



Research Article

***Helicobacter Pylori* Infection Triggers PERK-Associated Survivin Loss in Gastric Tissue Samples and Cell Lines**

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Abstract

Infection by *Helicobacter pylori* (*Hp*) is the main risk factor associated with the development and progression of precancerous lesions to gastric cancer. Protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) is activated by endoplasmic reticulum (ER) stress, and ER stress-induced apoptotic cell death has been associated with *Hp* infection. Survivin, an inhibitor of apoptosis, is down-regulated in the mucosa of *Hp* infected subjects and loss of survivin in gastric cells correlates with reduced viability. Here, we determined whether *Hp*-induced changes in PERK contribute to the loss of survivin, previously associated with the genesis of gastric cancer precancerous lesions. Our results show that PERK is activated in the early stages of *Hp* infection in the human gastric mucosa affected by gastritis and PERK activation coincided with reduced survivin levels compared with *Hp*-negative gastritis. Upon *Hp* infection *in vitro*, PERK silencing restored survivin abundance and its activity increased survivin loss, in parallel with a partial downstream activation of the α -subunit of eukaryotic initiation factor-2 (eIF2 α). Our results suggest a novel mechanism by which *Hp*-stimulated PERK reduces gastric cell viability/proliferation by down-regulating survivin and is likely to favour the genesis of gastric cancer precancerous lesions.

Keywords: *Helicobacter pylori*; PERK; survivin; ER stress; precancerous gastric lesion; gastritis; gastric cancer

1. Introduction

Gastric cancer constitutes the third leading cause of cancer-related mortality worldwide [1]. This high mortality rate is often related to a late diagnosis, due to the lack of early signs or symptoms [2]. Infection by *Helicobacter pylori* (*Hp*), a Gram-negative bacterium that colonises the gastric

mucosa [3], constitutes the main risk factor associated with the development of gastric cancer. On average, *Hp* colonizes the gastric epithelium of about 50% of the world population [4]. The intestinal-type adenocarcinoma is the most frequent type of gastric cancer [5]. The latter is preceded by a prolonged multistep process termed precancerous gastric lesions [6-8]. These precancerous lesions begin with the transition from normal gastric mucosa to chronic non-atrophic gastritis, triggered commonly by *Hp* infection, which then leads to atrophic gastritis, intestinal metaplasia, and finally to dysplasia and gastric cancer [6-9]. Thus, *Hp* infection is particularly relevant during early stages of gastric carcinogenesis since eradication with antibiotics considerably reduces the presence of gastric precancerous lesions [10] until the intestinal metaplasia stage; however, antibiotics are ineffective once the disease has progressed beyond this stage [11, 12].

Hp infection triggers an inflammatory response indirectly mediated by cytokines, which facilitate the induction of chronic inflammation [13], or directly in host gastric cells through the action of bacterial virulence factors [14-16]. In addition, *Hp* chronic infection induces cellular adaptive mechanisms, such as apoptosis of gastric cells and reduced epithelial turnover leading to atrophy [17, 18], compensatory increases in cell proliferation [19, 20], phenotypic changes [21, 22] and endoplasmic reticulum (ER) stress-induced apoptotic cell death [23, 24].

The accumulation of unfolded and/or misfolded proteins within the ER induces ER stress, which can be resolved by an adaptive mechanism termed the unfolded protein response (UPR) [25]. Amongst ER stress sensors, the serine/threonine protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), is a protein whose activity is

regulated by protein misfolding within the ER [26]. Upon perturbation of ER homeostasis and accumulation of unfolded/misfolded proteins, PERK is activated undergoing simultaneous dimerisation/oligomerisation and autophosphorylation [25]. The PERK canonical pathway inhibits global protein translation, hence the number of proteins entering the ER is reduced [27]. However, failure to resolve ER stress induces the UPR, which initiates stress signalling pathways including those involved in cell death [28-30]. Particularly, *Hp* infection triggers ER stress [23] and activates the UPR [23, 31]. In *Hp*-infected gastric mucosa, BiP/GRP78 (binding immunoglobulin protein), a marker of ER stress, is increased compared with healthy *Hp*-negative subjects [23].

We have previously shown that survivin, a member of the inhibitor of apoptosis family of proteins [32], which is over-expressed in many human cancers [33], is down-regulated in the mucosa of *Hp* infected subjects [34]. *In vitro*, loss of survivin correlated with increased apoptosis and reduced viability in gastric cell lines [15, 34]. Loss of survivin due to *Hp* is predicted to disrupt gastric mucosa homeostasis [15, 34] which could then exacerbate signalling pathways that favour the progression of precancerous lesions. Evidence suggests that *Hp* triggers ER stress [23] and activates the UPR [23, 31] leading to gastric cell death; however, it remains unclear whether these cellular mechanisms may be connected to *Hp*-induced down-regulation of survivin. Here we investigated whether *Hp*-induced changes in the ER stress sensor protein PERK may contribute to the loss of survivin, previously associated with gastric cell death. These findings represent the first molecular link that connects ER stress-triggered by *Hp* infection to gastric cell death and the genesis of gastric cancer precancerous lesions.

2. Materials and Methods

2.1 Tissue Samples

Gastric biopsy specimens from corpus and antrum of 50 subjects who underwent upper endoscopy between 2010 and 2012 at the CRS - San Rafael, SSMSO, Santiago were analysed. Written informed consent, approved by the Ethics Committee of the Pontificia Universidad Católica de Chile (project #14-280; date 4 June 2015) and the Ethics Committee of the Faculty of Medicine, Universidad de Chile (project #046-2017; date 9 May 2017), was obtained from each subject. The study was conducted in accordance with the rules of the Helsinki declaration. Gastric precancerous lesions were classified based on the Operative Link on Gastritis Assessment (OLGA) [35], a histological assessment of random biopsies taken from antrum and corpus areas of the stomach according to the Sydney protocol [36]. At least four sites were sampled from the stomach during upper endoscopy (two antral and two corpus). The OLGA scoring standard was used to grade and stage chronic gastric inflammation, gastric atrophy and intestinal metaplasia. The analysis of corpus and antrum biopsies corresponded to sections from early stages of gastritis (OLGA 0-II), clinically grouped in *Hp*-positive and *Hp*-negative cases. On average, intestinal metaplasia was undetectable or less than 30% in all samples. *Hp* status was evaluated by the urease test [37], Giemsa staining [38] and immunohistochemistry (see below).

Biopsies were collected at room temperature during the endoscopic procedure. Samples were fixed in zinc-formalin at 4°C for 24 h and then transferred to phosphate-buffered saline pH 7.2 and kept at 4°C. After standard histological processing, gastric tissue biopsies were embedded in paraffin and 3µm-thick sections were obtained, subsequently deparaffinised, rehydrated, and routinely

stained with haematoxylin-eosin for histopathological evaluation.

2.2 Immunohistochemistry analysis

We used Resource Identification Portal (RRID) to allow research resource identification where possible. The protein abundance of PERK (1:500; Cell Signaling Technology Cat# 3192, RRID:AB_2095847), phospho-PERK (P-PERK(Thr982)) (1:500; Abcam Cat# ab192591, RRID:AB_2728666), and survivin (1:300; R and D Systems Cat# AF886, RRID:AB_355684) was detected using the avidin-biotin-peroxidase complex method (VECTASTAIN® Universal HRP Kit; Vector Laboratories Cat# PK-7800, RRID:AB_2336829) in gastric biopsy sections and the 3,3'-diaminobenzidine (DAB)-Substrate Chromogen (Dako #K3468). The presence of *Hp* in samples was detected with an *Hp*-specific polyclonal antibody (Dako #B0471). All sections were counterstained with haematoxylin. Quantification and analysis of PERK, P-PERK(Thr982) and survivin-Q-score (intensity multiplied by percentage of positively stained cells) was performed by two independent pathologists who were unaware of the clinical data and the objectives of the study (G.C. and A.R.).

2.3 Cell lines, strains, and culture conditions

The gastric adenocarcinoma cell line AGS, obtained from the American Tissue Culture Collection (ATCC Cat# CRL-1739, RRID:CVCL_0139), and the non-transformed gastric epithelial cell line GES-1 (kindly provided by Dr. Armando Rojas, Universidad Católica del Maule, Chile) were cultured in Roswell Park Memorial Institute medium (RPMI 1640 medium; Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (Biological Industries) and antibiotics (10,000 U/mL penicillin, 10 µg/mL streptomycin), at 37°C in a humidified atmosphere

supplemented with 5% CO₂. The completely sequenced *Helicobacter pylori* (*Hp*) strain 26695 [KE26695] (ATCC Cat#700392) was used. *Hp* was routinely grown on trypticase soy agar (TSA) plates supplemented with 5% horse serum (Biological Industries), the culture supplement Vitox (Oxoid), and the antibiotic supplement Dent (Oxoid) for 24 h at 37°C in a humidified atmosphere supplemented with 5% CO₂ [34].

2.4 Infection of gastric cells with *Helicobacter pylori*

GES-1 and/or AGS cells were infected with a multiplicity of infection (MOI) of 100 (bacteria:cell ratio of 100:1) for 4 and 24 h as previously described by Valenzuela *et al.* [15, 34]. For infection experiments 8x10⁵ cells were seeded onto a 60-mm tissue culture plate in culture medium without antibiotics for 24 h and then infected with *Hp*.

2.5 siRNA transfection

GES-1 and AGS cells were transfected with 100 nM SignalSilence® PERK siRNA I (siRNA PERK) (Cell Signaling Technology #9024) or 100 nM non-specific Control siRNA (Cnt siRNA) (Unconjugated; Cell Signaling Technology #6568) 16 h before *Hp* infection using the Lipofectamine RNAi Max reagent (Thermo Scientific) following the manufacturer's instructions.

2.6 Inhibition of PERK activity

GES-1 and AGS cells were pre-incubated with 25 µM GSK2606414 (Tocris Bioscience #5107) [39, 40], a selective PERK inhibitor, 1 h before *Hp* infection.

2.7 Immunoblotting analysis

AGS or GES-1 cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 8.0; 150 mM NaCl, 1% NP-40 (IGEPAL #CA-630), 0.5%, sodium deoxycholate and 0.1% sodium dodecyl

sulphate (SDS)) with protease and phosphatase inhibitors and proteins (50 µg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes [16]. Blots were blocked for 1 h at room temperature using 3% bovine serum albumin (Rockland) in Tris-buffered saline (TBS)-Tween. Membranes were then incubated in primary antibody diluted in 3% bovine serum albumin in TBS-Tween. Protein abundance of PERK (1:1000; Cell Signaling Technology Cat# 3192, RRID:AB_2095847), phospho-PERK (P-PERK(Thr982)) (1:1000; Abcam Cat# ab192591, RRID:AB_2728666), survivin (1:3000; R and D Systems Cat# AF886, RRID:AB_355684), phospho-eIF2 α (P-eIF2 α (Ser51)) (1:1000; Abcam Cat# ab32157, RRID:AB_732117), eIF2 α (1:1000; Santa Cruz Biotechnology Cat# sc-133132, RRID:AB_1562699) and PUMA α/β (1:1000; Santa Cruz Biotechnology Cat# sc-374223, RRID:AB_10987708) was determined in GES-1 and AGS cells. Horseradish peroxidase conjugated secondary anti-rabbit and anti-mouse antibodies were used (Rockland) and immunolabeling was visualized with enhanced chemiluminescence detection solution (Thermo Scientific). Densitometry was performed using ImageJ software (<http://rsbweb.nih.gov/ij/>; National Institutes of Health). Target protein abundance in each individual lane was normalized to β -actin (1:10,000; Sigma-Aldrich Cat# A5316, RRID:AB_476743) or Hsp90 (1:5000; Santa Cruz Biotechnology Cat# sc-13119, RRID:AB_675659).

2.8 BrdU incorporation assay

Gastric cells were plated on 96-well plates at a density of 5×10^3 cells/well. Cell proliferation was detected after 24 h of *Hp* infection in uninfected and infected cells by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation using a colorimetric BrdU Cell Proliferation Assay Kit

(BioVision, Inc. #K306) according to the manufacturer's instructions.

2.9 Viability assay

Gastric cells were plated on 96-well plates at a density of 5×10^3 cells/well. Cell viability was evaluated after 24 h of incubation in uninfected and *Hp*-infected cells by the MTS assay (Promega #G5421) according to the manufacturer's instructions.

2.10 Statistical analysis

Clinical data were expressed as mean \pm standard error of the mean (SEM). Other data were expressed as the mean + SEM of at least 3 independent experiments. Statistical analysis was carried out with GraphPad Prism software version 5.0 (San Diego, CA, USA). To determine whether data distribution was normal, a Shapiro-Wilk test was initially performed. Clinical sample analyses were performed using Mann-Whitney test. Immunoblotting statistical analyses, using one-way ANOVA (or non-parametric) test with Bonferroni post-hoc test and assay data were analysed using two-way ANOVA. Statistical significance was defined as $p < 0.05$.

3. Results

3.1 PERK activity increased in human gastric tissue samples infected with *Helicobacter pylori*

Hp infection is relevant during the early stages of gastric carcinogenesis [10], particularly during gastritis. Thus, we examined stomach corpus and antrum biopsy sections from OLGA 0-II stage subjects, an equivalent to the early stages of gastritis, clinically grouped in *Hp*-positive and *Hp*-negative cases. PERK protein was detected in gastric glandular cells from corpus and antrum biopsy sections from subjects in OLGA 0-II stages (Figure 1). Although PERK staining varied in intensity between samples, total

PERK levels remained unchanged between *Hp*-infected and non-infected individuals (Figure 1A,B). Representative PERK immunostaining images are shown in Figure 1E. P-PERK (Thr982), used as a marker of PERK activation, showed a significant increase in subjects with *Hp*-associated gastritis in both antrum and corpus, compared with *Hp*-negative individuals (Figure 1C,D). P-PERK (Thr982) staining localized to the cytoplasm of gastric glandular cells and also to the surrounding mesenchymal tissue in *Hp* positive-OLGA 0-II gastric biopsy sections (Figure 1E). Whereas total PERK protein abundance remained independent of *Hp* infection, PERK activity was markedly increased in gastric tissue samples from early stages of *Hp*-associated gastritis. Given our previously published studies linking *Hp*-induced gastritis to loss of the inhibitor of apoptosis survivin, we sought to determine whether PERK activity and/or abundance changed in *Hp*-infected gastric cells *in vitro* and whether these changes might be linked to the loss of survivin.

3.2 Total PERK abundance remained unchanged following *Helicobacter pylori* infection of gastric cells

To determine whether *Hp* infection induces changes in total PERK abundance *in vitro*, we infected gastric cell lines GES-1 and AGS with the wild-type *Hp* strain 26695 for 24 h, at a multiplicity of infection (MOI) of 100. Immunoblotting analysis of cell lysates revealed that PERK abundance remained unaltered following *Hp* infection for 4 and 24 h, compared with uninfected controls for 24 h (Figure 2A,B). Silencing of PERK resulted in a significant reduction in PERK protein levels by 80% to 86% in GES-1 and AGS cells after 24 h of *Hp* infection, respectively (Figure 2A,B). In addition, PERK silencing significantly increased cell viability following *Hp* infection, by ~35% after 24 h (Suppl. Figure 1A). Pharmacological inhibition of PERK activity, using the PERK inhibitor GSK2606414, did

not have an effect on PERK abundance after *Hp* infection for 24 h (Figure 2C,D). Thus, as was observed in the clinical samples, total PERK protein abundance was not affected by *Hp* infection *in vitro*.

3.3 PERK activity following *Helicobacter pylori* infection of gastric cells

Hp infection for 24 h induced a significant 3-fold increase in PERK activation in the non-transformed gastric cell line GES-1, which was then inhibited, by the PERK kinase activity inhibitor GSK2606414 (Figure 3A). PERK activation status in transformed gastric AGS cells remained unchanged after 24 h *Hp* infection in (Figure 3B), though PERK activity was then reduced (~50%) after treatment with GSK2606414 (Figure 3B) suggesting that, after 24 h, *Hp* infection does not induce PERK activation in AGS cells. However, time-course experiments evaluating PERK activation in AGS cells suggest that PERK activity is significantly higher at earlier times of infection. After 4 h of *Hp* infection PERK activity increased compared to uninfected cells, and this increment was partly inhibited by GSK2606414 (Suppl. Figure 2).

3.4 Survivin abundance decreased in human gastric tissue samples infected with *Helicobacter pylori*

ER stress-induced apoptotic cell death, via the PERK pathway, has been associated with *Hp* infection [23]. Moreover, studies from our laboratory linked loss of survivin in *Hp* infected cells to apoptosis [15, 34]. With this in mind, we hypothesized that *Hp*-induced changes in PERK may contribute to the loss of survivin in gastric cells. Therefore, we determined whether survivin abundance also changed in the same clinical cohort evaluated in Figure 1. Survivin staining was predominantly located to the cytoplasm of gastric glandular cells in corpus and antrum gastric biopsy sections from OLGA 0-II stages of gastritis,

clinically grouped in *Hp*-positive and *Hp*-negative cases (Figure 4). Survivin levels decreased significantly in both the antrum and corpus of *Hp*-positive individuals, compared with individuals with *Hp*-negative gastritis (Figure 4A,B). Representative immunostaining images are shown in Figure 4C. Therefore, increased PERK activity in biopsy sections from OLGA 0-II stages of gastritis of *Hp* infected subjects coincides with down-regulation of survivin levels.

3.5 *Helicobacter pylori*-stimulated PERK activity down-regulated survivin abundance

Immunoblotting analysis of gastric cell lysates revealed that survivin was reduced 30% in GES-1 cells (Figure 5A), and decreased 50% in AGS cells (Figure 5B) after 24 h of *Hp* infection. This effect was reverted upon PERK silencing, where we detected a 21% and 50% recovery in survivin levels after 24 h *Hp* infection in GES-1 and AGS cells, respectively. Restoration of survivin levels by silencing PERK correlated with increased proliferation of AGS cells (Suppl. Figure 1B). The pharmacological inhibition of PERK activity caused a marked restoration of survivin abundance by increasing levels 23% in GES-1 cells (Figure 5C), and completely restoring survivin abundance in AGS cells (Figure 5D). In addition, activation of the PERK pathway initiates expression of pro-apoptotic BH3-only proteins [29], which control cellular homeostasis and also participate in mitochondria-dependent cell death [30]. Particularly, p53 upregulated modulator of apoptosis α/β (PUMA α/β) is a master regulator of apoptosis that inhibits anti-apoptotic Bcl-2 family members [41]. Preliminary data show that the abundance of PUMA α/β is increased after 24 h of *Hp* infection, but PERK silencing did not have an effect on PUMA α/β abundance in gastric cells infected with *Hp* (Suppl. Figure 3).

3.6 Activation of eIF2 α partly contributed to the reduction in survivin protein levels

The PERK canonical pathway inhibits global protein translation by direct phosphorylation of the α -subunit of eukaryotic initiation factor (eIF2 α). In response to eIF2 α phosphorylation, the number of proteins entering the ER is reduced [27], but translation of other mRNAs, such as activating transcription factor 4 (ATF4) [42] and C/EBP-homologous protein (CHOP) increases, leading to cell death (49). Thus, we tested whether the activation of eIF2 α via the PERK pathway is a potential mechanism linking PERK to the loss of survivin after *Hp* infection in gastric cells. Non-transformed GES-1 cells displayed significant eIF2 α activation (~50% increase) 24 h after *Hp* infection, compared with control uninfected cells, which was partially reduced (32%) when PERK was silenced (Figure 6A). Inhibition of PERK activity during *Hp* infection for 24 h led to a mild but significant decrease in eIF2 α activation (Figure 6B). Total eIF2 α protein abundance remained unaffected by *Hp* infection for 24 h in GES-1 cells (Suppl. Figure 5A). On the contrary, the transformed gastric adenocarcinoma cell line AGS displayed eIF2 α activation after 4 and 24 h *Hp* infection to a lesser extent compared with uninfected controls (Suppl. Figure 4A). This response was unaffected by either PERK silencing (Suppl. Figure 4A) or the pharmacological inhibition of PERK activity (Suppl. Figure 4B). Likewise, total eIF2 α abundance was unchanged by *Hp* infection after 24 h (Suppl. Figure 5B). As in Figure 3B, where PERK activation remained unchanged, the reduced eIF2 α downstream activation after *Hp* infection suggests that the tumour-derived AGS cells already display high ER stress levels.

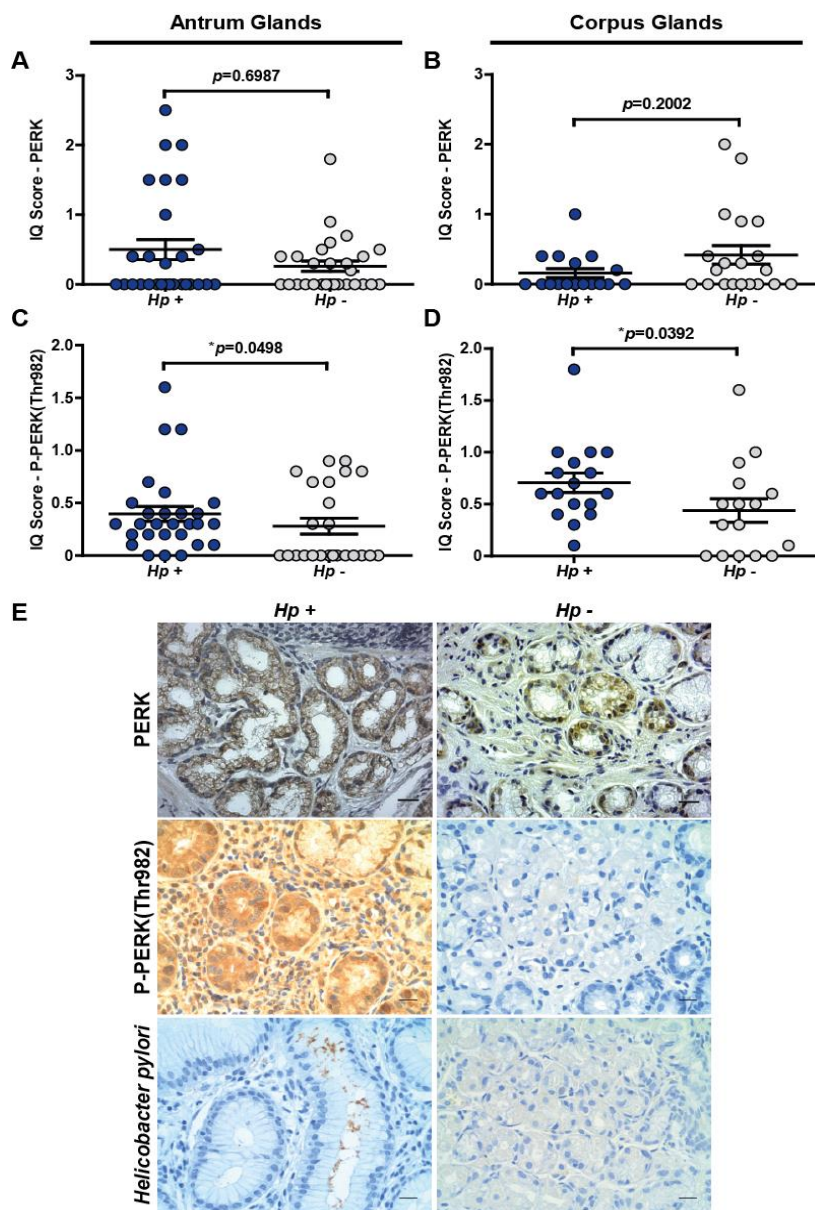


Figure 1: Immunohistochemical detection and analysis of PERK and P-PERK(Thr982) in gastric tissue samples of corpus and antrum from OLGA 0-II stages grouped in *Hp*-positive and *Hp*-negative individuals. (A) Semi-quantitative scoring (IQ) of PERK staining in antrum glands in *Hp*-positive and *Hp*-negative individuals; n=28, $p=0.6987$, mean \pm SEM; (B) IQ score of PERK staining in corpus glands in *Hp*-positive and *Hp*-negative individuals; n=17, $p=0.2002$, mean \pm SEM; (C) IQ score of P-PERK(Thr982) staining in antrum glands in *Hp*-positive and *Hp*-negative individuals; n=24, $*p=0.0498$, mean \pm SEM; (D) IQ score of P-PERK(Thr982) staining in corpus glands in *Hp*-positive and *Hp*-negative individuals; n=16, $*p=0.0392$, mean \pm SEM; (E) Representative PERK, P-PERK(Thr982) and *Helicobacter pylori* (*Hp*) immunostaining in gastric tissue. Immunostaining is visualised as a brown pigment. All samples were counterstained with haematoxylin (blue: nuclei). Scale bar: 50 μ m.

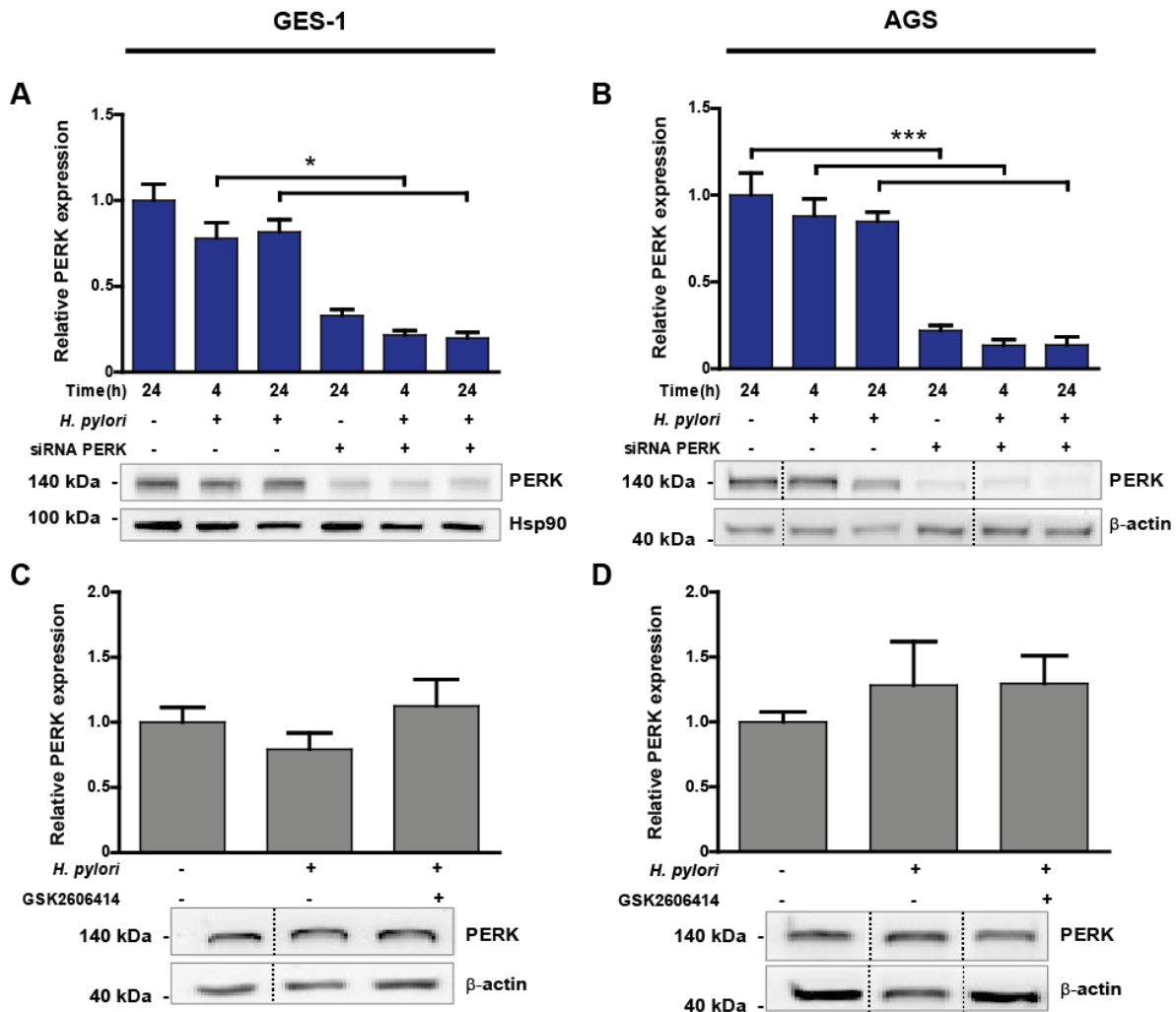


Figure 2: (A-B) Representative immunoblots with the corresponding graphic quantifications showing the mean relative protein abundance of PERK (normalised to β-actin or Hsp90 abundance) in uninfected (*H. pylori* -) and *Hp*-infected (*H. pylori* +) GES-1 (A) and AGS (B) cells. Gastric cells were infected with *Hp* for 4 and 24 h; in parallel, uninfected control cells were kept in medium for 24 h. PERK silencing (siRNA PERK) was achieved by transfecting GES-1 or AGS cells with 100 nM PERK siRNA 16 h before *Hp* infection. Non-specific siRNA (100 nM) was used as control (-). (A) n=5, *p<0.05; mean + SEM; (B) n=7, ***p<0.001; mean + SEM. (C-D) PERK protein abundance in uninfected (*H. pylori* -) and *Hp*-infected (*H. pylori* +) GES-1 (C) and AGS (D) cells after 24 h. Representative immunoblots with the corresponding graphic quantifications showing the mean relative protein abundance of PERK (normalised to β-actin abundance). GES-1 and AGS cells were pre-incubated with 25 μM GSK2606414, a selective PERK inhibitor, 1 h before *Hp* infection. (C) n=5; mean + SEM; (D) n=7; mean + SEM. Dotted lines indicate where lanes, from the same immunoblot, have been removed for clarity.

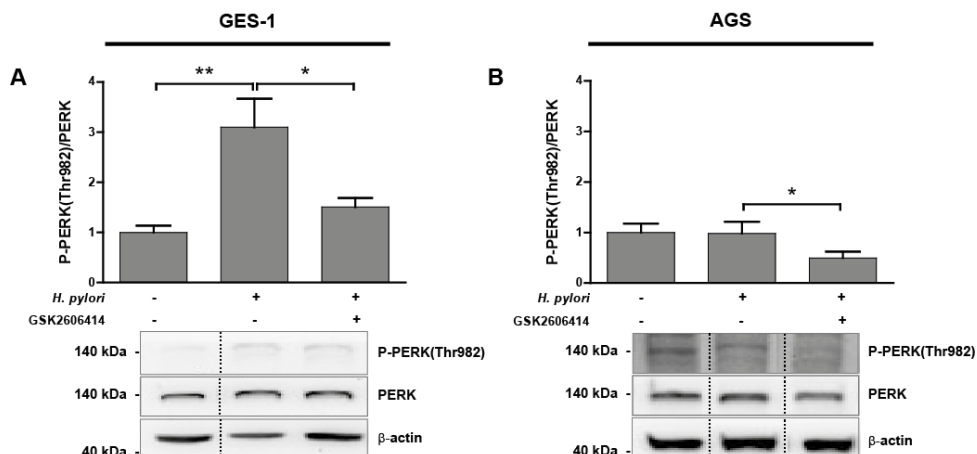


Figure 3: (A-B) P-PERK(Thr982) protein levels in uninfected (*H. pylori* -) and *Hp* infected (*H. pylori* +) GES-1 (A) and AGS (B) cells after 24 h. Representative immunoblots with the corresponding quantifications from several experiments showing P-PERK(Thr982)/PERK ratios. Values for P-PERK(Thr982) and PERK were normalised to β -actin abundance. GES-1 and AGS cells were pre-incubated for 1 h with 25 μ M GSK2606414 before *Hp* infection. (A) n=5, ** p <0.01, * p <0.05; mean + SEM; (B) n=4, * p <0.05; mean + SEM. Dotted lines indicate where lanes, from the same immunoblot, have been removed for clarity.

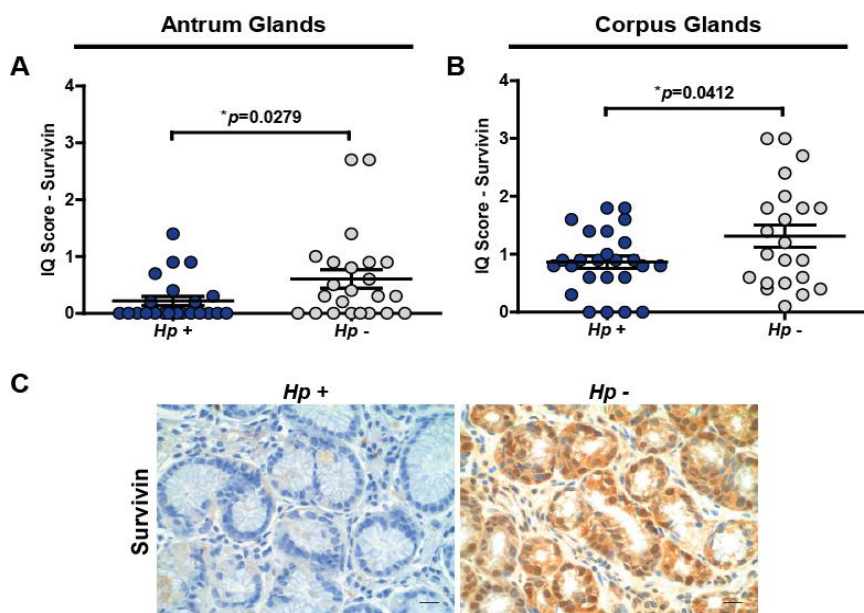


Figure 4: Immunohistochemical detection and analysis of survivin in gastric tissue samples from corpus and antrum of OLGA 0-II stages grouped in *Hp*-positive and *Hp*-negative individuals. (A) Semi-quantitative scoring (IQ) of survivin staining in antrum glands in *Hp*-positive and *Hp*-negative individuals; n=23, * p =0.0279; mean \pm SEM; (B) IQ score of survivin staining in corpus glands in *Hp*-positive and *Hp*-negative individuals; n=22, * p =0.0412, mean \pm SEM; (C) Representative survivin immunostaining in antrum and corpus gastric tissue. Staining is visualised as a brown pigment. All samples were counterstained with haematoxylin (blue: nuclei). Scale bar: 50 μ m.

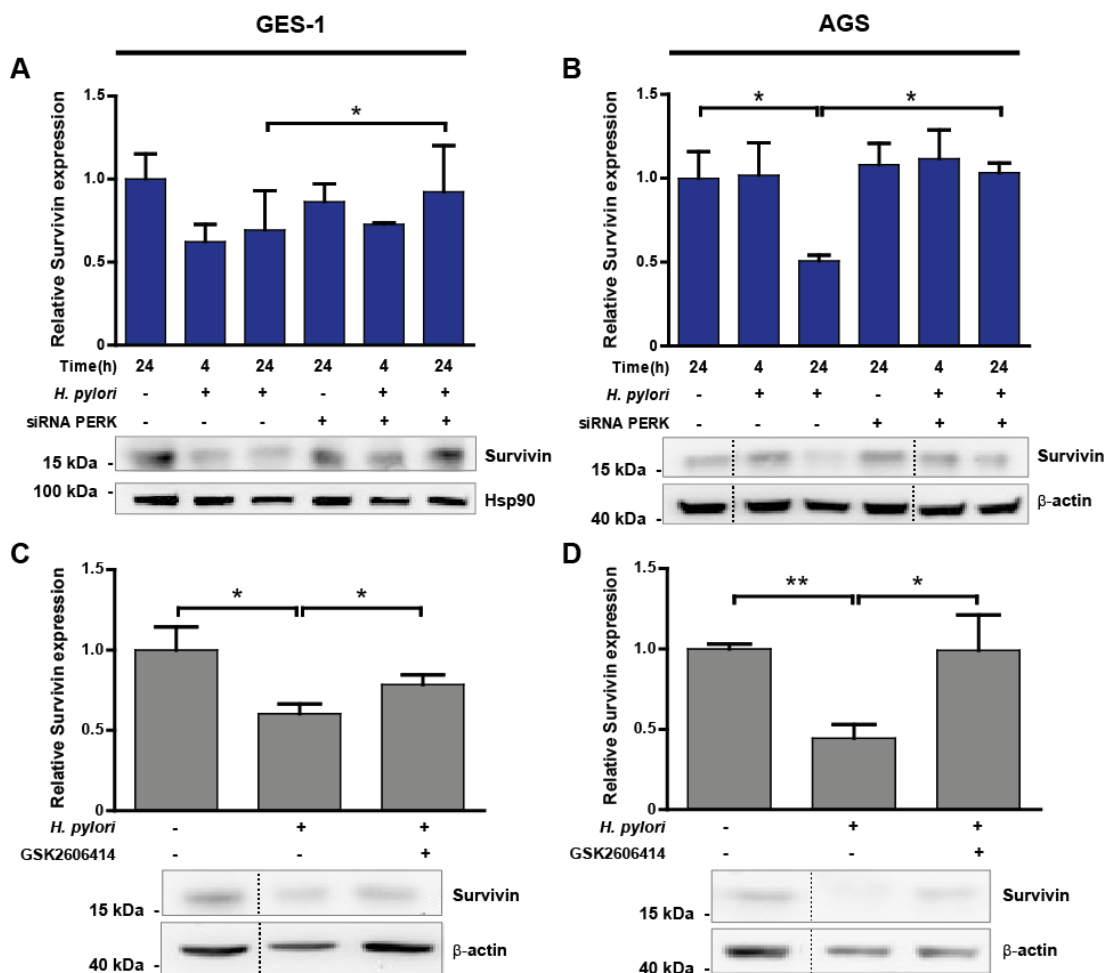


Figure 5: (A-B) Representative immunoblots with the corresponding graphic quantifications showing the mean relative protein abundance of survivin (normalised to β -actin or Hsp90 abundance) in uninfected (*H. pylori* -) and *Hp*-infected (*H. pylori* +) GES-1 (A) and AGS (B) cells. Gastric cells were infected with *Hp* for 4 and 24 h; in parallel, uninfected control cells were kept in medium for 24 h. PERK silencing (siRNA PERK) was achieved by transfecting GES-1 or AGS cells with 100 nM PERK siRNA 16 h before *Hp* infection. Non-specific siRNA (100 nM) was used as control (-). (A) $n=3$, $*p<0.05$; mean + SEM; (B) $n=3$, $*p<0.05$; mean + SEM. (C-D) Survivin protein abundance in uninfected (*H. pylori* -) and *Hp*-infected (*H. pylori* +) GES-1 (C) and AGS (D) cells after 24 h. Representative immunoblots with the corresponding graphic quantifications showing the mean relative protein abundance of survivin (normalised to β -actin abundance). GES-1 and AGS cells were pre-incubated with 25 μ M GSK2606414, a selective PERK inhibitor, 1 h before *Hp* infection. (C) $n=4$, $*p<0.05$; mean + SEM; (D) $n=3$, $**p<0.01$, $*p<0.05$; mean + SEM. Dotted lines indicate where lanes, from the same immunoblot, have been removed for clarity.

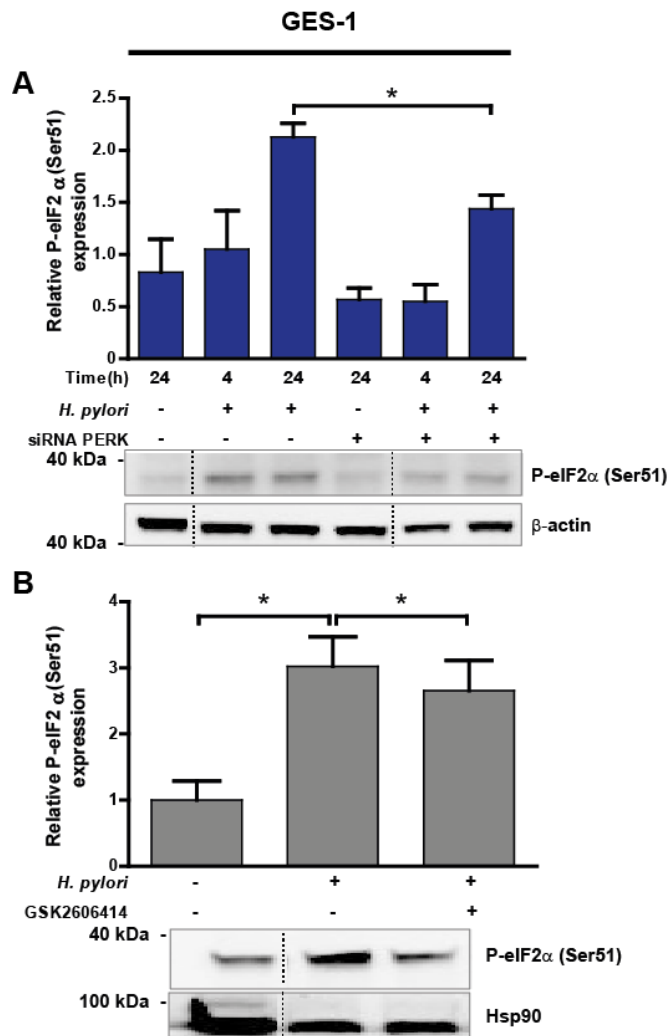


Figure 6: (A) eIF2α protein activation, measured as P-eIF2α(Ser51), in uninfected (*H. pylori* -) and *Hp*-treated (*H. pylori* +) GES-1 cells after 4 and 24 h. Representative immunoblots with corresponding graphic quantification showing mean relative protein abundance of P-eIF2α(Ser51) (normalised to β-actin abundance). Gastric cells were infected with *Hp* for 4 and 24 h; in parallel, uninfected control cells were kept in medium for 24 h. PERK silencing (siRNA PERK) was achieved by transfecting GES-1 cells with 100 nM PERK siRNA 16 h before *Hp* infection. Non-specific siRNA (100 nM) was used as a control (-). n=3, **p*<0.05; mean + SEM. (B) P-eIF2α(Ser51) abundance in uninfected (*H. pylori* -) and *Hp*-treated (*H. pylori* +) GES-1 cells after 24 h. Representative immunoblots with the corresponding graphic quantification showing mean relative protein abundance of P-eIF2α(Ser51) (normalised to Hsp90 abundance). GES-1 cells were pre-incubated with 25 μM GSK2606414, a selective PERK inhibitor, 1 h before *Hp* infection. n=4, **p*<0.05; mean + SEM. Dotted lines indicate where lanes, from the same immunoblot, have been removed for clarity.

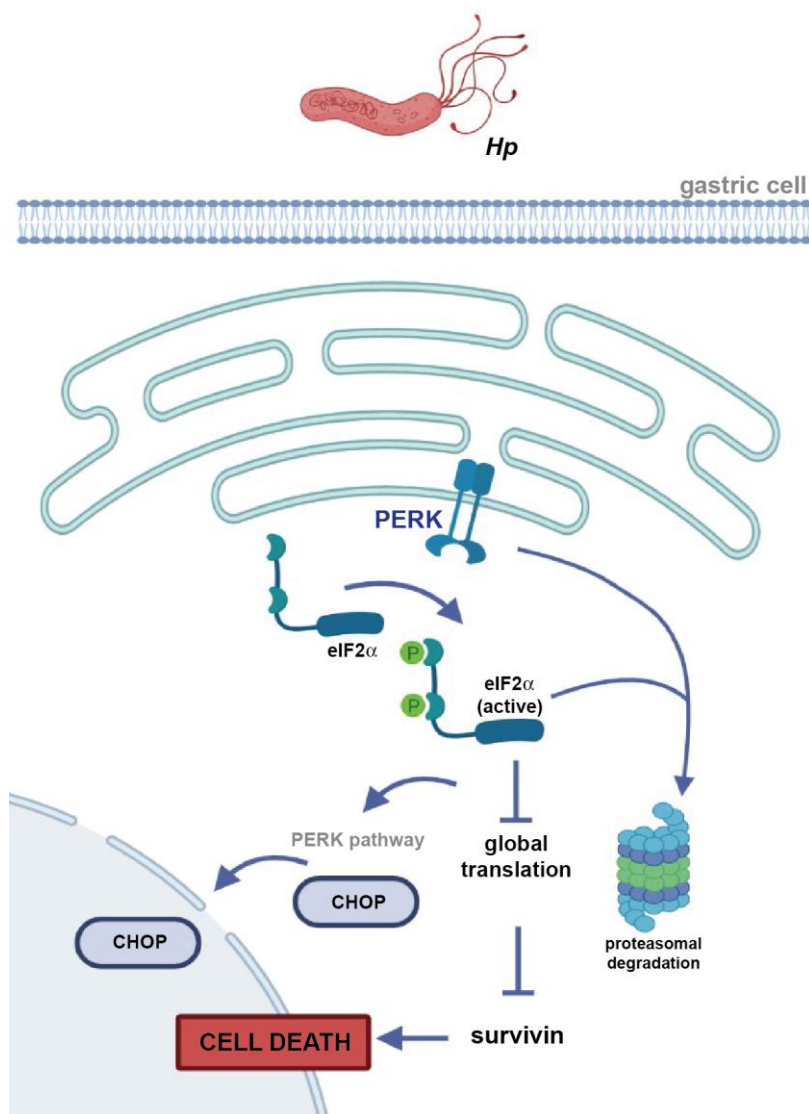


Figure 7: Schematic illustration depicting how PERK activity and/or abundance could contribute to the loss of survivin in gastric cell during *Helicobacter pylori* (*Hp*) infection. In non-transformed gastric cells, *Hp* induces ER stress sensor PERK activation and downstream eIF2 α , leading to a reduction in global protein translation and/or increased PERK-dependent proteasomal degradation, which could explain how survivin abundance is reduced. Activation of eIF2 α downstream of PERK triggers the canonical apoptosis pathway (via transcription factor C/EBP-homologous protein (CHOP)) resulting in cell death. This sequence of events reported reflects what was observed in tissue samples given that there, *Hp* infection correlated with increased PERK phosphorylation and loss of survivin. Partly created with BioRender.com.

4. Discussion

Here we determined whether *Hp*-induced changes in the ER stress sensor PERK contributed to the loss of survivin, previously associated with gastric cell death and the genesis of gastric cancer precancerous lesions. Our results showed that PERK is activated in *Hp* infection during gastritis in precancerous human gastric mucosa, which correlated with reduced survivin levels compared with *Hp*-negative gastritis. *In vitro*, when infected with *Hp* for 24 h, PERK silencing restored survivin abundance in gastric cell lines, thus mechanistically linking PERK activation to the loss of survivin. Finally, we propose that activation of eIF2 α , downstream of the PERK canonical pathway, could represent one mechanism connecting *Hp* infection to PERK activation, downregulation of survivin and gastric cell death. Consequently, the cellular adaptive ER stress sensor PERK is connected to *Hp* infection and the reduction in survivin abundance, both in clinical samples and *in vitro* in gastric cells. Following *Hp* infection, the loss of survivin via PERK-dependent mechanisms results in gastric cell death, which could favour the genesis and progression of gastric cancer precancerous lesions.

Our data are the first to demonstrate that the UPR sensor, PERK is activated in clinical samples from *Hp*-positive individuals during OLGA 0-II staged gastritis, suggesting that PERK is chronically activated in *Hp*-positive compared with *Hp*-negative gastritis. Total PERK abundance remained unchanged in these samples. These observations agree with our *in vitro* data, where total PERK abundance was comparable between *Hp*-infected and non-infected gastric cells. *Hp* infection induced PERK activation in GES-1 cells, but it decreased in the AGS cell line after 24 h of infection. Instead, discrete activation of PERK was observed at earlier time points following infection. The

non-transformed gastric cell line GES-1 used here represents the best available model of a “normal” gastric cell line and the response of these cells to infection likely reflects more closely what happens in the gastric mucosa. As for other gastric cancer cell lines, AGS are a commonly employed gastric adenocarcinoma cell line, with higher basal ER stress levels [43], in which PERK activation upon *Hp* infection was detected but occurred with different kinetics.

Although PERK activity was markedly increased in GES-1 cells infected by *Hp* for 24 h, the inhibitory effect of GSK2606414 was partial. GSK2606414 was initially identified as a selective PERK inhibitor [39], by blocking PERK activation in cells and inhibiting tumour growth in human xenograft models in mice [39]. However, for *Hp* infection of gastric cells the situation appears to be complex. Indeed, GSK2606414 was also demonstrated to inhibit RIPK1, a kinase involved in TNF α -mediated cell death [44] and more recently, KIT, a type III receptor tyrosine kinase, expressed amongst others in the intestinal epithelium [45, 46]. Inhibition of these shared targets by GSK2606414 may lead to compensatory mechanisms that explain the only partial effect of GSK2606414 in *Hp*-infected gastric GES-1 cells. While interesting, such possibilities remain to be explored in future studies.

So far, other studies have focused on *Hp* infection, ER stress, UPR activation and gastric cell death [23, 24, 47]. Other UPR components also contribute to cell homeostasis; however, PERK directly contributes to checkpoint function and cell survival through its capacity to regulate cell division [48, 49]. Besides its classical role in the UPR, PERK more recently has been shown to contribute to tumour progression and cell survival [49-51]. However, the role of PERK signalling in tumour development remains

controversial [52]. Whereas some studies suggest that PERK activation inhibits tumour cell proliferation leading to apoptosis, in agreement with the classical role of PERK in the UPR [53, 54], others have shown that PERK activation facilitates tumour development by promoting tumour cell survival and enhancing angiogenesis [51, 55, 56]. Consistent with this second role for PERK, there is growing evidence to suggest that PERK is also involved in signalling pathways through UPR-independent functions (PERK non-canonical pathways) [49]. Overall, these observations support the idea that, besides its classical role in UPR and apoptosis, following *Hp* infection, PERK could also promote pro-survival traits in human gastric cells, such as migration and invasion and thus, the acquisition of a more aggressive phenotype [18].

Here we report that *Hp*-induced changes in the ER stress sensor PERK contributed to the loss of survivin, an inhibitor of apoptosis, previously associated with gastric cell death and the genesis of gastric cancer precancerous lesions [15, 34]. In addition, our preliminary experiments revealed an increase in the abundance of the pro-apoptotic protein PUMA α/β after exposure to *Hp*, suggesting that PERK-induced reduced cell viability is specifically linked to decreased survivin abundance and is likely to be independent of other pro-apoptotic BH3-only protein members. Akazawa *et al.* [23] reported comparable levels of pro-apoptotic BH3-only proteins; however they did so using purified virulence factor vacuolating cytotoxin A on AZ-521 cells. Therefore, we cannot rule out the possibility that the differences with our results relate to the infection of gastric cells with the intact *Hp* WT26695 strain, which not only expresses vacuolating cytotoxin A, but also other bacterial virulence factors. In contrast to survivin, there was increased abundance of PUMA α/β after 24 h of *Hp* infection. Thus, the decline in survivin abundance after

exposure to *Hp* points toward a unique mode of regulation involving the ER stress sensor PERK. In agreement, our group has previously reported that the onset of DNA fragmentation and annexin-V/propidium iodide staining were significantly elevated in gastric cells infected with *Hp* at a MOI 100 for 24 h [34]. Thus, survivin down-regulation upon *Hp* infection coincides with reduced viability and augmented apoptotic cell death in gastric cells [34].

As yet, both transcriptional regulation [57, 58] and posttranslational degradation via the proteasome [59] have been proposed to play important roles in controlling survivin levels. A previous report from our laboratory showed that, on the one hand, survivin messenger RNA levels are unchanged in the presence of *Hp*, implying that a posttranscriptional mechanism is responsible for the loss of survivin and, on the other hand, the *Hp* virulence factor gamma-glutamyl transpeptidase enhanced survivin degradation via the proteasome by a Fe³⁺-dependent pathway in gastric cells [15]. Our results provide one alternative mechanism, whereby following induction of ER stress and UPR activation, known to occur following *Hp* infection, both *in vivo* and *in vitro*, PERK activity and/or abundance contributes to the loss of survivin (Figure 7). In the non-transformed epithelial gastric cell line GES-1, *Hp* induces activation of the ER stress sensor PERK, which could partly activate downstream eIF2 α , leading to a reduction in global protein translation. This is likely to represent a mechanism by which survivin abundance is reduced. In parallel, eIF2 α activation triggers downstream of PERK the canonical apoptosis-related pathway (via CHOP) resulting in cell death (Figure 7). In agreement, an increase in eIF2 α activation up to 12 h has been shown to be dependent on the *Hp* virulence factor vacuolating cytotoxin A in AZ-521 cells [24].

Although *Hp*-triggered PERK activation in GES-1 cells could lead to increased eIF2 α activation and a reduction in survivin abundance, the results from this work also suggest there are additional PERK-dependent mechanisms involved in survivin loss. In accordance, a functional link between eIF2 α (Ser51) and proteasomal degradation has been observed [60, 61]. Thus, PERK induces proteasome-dependent degradation via a mechanism requiring eIF2 α during ER stress [60]. In addition, PERK activity is a positive regulator of proteasomal activity [62, 63]. This suggests that, in parallel to diminished global protein synthesis due to eIF2 α activation, also increased PERK-dependent proteasomal degradation could contribute to survivin down-regulation in GES-1 cells infected by *Hp* (Figure 7).

5. Conclusion

Our results confirmed that PERK is activated in *Hp*-positive gastritis and that this correlated with reduced survivin presence as compared to *Hp*-negative gastritis subject samples. *In vitro*, we demonstrate that PERK silencing restores survivin abundance following *Hp* infection, thereby positioning PERK as a key intermediate in the sequence post-infection leading to survivin loss, which could favour the genesis of precancerous gastric lesions. Reduced global protein translation due to PERK and eIF2 α activation provide interesting novel targets to prevent survivin protein loss in the gastric mucosa and disease onset.

Author Contributions

Conceptualization, P.D., A.H.C., S.L and A.F.G.Q.; methodology, P.D. and A.Ro.; formal analysis, P.D., A.R. and G. C.; investigation, P.D.; resources, A.H.C., S.L. and A.F.G.Q.; data curation, A.F.G.Q.; writing-original draft preparation, P.D.; writing-review and editing, P.D., A.H.C.,

S.L. and A.F.G.Q.; visualization, P.D.; supervision, S.L and A.F.G.Q.; project administration, S.L. and A.F.G.Q.; funding acquisition, P.D., A.H.C., S.L. and A.F.G.Q. All authors have read and agree to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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