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Supplementary Information for Targeted mobilization of Lrig1$^+$ gastric epithelial stem cell populations by a carcinogenic *Helicobacter pylori* type IV secretion system

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Supplementary Information Text

MATERIALS AND METHODS.

Animals and gastroid culture. All procedures were approved by the Institutional Animal Care Committee of Vanderbilt University Medical Center. The generation of *Lrig1-CreERT2/+* mice has previously been described (1). For lineage-tracing experiments of Lrig1-expressing cells, *Lrig1-CreERT2/+* mice were crossed with *R26R-YFP/+* mice, hereafter referred to as Lrig1$^+$/YFP mice. Mice were administered 2 mg of tamoxifen (Sigma) in corn oil by intraperitoneal injection and were orogastrically challenged one week later by oral gavage with Brucella broth (uninfected control; n=7 at 2 weeks, n=5 at 8 weeks), the mouse-adapted wild-type *cag*$^+$ *H. pylori* strain PMSS1 (n=10 at 2 weeks, n=6 at 8 weeks), or with a PMSS1 *cagE*$^-$ isogenic mutant (n=10 at 2 weeks, n=7 at 8 weeks). Mice in each treatment group were caged separately. Mice were euthanized 2 or 8 weeks post-*H. pylori* challenge and gastric tissue was harvested for quantitative culture and immunohistochemistry. For gastroid cultures, mice were euthanized 6 weeks post-*H. pylori* challenge and gastroids were isolated as previously described (2).

Primary human gastric organoid 2D monolayers.

Human fundus was collected during sleeve gastrectomies according to an approved University of Cincinnati IRB protocol (IRB protocol number: 2015–4869). Gastric tissue was washed and digested, and isolated glands were incubated in Matrigel, as previously described (3). Primary human gastric organoids were then converted to 2D epithelial cell monolayers, as previously described (3). Briefly, Matrigel was removed and 3D gastric organoids were plated on collagen-coated plates (3). Primary 2D gastric monolayers were then co-cultured with wild-type *cag*$^+$ *H. pylori* strain 7.13 or isogenic 7.13 *cagA*$^-$ or *cag*E$^-$ mutant strains at a multiplicity of infection (MOI) of 100:1 for 6 or 24 h. RNA and protein were then isolated from gastric organoid co-cultures for real-time PCR and Western blot analysis respectively.

***H. pylori* quantitative culture**

To assess *H. pylori* colonization, one quarter of the stomach was harvested and homogenized in sterile PBS. Serially diluted samples were plated on selective trypticase soy agar plates (BD Biosciences) with 5% sheep blood (Fisher Scientific) containing vancomycin (Sigma-Aldrich, 20 μg/ml), nalidixic acid (Sigma-Aldrich, 10 μg/ml), bacitracin (Calbiochem, 30 μg/ml), and amphotericin B (Sigma-Aldrich, 2 μg/ml) and were incubated at 37°C with 5% CO_2 for 5–6 days for isolation of *H. pylori*. Colonization density was quantified as log colony forming units per gram of gastric tissue (log CFU/g).

Analysis of inflammation in murine gastric tissue

Gastric tissue was fixed in 10% neutral-buffered formalin (Azer Scientific), paraffin-embedded, and stained with hematoxylin and eosin (H&E). A single pathologist, blinded to treatment groups, scored indices of inflammation.

Immunofluorescence

To assess YFP expression in murine gastric tissue, immunofluorescence was performed on deparaffinized gastric tissue sections using a rabbit polyclonal anti-GFP antibody (1:500, Life Technologies), or a chicken polyclonal anti-GFP antibody (1:200, Abcam). To investigate the relationship between YFP expression and terminally differentiated cells, dual IHC was performed for GFP in combination with antibodies targeting UEA1 to detect surface mucus cells (1:2000, Sigma), or intrinsic factor to detect chief cells (1:2000, gift from David Alpers, Washington University, St. Louis), or GS-II Lectin to detect mucus neck cells and SPEM (1:2500, ThermoFisher). Proliferation was analyzed using a rat polyclonal anti-Ki67 antibody (1:200, eBioscience), and *H. pylori* localization was determined using a rabbit polyclonal anti-*H. pylori* antibody (1:200 Dako). Primary antibodies were detected with Alexa-fluor secondary antibodies (Invitrogen). Nuclei were detected using Hoescht (Invitrogen). Imaging was performed in the Digital Histology Shared Resource at Vanderbilt University Medical Center. Cells were quantified from at least 2 high-powered fields per animal from both the antrum and corpus and were then normalized to the total number of cell nuclei in the same fields.

Immunohistochemistry

To assess Lrig1 expression in human gastric tissue, immunohistochemistry was performed on deparaffinized gastric tissue sections using a rabbit polyclonal anti-Lrig1 antibody (1:100, ThermoFisher). Human tissue was obtained from Vanderbilt University Medical Center, Nashville, TN and New Orleans, LA, and included control cases from normal gastric antrum and corpus, *H. pylori*-associated non-atrophic gastritis (NAG), chronic atrophic gastritis without intestinal metaplasia (CAG), and atrophic gastritis with intestinal metaplasia (IM) (SI Appendix, Table S1). A single pathologist (MBP) scored the percentage of Lrig1$^+$ epithelial cells in well-oriented gastric sections as previously described (4). Apoptosis was analyzed using a rabbit polyclonal anti-cleaved caspase-3 antibody (1:100, Cell Signaling). Primary antibodies were detected with Envision system-HRP Polymer Rabbit (DAB Staining IHC kits, DakoCytomation) and counter-stained with CAT Hematoxylin (Biocare Medical). Imaging was performed in the Digital Histology Shared Resource at Vanderbilt University Medical Center.

Western blot analysis. For analysis of cellular protein, human gastric monolayers were co-cultured with or without *H. pylori* for 24 hours. Cells were lysed in RIPA buffer (50mM Tris, 150mM NaCl, 1% Triton X-100, 0.1% SDS) for 10 minutes at 4°C, centrifuged at 13,000 rpm for 10 minutes and supernatants were collected. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Lrig1 was detected using an anti-Lrig1 antibody (0.4μg/ml, ThermoFisher) in combination with goat anti-rabbit IgG (1:10,000, Promega) HRP-conjugated secondary antibody. Samples were re-probed using a mouse anti-GAPDH antibody (Santa Cruz Biotechnology) as a control for equal loading. Primary antibodies were visualized by Western Lightning Chemiluminescence Reagent Plus according to the manufacturer's instructions.

Quantitative real-time reverse transcriptase-polymerase chain reaction. Human gastric monolayers were co-cultured with or without *H. pylori* for 6 hours. RNA was isolated using the RNeasy® RNA isolation kit (Qiagen), according to the manufacturer's instructions. Reverse

transcriptase PCR and quantitative real-time PCR (Applied Biosystems, 7300 Real-Time PCR System) were performed, according to the manufacturer's instructions. Levels of human *LRIG1* mRNA expression (TaqMan®, Applied Biosystems) were standardized to levels of human *GAPDH* mRNA expression (TaqMan®, Applied Biosystems).

Fig. S1.

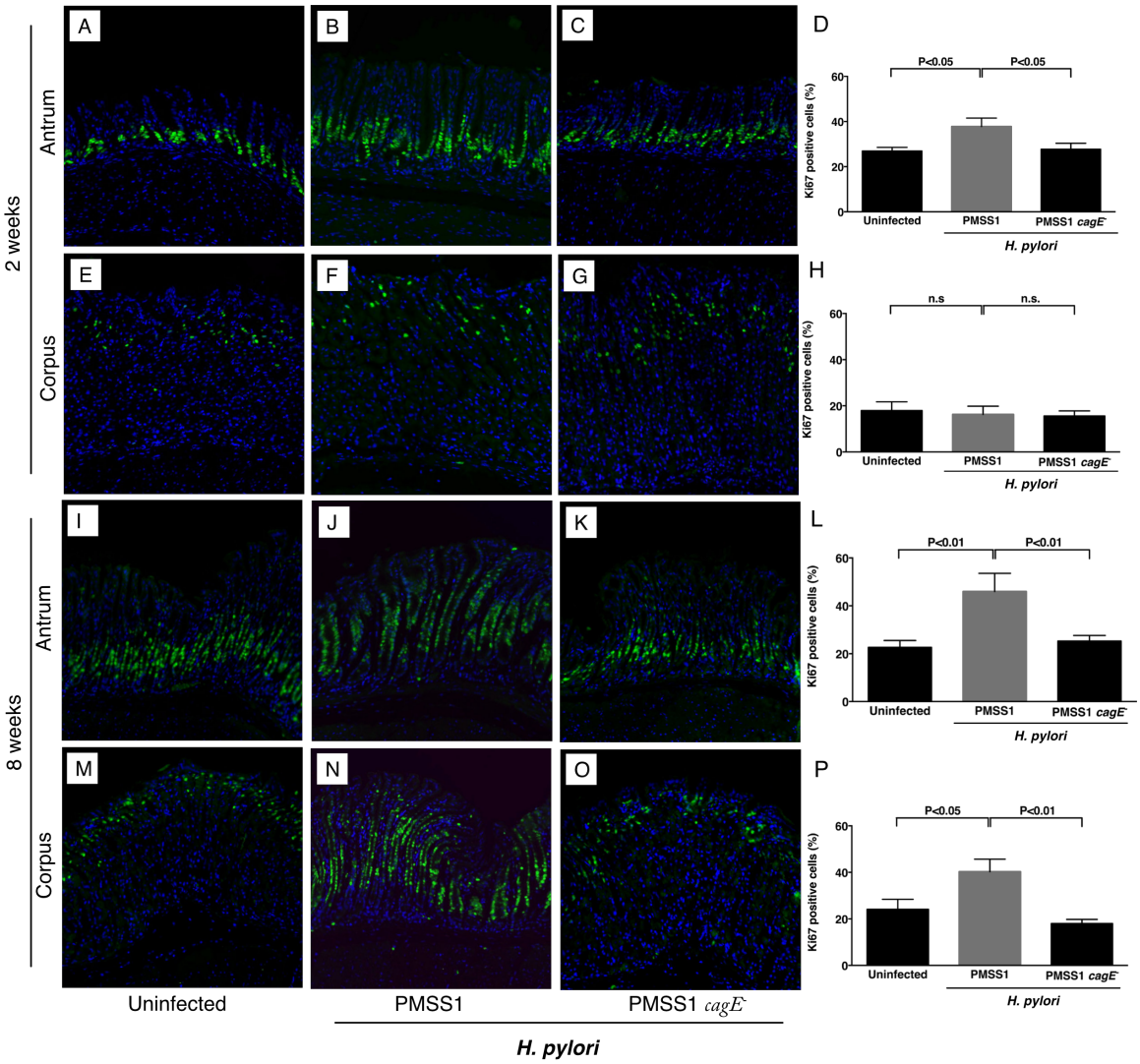
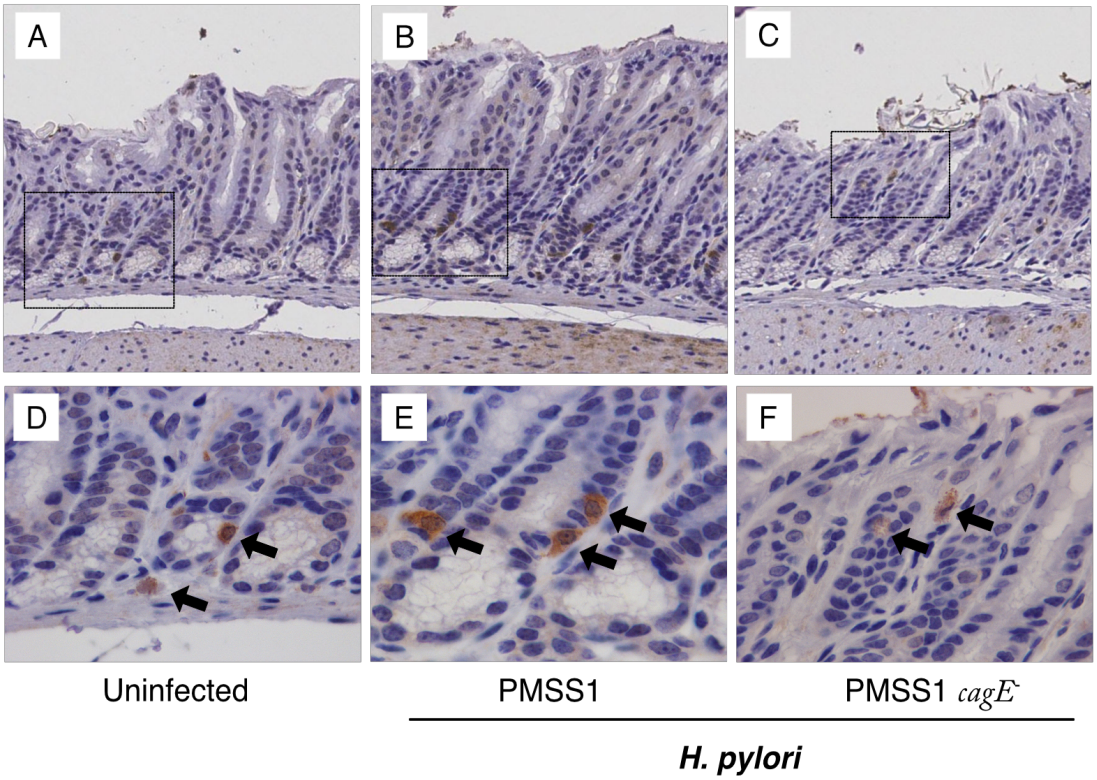


Fig. S1. *H. pylori* increases proliferation in a *cag*-dependent manner. Lrig1/YFP mice were challenged for 2 weeks (A-H) or 8 weeks (I-P) with Brucella broth (A, E, I, M), with *H. pylori* WT strain PMSS1 (B, F, J, N), or with a PMSS1 *cagE*$^{-}$ isogenic mutant (C, G, K, O). Proliferation was assessed by Ki67 (green) immunostaining, nuclei are stained blue. Proliferation was quantified from at least 2 fields per animal in both the antrum and corpus (D, H, L, P). ANOVA and Bonferroni tests were used to determine statistical significance between groups.

Fig. S2.


A
B
C
D
E
F
Uninfected
PMSS1
PMSS1 cagE-
H. pylori

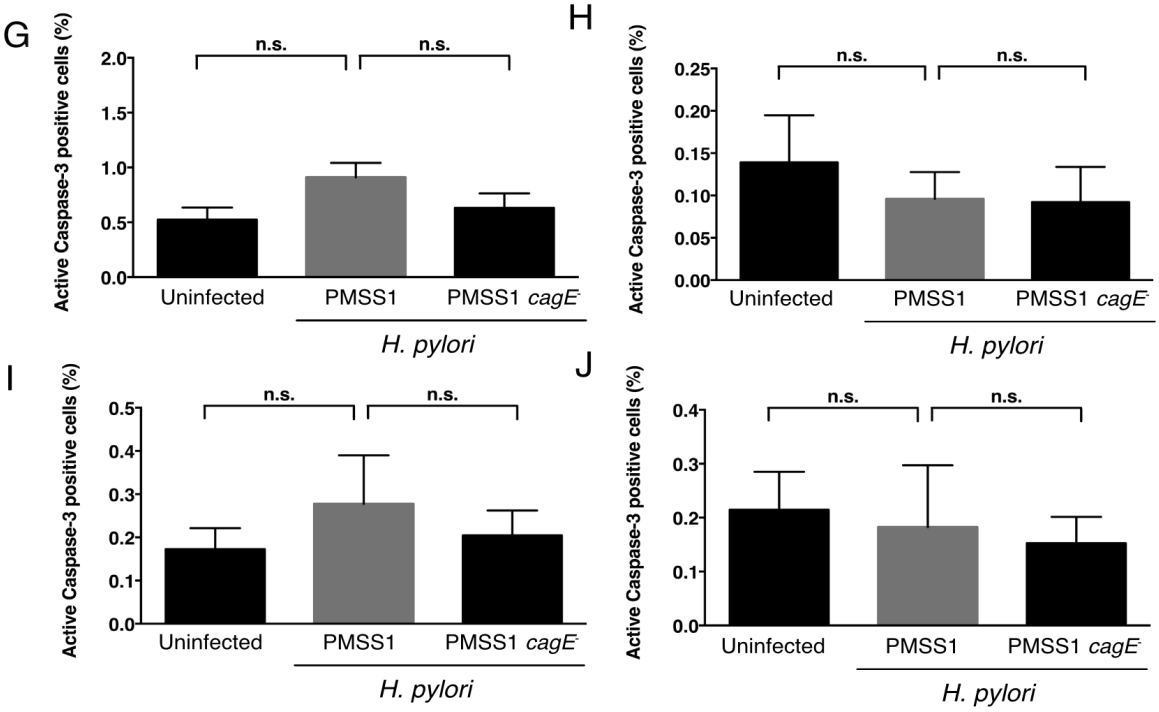

G
H
I
J
Active Caspase-3 positive cells (%)
n.s.
n.s.
Uninfected
PMSS1
PMSS1 cagE-
H. pylori

Fig. S2. Apoptosis is not altered by *H. pylori* infection. Lrig1/YFP mice were challenged for 2 weeks (A-H) or 8 weeks (I-J) with Brucella broth (A, D), *H. pylori* WT strain PMSS1 (B, E), or a PMSS1 *cagE*$^{-}$ isogenic mutant (C, F). Apoptosis was assessed by activated caspase-3 staining. Apoptotic cells were quantified by enumerating positively stained cells in 3 high-powered fields per animal in both the antrum (G, I) and corpus (H, J) and these data were then expressed as a percentage of total counted cells. Representative staining from antrum 2 weeks post-challenge is shown as 20X images (A-C), and as higher magnification images (100X) (D-F) with selected areas denoted by rectangles. Apoptotic cells are indicated by arrows. ANOVA and Bonferroni tests were used to determine statistical significance between groups.

Fig. S3.

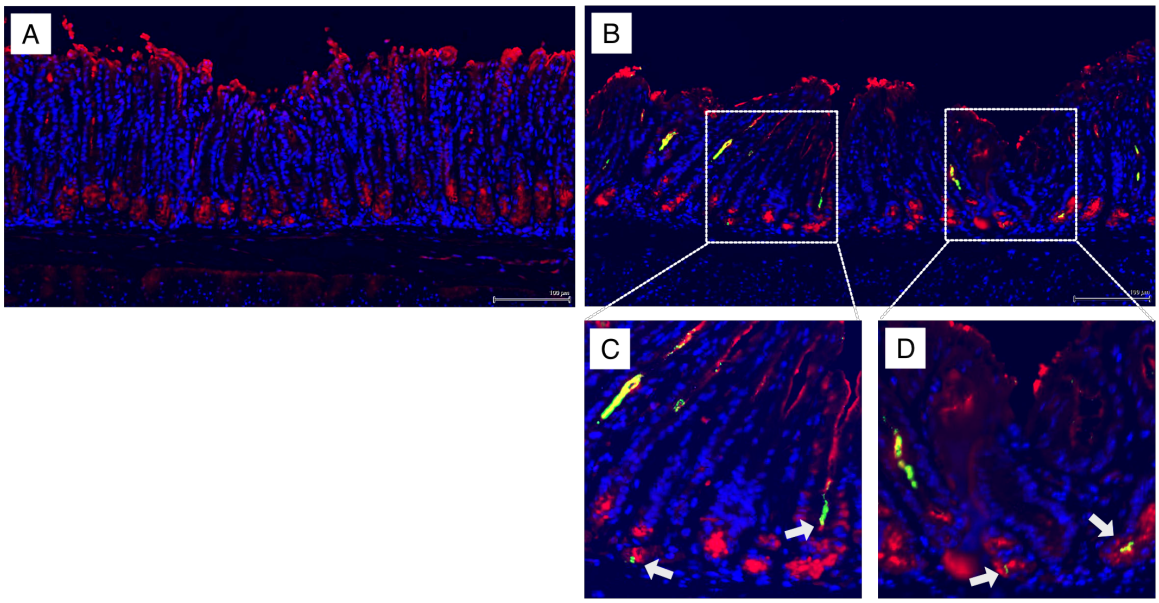


Fig. S3. *H. pylori* is closely juxtaposed with Lrig1 lineage-traced cells in the base of the antrum. Lrig1/YFP mice were challenged for 8 weeks with Brucella broth (A), or with *H. pylori* WT strain PMSS1 (B-D). Induced Lrig1 is labeled in red, *H. pylori* in green, and nuclei in blue. *H. pylori* was not detected in uninfected antrum and there was minimal lineage tracing (A). Wild-type *H. pylori* increased Lrig1 marked cells and *H. pylori* was closely juxtaposed with some of these cells in the proliferative zone in the antral base (B-D, denoted by arrows). Low magnification (10X) images are shown in A and B, and higher magnification images (20X) are shown in C and D and represent areas denoted by the rectangles.

Fig. S4.

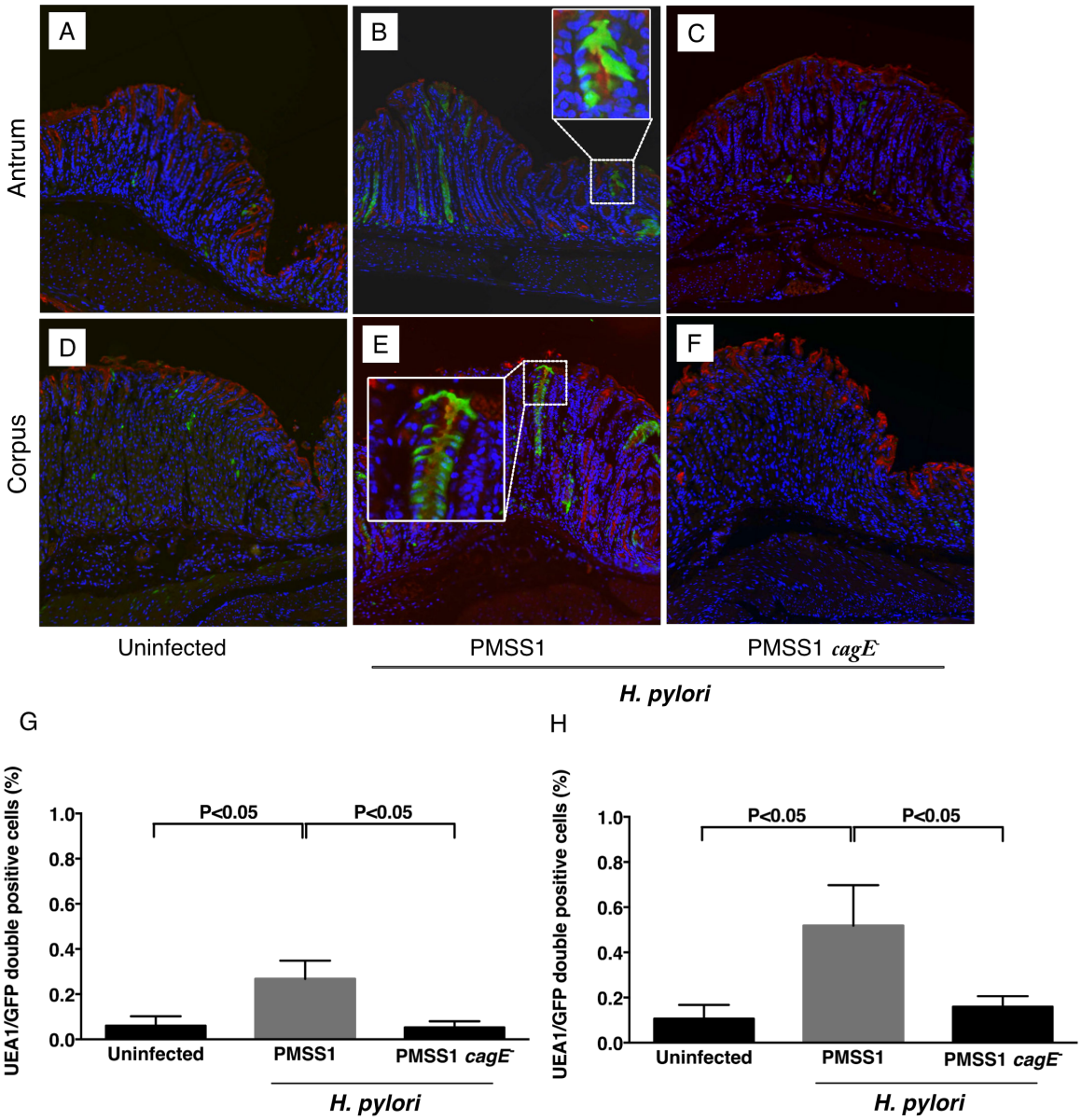

A
B
C
D
E
F
Antrum
Corpus
Uninfected
PMSS1
PMSS1 cagE-
H. pylori
G
H
UEA1/GFP double positive cells (%)
P<0.05
P<0.05
1.0
0.8
0.6
0.4
0.2
0.0
Uninfected
PMSS1
PMSS1 cagE-
H. pylori

Fig. S4. Lrig1 lineage-traced cells co-localize with surface mucus cells in response to *cag*$^{+}$ *H. pylori.* Lrig1/YFP mice were challenged for 8 weeks with Brucella broth, with *H. pylori* WT strain PMSS1, or with a PMSS1 *cagE*$^{-}$ isogenic mutant. Lrig1 lineage mapping derivation is labeled in green, nuclei in blue, and UEA1 in red. In uninfected antrum (A) and corpus (D), UEA1 localized to the pit and there was minimal lineage tracing. Wild-type *H. pylori* strain PMSS1 increased Lrig1 marked cells and a subset of these cells co-expressed UEA1 (B and E). This was not seen in response to the PMSS1 *cagE*$^{-}$ isogenic mutant in antrum (C) or corpus (F). Quantification of cells double positive for UEA1 and GFP is shown in (G) antrum and (H) corpus, and cells were quantified from at least 2 fields per animal from both the antrum and corpus. ANOVA and Bonferroni tests were used to determine statistical significance between groups.

Table S1. Human subject information, histological diagnoses, and *H. pylori* status.

Case	Sample Origin	Age	Sex	HP status[1]	Histological Diagnosis[2]
NIH-1204	USA-New Orleans	85	F	positive	CAG
NIH-1932	USA-New Orleans	42	M	positive	CAG
NIH-2052	USA-New Orleans	50	M	positive	IM
NIH-2072	USA-New Orleans	42	M	positive	IM
NIH-2102	USA-New Orleans	35	F	negative	Normal
NIH-2512	USA-New Orleans	50	F	positive	IM
NIH-2652	USA-New Orleans	47	F	positive	CAG
NIH-2852	USA-New Orleans	61	M	positive	NAG
NIH-3012	USA-New Orleans	26	F	positive	NAG
NIH-3032	USA-New Orleans	36	F	positive	CAG
NIH-3512	USA-New Orleans	48	M	positive	CAG
NIH-4142	USA-New Orleans	42	F	positive	NAG
NIH-4162	USA-New Orleans	43	F	positive	IM
NIH-4222	USA-New Orleans	57	F	positive	NAG
NIH-4322	USA-New Orleans	60	M	positive	CAG
NIH-4382	USA-New Orleans	51	F	positive	NAG
NIH-4432	USA-New Orleans	66	F	positive	NAG
NIH-4562	USA-New Orleans	37	F	positive	NAG
NIH-4702	USA-New Orleans	65	F	positive	IM
NIH-4762	USA-New Orleans	31	F	negative	Normal
NIH-4822	USA-New Orleans	41	F	positive	CAG
NIH-4842	USA-New Orleans	62	F	positive	IM
NIH-4892	USA-New Orleans	64	F	negative	Normal
NIH-5102	USA-New Orleans	47	F	positive	NAG
NIH-5152	USA-New Orleans	37	F	positive	NAG
NIH-5372	USA-New Orleans	44	F	positive	NAG
NIH-5432	USA-New Orleans	29	F	positive	NAG
NIH-5472	USA-New Orleans	61	M	positive	NAG
NIH-5682	USA-New Orleans	38	M	positive	NAG
NIH-6042	USA-New Orleans	44	M	positive	NAG
NIH-6052	USA-New Orleans	59	M	positive	IM
08-22326	USA-Nashville	60	M	unknown	Normal
09-25035	USA-Nashville	71	M	unknown	Normal
15-02002	USA-Nashville	74	M	unknown	Normal
06-28264	USA-Nashville	62	F	unknown	NAG
10-24011	USA-Nashville	60	F	unknown	NAG
11-04500	USA-Nashville	75	F	unknown	NAG
12-13205	USA-Nashville	47	M	positive	NAG
09-10894	USA-Nashville	66	F	unknown	CAG

13-09899	USA-Nashville	70	M	unknown	CAG
03-12052	USA-Nashville	59	F	unknown	Normal
09-37576	USA-Nashville	51	M	unknown	Normal
10-23193	USA-Nashville	76	M	unknown	Normal
15-02024	USA-Nashville	45	F	unknown	Normal
15-02616	USA-Nashville	68	F	unknown	Normal
09-13807	USA-Nashville	86	F	unknown	NAG
14-01288	USA-Nashville	66	F	positive	NAG
14-20959	USA-Nashville	35	F	positive	NAG
12-13150	USA-Nashville	38	F	positive	NAG
12-16259	USA-Nashville	46	M	positive	NAG
13-09350	USA-Nashville	40	F	positive	NAG
13-15570	USA-Nashville	48	F	positive	NAG
13-29885	USA-Nashville	46	M	positive	NAG
13-31911	USA-Nashville	58	F	positive	NAG
13-31978	USA-Nashville	63	F	positive	NAG
11-40989	USA-Nashville	56	M	unknown	CAG
14-29179	USA-Nashville	49	M	unknown	CAG
09-13303	USA-Nashville	74	M	unknown	CAG
14-26936	USA-Nashville	61	M	positive	CAG
12-24945	USA-Nashville	49	M	positive	CAG
12-14900	USA-Nashville	49	F	unknown	IM
14-26887	USA-Nashville	63	F	unknown	IM

[1]*H. pylori* status was determined by histologic assessment.
[2]Histological diagnosis represents the most advanced lesion reported.
NAG=non-atrophic gastritis, CAG=chronic atrophic gastritis without IM, IM=Intestinal metaplasia.

References

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