β-catenin and p120-catenin mediate PPARδ-dependent epithelial proliferation induced by carcinogenic *Helicobacter pylori*

Supplemental Data

Supplemental Methods

H. pylori strains

H. pylori cag$^+$ strains 7.13, 60190, G27, and J166[1-3], and the *cag*$^-$ strain J68[1], were grown in *Brucella* broth and added to gastric cells at a bacteria-to-cell ratio of 100:1. *H. pylori* strain 7.13 does not produce a VacA protein (data not shown), similar to previously published results focused on its parental strain B128[4]. Since VacA has been reported to counter many of the effects that the *cag* pathogenicity island exerts on gastric epithelial cells[5,6], utilization of strain 7.13 provides a unique opportunity to more directly examine gastric epithelial proliferative responses that are induced by the *cag* island. Isogenic 7.13 *cagA*$^-$, *cagE*$^-$, and *slt*$^-$ null mutants were constructed by insertional mutagenesis using *aphA*[2]. The *cagA*$^-$/*slt*$^-$ double mutant was constructed by insertional mutagenesis using *aphA* and the chloramphenicol (*cat*) resistance cassette from pBSC103, respectively[1].

Cell culture, plasmids and reagents

MKN28, NCI-87 and KATO III human gastric epithelial cells were grown in RPMI medium 1640 (GIBCO/BRL) with 10% FBS (Sigma) and 20 μg/ml gentamicin (GIBCO/BRL) under 5% CO_2 at 37°C. The PPRE3-tk-luciferase reporter plasmid and dominant-negative PPARδ construct were kind gifts from D. Wang (Vanderbilt University). Topflash and Fopflash reporter plasmids were kind gifts from K. Kinzler and B. Vogelstein (Johns Hopkins University). The PPARδ-specific ligand, GW501516 (Cayman Chemical Co., Ann Arbor, MI) was used at a final concentration of 10 nM. The

PI3K inhibitor LY294002 (Cell Signaling Technology) was used at a concentration of 50μM/L.

Viral production and retroviral transduction

Phoenix 293 packaging cell lines at 50% confluence were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Fresh medium was added 24 hours after transfection, and tissue culture medium was collected and filtered through a 0.45-μm filter 72 hours after transfection. For retroviral transduction, MKN28 cells at 50% confluence were incubated overnight with freshly harvested virus containing 4 μg/ml Polybrene (American Bioanalytical). To generate stable cell lines, cells transduced with the pSUPER.retro.puro virus were selected with 1.5 μg/ml puromycin for 48 hours. Clonal populations were selected using cloning rings and limiting dilution techniques[7].

Transient transfection of siRNA

MKN28 cells (4×10^5) in 12-well plates were transiently transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, transfection reagent (2.0 μl/well) was mixed with siRNA oligos (5 μl of 20 μM solution/well) in 200 μl Opti-MEM (Life Technologies). Cells were incubated with the transfection mixture for 24 hours, fresh medium was added, and bacterial co-cultures were performed 24 hours later.

Western blot analysis

Cells or gastric mucosal lysates were lysed in RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS) containing protease inhibitor cocktail. Proteins

(30 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Pall, Ann Arbor, MI). Protein levels were assessed by Western blotting using anti-PPARδ (1:500, Santa Cruz Biotechnology), anti-β-catenin (1:1000, Sigma Aldrich), anti-p120 (1:1000, Abcam), anti-Cyclin E1 (1:500, Santa Cruz Biotechnology) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (1:2000; Millipore Bioscience Research Reagents). Primary antibodies were detected using goat anti-mouse, goat anti-rabbit, or donkey anti-goat (1:5000; Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibodies and visualized by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life and Analytical Sciences) according to the manufacturer's instructions on a Chemigenius system (Syngene). For cellular fractionation, cytoplasmic and nuclear fractions were obtained using the Q-Proteome Cell Compartment Kit (Qiagen).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Gastric cells were grown to confluence and then co-cultured with *H. pylori* or medium alone for 6, 12, 24 and 48 hours. RNA was prepared from co-culture lysates using RNAeasy kit (Qiagen) following the manufacturer's instructions. Reverse transcriptase-PCR was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), which was followed by real-time quantitative PCR using SYBR green (Applied Biosystems, Foster City, CA) and the 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Relative differences between treatment groups were calculated based on values for the gene of interest normalized to values of the hypoxanthine phosphoribosyltransferase 1 gene (*hprt1)*. The primers used were: *ppar*δ, forward 5' GAGGAAGTGGCCACGGGTGAC 3' and reverse 5' CCACCTGAGGCCCCATCACAG 3'; *hprt,* forward 5' TTGGAAAGGGTGTTTATTCCTCA 3' and reverse 5' TCCAGCAGGTCAGCAAAGAA 3'.

Three-dimensional Matrigel proliferation assay

Tissue culture plates were coated with 100 µl of thawed BD Matrigel™ Basement Membrane Matrix (catalog # 354234) which contains insulin-like growth factor along with other growth factors and placed at 37°C for 30 minutes for Matrigel solidification. MKN28 cells (2 x 10^4) transfected with empty vector, dominant-negative PPARδ, scrambled siRNA, PPARδ-specific siRNA, or Cyclin E1-specific siRNA were overlayed on Matrigel™ and, after 24 hours, cells were infected with *H. pylori,* medium alone and/or GW501516. Cells were removed every 24 hours using BD Cell Recovery Solution according to the manufacturer's protocol and enumerated using Trypan Blue or BrdU incorporation (Roche).

PPARδ and β-catenin transcriptional assays

MKN28 cells (2 x 10^5) plated in 12-well plates were transiently transfected with 4 µl Lipofectamine 2000 (Invitrogen), 0.3 µg PPRE-tk-luciferase/ 5 ng of pRL-SV40, and 0.4 µg empty vector or dominant-negative PPARδ for 5 hours. Expression levels of dominant negative PPARδ were determined by Western blotting (Supplemental Figure 1A). Twenty-four hours post-transfection, cells were co-cultured with *H. pylori* and then harvested in 1x Passive Lysis Buffer (Promega). For β-catenin studies, MKN28 cells (2 x 10^5) were transfected with 4 µl Lipofectamine 2000, 1 µg/ml Topflash or 1 µg/ml Fopflash, and 5 ng pRL-SV40 in Opti-MEM (Life Technologies) for 5 hours. Transfection mixtures were then replaced with complete medium containing *H. pylori* or medium alone. After 24 hours, cells were harvested in 1x Passive Lysis Buffer (Promega). Luciferase activity was determined using a luminometer and normalized to *Renilla* luciferase using the Dual-Luciferase assay kit (Promega).

Primary gastric cell extraction and culture

All animal studies were approved by the Vanderbilt Institutional Animal Care and Usage Committee. Stomachs were removed from 8-week-old male wild-type C57Bl/6 and PPARδ$^{-/-}$ C57Bl/6 mice (provided by D. Wang, Vanderbilt University), ligated at the pylorus and esophagus, inverted, and injected with 1 ml of 0.5 mg/ml collagenase A as described previously[7]. Stomachs were then washed in Hanks' balanced salt solution (HBSS) three times at 37°C. Tissues were incubated in 10 ml of 1 mM dithiothreitol for 15 minutes at 37°C with shaking, washed in HBSS, and incubated in 0.37 mg/ml collagenase for 30 minutes at 37°C. After the first collagenase digestion, samples were washed again in HBSS and incubated for a further 30 minutes in collagenase (0.37 mg/ml; 37°C). Tissue was triturated and larger fragments of tissue were allowed to settle under gravity for 45 seconds. The supernatant containing isolated gastric cell colonies was removed and transferred to a clean 50-ml conical tube, shaken vigorously to release additional colonies, and left on ice to sediment for 30 minutes. The supernatant was then carefully removed and discarded, and isolated cell colonies were plated on chamber slides for immunofluorescence or 12-well plates for Western blot analysis.

Immunofluorescence

Primary gastric colonies were cultured in glass chamber slides and were then co-cultured with *H. pylori* or medium alone for 48 hours. Cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 4% paraformaldehyde in DPBS for 10 minutes. Cells were rinsed with DPBS and subsequently permeabilized for 30 minutes with DPBS containing 0.1% Triton X-100, followed by incubation in 3% BSA for 1 hour at room temperature. Slides were immunostained with goat monoclonal anti-PPARδ antibody (Santa Cruz) or rabbit polyclonal anti-Cyclin E antibody (Santa Cruz) at a concentration of 1:100, overnight at 4°C. Washed slides were incubated with rabbit

anti-goat AlexaFluor 546-conjugated antibody (Invitrogen) or goat anti-rabbit AlexaFluor 488-conjugated antibody (Invitrogen), at a concentration of 1:100 for 1 hour at room temperature. Nuclei were stained with DAPI or TO-PRO3. Slides were washed and then mounted using ProLong Gold (Invitrogen). Imaging was performed on an Olympus 1X71 microscope (Olympus, Center Valley, PA) by using 40x/1.30 Plan-NeoFluar oil objective at 37°C, and acquisition and data analysis were performed with Delta Vision SoftWoRx Image Analysis Software 3.7.0 (Stress Photonics, Madison, WI). Confocal imaging was performed on an LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY) by using a 40x/1.40 Pan-APOCHROMAT oil objective at room temperature, and acquisition was performed using the manufacturer's proprietary software.

Immunohistochemistry

A single observer blinded to *H. pylori* infection status and mouse genetic background evaluated PPARδ and Ki67 immunolabeling within human, gerbil, and mouse gastric mucosa. Immunohistochemical analysis was performed on paraffin-embedded gastric tissue by using an anti-PPARδ antibody (1:5000, Novus Biologicals) or an anti-Ki67 antibody (1:100, Biocare Medical). In human mucosa, the percentage of PPARδ-positive surface epithelial cells was quantified by evaluating 100 consecutive cells in 3 well-oriented, randomly selected specimen areas. In gerbil and mouse mucosa, PPARδ immunostaining was evaluated semiquantitatively in the epithelium of antral mucosa. Epithelial cells were scored for intensity of staining on a scale of 0 (absent), 1 (weak), 2 (moderate), and 3 (strong). The percentage of positively stained epithelial cells was multiplied by the intensity score, resulting in a scoring range of 0–300. The percentage of epithelial cells positive for Ki67 was calculated quantitatively by evaluating epithelial cells in 10 high-power fields in randomly selected, well-oriented sections.

H. pylori human clinical samples

The *H. pylori* clinical samples used in this study were randomly selected from a larger repository of biopsies harvested from patients between ages 29 and 69 years enrolled in an ongoing prospective study in Colombia designed to study mechanisms of *H. pylori* carcinogenesis *in vivo*[8]. The protocol was approved by the Institutional Review Board of Louisiana State University Health Sciences Center and the Committees on Ethics of Universidad del Valle and Hospital Departamental de Nariño in Colombia. Four gastric biopsy specimens were obtained during endoscopy and were used for histology[8]. One additional gastric antral biopsy specimen was placed immediately in liquid nitrogen and stored at - 80°C and used for total DNA extraction[9]. The presence of the *cag* island was determined by PCR amplification as described[9].

Statistical Analysis.

All *in vitro* experiments were performed on at least three independent occasions. Statistical analysis was performed by Student's t test and ANOVA using Prism Graph Pad. A P-value < .05 was defined as statistically significant.

Supplemental Figure Legends

Supplemental Figure 1. Expression of dominant-negative PPARδ and siRNA inhibition of β-catenin and p120-catenin in MKN28 gastric epithelial cells. ***(A)*** MKN28 cells were transfected with medium alone, empty vector or dominant-negative PPARδ plasmids. Twenty-four hours post-infection, protein was analyzed by Western blot using an anti-PPARδ antibody. Anti-GAPDH antibodies served as normalization controls. Densitometric analysis of Western blots performed on two different occasions is shown below representative blots. *p< .05 versus medium alone or empty vector control. ***(B)*** MKN28 cells were transiently transfected with scrambled or β-catenin-specific siRNA; total protein was extracted and subjected to Western blot analysis using an anti-β-catenin antibody. Densitometric analysis of Western blots performed on at least three occasions is shown below representative blots. *p< .005 versus scrambled control. ***(C)*** MKN28 cells were retrovirally transduced with either scrambled or human p120-specific siRNA (p120i), and clonal populations were selected. Total protein was extracted from control or p120i cells and analyzed by Western blot using a monoclonal anti-p120 antibody. Densitometric analysis of Western blots performed on at least three occasions is shown below representative blots. *p< .05 versus scrambled control.

Supplemental Figure 2. *H. pylori* promotes nuclear β-catenin accumulation in a PI3K-dependent manner. ***(A)*** *H. pylori* strain 7.13 was added to MKN28 cells in the absence or presence of vehicle alone (DMSO) or 50 μmol/L LY294002 (PI3Ki). Two hours after infection, whole-cell lysates were subjected to Western blot analysis using an anti–phospho-GSK3β antibody. (-), cells incubated with medium alone. A representative blot is shown. Western blots for total GSK3β served as normalization controls for MKN28 cell viability under different experimental conditions, and Western blots for GAPDH

served as loading controls. Densitometric analysis of multiple Western blot repetitions performed on at least 3 occasions is shown below representative blots. Error bars indicate SEMs. *p< .001 vs. uninfected untreated control. # p< .001 vs. 7.13-infected MKN28 cells in the absence of PI3Ki. ***(B)*** MKN28 cells were co-cultured with strain 7.13 in the absence or presence of 50 μmol/L LY294002 (PI3Ki), or medium alone for 24 hours. Cells were stained with anti-β-catenin and AlexaFluor-488 antibodies and analyzed by immunofluorescence microscopy. Arrowheads, nuclear ß-catenin. ***(C)*** MKN28 cells were transiently transfected with $PPRE_3$-tk-luciferase and pRL-SV40, followed by infection with strain 7.13 in the absence or presence of 50 μmol/L LY294002 (PI3Ki). Dual luciferase assays were then performed. *p < .001 versus 7.13-infected samples in the absence of PI3Ki.

Supplemental Figure 3. *H. pylori*-induced proliferation does not exhibit synergy with GW501516-induced PPARδ activation. Following infection with strain 7.13, GW501516, strain 7.13 and GW501516, or medium alone, cells were removed from Matrigel after 4 days and enumerated using Trypan blue staining. Error bars = SEM for experiments performed on at least 3 occasions. *p < .05 versus uninfected control.

Supplemental Figure 4. *H. pylori* induces PPARδ expression in *ex vivo* gastric cell colonies in a *cagA*$^-$ and *slt*$^-$-dependent manner, and PPARδ$^{+/-}$ mice exhibit *pparδ* haploinsufficiency. ***(A)*** Primary murine gastric cell colonies were co-cultured with strain 7.13 or isogenic 7.13 *cagA*$^-$ or *slt*$^-$ mutants. Twenty-four hours post-infection, protein was extracted and analyzed by Western blot using an anti-PPARδ antibody. Graph represents densitometric analysis of Western blots performed on at least three occasions. *p< .02 versus uninfected control. ***(B)*** Gastric mucosal lysates were obtained from wild-type PPARδ$^{+/+}$ and heterozygous PPARδ$^{+/-}$ mice, protein was extracted and

analyzed by Western blot using an anti-PPARδ antibody. Western blots for GAPDH served as loading controls. A representative blot is shown.

Supplemental References

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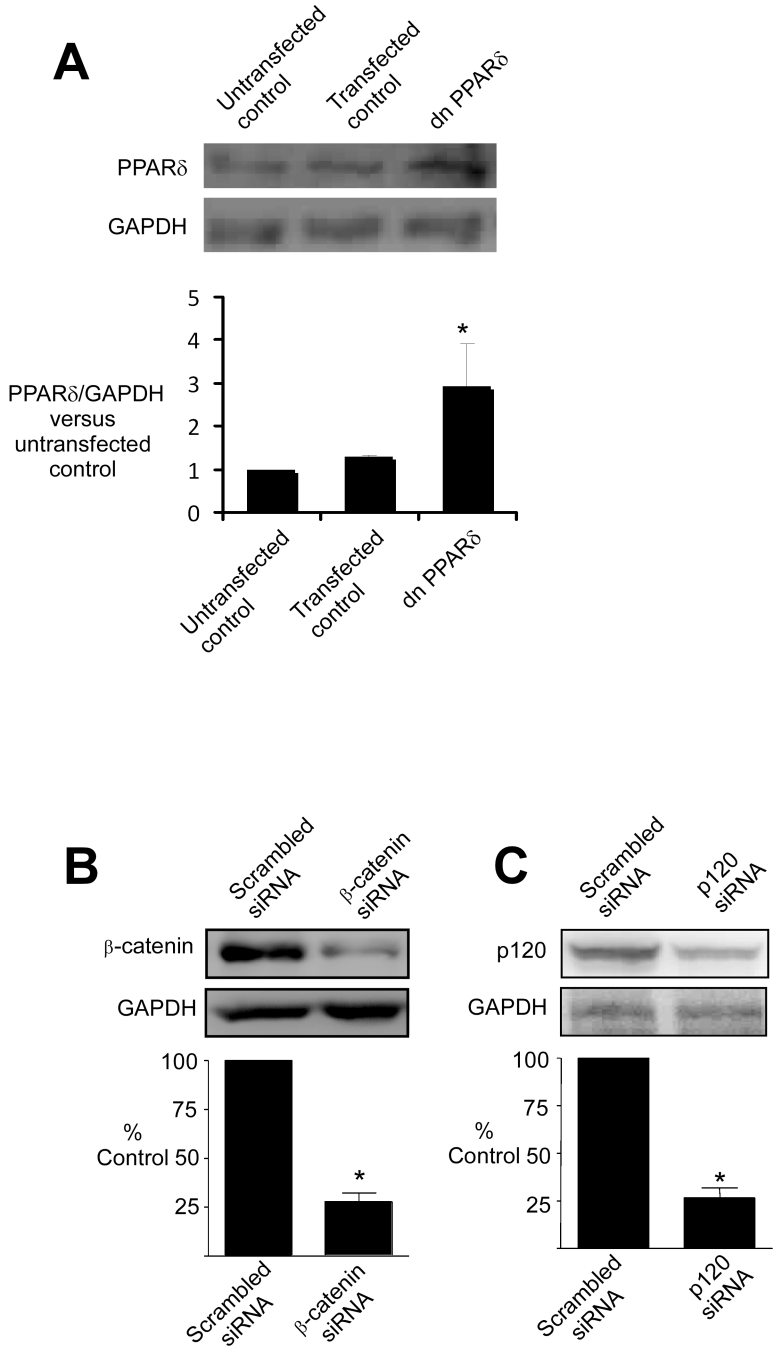
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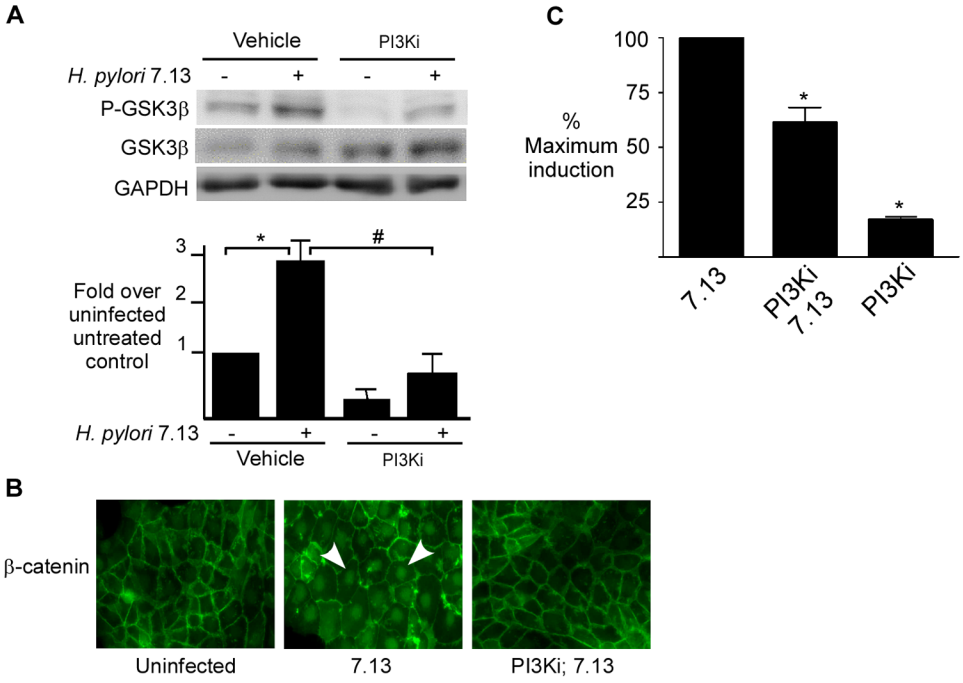
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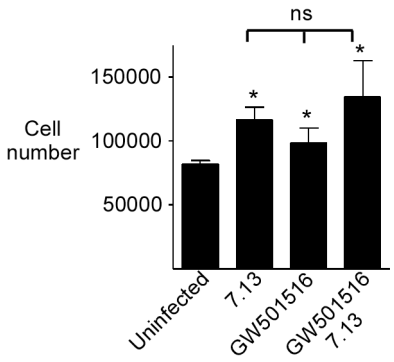
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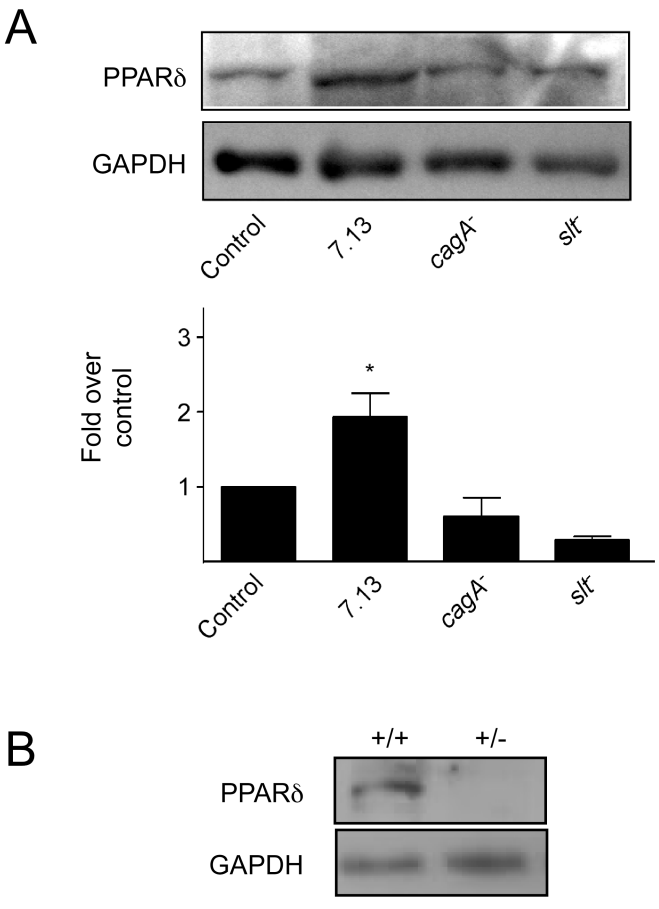
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