Published in final edited form as:

Gastroenterology. 2008 May ; 134(5): 1412–1423.

Interaction of *Helicobacter pylori* with gastric epithelial cells is mediated by the p53 protein family

Jinxiong Wei^{*}, Daniel O'Brien[‡], Anna Vilgelm^{*}, Maria B. Piazzuelo[‡], Pelayo Correa[‡], Mary K. Washington[§], Wael El-Rifai^{*,||}, Richard M. Peek^{‡,||,*}, and Alexander Zaika^{*,||}

^{*}Department of Surgery, Vanderbilt University Medical Center and Vanderbilt-Ingram Cancer Center, Nashville, TN

[‡]Division of Gastroenterology, Vanderbilt University Medical Center and Vanderbilt-Ingram Cancer Center, Nashville, TN

[§]Department of Pathology, Vanderbilt University Medical Center and Vanderbilt-Ingram Cancer Center, Nashville, TN

^{||}Department of Cancer Biology, Vanderbilt University Medical Center and Vanderbilt-Ingram Cancer Center, Nashville, TN

Abstract

Background & Aims—While the p53 tumor suppressor has been extensively studied, many critical questions remain unanswered about the biological functions of p53 homologs, p73 and p63. Accumulating evidence suggests that both p73 and p63 play important roles in regulation of apoptosis, cell differentiation, and therapeutic drug sensitivity.

Methods—Gastric epithelial cells were co-cultured with *H. pylori*, and the roles of p63 and p73 proteins were assessed by luciferase reporter, real-time PCR, immunoblotting, and cell survival assays. shRNA and dominant-negative mutants were employed to inhibit activity of p73 and p63 isoforms. Human and murine gastric tissues were analyzed by immunohistochemistry with p73 and p63 antibodies, and modified Steiner's silver method.

Results—Interaction of *H. pylori* with gastric epithelial cells leads to robust up-regulation of p73 protein *in vitro* and *in vivo* in human gastritis specimens and *H. pylori*-infected mice. The p73 increase resulted in up-regulation of pro-apoptotic genes, NOXA, PUMA, and Fas receptor in gastric epithelial cells. Down-regulation of p73 activity suppressed cell death and Fas receptor induced by *H. pylori*. Bacterial virulence factors within the *cag* pathogenicity island, c-Abl tyrosine kinase and interaction with p63 isoforms control the activity of p73.

Conclusion—Our findings implicate p73 in *H. pylori*-induced apoptosis, and more generally suggest that the p53 family may play a role in the epithelial cell response to *H. pylori* infection.

Introduction

Helicobacter pylori (*Hp*) is a Gram-negative pathogen that colonizes the stomach of approximately half of the world's population. *Hp* infection has been implicated in the

Corresponding author: Alexander Zaika, Department of Surgery and Cancer Biology, Vanderbilt University Medical Center, 1255 Light Hall, 2215 Garland Ave., Nashville, TN 37232. E-mail: alex.zaika@vanderbilt.edu.

The authors have no conflict of interest to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

pathogenesis of gastritis, peptic ulcer disease and gastric cancer. The *cag* pathogenicity island (*cag* PAI) is one of the major virulence determinants of *Hp*. It encodes a type IV secretion system. A product of the *cag* PAI, CagA is delivered by this secretion system into epithelial cells after bacterial attachment and subsequently activates multiple intracellular signaling cascades, eventuating in cellular morphological changes and alteration in apoptotic response.

Bacterial factors allow *Hp* to persist invoking an intense inflammatory response, which leads to gastric tissue damage accompanied by apoptosis.¹ Sustained large-scale apoptosis induced by *Hp* may result in atrophy of the gastric glands, a premalignant lesion of the stomach. *Hp*-induced apoptosis can be recapitulated by co-culture of *Hp* and epithelial cells *in vitro*.² *In vivo*, increased apoptosis secondary to *Helicobacter* infection was demonstrated in mice, and in a Mongolian gerbil model.^{3, 4} In human tissues, increased apoptosis have also been demonstrated in patients with gastroduodenal ulcers and gastritis associated with *Hp* infection.^{5, 6} It has been suggested that apoptosis associated with the *Hp* infection is closely linked to increases in cellular proliferation, which in compensation of a disproportionate damage of gastric epithelium, causes dysplastic changes.⁷

Hp leads to a number of primary and secondary effects that could potentially activate apoptotic pathways. It is thought that the Fas/FasL system plays an important role in apoptotic response, given that *Helicobacter*-induced apoptosis and gastric atrophy were substantially decreased in Fas-knockout mice.⁸ Suppression of Fas-mediated apoptosis accelerates *Helicobacter*-induced gastric cancer in mice. Another potential mediator of cellular apoptosis is p53; however, its role in interaction with *Hp* remains not well understood.

p53 is the founding member of a family of proteins that also includes p73 and p63. Extensive structural similarity exists in all of these proteins, but the highest is found in the DNA-binding domain in which p63 and p73 share an approximate 60% amino-acid identity with p53. When over-expressed, p73 and p63 can mimic biological activities attributed to p53. p73 and p63 activate transcription of many p53-target genes that are involved in cell-cycle regulation and apoptosis. Analogous to p53, activated p73 mediates a cellular response to DNA damage induced by γ -irradiation or treatment with chemotherapeutic drugs.⁹ p73^{+/-} and p63^{+/-} heterozygous mice develop both malignant and benign lesions suggesting that these proteins may play a tumor suppressor role.¹⁰ p73 and p63 are expressed as a complex variety of protein isoforms. Isoforms without N-terminal transactivation domain (TAD) are termed Δ N isoforms, while isoforms with TAD are known as TA isoforms. In contrast to TA isoforms, which have "p53-like" properties, Δ N-isoforms, Δ Np73 α and Δ Np63 α , may function as transcriptional inhibitors of TAp73, TAp63 and p53.¹¹

p73 knockout mice exhibit a spectrum of profound defects, including aberrant neurogenesis, inflammation and sustained chronic bacterial infections.¹² The majority of p73-deficient mice live only 4 to 6 weeks and die of chronic infections, preceded by an erosion of intestinal epithelium and massive gastrointestinal hemorrhage.¹⁰ At the present time, the cause of the increased susceptibility to infections in p73-deficient animals is not well understood.

In our study, we demonstrated for the first time that p73 and p63 play a role in the regulation of the interaction between *Hp* and gastric epithelial cells. Our findings provide evidence that p73 signaling may be a previously uncharacterized component of the host response to *H. pylori*, which may play an important role in the pathogenesis associated with *Hp* infection.

Materials and Methods

Cell and *H. pylori* cultures

The human gastric cancer cell lines AGS, Kato III, MKN45 and MKN28 were maintained in RPMI 1640 medium (Invitrogen, CA) supplemented with 10% FBS. Mouse primary gastric epithelial cells were harvested from a transgenic mouse, bearing a temperature-sensitive mutant of SV40 large T antigen and cultured in RPMI medium 1640 with 5% FBS and 20 µg/ml gentamycin at 33°C.¹³

The *Hp cagA*⁺ clinical strains (J166, J291, 26695), *cagA*⁻ strains (J63, J68, J188) and rodent-adapted strain 7.13 were grown in *Brucella* broth with 5% FBS for 18 hours, harvested by centrifugation, and added to gastric cells at a bacteria-to-cell ratio of 100:1. Isogenic *cagA*⁻, *cagE*⁻, and *vacA*-null mutants were constructed within strain J166 by insertional mutagenesis using *aphA* and selected with kanamycin.

Vectors and antibodies

Plasmids expressing human TAp73α, TAp73β and p73 mutants DD, mtDD, and PG13Luc, a p53/p73 reporter plasmid, were described previously.^{14, 15} Plasmids expressing mouse c-Abl and kinase-defective c-Abl mutant (K290H) were kind gifts from Drs. J. Wang and Y. Haupt (UCSD, USA and the Hebrew University, Israel). pCEP-H1 vector expressing short hairpin RNA (shRNA) against p63 was kindly provided by Dr. J. Pietenpol (Vanderbilt University, TN). shRNA construct directed against c-Abl was a gift from Dr. S. Wessler.¹⁶ CagA expression vector was described previously.¹³ p73DD-IRES-GFP and p73mtDD-IRES-GFP were generated by subcloning the aforementioned mutants into the MSCV-IRES-GFP vector.

Antibodies to the following proteins were employed in this study: Fas (C-20), p63 (4A4), c-Abl (K-12), and p73 (H-79) from Santa Cruz Biotechnology (Santa Cruz, CA); p-Tyr (4G10) from Upstate Biotechnology (Lake Placid, NY); p53 (DO-1), p21 (Ab-1), and p73 (Ab-3) from Calbiochem (San Diego, CA); PUMA (ab9643) from Abcam Inc. (Cambridge, MA), and Noxa from Imgenex (San Diego, CA). Protein loading was monitored using the anti-β-actin antibody (Cell Signaling, MA).

RNA extraction and real-time RT-PCR analysis

Cells were harvested at indicated time points after infection. Total RNA was extracted using Trizol reagent (Invitrogen, CA). Quantitative PCR was performed as described previously¹⁷ with the following specific primers: TAp73 (CACGTTTGAGCACCTCTGGA, GAACTGGGCCATGACAGATG), ΔNp73 (TGTACGTCGGTGACCCCGCAC, TCGGTGTTGGAGGGGATGACA); TAp63 (TCAGAAGATGGTGCGACAAAC, GCGTGGTCTGTGTTATAGGGAC), ΔNp63 (GAAAACAATGCCCAGACTCAA, TGCGCGTGGTCTGTGTGA), NOXA (AGATGCCTGGGAAGAAG, AGTCCCCTCATGCAAGT), p21 (CTGGAGACTCTCAGGGTCGAAA, GATTAGGGCTTCCTCTTGGAGAA) and PUMA (ACGACCTCAACGCACAGTACG, TCCCATGATGAGATTGTACAGGAC) using the iCycler (Bio-Rad, Hercules, CA). Results were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) expression.

Co-immunoprecipitation

AGS cells were transfected with plasmid expressing flag-tagged TAp73α. Twenty-four hours post-transfection cells were co-cultured with *Hp* or left untreated. An equal amount of cell lysates were immunoprecipitated with either Flag antibody or nonspecific IgG. Binding of ΔNp63α to TAp73 was analyzed by Western blotting.

Human tissues, mice, experimental infections and immunohistochemistry

Gastric biopsy samples were taken from human subjects undergoing medically indicated endoscopies at Vanderbilt University (Nashville, TN) and the Memorial Medical Center (New Orleans, LA) in accordance with the Institutional Review Board-approved protocols. Eighteen samples (7 histologically normal and 11 with *Hp*-associated gastritis) were randomly selected for immunohistochemical studies. Serial 4 μm -thick sections were stained with hematoxylin and eosin for routine histology, and modified Steiner silver stain for evaluation of *Hp*. Staining with a p73 antibody was performed following previously described techniques.¹⁸ As an additional control, slides were equally treated with the primary antibody omitted.

Eight to 12-week-old C57BK/6 mice were used ($n=10$). *Brucella* broth containing 1×10^8 cfu of the *Hp* rodent-adapted strains 7.13 or SS1 were used as inoculum and delivered by gastric incubation. Eight weeks post-challenge, the mice were euthanized. At necropsy, linear strips extending from the squamocolumnar junction through the proximal duodenum were fixed in 10% neutral buffered formalin, paraffin-embedded and stained with hematoxylin and eosin and with a p73 antibody. An additional group of mice ($n=5$) were euthanized at day three after infection in order to detect early changes in the p73 levels. All experiments were approved by the Vanderbilt University Animal Care and Use Committee.

p73 half-life determination

Subconfluent Kato III cells were co-cultured with *Hp* for 4 hours. Cells were treated with 25 $\mu\text{g}/\text{ml}$ cycloheximide (CHX) for 0.5, 1.5, 3.5, or 5.5 hours, washed with ice-cold PBS and lysed in RIPA buffer. Cell lysates were centrifuged, and equal amounts of protein were subjected to Western blot analysis. The p73 protein was quantitated using the NIH Image software and plotted as a percentage of the p73 remaining.

Apoptosis and cell survival assays

Cell death was measured by flow cytometry. Briefly, AGS cells were seeded into 60mm plates and transfected with p73DD-IRES-GFP, p73mutDD-IRES-GFP or empty MSCV-IRES-GFP vectors for 24 hours. The transfected cells were then co-cultured with *Hp* for an additional 24 hours. Cells were trypsinized and stained with 50 $\mu\text{g}/\text{mL}$ propidium iodide, and DNA content was measured in GFP-positive cells by FACS.

Apoptosis was also measured by TUNEL assay as described previously.¹⁵

Statistical analysis

Statistical analysis was performed using the Student's t-test and the Mann Whitney test depending on the data set. Results were expressed as mean values ($\pm$ SEM unless otherwise noted). Results were considered significant if $p < 0.05$.

Results

p73 protein is up-regulated in *H. pylori*-infected human and murine gastric tissues

To investigate the role of p73 in *Hp* infection, we performed immunohistochemical analysis of p73 in human gastric tissue infected with *Hp* and found that the p73 protein was up-regulated in epithelial cells of all ($n=11$) analyzed specimens (Figure 1A). Normal gastric epithelium from uninfected persons ($n=7$) was negative for p73 with the exception of one specimen, which showed weak immunoreactivity (Figure 1B). The p73 immunoreactivity was present in the nuclei of epithelial cells localized to the superficial and neck regions of the gastric glands, predominantly in the antrum of gastritis patients. Staining of serial sections from the same patient for both p73 protein and *Hp* revealed populations of epithelial cells with attached *Hp*

bacteria that exhibited elevated levels of p73 (insets in Figures 1C and 1D). In contrast to p73, immunostaining for p63 protein was negative in any of the *Hp*-positive subjects studied (data not shown).

To explore the effect of *Hp* on p73 in a more controlled environment, C57BL/6 mice were infected with rodent-adapted *Hp*, and disease outcome was followed at different time points. All mice challenged with *Hp* were successfully infected and developed gastritis. Similar to human tissues, the *Hp* infection resulted in a significant increase in p73 protein levels in the infected mice, compared to uninfected control animals inoculated with broth alone (Figures 1E). At an early time point (3 days post-infection), the immunostaining also revealed an increased p73 levels, though its expression was weaker and predominately localized to the superficial epithelium (Figure 1E). Combined, these results show that *Hp* upregulates p73 protein *in vivo*.

H. pylori* up-regulates p73 protein and down-regulates Δ Np63 α *in vitro

We next examined whether *Hp* infection affects the p73 protein levels *in vitro*. Co-culture of AGS cells with *Hp* for 2 to 24 hours led to a significant increase of endogenous levels of p73 compared to uninfected control (Figure 2A). Similar to AGS cells, *Hp* infection led to up-regulation of p73 in conditionally immortalized murine gastric epithelial cells grown under primary conditions (Figure 2B) and another human gastric epithelial cell line, Kato III, (Figure 2C), thereby confirming our finding.

It has previously been reported that TAp73 protein is up-regulated by transcriptional and/or post-translational mechanisms after cellular stress.⁹ Based on these observations, we next asked whether *Hp* altered the p73 gene transcription. As shown in Figures 3A and 3B, TAp73 transcript levels were not increased after co-culture of AGS cells with *Hp*, and similar effects were seen in Kato III cells (data not shown) indicating that TAp73 protein was up-regulated by post-translational mechanisms. Indeed, our analysis revealed that protein stability of TAp73 is significantly increased by *Hp* ($p < 0.05$). The half-life of TAp73 β was more than 5.5 hours in infected Kato III cells compared to 1.3 hours in control untreated cells (Figure 3C).

Similar to TAp73 mRNA, co-culture of *Hp* with AGS or Kato III cells resulted in a slight decrease of TAp63 and Δ Np73 transcripts in a time-dependent manner (Figure 3A, B). Protein levels of TAp63 and Δ Np73 α were also changed insignificantly (data not shown). Notably, *Hp* had a dramatic effect on Δ Np63 mRNA levels. Δ Np63 transcript was decreased 2 hours after *Hp* infection, and then further decreased to undetectable levels after 6 hours (Figure 3A, B). Consistent with the mRNA changes, Δ Np63 α protein was also down-regulated (Figure 3D), although changes in Δ Np63 mRNA was more evident than in the protein, most likely reflecting high stability of the Δ Np63 protein.

Up-regulation of p73 protein is linked to activation of p73-p53 target genes

We and others have previously reported that TAp73 isoforms bind to p53-responsive promoters and transcriptionally up-regulate a spectrum of p53 target genes.^{17, 19} Therefore, we asked whether p73 protein up-regulation leads to activation of transcription in *Hp*-infected cells. To exclude any potential effects of p53, our analysis was conducted in Kato III cells, which lack p53 expression. Luciferase reporter analysis using PG-13LUC reporter, which has p53/p73 binding sites within the promoter, revealed that *Hp* induces this reporter in a time-dependent manner (Figure 4A). To confirm these data, expressions of the endogenous p73/p53 transcriptional targets NOXA and p21/Waf1 were assessed by real-time PCR and Western blotting in Kato III cells. The analyzed transcripts and proteins were up-regulated as a result of co-incubation of Kato III cells with *Hp* (Figure 4B and 4C). To confirm these data, we analyzed the expressions of NOXA and p21/Waf1 in another cell line, AGS. Similar to Kato

III cells, NOXA and p21/Waf1 proteins were upregulated in AGS cells after 24 hours of co-incubation with *Hp* (Figure 4D). Another pro-apoptotic target of p73, PUMA, was also up-regulated by *Hp* (Figure 4D). Thus, two p73 transcriptional targets, which mediate apoptosis, PUMA and NOXA, are induced by *Hp*.

To rule out a potential effect of p53 in wild-type p53 cell line AGS, this protein was analyzed by Western blotting in cells infected with *Hp*. p53 levels were either unchanged or slightly decreased in these cells (Figure 4E). Combined, these data suggest that p73 is involved in up-regulation of p53 target genes in *Hp*-infected cells.

p73 is involved in up-regulation of FasR by *H. pylori*

A significant decrease in apoptosis in Fas receptor knockout mice strongly suggests that the FasR plays a critical role in *Hp*-induced apoptosis.⁸ Therefore, we examined the FasR protein expression in AGS cells transfected with TAp73 isoforms. The FasR protein were markedly elevated following the transfections with TAp73 α and TAp73 β (Figure 5A). Co-culture of AGS cells with *Hp* also significantly induced the FasR protein (Figure 5B). To directly assess the role of p73, we employed a dominant-negative p73 mutant termed DD, which specifically suppresses activity of TAp73 isoforms without affecting p53-dependent transcription and apoptosis.¹⁴ As shown in Figure 5C (compare lanes 1 and 3), expression of FasR was significantly decreased in *Hp*-infected cells, which express DD, implicating TAp73 in regulation of the FasR protein in these cells. These data were confirmed using cells that stably express the DD mutant (data not shown).

Role of p73 in apoptosis induced by *Hp*

We next examined the effect of *Hp* on the viability of gastric epithelial cells. Apoptosis was assessed using TUNEL assay in AGS, Kato III and MKN45 cells co-cultured with *Hp*. Marked cell death was observed in all tested cell lines (Figure 6A). Interestingly, apoptosis in p53-null Kato III cells was comparable to apoptosis in the p53 wild-type cell lines, AGS and MKN45; thus, supporting our observation that p73 may play an important role in *Hp*-induced apoptosis.

To investigate the role of p73 in apoptosis induced by *Hp*, we employed expression vectors that bi-cistronically expressed GFP and either DD or mtDD(L371P). The latter mutant bears a point mutation at codon 371(L->P) that inactivates its dominant-negative properties.¹⁴ AGS cells, transfected with either DD, mtDD or GFP alone, were co-cultured with *Hp* and analyzed by FACS using GFP as a sorting marker. Notably, cells expressing DD mutant were significantly more resistant to *Hp*-induced apoptosis compared to mtDD or empty vector (MSCV-GFP) (Figure 6B). These findings demonstrate that inactivation of TAp73 leads to suppression of *Hp*-induced apoptosis.

Role of cellular and bacterial virulence-related factors in activation of TAp73

The results described above show that in addition to activation of p73, *Hp* infection leads to robust down-regulation of Δ Np63 (Figure 3). Since the truncated form of p63 is known to negatively regulate the pro-apoptotic function of TAp73 through the direct protein-protein interaction²⁰ we examine whether Δ Np63 α inhibits TAp73 in gastric epithelial cells using a reporter assay. Increasing ratios of Δ Np63 resulted in complete suppression of the TAp73 β transcriptional activity in a dose-dependent manner (Figure 7A). Furthermore, when Δ Np63 α and TAp73 β were co-expressed, Δ Np63 β efficiently suppressed protein expression of TAp73 transcriptional targets, PUMA and NOXA. (Figure 7B). A similar inhibitory effect of Δ Np63 α was observed in experiments with TAp73 α (data not shown). Thus, when expressed, Δ Np63 α inhibits transcriptional activity of TAp73.

To mimic Δ Np63 down-regulation by *Hp*, we next suppressed endogenous Δ Np63 protein using shRNA in AGS cells ectopically expressing TAp73. The siRNA efficiently inhibited the Δ Np63 α protein expression in these cells (Figure 7C, bottom panel). Concomitantly, the transcriptional activities of TAp73 α and TAp73 β were increased as was detected by p53/p73 luciferase reporter (Figure 7C). These data confirmed that exogenous and endogenous Δ Np63 are potent transcriptional inhibitors of TAp73.

To further analyze the effect of Δ Np63, AGS cells were transfected with either empty vector or Δ Np63 α and co-cultured with *Hp*. As shown in Figure 7D, Δ Np63 α significantly decreases protein expression of NOXA and PUMA, induced by *Hp* (Figure 7D). Moreover, Δ Np63 α -transfected cells were considerably more resistant to cell death induced by *Hp* (Figure 7E).

We next examined whether *Hp* affects the binding of Δ Np63 to TAp73. Using a co-immunoprecipitation approach, a significant decrease in TAp73- Δ Np63 complex was detected in cells co-cultured with *Hp* (Figure 7F, compare lanes 2 and 3). When combined, these findings suggest that down-regulation of Δ Np63 triggered by *Hp* reduces the inhibitory binding of Δ Np63 to TAp73 that results in increased transcriptional activity of TAp73.

To determine whether *Hp* virulence proteins, which have been shown to alter cell viability, regulate TAp73 protein, isogenic *cagA*-, *vacA*-, and *cagE*-null *Hp* mutants were generated and their ability to modulate the p73 protein were tested. The *vacA*-mutant had a similar effect on p73 as the wild-type strain (Figure 8A). In contrast, the ability to up-regulate TAp73 was compromised by the loss of either *cagA* or *cagE*, components of the *cag* PAI. Only a weak p73 increase was observed after 24 hours of co-culture with the *cagA* or *cagE* mutants compared to notable up-regulation after 12 hours with wild-type bacteria (Figure 8A, B). To expand this analysis, we co-cultured another gastric epithelial cell line MKN28 with six well-characterized *cag*+ or *cag*- *Hp* clinical isolates and analyzed the p73 protein. TAp73 α was predominantly up-regulated by *cag*+ strains confirming our results obtained with the isogenic mutants (Figure 8C).

c-Abl tyrosine kinase is known to regulate activity of p73⁹ and it is also activated by *Hp*.^{16, 21} To determine whether c-Abl is involved in the *Hp*-induced up-regulation of p73, *c-Abl* expression was silenced by shRNA (Figure 8D, lower panel). The inhibition of c-Abl significantly inhibited p73 induced by *Hp* suggesting that c-Abl mediates the p73 up-regulation (Figure 8D). To further examine the role of c-Abl, AGS cells were transiently transfected with CagA and c-Abl. The levels of p73 remained unchanged after transfections of CagA or c-Abl alone. However, co-transfection of c-Abl and CagA recapitulated the effect of *Hp* and led to up-regulation of p73 (Figure 8E). Moreover, c-Abl kinase activity is essential for the p73 up-regulation as kinase-deficient c-Abl mutant (KD) was unable to increase the p73 levels (Figure 8E). Together, these data implicate *cag* PAI and c-Abl kinase as important regulators of the p73 protein in *Hp*-infected gastric epithelial cells.

Discussion

Apoptosis is an important determinant of epithelial cell response to *Hp* infection, which has been implicated in the pathogenesis of gastritis and gastric cancer.⁵ In this study, we investigated the role of the p53 protein family in cell response to *Hp* infection. We found that TAp73 isoforms are strongly up-regulated by *Hp* in gastric epithelial cells *in vitro* and *in vivo*, and p73, as well as p63, are involved in *Hp*-induced apoptosis. Interestingly, p73 is up-regulated by post-translational mechanisms rather than by an increase of p73 mRNA. Our data suggest that direct interaction of epithelial cells with *Hp* leads to the TAp73 increase *in-vitro*. However, we cannot exclude that *in vivo* p73 levels are also controlled by inflammatory

processes associated with *Hp* infection. Indeed, it has been reported that p73 is induced by pro-inflammatory cytokine TNF α .²²

Currently, the biological function(s) of p73 in normal tissues is not well understood. Our results demonstrate that down-regulation of p73 activity suppresses apoptosis induced by *Hp*. In addition, co-culture of gastric epithelial cells with *Hp* lead to up-regulation of p53/p73 target genes involved in apoptosis and cell-cycle regulation, NOXA, PUMA, and p21/Waf1, in p53-deficient, as well as in wild-type p53 cell lines. Our analysis also shows that TAp73 up-regulates the Fas receptor in gastric epithelial cells, and inhibition of the TAp73 activity results in suppression of the FasR protein induced by *Hp*.

Previous studies have shown varied results as to whether p53 is activated by *Hp*.^{23, 24} In our experiments *in vitro*, we found that co-culture of epithelial cells with *Hp* resulted in unchanged and even slightly reduced levels of the p53 protein. However, our immunohistochemical analysis confirmed previously reported observations that the p53 protein level is elevated in gastric mucosa of *Hp*-infected patients (data not shown). It suggests that both p53 and p73 are involved in response to *Hp* infection, though they might have different functional roles. Additional studies are necessary to clarify this issue.

Our data suggest that p73 may be a novel component of the host-defense mechanism. Indeed, p73 knockout mice have been shown to be highly sensitive to chronic bacterial infections without obvious deficiencies in immune cell populations.¹² In mouse tissues, p73 is primarily expressed in the epithelia bordering the sites of infections.¹² In addition, our preliminary data suggest that p73 can be up-regulated by bacterial species other than *Hp* (data not shown). This hypothesis is also supported by recent studies showing that several pathogenic viruses, such as measles, adenovirus, hepatitis C and HTLV-1 have inherent mechanisms that suppress p73 activity.^{25–27} Moreover, the measles virus V protein specifically inhibits p73-mediated transcription and apoptosis, thereby suppressing PUMA gene expression.²⁶

p73 and p63 bind directly through the tetramerization domain. It also has been shown that the binding of Δ Np63 to TAp73 negatively regulates the p73 pro-apoptotic activity in cancer cells.²⁰ Our analysis suggests that *Hp*-induced apoptosis is regulated by TAp73- Δ Np63 interaction. Indeed, ectopic and endogenous Δ Np63 α inhibit the transcriptional activity of TAp73 isoforms in gastric epithelial cells. Δ Np63 α suppresses the up-regulation of p53/p73 pro-apoptotic targets induced by *Hp* and increases survival of epithelial cells. In addition, we found that the co-culture of gastric epithelial cells with *Hp* leads to strong down-regulation of Δ Np63 transcript and protein, decrease of TAp73- Δ Np63 binding, and up-regulation of TAp73. These cumulative changes activate the TAp73 protein.

Another important angle to the mechanism of p73 up-regulation is the role of bacterial factors. We found that *cagA* and *cagE*, components of the *cag* PAI, had significant effects on p73 protein levels. Deletion of *cagA* or *cagE* attenuated up-regulation of p73 protein that indicates the important role of the functional *cag* PAI, a genetic locus associated with gastric cancer. These data are consistent with early observations that the *cag* PAI is associated with up-regulation of pro-apoptotic proteins and increased apoptosis in human gastric epithelium *in vitro* and *in vivo*.^{24, 28} Activation of c-Abl protein kinase by *Hp* is also dependent on the functional *cag* PAI and specifically, *CagA* protein.^{16, 21} We found that p73 induction is attenuated in cells deficient in c-Abl expression. Thus, our data suggest that c-Abl mediates up-regulation of p73 in *Hp*-infected cells.

In summary, we have shown that apoptosis associated with *H. pylori* infection is mediated by p73 protein. p73 activation is controlled by the cellular proteins, Δ Np63 and c-Abl, and the *cag* PAI. These results suggest that p73 may play an important role in the pathogenesis associated with *H. pylori* infection.

Acknowledgements

Grant support: This work was supported by the National Cancer Institute grants NIH CA108956 and NIH CA129655.

References

1. Ernst PB, Peura DA, Crowe SE. The translation of *Helicobacter pylori* basic research to patient care. *Gastroenterology* 2006;130:188–206. [PubMed: 16401482]quiz 212-3
2. Shirin H, Sordillo EM, Oh SH, et al. *Helicobacter pylori* inhibits the G1 to S transition in AGS gastric epithelial cells. *Cancer Res* 1999;59:2277–2281. [PubMed: 10344728]
3. Peek RM Jr, Wirth HP, Moss SF, et al. *Helicobacter pylori* alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. *Gastroenterology* 2000;118:48–59. [PubMed: 10611153]
4. Wang TC, Goldenring JR, Dangler C, et al. Mice lacking secretory phospholipase A2 show altered apoptosis and differentiation with *Helicobacter felis* infection. *Gastroenterology* 1998;114:675–689. [PubMed: 9516388]
5. Moss SF, Calam J, Agarwal B, et al. Induction of gastric epithelial apoptosis by *Helicobacter pylori*. *Gut* 1996;38:498–501. [PubMed: 8707076]
6. Houghton J, Korah RM, Condon MR, et al. Apoptosis in *Helicobacter pylori*-associated gastric and duodenal ulcer disease is mediated via the Fas antigen pathway. *Dig Dis Sci* 1999;44:465–478. [PubMed: 10080136]
7. Fox JG, Wang TC. *Helicobacter pylori* infection: pathogenesis. *Curr Opin Gastroenterol* 2002;18:15–25. [PubMed: 17031225]
8. Houghton JM, Bloch LM, Goldstein M, et al. In vivo disruption of the fas pathway abrogates gastric growth alterations secondary to *Helicobacter* infection. *J Infect Dis* 2000;182:856–864. [PubMed: 10950781]
9. Gong JG, Costanzo A, Yang HQ, et al. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. *Nature* 1999;399:806–809. [PubMed: 10391249]
10. Flores ER, Sengupta S, Miller JB, et al. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell* 2005;7:363–373. [PubMed: 15837625]
11. Zaika AI, El-Rifai W. The role of p53 protein family in gastrointestinal malignancies. *Cell Death Differ* 2006;13:935–940. [PubMed: 16557273]
12. Yang A, Walker N, Bronson R, et al. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 2000;404:99–103. [PubMed: 10716451]
13. Franco AT, Israel DA, Washington MK, et al. Activation of beta-catenin by carcinogenic *Helicobacter pylori*. *Proc Natl Acad Sci U S A* 2005;102:10646–10651. [PubMed: 16027366]
14. Irwin M, Marin MC, Phillips AC, Seelan RS, et al. Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature* 2000;407:645–648. [PubMed: 11034215]
15. Tomkova K, Belkhir A, El-Rifai W, et al. p73 isoforms can induce T-cell factor-dependent transcription in gastrointestinal cells. *Cancer Res* 2004;64:6390–6393. [PubMed: 15374945]
16. Poppe M, Feller SM, Romer G, et al. Phosphorylation of *Helicobacter pylori* CagA by c-Abl leads to cell motility. *Oncogene* 2007;26:3462–3472. [PubMed: 17160020]
17. Tomkova K, El-Rifai W, Vilgelm A, et al. The gastrin gene promoter is regulated by p73 isoforms in tumor cells. *Oncogene* 2006;25:6032–6036. [PubMed: 16636659]
18. Hong SM, Cho H, Moskaluk CA, et al. p63 and p73 expression in extrahepatic bile duct carcinoma and their clinical significance. *J Mol Histol* 2007;38:167–175. [PubMed: 17385050]
19. Thottassery JV, Westbrook L, Someya H, et al. WB. c-Abl-independent p73 stabilization during gemcitabine- or 4'-thio-beta-D-arabinofuranosylcytosine-induced apoptosis in wild-type and p53-null colorectal cancer cells. *Mol Cancer Ther* 2006;5:400–410. [PubMed: 16505115]
20. Rocco JW, Leong CO, Kuperwasser N, et al. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell* 2006;9:45–56. [PubMed: 16413471]
21. Tammer I, Brandt S, Hartig R, et al. Activation of Abl by *Helicobacter pylori*: a novel kinase for CagA and crucial mediator of host cell scattering. *Gastroenterology* 2007;132:1309–1319. [PubMed: 17408661]

22. Chau BN, Chen TT, Wan YY, et al. Tumor necrosis factor alpha-induced apoptosis requires p73 and c-ABL activation downstream of RB degradation. *Mol Cell Biol* 2004;24:4438–4447. [PubMed: 15121862]
23. Ahmed A, Smoot D, Littleton G, et al. *Helicobacter pylori* inhibits gastric cell cycle progression. *Microbes Infect* 2000;2:1159–1169. [PubMed: 11008106]
24. Peek RM Jr, Blaser MJ, Mays DJ, et al. *Helicobacter pylori* strain-specific genotypes and modulation of the gastric epithelial cell cycle. *Cancer Res* 1999;59:6124–6131. [PubMed: 10626802]
25. Alisi A, Giambartolomei S, Cupelli F, et al. Physical and functional interaction between HCV core protein and the different p73 isoforms. *Oncogene* 2003;22:2573–2580. [PubMed: 12730672]
26. Cruz CD, Palosaari H, Parisien JP, et al. Measles virus V protein inhibits p53 family member p73. *J Virol* 2006;80:5644–5650. [PubMed: 16699046]
27. Kaida A, Ariumi Y, Ueda Y, et al. Functional impairment of p73 and p51, the p53-related proteins, by the human T-cell leukemia virus type 1 Tax oncoprotein. *Oncogene* 2000;19:827–830. [PubMed: 10698501]
28. Moss SF, Sordillo EM, Abdalla AM, et al. Increased gastric epithelial cell apoptosis associated with colonization with cagA + *Helicobacter pylori* strains. *Cancer Res* 2001;61:1406–1411. [PubMed: 11245442]

Abbreviations

FasR, Fas receptor; cagA, cytotoxin-associated gene A; vacA, vacuolating cytotoxin A; cagE, cytotoxin-associated gene E..

Figure 1. p73 protein levels are increased in gastric mucosa harvested from both gastritis patients positive for *H. pylori* and mice infected with *H. pylori*

Representative staining for p73 is shown for *Hp*-infected (A and C) and uninfected (B) patients (original magnification; $\times 20$). Black arrows show nuclear p73. Serial sections from the same patient were stained for p73 (C) and *Hp* (D). Insets depict magnified views of the gastric gland positive for *Hp* and p73 ($\times 40$). White arrows show *Hp* attached to the gastric epithelium. Morphological differences between C and D reflect thickness of serial sections. (E) p73 immunostaining ($\times 40$) in the antrum of a mouse infected with *Hp* for eight weeks reveals strong nuclear expression of p73 in epithelial cells (left panel). Inflammation is also observed within the lamina propria. p73 protein was up-regulated in the stomach of a mouse infected with

Hp for 3 days (right panel). The central microphotograph shows staining for p73 in the antrum of control uninfected mice. A weak cytoplasmic staining of mucus producing cells is present, but most likely non-specific.

Figure 2. Co-incubation with *H. pylori* increases endogenous protein levels of TAp73 in gastric epithelial cells

Protein lysates were prepared from control cells (–) or those co-cultured with *Hp* (+) for the indicated time and analyzed by Western blotting. (A) Protein levels of TAp73 α and TAp73 β isoforms were increased after co-culture of AGS cells with *Hp*. (B) Conditionally immortalized murine gastric epithelial cells grown under primary conditions were co-cultured with *Hp* strain 7.13 and analyzed using an anti-p73 β antibody. (C) Same as (A) except Kato III cells were analyzed.

Figure 3. Expression analysis of p73 and p63 isoforms in gastric epithelial cells co-cultured with *H. pylori*

(A) mRNA was prepared from control AGS cells (–) or those co-cultured with *Hp* (+) for the indicated time. mRNA expression of p73 and p63 isoforms was analyzed by RT-PCR. TAp73 mRNA levels did not change significantly; however, *Hp* dramatically decreased ΔNp63 mRNA. (B) The bar graph represents quantitative real-time RT-PCR analysis of p63 and p73 transcripts in AGS cells co-cultured with *Hp* for the indicated time. Data were normalized to HPRT1 mRNA expression. Expression of p73 and p63 isoforms in uninfected cells was arbitrarily set at 1. (C) Co-culture of Kato III cells with *Hp* prolongs the half-life of endogenous p73 protein (see the Material and Methods). Data depicted as mean ± SEM (n=3). (D) A representative Western blot of four separate experiments showing a decrease of the ΔNp63α protein in AGS cells co-cultured with *Hp*.

Figure 4. TAp73/p53 transcriptional targets are up-regulated in *H. pylori*-infected gastric epithelial cells

(A) p53-null, Kato III cells were transfected with the p53 luciferase reporter, PG-13LUC, and co-cultured with *Hp* for the indicated time intervals. Data are expressed as fold induction normalized to Renilla luciferase activity (mean $\pm$ SD; n=3). *Hp* increased the reporter activity. *, p<0.05 vs. uninfected cells. (B) Real-time PCR analysis of p21 and NOXA transcripts after co-culture of Kato III cells with *Hp*. The graph depicts the fold-induction of normalized gene expression. *Hp* significantly induced p21 and NOXA mRNAs. *, p<0.05 vs. uninfected cells (n=3). (C) Western blot analysis of p73 target genes, p21/Waf1 and NOXA, in Kato III cells. Protein lysates were prepared from control cells (–) or those co-cultured with *Hp* (+) for the indicated time. (D) Same as (C) except AGS cells were analyzed. (E) A representative immunoblot demonstrating that p53 is not up-regulated by *Hp* in AGS cells. Changes of p53 levels in uninfected cells at different time points likely reflect changes in cell density. As a positive control for p53 induction, cells were treated with 5mM camptothecin (Camp) for 24 hours. The graphs (C, D and E) represent the results of densitometric analysis of immunoblots

from three experiments and depict actin-normalized protein expression (mean $\pm$ SEM). *Hp* significantly increased expression of p53/p73 target genes. *, $p < 0.05$ vs. uninfected cells.

Figure 5. TAp73 isoforms up-regulate Fas receptor in gastric epithelial cells

(A) AGS cells were co-transfected with GFP and either pcDNA3 (Vect), TAp73 α or TAp73 β expression vectors. Gel loading was normalized for GFP protein expression. Transfection of p73 isoforms increased FasR protein levels. *, $p < 0.05$ vs. vector-transfected cells. (B) Protein lysates prepared from infected (+) and control (-) cells were analyzed by Western blotting using FasR-specific antibody. Co-culture of AGS cells with *Hp* led to up-regulation of FasR. *, $p < 0.05$ vs. uninfected cells. (C) AGS cells were transfected with either dominant-negative p73 mutant (DD) or pcDNA3 vector (Vect) or left uninfected (uninfected), and the levels of endogenous FasR were analyzed by Western blotting. The bottom panel shows expression of p53 protein. Inhibition of TAp73 activity by DD causes down-regulation of FasR. *, $p < 0.05$ vs. vector-transfected. Graphs show the results of densitometric analysis of immunoblots from three experiments and depict normalized FasR protein expression (mean $\pm$ SEM).

Figure 6. TAp73 mediates cell death induced by *H. pylori*

(A) AGS, Kato III or MKN45 cells were incubated alone or in the presence of *Hp* for 60 hours, apoptosis was then examined by TUNEL assay. As a positive control for apoptosis, AGS cells were treated with 10 μ M camptothecin (Camp) for 48 hours. Results are expressed as a percentage of TUNEL-positive cells. Co-culture of gastric epithelial cells with *Hp* induced apoptosis. **, $p < 0.01$ vs. uninfected cells ($n=4$). (B) AGS cells were transfected with the indicated vectors and co-cultured with *Hp* for 24 hours. Apoptosis was assessed by flow cytometry as described in the Material and Methods section. The proportion of GFP-positive cells in subG1 is shown. Cells were treated with camptothecin (Camp) as an additional control. Inhibition of TAp73 activity suppressed apoptosis induced by *Hp* (*, $p < 0.05$).

Figure 7. Cellular and bacterial factors play roles in up-regulation and activation of TAp73 by *H. pylori*

(A) Δ Np63 α inhibits transcriptional activity of TAp73 β as detected by the luciferase reporter, PG13Luc, in AGS cells. Suppression by Δ Np63 α for the molar ratios of TAp73 β to Δ Np63 α is indicated. Luciferase activity was normalized to the Renilla luciferase activity (mean $\pm$ SD; n=3). *, p<0.05 vs. cells co-transfected with TAp73 β and vector. (B) A representative Western blot demonstrating inhibition of endogenous PUMA and NOXA proteins by Δ Np63 α in AGS cells. Cells were co-transfected with plasmids expressing wild-type TAp73 β and either empty vector or Δ Np63 α at a 1:3 molar ratio. Cells in the "Vect" lane are transfected with pcDNA3 only. (C) Inhibition of Δ Np63 α by shRNA activated TAp73 α or TAp73 β proteins as assessed

by a luciferase reporter PG13Luc. *, $p < 0.05$ vs. cells transfected with a scrambled shRNA. Bottom panel: p63 shRNA vector inhibited expression of the Δ Np63 α protein as was detected by Western blotting. (D) A representative Western blot demonstrating that Δ Np63 α suppressed endogenous transcriptional targets of TAp73, NOXA and PUMA, induced by *Hp* in AGS cells. "Vect" and "Control" lanes represent pcDNA3-transfected and untransfected cells, respectively. (E) pcDNA3 (Vect)- or Δ Np63 α -transfected cells were co-cultured with *Hp* for 24 or 48 hours and cell survival was measured using MTT assay. Δ Np63 α increased survival of AGS cells co-cultured with *Hp*. *, $p < 0.05$ vs. pcDNA3-transfected cells (mean $\pm$ SD; $n=6$). (F) A representative immunoblot demonstrating inhibition of TAp73 binding to Δ Np63 α in *Hp*-infected cells (see the Material and Methods for details). Cisplatin treatment was used as a positive control.

Figure 8. The *cag* pathogenicity island and c-Abl protein kinase play a role in up-regulation of TAp73

(A) AGS cells were cultured in the presence of the wild-type *Hp* toxigenic strain J166 or isogenic *cagA*- or *vacA*- null mutants and protein levels of TAp73 were assessed by Western blotting. (B) Same as (A) except *cagE* mutant was analyzed. (C) MKN28 cells were co-cultured with *Hp* clinical isolates for 12 hours, and TAp73 levels were determined by Western blotting. (D) AGS cells stably transfected with either shRNA against c-Abl (clones N1-N3) or scrambled shRNA (clones C1-C3) were co-cultured with *Hp* or left untreated. Down-regulation of c-Abl inhibited p73 in *Hp*-infected cells. (E) AGS cells were transfected with indicated plasmids and analyzed for expression of p73, c-Abl, CagA, and phosphorylation of CagA by Western blotting. A co-transfection of c-Abl and CagA led to up-regulation of TAp73 and phosphorylation of CagA that was detected with an anti-P-Tyr antibody. Expression of HA-tagged CagA was determined using anti-HA antibody.