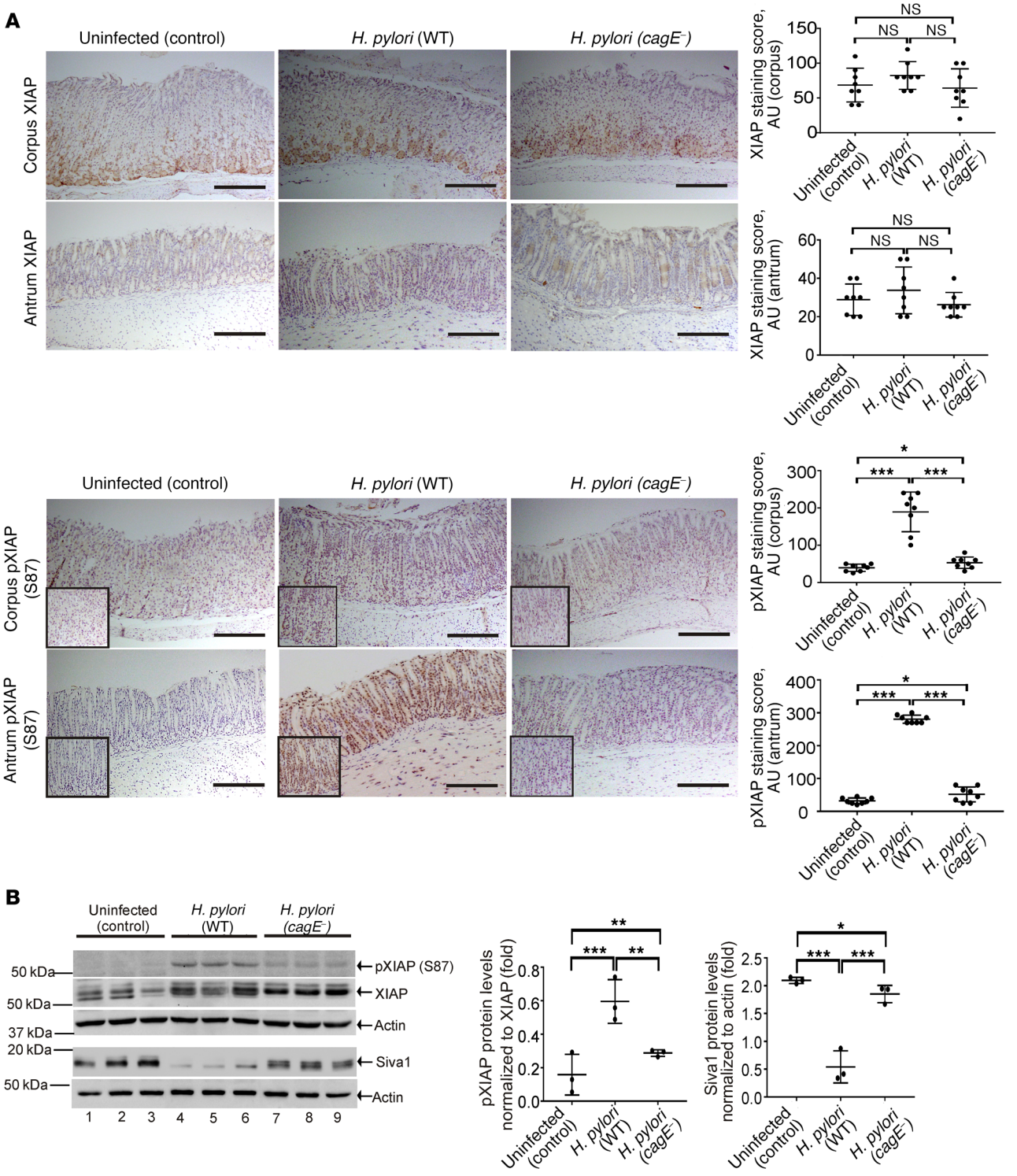

Figure 8. Analyses of Siva1 and XIAP proteins in vivo. (**A**) Representative IHC staining for XIAP and pXIAP proteins in the corpus and the antrum of uninfected mice and ones infected with WT *H. pylori* strain PMSS1 or its *cagE*⁻ isogenic mutant for 8 weeks. Insets show magnified views, 40x. Scale bars: 50 μm. Histograms show IHC scores for expression of XIAP and pXIAP proteins (n = 8/group). (**B**) Western blot analysis of XIAP, pXIAP, and Siva1 proteins in gastric tissues collected from control uninfected mice and ones infected with WT *H. pylori* strain PMSS1 and its *cagE*⁻ isogenic mutant for 8 weeks (3 mice/group). Graphs show the densitometric analysis. Western blot analysis of Siva1 protein is also shown in Figure 1B. Statistical significance was calculated using 1-way ANOVA followed by Tukey's multiple comparison test. Data are displayed as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

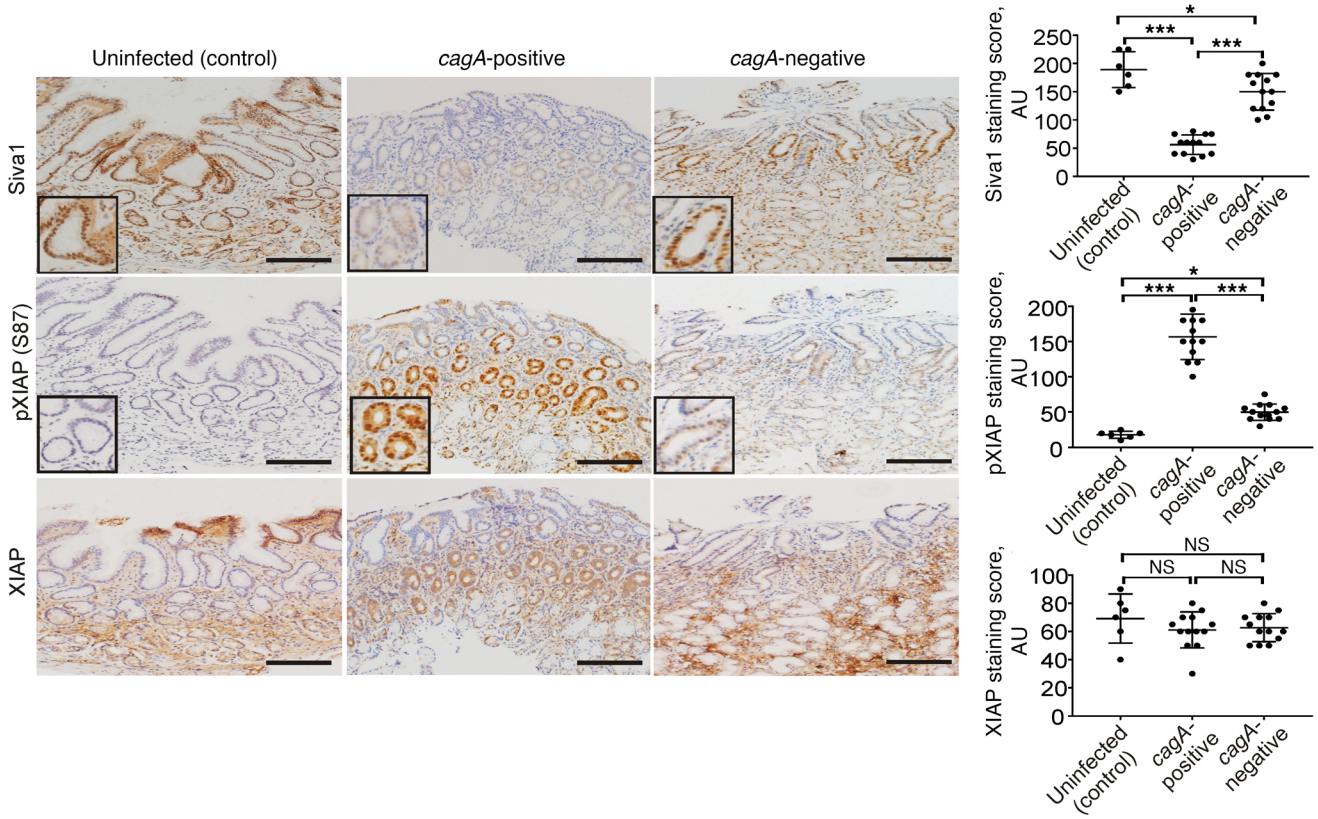

Figure 9. Analyses of Sva1 and XIAP proteins in the human gastric mucosa. Representative IHC staining for Siva1, XIAP, and pXIAP(S87) proteins in gastric biopsies collected from uninfected patients and subjects infected with *cagA*$^+$ and *cagA*$^-$ *H. pylori* bacteria. Insets show magnified views, ×40. Scale bars: 50 μm. Histograms show IHC scores for expression of the indicated proteins (n = 32) Statistical significance was calculated using 1-way ANOVA followed by Tukey's multiple comparison test. Data are displayed as mean ± SD. *P < 0.05; ***P < 0.001.

cient in delivery of CagA, has a significantly weaker effect on these proteins. Similar differences were found analyzing human individuals infected with *cagA*$^+$ and *cagA*$^-$ bacteria. We also found significant differences in the regulation of Siva1 and XIAP by 2 related strains B128 and 7.13. The latter strain, which induces premalignant and malignant lesions in different rodent models (20, 21), was significantly more potent in induction of pXIAP and inhibition of Siva1 than its counterpart strain B128. This effect can be plausibly explained by levels of CagA protein. Several studies, including ours, have previously reported that strain 7.13 injects a significantly greater amount of CagA protein into human cells than strain B128 (6, 20, 21, 28). However, we can't exclude that additional *H. pylori* virulence factors may contribute to Siva1 protein regulation.

CagA functions as a bacterial oncoprotein. Oncogenic potential of CagA has been demonstrated using Drosophila, zebrafish, and mouse models (32–34). Epidemiological studies also showed that the presence of CagA significantly increases the risk of gastric cancer development in infected patients (35–37). In agreement, our studies consistently demonstrate that CagA inhibits apoptosis that may promote survival of damaged epithelial cells and contribute to gastric tumorigenesis. This is also consistent with our finding that tumorigenic *H. pylori* strain 7.13 strongly inhibits apoptosis compared with related nontumorigenic strain B128. However, cellular response to *H. pylori* infection is a complex process that reflects multiple interactions between bacterial virulence factors, intracellular signaling pathways, and the host immune responses. It has been reported that CagA promotes chronic inflammation in infected patients that exacerbates gastric tissue damage (38, 39). It is plausible that similar to other oncogenes, CagA provides proliferation and survival signals, but in certain circumstances may induce cell death, triggering the oncogenic stress response in normal cells. This complexity may explain why studies of patients infected with *cagA*$^+$ bacteria showed a decreased apoptosis but extensive gastric tissue damage (39–41). Additional studies are needed to clarify this issue.

Using small molecule chemical screens, the prosurvival PI3K/Akt pathway was found to regulate Siva1 protein in infected cells. Among multiple proteins regulated by the PI3K/Akt pathway, our studies identified XIAP E3 ubiquitin ligase, which is phosphorylated at Ser87, leading to ubiquitination and proteasomal degradation of Siva1 protein. We found that inhibition of XIAP using RNAi abrogates degradation of Siva1 by *H. pylori*. This inhibitory mechanism appears to be unique to *H. pylori*, as human papillomavirus-16 (HPV-16) and coxsackievirus B3, which are known to inhibit Siva1, produce specialized viral proteins E7 and VP2 that interact with Siva1, causing its inhibition without protein degradation (42, 43). Notably, HPV-16 belong to a high-risk group of papillomaviruses that causes the formation of various neoplasms.

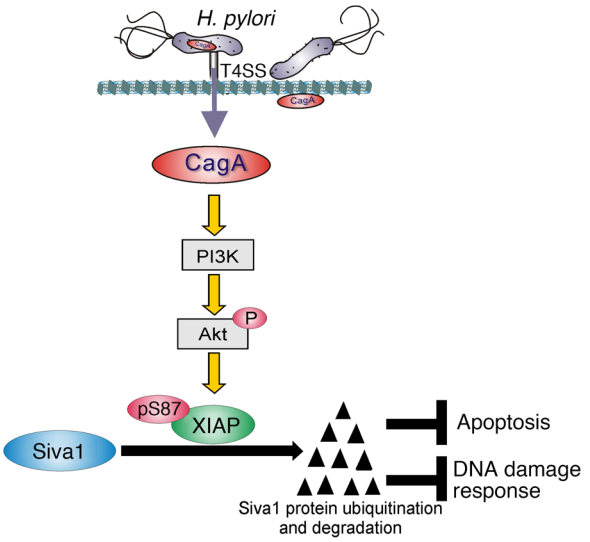

Figure 10. Schematic depiction of Siva1 protein regulation in *H. pylori*–infected cells.

Previous studies have found that *Siva1* gene is transcriptionally regulated by p53 (12). Given that p53 is inhibited by *H. pylori* (28), we tested whether p53 is responsible for downregulation of Siva. Our experiments showed that *H. pylori* downregulates Siva1 in a p53-independent manner. Thus, *H. pylori* independently targets both Siva1 and p53 proteins. It is plausible that inhibition of multiple apoptotic pathways helps bacteria to modulate cellular stress in the stomach, resulting in a more successful persistent infection.

Interestingly, *H. pylori* strongly affects expression of Siva1 protein in gastric chief cells (Figure 1A). These cells are known to be involved in the formation of spasmolytic polypeptide-expressing metaplasia, which occurs as a result of damage of the gastric oxyntic mucosa (44–46). Conceivably, inhibition of apoptosis in chief cells may potentiate metaplasia and lead to accumulation of mutations, resulting in tumorigenic alterations in the stomach.

In conclusion, we found that bacterial virulence factor CagA affects Siva1 protein by increasing its ubiquitination and proteasomal degradation. This process is mediated by activation of the PI3K/Akt pathway and XIAP E3 ubiquitin ligase induced by *H. pylori* (Figure 10). Our data also demonstrated that *H. pylori*–induced downregulation of Siva1 causes inhibition of the apoptotic and DNA damage responses.

Methods

Cell lines and H. pylori strains. Human gastric epithelial cell lines AGS and SNU1 were purchased from ATCC. The ATCC uses PCR-based assays, karyotyping and other approaches to confirm the identity of cell lines. Cells were maintained in F12 medium, containing 10% FBS (Thermo Fisher Scientific). Colon cancer cell lines HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ (provided by Bert Vogelstein, Johns Hopkins University, Baltimore, Maryland, USA) were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) with 10% FBS (25). All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. AGS cells express WT p53 protein (47).

H. pylori strains B128, 7.13, J166, and their *cagA*$^-$ and *cagE*$^-$ isogenic mutants were described previously (20, 21, 28, 48). *H. pylori* strain 7.13 was generated via in vivo adaptation of the B128 clinical isolate as described previously (20). Bacteria were grown on Trypticase Soy Agar plates with 5% sheep blood (BD Biosciences) or in *Brucella* broth (BD Biosciences) containing 10% FBS at 37°C under 5% CO_2.

H. pylori from liquid culture was harvested by centrifugation and used for infection of gastric cells at a bacteria to cell ratio of 100:1 (MOI 100). After infection, cells were cocultured with *H. pylori* for the indicated period of time, harvested, and lysed in the RIPA buffer. Lysates were analyzed by Western blotting or used in flow cytometry and immunoprecipitation experiments. Densitometry analysis of Western blots was performed using the ImageJ program (NIH).

Vectors, RNAi, and chemicals. pcDNA3-FLAG-Siva1 expression plasmid was purchased from GenScript (catalog OHu18390D). pEBB-FLAG-XIAP was a gift from Jon Ashwell (Addgene plasmid 11558). Siva shRNA (catalog sc-37385-SH), XIAP shRNA (catalog sc-37508-SH) and control shRNA (catalog sc-108060) plasmids were from Santa Cruz Biotechnology. GFP expression vector (EGFP-N1) was from Clontech Laboratories and used for normalization of transfection efficiency. Plasmids expressing HA-ubiquitin and CagA were described previously (5, 28, 49).

Siva1 siRNA (5′-CACAGCACUUUCUCGUACAUGUCACUG-3′) was synthesized by Integrated DNA Technologies. PI3K (catalog sc-37269), XIAP (catalog sc-37508), Cbl-b (catalog sc-29950), and Akt (catalog sc-43609) siRNA were purchased from Santa Cruz Biotechnology. Control (catalog AM4636) and XIAP (catalog AM51331) siRNA were purchased from Ambion. siRNAs targeting the 3′UTR of Siva1 (catalog A-012262-18-0005) and XIAP (catalog A-004098-24-0005) mRNAs were from Dharmacon.

Proteasomal inhibitor MG132 was purchased from MilliporeSigma. Cycloheximide (CHX), bafilomycin A (BAF A), camptothecin (CPT), and Protease Inhibitor Cocktail were from Sigma-Aldrich.

The following kinase and phosphatase inhibitors were used: LY 2944002(PI3K), PP2(Src), BAY 11-7085(NF-κB) from Calbiochem; U0126(MEK1/2) from Cell Signaling Technology; GSK690693(Akt), Go6983(PKC), PF-562271(FAK), CHIR-98014(GSK-3α/β), SHP099(SHP2) from Selleck Chemicals; imatinib mesylate(c-Abl) from Sigma-Aldrich.

H. pylori infections, IHC, and collection of murine and human tissues. C57BL/6 mice (4–8 weeks old) from Charles River were randomly assigned into 3 groups and orogastrically challenged with either sterile *Brucella* broth or rodent-adapted *H. pylori* strain PMSS1 or its isogenic *cagE*$^-$ mutant (19). Gastric tissues were collected 8 weeks after infection. At necropsy, linear tissue strips extending from gastroesophageal junction and ending just beyond the gastroduodenal junction were fixed in 10% neutral-buffered formalin, paraffin-embedded, and used for immunohistochemical and apoptosis analyses.

Adult individuals from Nariño, Colombia, with dyspeptic symptoms were invited to participate. Exclusion criteria were previous gastrectomy, serious chronic diseases, or ingestion of any of the following in the 4 weeks before the endoscopic procedure: H2 receptor antagonists, proton pump inhibitors, or antimicrobials. Gastric biopsies were collected from the antrum and embedded in paraffin for histology. One additional biopsy from the antrum was immediately frozen in glycerol and thioglycolate and used for *H. pylori* culture. *H. pylori cagA* status was assessed by PCR using a single colony per individual (50). Expression of Siva1, XIAP, and pXIAP(S87) proteins

in murine and human gastric tissues was assessed by IHC using the following antibodies: Siva1 (catalog sc-514375, catalog sc-376260) and XIAP (catalog sc-55550) from Santa Cruz Biotechnology, and pXIAP-S87 (catalog SAB4504010) from Sigma-Aldrich.

All antibodies were used at a dilution 1:200. Immunohistochemical results were evaluated for intensity and staining frequency. The intensity of staining was graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The frequency was graded according to the percentage of positive cells. Total scores were calculated by multiplying the intensity score by the percentage of positive cells.

Antibodies and reagents. The following antibodies were used: rabbit polyclonal antibody against proteins H2A.X (catalog 2595), cleaved PARP (catalog 9541), cleaved caspase-3 (catalog 9661), caspase-3 (catalog 9662), Akt (catalog 9272), and rabbit monoclonal antibody pH2A.X (S139) (catalog 9718) from Cell Signaling Technology. pAkt-T308 (catalog 2214-1) antibody was from Epitomics. Mouse monoclonal Siva1 (catalog sc-514375, sc-376260), XIAP (catalog sc-55550), p-Tyr (catalog sc-7020), PI3K (catalog sc-376641), and normal mouse IgG (catalog sc-2025) antibodies were from Santa Cruz Biotechnology. Rabbit polyclonal pXIAP(S87) (catalog SAB4504010) and mouse monoclonal β-Actin (catalog A5441) antibodies were from Sigma-Aldrich. Mouse monoclonal HA.11 (catalog 901501) was from BioLegend. Rabbit polyclonal CagA (catalog HPP-5003-9) antibody was from Austral Biologicals. Bacterial Heat Shock Protein B (HspB) antibody was a gift from Timothy L. Cover (Vanderbilt University, Nashville, Tennessee, USA). The following secondary antibodies were used: HRP-conjugated anti-mouse IgG (catalog W4028) from Promega and HRP-conjugated anti-rabbit IgG (catalog 7074) from Cell Signaling Technology. Cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's manual.

Generation of stable cell lines. shRNA stable cell lines were generated by transfection of AGS cells with control, Siva1, or XIAP shRNA vectors (Santa Cruz Biotechnology), followed by selection with puromycin (2 μg/mL). AGS cells stably expressing Siva1 or XIAP proteins were selected with G418 (400 μg/mL).

siRNA rescue experiments. AGS cells were transfected with scrambled control siRNA or siRNA targeting the 3′UTR of the Siva1 or XIAP transcripts. Twenty-four hours after first transfection, cells were transfected again with either control empty vector or Siva1 or XIAP expression plasmids for an additional 24 hours. The transfected cells were then cocultured with *H. pylori* strains 7.13 or B128 for 4 hours, collected, and analyzed by Western blotting.

Apoptosis analysis. AGS cells were cocultured with *H. pylori* strains 7.13 or B128 for 18 hours, harvested by centrifugation (400*g* for 1 minute), washed with PBS, and analyzed using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's protocol. Cells were analyzed by flow cytometry using the BD FACS Aria fusion II system. Apoptosis was quantified using the BD FACSDiva software (Becton Dickinson Biosciences).

Apoptosis in tissue specimens was measured by TUNEL assay using the In Situ Cell Death Detection Kit from Sigma-Aldrich, according to the manufacturer's protocol.

Determination of Siva1 protein stability. Cycloheximide chase assay was performed to measure stability of Siva1 protein, as described previously (6, 28). Briefly, AGS cells were either left uninfected or cocultured with *H. pylori* strain 7.13 or B128 and treated with cycloheximide (150 μM) to suppress de novo protein synthesis. Cells were then collected at the indicated time points and analyzed for Siva1 protein expression by Western blotting.

Determination of Siva1 protein ubiquitination. AGS cells were transfected with plasmid expressing HA-ubiquitin for 24 hours, cocultured with *H. pylori* strain 7.13 or B128 for 60 minutes, and treated with proteasomal inhibitor MG13 (20 μM) for an additional 8 hours. Cells were then harvested in RIPA buffer, sonicated for 30 seconds, and centrifuged (15000*g* for 20 minutes) at 4°C. Siva1 protein was immunoprecipitated from cell lysates and analyzed by Western blotting using HA-tag and Siva1 antibodies.

Statistics. Statistical analysis was performed using GraphPad Prism 7. The immunohistochemical results and the densitometric measurements of Western blot bands were statistically analyzed by 1-way ANOVA followed by Tukey's multiple comparison test or 2-tailed Student's *t* test, depending on the data set. Pearson's correlation analysis was used to investigate the relationship between protein expression and apoptosis. Results were shown as mean ± SD (or SE). Results were considered significant if P was less than 0.05. In figures, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, NS = $P > .05$.

Study approval. The ethics committees of the participating hospitals and the Universidad del Valle in Cali, Colombia, and the Institutional Review Board of Vanderbilt University approved the protocols for human studies. All participants provided informed consent. Animal studies were performed according to the protocols approved by the Institutional Animal Care Committee of the University of Miami.

Author contributions

MP and EZ performed experiments, analyzed data, and wrote the manuscript. MBP, RMP, and KTW provided clinical specimens and discussed results. MGB and MP performed immunohistochemical assessment. RMP and WER helped to interpret results. AIZ coordinated the project, interpreted results, and corrected the manuscript.

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1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394–424.
2. Hooi JKY, et al. Global prevalence of Helicobacter pylori infection: systematic review and meta-analysis. *Gastroenterology*. 2017;153(2):420–429.
3. Hatakeyama M. Helicobacter pylori CagA and gastric cancer: a paradigm for hit-and-run carcinogenesis. *Cell Host Microbe*. 2014;15(3):306–316.

4. Zaika AI, Wei J, Noto JM, Peek RM. Microbial regulation of p53 tumor suppressor. *PLoS Pathog*. 2015;11(9):e1005099.
5. Wei J, et al. Bacterial CagA protein induces degradation of p53 protein in a p14ARF-dependent manner. *Gut*. 2015;64(7):1040–1048.
6. Horvat A, et al. Helicobacter pylori pathogen regulates p14ARF tumor suppressor and autophagy in gastric epithelial cells. *Oncogene*. 2018;37(37):5054–5065.
7. Toller IM, et al. Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells. *Proc Natl Acad Sci U S A*. 2011;108(36):14944–14949.
8. Shi Y, Wang P, Guo Y, Liang X, Li Y, Ding S. Helicobacter pylori-induced DNA damage is a potential driver for human gastric cancer AGS cells. *DNA Cell Biol*. 2019;38(3):272–280.
9. Hartung ML, et al. H. pylori-induced DNA strand breaks are introduced by nucleotide excision repair endonucleases and promote NF-κB target gene expression. *Cell Rep*. 2015;13(1):70–79.
10. Xue L, et al. Siva-1 binds to and inhibits BCL-X(L)-mediated protection against UV radiation-induced apoptosis. *Proc Natl Acad Sci USA*. 2002;99(10):6925–6930.
11. Prasad KV, et al. CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. *Proc Natl Acad Sci U S A*. 1997;94(12):6346–6351.
12. Fortin A, et al. The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. *J Biol Chem*. 2004;279(27):28706–28714.
13. Lin FT, Lai YJ, Makarova N, Tigyi G, Lin WC. The lysophosphatidic acid 2 receptor mediates down-regulation of Siva-1 to promote cell survival. *J Biol Chem*. 2007;282(52):37759–37769.
14. Li N, et al. Siva1 suppresses epithelial-mesenchymal transition and metastasis of tumor cells by inhibiting stathmin and stabilizing microtubules. *Proc Natl Acad Sci U S A*. 2011;108(31):12851–12856.
15. Van Nostrand JL, Brisac A, Mello SS, Jacobs SB, Luong R, Attardi LD. The p53 target gene SIVA enables non-small cell lung cancer development. *Cancer Discov*. 2015;5(6):622–635.
16. Ray RM, Bhattacharya S, Johnson LR. Mdm2 inhibition induces apoptosis in p53 deficient human colon cancer cells by activating p73- and E2F1-mediated expression of PUMA and Siva-1. *Apoptosis*. 2011;16(1):35–44.
17. Wang X, et al. Siva1 inhibits p53 function by acting as an ARF E3 ubiquitin ligase. *Nat Commun*. 2013;4:1551.
18. Park IK, Blum W, Baker SD, Caligiuri MA. E3 ubiquitin ligase Cbl-b activates the p53 pathway by targeting Siva1, a negative regulator of ARF, in FLT3 inhibitor-resistant acute myeloid leukemia. *Leukemia*. 2017;31(2):502–505.
19. Arnold IC, et al. Tolerance rather than immunity protects from Helicobacter pylori-induced gastric preneoplasia. *Gastroenterology*. 2011;140(1):199–209.
20. Franco AT, et al. Activation of beta-catenin by carcinogenic Helicobacter pylori. *Proc Natl Acad Sci U S A*. 2005;102(30):10646–10651.
21. Fox JG, et al. Helicobacter pylori-associated gastric cancer in INS-GAS mice is gender specific. *Cancer Res*. 2003;63(5):942–950.
22. Chen G, et al. Apoptosis in gastric epithelial cells is induced by Helicobacter pylori and accompanied by increased expression of BAK. *Biochem Biophys Res Commun*. 1997;239(2):626–632.
23. Rudi J, et al. Involvement of the CD95 (APO-1/Fas) receptor and ligand system in Helicobacter pylori-induced gastric epithelial apoptosis. *J Clin Invest*. 1998;102(8):1506–1514.
24. Baik SC, et al. Increased oxidative DNA damage in Helicobacter pylori-infected human gastric mucosa. *Cancer Res*. 1996;56(6):1279–1282.
25. Bunz F, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*. 1998;282(5393):1497–1501.
26. Cohen-Kaplan V, Livneh I, Avni N, Cohen-Rosenzweig C, Ciechanover A. The ubiquitin-proteasome system and autophagy: Coordinated and independent activities. *Int J Biochem Cell Biol*. 2016;79:403–418.
27. Eskelinen EL, Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta*. 2009;1793(4):664–673.
28. Wei J, et al. Regulation of p53 tumor suppressor by Helicobacter pylori in gastric epithelial cells. *Gastroenterology*. 2010;139(4):1333–1343.
29. Resch U, Schichl YM, Winsauer G, Gudi R, Prasad K, de Martin R. Siva1 is a XIAP-interacting protein that balances NFkappaB and JNK signalling to promote apoptosis. *J Cell Sci*. 2009;122(Pt 15):2651–2661.
30. Odenbreit S, Püls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science*. 2000;287(5457):1497–1500.
31. Argent RH, Thomas RJ, Letley DP, Rittig MG, Hardie KR, Atherton JC. Functional association between the Helicobacter pylori virulence factors VacA and CagA. *J Med Microbiol*. 2008;57(Pt 2):145–150.
32. Neal JT, Peterson TS, Kent ML, Guillemin K. H. pylori virulence factor CagA increases intestinal cell proliferation by Wnt pathway activation in a transgenic zebrafish model. *Dis Model Mech*. 2013;6(3):802–810.
33. Wandler AM, Guillemin K. Transgenic expression of the Helicobacter pylori virulence factor CagA promotes apoptosis or tumorigenesis through JNK activation in Drosophila. *PLoS Pathog*. 2012;8(10):e1002939.
34. Ohnishi N, et al. Transgenic expression of Helicobacter pylori CagA induces gastrointestinal and hematopoietic neoplasms in mouse. *Proc Natl Acad Sci U S A*. 2008;105(3):1003–1008.
35. Blaser MJ, et al. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res*. 1995;55(10):2111–2115.
36. Kuipers EJ, Pena AS, Meuwissen SG. [Helicobacter pylori infection as causal factor in the development of carcinoma and lymphoma of the stomach; report WHO consensus conference]. *Ned Tijdschr Geneeskd*. 1995;139(14):709–712.
37. Peek RM. Helicobacter pylori strain-specific modulation of gastric mucosal cellular turnover: implications for carcinogenesis. *J Gastroenterol*. 2002;37 Suppl 13:10–16.
38. Chuang CH, et al. Helicobacter pylori with stronger intensity of CagA phosphorylation lead to an increased risk of gastric intestinal metaplasia and cancer. *BMC Microbiol*. 2011;11:121.
39. Teh M, Tan KB, Seet BL, Yeoh KG. Study of p53 immunostaining in the gastric epithelium of cagA-positive and cagA-negative Helicobacter pylori gastritis. *Cancer*. 2002;95(3):499–505.
40. Peek RM, et al. Helicobacter pylori cagA+ strains and dissociation of gastric epithelial cell proliferation from apoptosis. *J Natl Cancer Inst*. 1997;89(12):863–868.
41. Rokkas T, et al. Relationship of Helicobacter pylori CagA status to gastric cell proliferation and apoptosis. *Dig Dis Sci*. 1999;44(3):487–493.
42. Henke A, Launhardt H, Klement K, Stelzner A, Zell R, Munder T. Apoptosis in coxsackievirus B3-caused diseases: interaction between the capsid protein VP2 and the proapoptotic protein siva. *J Virol*. 2000;74(9):4284–4290.
43. Severino A, et al. Human papillomavirus-16 E7 interacts with Siva-1 and modulates apoptosis in HaCaT human immortalized keratinocytes. *J Cell Physiol*. 2007;212(1):118–125.
44. Meyer AR, Goldenring JR. Injury, repair, inflammation and metaplasia in the stomach. *J Physiol (Lond)*. 2018;596(17):3861–3867.
45. Sáenz JB, Mills JC. Acid and the basis for cellular plasticity and reprogramming in gastric repair and cancer. *Nat Rev Gastroenterol Hepatol*. 2018;15(5):257–273.
46. Sáenz JB, Vargas N, Mills JC. Tropism for spasmolytic polypeptide-expressing metaplasia allows Helicobacter pylori to expand its intragastric niche. *Gastroenterology*. 2019;156(1):160–174.e7.
47. Cheng LL, et al. TP53 genomic status regulates sensitivity of gastric cancer cells to the histone methylation inhibitor 3-deazaneplanocin A (DZNep). *Clin Cancer Res*. 2012;18(15):4201–4212.
48. Peek RM, et al. Helicobacter pylori strain-specific genotypes and modulation of the gastric epithelial cell cycle. *Cancer Res*. 1999;59(24):6124–6131.
49. Treier M, Staszewski LM, Bohmann D. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. *Cell*. 1994;78(5):787–798.
50. de Sablet T, et al. Phylogeographic origin of Helicobacter pylori is a determinant of gastric cancer risk. *Gut*. 2011;60(9):1189–1195.