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Spermine Oxidase Mediates *Helicobacter pylori*-induced Gastric Inflammation, DNA Damage, and Carcinogenic Signaling

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Abstract

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY INFORMATION

Supplementary information is available at Oncogene's website.

Helicobacter pylori infection is the main risk factor for development of gastric cancer, the third leading cause of cancer death worldwide. *H. pylori* colonizes the human gastric mucosa and persists for decades. The inflammatory response is ineffective in clearing the infection, leading to disease progression that may result in gastric adenocarcinoma. We have shown that polyamines are regulators of the host response to *H. pylori*, and that spermine oxidase (SMOX), which metabolizes the polyamine spermine into spermidine plus H_2O_2, is associated with increased human gastric cancer risk. We now used a molecular approach to directly address the role of SMOX, and demonstrate that *Smox*-deficient mice exhibit significant reductions of gastric spermidine levels and *H. pylori*-induced inflammation. Proteomic analysis revealed that cancer was the most significantly altered functional pathway in $Smox^{-/-}$ gastric organoids. Moreover, there was also less DNA damage and β-catenin activation in *H. pylori*-infected $Smox^{-/-}$ mice or gastric organoids, compared to infected wild-type animals or gastroids. The link between SMOX and β-catenin activation was confirmed in human gastric organoids that were treated with a novel SMOX inhibitor. These findings indicate that SMOX promotes *H. pylori*-induced carcinogenesis by causing inflammation, DNA damage, and activation of β-catenin signaling.

INTRODUCTION

Gastric cancer is the third most common cause of cancer death worldwide [1] and *Helicobacter pylori* infection of the stomach is the strongest risk factor for disease development [2]. Although approximately half of the world's population is colonized by *H. pylori* [3, 4] only a subgroup of those infected progress through a cascade of histological lesions from atrophic gastritis to intestinal metaplasia, dysplasia, and gastric adenocarcinoma [5, 6].

The polyamines putrescine, spermidine and spermine are generated through a sequential process that starts with the conversion of L-ornithine into putrescine by the enzyme ornithine decarboxylase (ODC). Putrescine is then metabolized to spermidine, spermidine to spermine by spermidine synthase and spermine synthase, respectively [7]. Notably, *H. pylori* infection increases ODC expression [8] and polyamine levels [9] in gastric tissues of *H. pylori*-infected individuals as compared with uninfected subjects. Further, ODC-derived putrescine alters the chromatin landscape in macrophages, suppressing gene expression, and thus dampening the gastric immune response [10]. Additionally, inhibition of ODC activity in Mongolian gerbils infected with *H. pylori* reduces the level of oxidative DNA damage in gastric epithelial cells (GECs) and also decreases cancer incidence [11, 12]. Spermine oxidase (SMOX), specifically back-converts the polyamine spermine to spermidine, generating H_2O_2 in the process [13, 14], which leads to DNA damage in gastric cells [15, 16]. We have demonstrated that *H. pylori* infection increases expression of SMOX in human and rodent gastric tissues and this is associated with oxidative DNA damage [17, 18]. Inhibition of SMOX by treatment with the SMOX inhibitor MDL 72527 reduces dysplasia and carcinoma in gerbils infected with *H. pylori* [11], suggesting that SMOX activity supports *H. pylori*-mediated carcinogenesis in the stomach. However, the effect of genetic ablation of *Smox* on the outcome of *H. pylori* infection has not been explored.

In the present study, we used *Smox*-deficient mice to directly evaluate the contribution of SMOX to *H. pylori*-associated pathology. Here we show that *Smox* deletion reduces spermidine levels in gastric tissues and also decreases inflammation and DNA damage in mice infected with *H. pylori*. DNA damage and activation of β-catenin, a signaling event strongly linked to gastric carcinogenesis [19–21], were diminished in $Smox^{-/-}$ mice and in murine gastric-derived organoids in response to *H. pylori* infection. Proteomic analysis implicated multiple cancer pathways as the most affected by *Smox* deletion in infected gastric organoids. Moreover, treatment of human-derived gastric organoids with a second-generation SMOX inhibitor (SLH150–54) leads to decreased β-catenin activation in response to *H. pylori*.

RESULTS

SMOX regulates spermidine levels in gastric tissues

First, we confirmed that *Smox* mRNA expression is completely eliminated in the gastric tissues of $Smox^{-/-}$ mice (Fig. 1A). We also verified that the deletion of *Smox* was associated with dysregulation of polyamine metabolism in the stomach (Fig. 1B–D). Spermidine was the most abundant polyamine in tissues from wild-type (WT) mice (Fig. 1C), and deletion of *Smox* led to significant reductions of gastric spermidine levels in both uninfected and infected mice (Fig. 1C). Putrescine levels were decreased in *H. pylori*-infected $Smox^{-/-}$ tissues, likely from loss of spermidine back-conversion, and spermine was not significantly altered between the different groups (Fig. 1B and D).

To verify that SMOX activity also affects spermidine levels in GECs, we generated monolayers of gastric organoids from WT and $Smox^{-/-}$ mice. The expression of *Smox* was eliminated in $Smox^{-/-}$ GECs (Fig. 1E). When the monolayers were infected with *H. pylori*, there were no significant changes in the production of the three polyamines (Fig. 1F–H). However, we confirmed that primary GECs from $Smox^{-/-}$ mice, infected or not with *H. pylori*, had reduced spermidine levels compared to WT organoids (Fig. 1G). Putrescine concentrations were not significantly affected by *Smox* deletion and we observed a slight accumulation of spermine in $Smox^{-/-}$ organoids that was not significant (Fig. 1F and H).

Reduction of *H. pylori*-induced gastritis in $Smox^{-/-}$ mice

C57BL/6 WT and $Smox^{-/-}$ mice were infected with the *H. pylori* strain PMSS1 for 4 weeks and gastric inflammation was evaluated by scoring hematoxylin and eosin (H&E) staining (Fig. 2A). There was reduced infiltration of immune cells in the gastric mucosa of *H. pylori*-infected $Smox^{-/-}$ mice compared to infected WT (Fig. 2A). Using a score combining acute and chronic inflammation in the gastric antrum and corpus [10, 22, 23], we found a significant increase in histologic gastritis in infected WT mice relative to uninfected animals (Fig. 2B). Compared to WT mice, $Smox^{-/-}$ mice showed significantly decreased inflammation (Fig. 2B) and reduced infiltration of polymorphonuclear cells (PMNs; Fig. 2C). When acute and chronic inflammation was assessed individually, *H. pylori*-infected $Smox^{-/-}$ mice exhibited significantly decreased acute inflammation (Supplementary Fig. 1). We then confirmed these results by assessing myeloperoxidase (MPO) expression in gastric tissues using immunostaining (Fig. 2D). Reduced levels of MPO-positive cells were

observed in *Smox*$^{-/-}$ mice infected for 8 weeks compared with WT animals (Fig. 2D); these observations were confirmed by quantifying the infiltrating cells expressing MPO (Fig. 2E). There was increased *H. pylori* colonization of the gastric tissue in *Smox*$^{-/-}$ mice compared with that observed in WT animals (Fig. 2F), an inverse relationship with inflammatory response that we have found in other studies [10, 22, 23]. Finally, in accordance with the level of inflammation in both genotypes, the expression of the genes encoding the chemokines *Cxcl1*, *Cxcl2*, and *Ccl5* was significantly increased in the WT animals infected with *H. pylori* and significantly reduced in infected *Smox*$^{-/-}$ versus WT mice (Figure 2G).

Oncogenic signaling in *H. pylori*-stimulated gastric epithelial cells is regulated by SMOX

To determine the involvement of SMOX in the global response of GECs to *H. pylori*, we used a proteomic approach. Gastric organoids were generated from WT and *Smox*$^{-/-}$ mice, monolayers of these GECs were infected or not with *H. pylori,* and the proteins were quantified using isobaric tag for relative and absolute quantification (iTRAQ) technology and LC-MS/MS analysis. There were 44 proteins significantly increased by *H. pylori* in organoid monolayers from *Smox*$^{-/-}$ mice as compared with infected WT cells (see data deposition information in Supplementary Methods). We also identified 23 proteins significantly downregulated by *H. pylori* in *Smox*$^{-/-}$ organoid monolayers when compared to WT cells infected with *H. pylori*. We then used Ingenuity Pathway Analysis and found that Cancer and RNA-Post-Transcriptional Modifications were the disease and functions most affected by *Smox* deletion in *H. pylori*-infected GECs (Fig. 3A). Importantly, other pathways involved in carcinogenesis, including cell death and survival, cellular movement, cellular function and maintenance, and cell cycle were significantly altered (Fig. 3A). Lastly, the gastrointestinal disease and immunological disease pathways, which are directly related to *H. pylori* infection, were also regulated by SMOX in infected cells.

Based on these data, we assessed the effect of SMOX on different molecular events associated with *H. pylori*-induced carcinogenesis. We have reported that SMOX is associated with DNA damage in patients infected with *H. pylori* [11] and that SMOX inhibition by MDL 72527 reduces *H. pylori*-induced gastric cancer in Mongolian gerbils [11]. Accordingly, the percentage of GECs expressing phosphoserine 139 of H2FA histone family member X (pH2AFX), a reliable marker of DNA damage [24, 25] was reduced in *Smox*$^{-/-}$ infected mice when assessed by flow cytometry in isolated GECs (Fig. 3B). We then confirmed that *H. pylori* infection *ex vivo* of gastric organoid monolayers from WT mice induced DNA damage, as evidenced by the presence of robust punctate nuclear staining for pH2AFX (Fig. 3C). This staining was less abundant in the monolayers of primary GECs from *Smox*$^{-/-}$ mice infected with *H. pylori* (Fig. 3C). Further, the quantification of DNA damage by flow cytometry also demonstrated a significant reduction of pH2AFX^{+} cells in *H. pylori*-infected *Smox*$^{-/-}$ monolayers compared with WT cells (Fig. 3D).

β-catenin/WNT signaling plays an important role in cell transformation and *H. pylori*-positive gastric tissues have increased β-catenin expression [19, 26, 27]. From the proteomic analysis, we identified proteins involved in WNT, NOTCH, and TGF-β signaling pathways, which are linked to β-catenin activation, and were differentially expressed in *Smox*$^{-/-}$

monolayers compared with WT. In particular, E3 ubiquitin-protein ligase TRIM33 was significantly upregulated in *Smox*$^{-/-}$ GECs infected with *H. pylori* compared to infected WT. TRIM33 ubiquitylates β-catenin and promotes its degradation, reducing nuclear β-catenin levels [28]. β-catenin expression was therefore assessed by immunofluorescence in gastric tissues from WT and *Smox*$^{-/-}$ mice and we observed β-catenin accumulated in the cytoplasm and nuclei in tissues from WT mice infected with *H. pylori* (Fig. 3E), indicative of activation. In contrast, β-catenin was mainly membrane-associated in the gastric tissues from *Smox*$^{-/-}$ mice (Fig. 3E), indicating abrogation of β-catenin activation. Quantification of the staining showed a significant increase in β-catenin activation in the infected WT mice and decreased activation in tissues from infected *Smox*$^{-/-}$ mice (Fig. 3F). Similarly, we observed abundant cytoplasmic β-catenin staining in 2-D monolayers of WT GECs after infection with *H. pylori* and mostly cell membrane-associated staining in the *Smox*$^{-/-}$ GECs (Fig. 3G). Image analysis of the staining in organoids replicated our findings in the tissues from WT and *Smox*$^{-/-}$ mice (Fig. 3H).

SMOX activity contributes to β-catenin signaling pathway activation in human-derived gastric organoids

To test if SMOX affects β-catenin activation in a human model system, gastric organoids were generated from human surgical samples as described [29], cultured as monolayers, and then infected with *H. pylori*, in the presence or absence of the novel SMOX inhibitor SLH150–54 [30]. *H. pylori* infection increased spermidine levels in these human GECS, and SLH150–54 significantly inhibited this response (Fig. 4A). Putrescine levels were reduced in *H. pylori*-infected cells and spermine was not altered (Fig. 4A). The SMOX inhibitor had no effect on putrescine or spermine concentrations (Fig. 4A).

β-catenin activation was assessed in human organoids transduced with a TCF/LEF reporter. Infection with *H. pylori* significantly increased β-catenin activation and SLH150–54 significantly reduced TCF/LEF reporter activity (Fig. 4B). The expression of *AXIN2*, a β-catenin target gene [31], was increased in response to *H. pylori* infection and then significantly decreased when SMOX activity was inhibited by SLH150–54 (Fig. 4C). Immunofluorescence staining for β-catenin also showed that *H. pylori* infection induced β-catenin activation in human organoids, evidenced by increased cytoplasmic and nuclear staining, which was reduced by SLH150–54 (Fig. 4D). Quantification of the fluorescence confirmed that the *H. pylori*-induced β-catenin nuclear translocation was decreased by SLH150–54 (Fig. 4E). Spermidine supplementation in the infected SLH150–54-treated cells increased β-catenin activation as compared with the infected cells treated with the inhibitor alone (Fig. 4D–E).

DISCUSSION

Global antibiotic eradication for *H. pylori* has been proposed as a strategy to decrease gastric cancer incidence, but antibiotic resistance and recurrence and/or recrudescence is becoming more common [32, 33]. Thus, alternative molecular strategies are needed to prevent disease progression in *H. pylori*-infected subjects. In the present study, we demonstrated that genetic deletion of *Smox* in mice attenuates *H. pylori*-induced inflammation and carcinogenic

signaling, including oxidative DNA damage and β-catenin activation. In addition, β-catenin activation is also repressed in *H. pylori*-infected human gastroids by a second-generation SMOX inhibitor, highlighting the potential clinical relevance of our findings.

Using a genetic model of *Smox* deletion, here we have shown consistent reduction in PMN infiltration and chemokine production in $Smox^{-/-}$ mice infected with *H. pylori*. These results further support the concept that SMOX is an important mediator of inflammation in response to bacterial infection. In the same way, it has been demonstrated that *Smox* deletion or a SMOX inhibitor dampen *Citrobacter rodentium*- and enterotoxigenic *Bacteroides fragilis*-induced colon inflammation, respectively [34, 35]. Further, we have reported a positive correlation between *i*) gastric polyamines and gastritis in *H. pylori*-infected gerbils [11], and *ii*) colon spermidine concentration and histological damage in *C. rodentium*-infected WT mice [34]. Taken together with the current results, these findings implicate SMOX as a regulator of mucosal inflammation in infectious models of the gastrointestinal tract, and suggest that the protective effect of *Smox* deletion is associated with the decreased levels of spermidine. The specific molecular/cellular mechanism responsible for the potentially deleterious effect of spermidine in these infections is under investigation.

Disease progression from gastritis to gastric cancer in *H. pylori*-infected individuals has been associated with increased levels of pro-inflammatory mediators including reactive oxygen and nitrogen species that induce DNA damage [11, 17, 36]. Previous work from our laboratory demonstrated that SMOX is induced during *H. pylori* infection in macrophages leading to increased production of H_2O_2 and oxidative DNA damage [37]. Herein, our data showed that DNA damage was reduced in the infected $Smox^{-/-}$ mice. In these animals, there is less reactive oxygen species (ROS) since there is less recruitment of PMNs, a major source of ROS, and no SMOX activity. Our findings are in agreement with a murine model of enterotoxigenic *Bacteroides fragilis* infection in which ROS and DNA damage were dependent on SMOX [35]. Our results indicate that SMOX is a main component of the pro-carcinogenic signaling in *H. pylori*-induced gastric cancer.

Proteomic analysis indicated that SMOX plays a prominent role in pathways activated by *H. pylori* that drive gastric carcinogenesis. One key signaling pathway linked to carcinogenesis in *H. pylori* infection is Wnt/β-catenin activation [20]. *H. pylori* induces β-catenin nuclear accumulation in GECs, promoting the emergence of cells with cancer stem cell-like properties [38]. It has been shown that the cytotoxin-associated gene A (CagA) from *H. pylori* interacts with E-cadherin disrupting its association with β-catenin [39]. Once this interaction is lost, β-catenin accumulates in the cytoplasm and the nucleus where it induces expression of target genes like *AXIN2* [31]. Strikingly, our data show that *Smox* deletion or chemical inhibition in murine or human gastroids reduces β-catenin activation, suggesting that activation of this oncogenic signaling circuit in the stomach is supported by SMOX. *Smox* deletion or SMOX inhibition had no effect in uninfected cells, which may indicate that SMOX activity favors, but does not initiate, β-catenin activation. We are describing in this report a novel link between polyamines and β-catenin activation in the context of *H. pylori* infection. Polyamine quantification in murine tissues and organoids consistently showed reduced levels of spermidine in $Smox^{-/-}$ mice as well as in human organoids treated with SLH150–54. Our result showing that spermidine supplementation can increase β-

catenin activation in SLH150–54-treated cells suggests that spermidine could play a role in this process. Supporting our findings, it has been shown that β-catenin tyrosine phosphorylation in colonic epithelial cells is reduced when polyamines are depleted, leading to reorganization of cytoskeletal proteins and inhibition of cell migration [40].

In summary, using a genetic model, we have demonstrated that *Smox* deletion reduces spermidine levels in gastric tissues, diminishes immune cell infiltration and prevents DNA damage. By using human and mouse gastric-derived organoids we identified β-catenin as one of the oncogenic signaling pathways supported by SMOX activity. Chronic inflammation and dysregulation of homeostatic signaling by *H. pylori* are essential etiological factors for disease progression. Taken together, our data implicate SMOX as a pro-carcinogenic enzyme in the infected stomach. Thus, targeted inhibition of SMOX could be considered as a chemopreventive strategy for gastric cancer.

Supplementary Material

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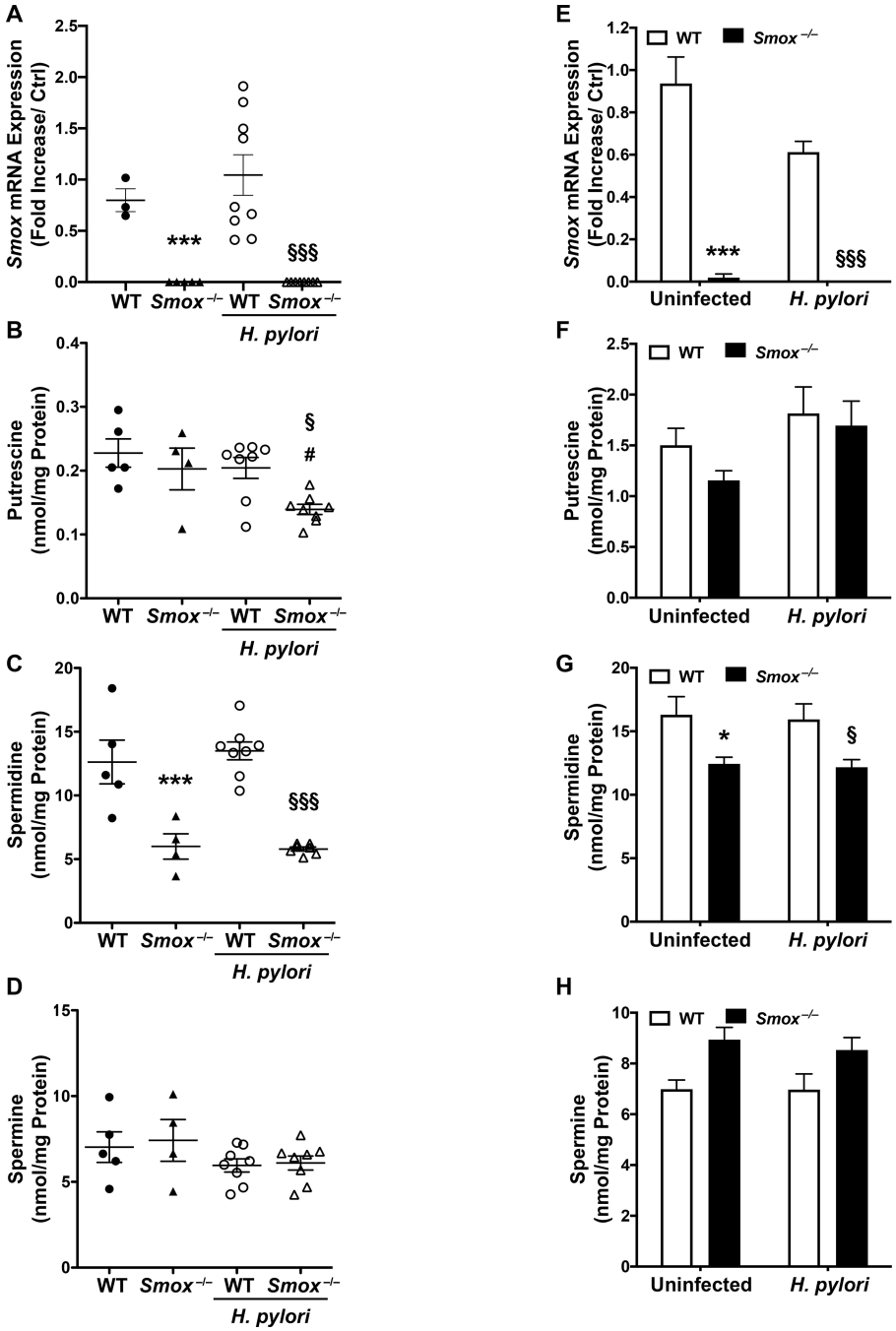

Figure 1.

Smox expression and polyamine concentrations in gastric tissues and murine gastroids. (**A**) *Smox* mRNA expression by real time PCR, and (**B**) putrescine, (**C**) spermidine, and (**D**) spermine quantification by mass spectrometry in the stomach tissues of WT and *Smox*$^{-/-}$ mice, infected or not with *H. pylori* PMSS1 for 4 weeks. Monolayers of murine gastroids were infected with *H. pylori* PMSS1 for 24 h, and *Smox* mRNA expression (**E**) as well as the three polyamines (**F**-**H**) were analyzed in the cell lysates. **P*<0.05 and ****P*<0.001 versus uninfected WT; §*P*<0.05 and §§§*P*<0.001 versus infected WT; #*P*<0.05 compared to

uninfected *Smox*$^{-/-}$. In (**A**-**D**), each dot represents a mouse and (**E**-**H**) is the mean ± SEM of 3 independent experiments, each performed with gastroids from 2 different mice.

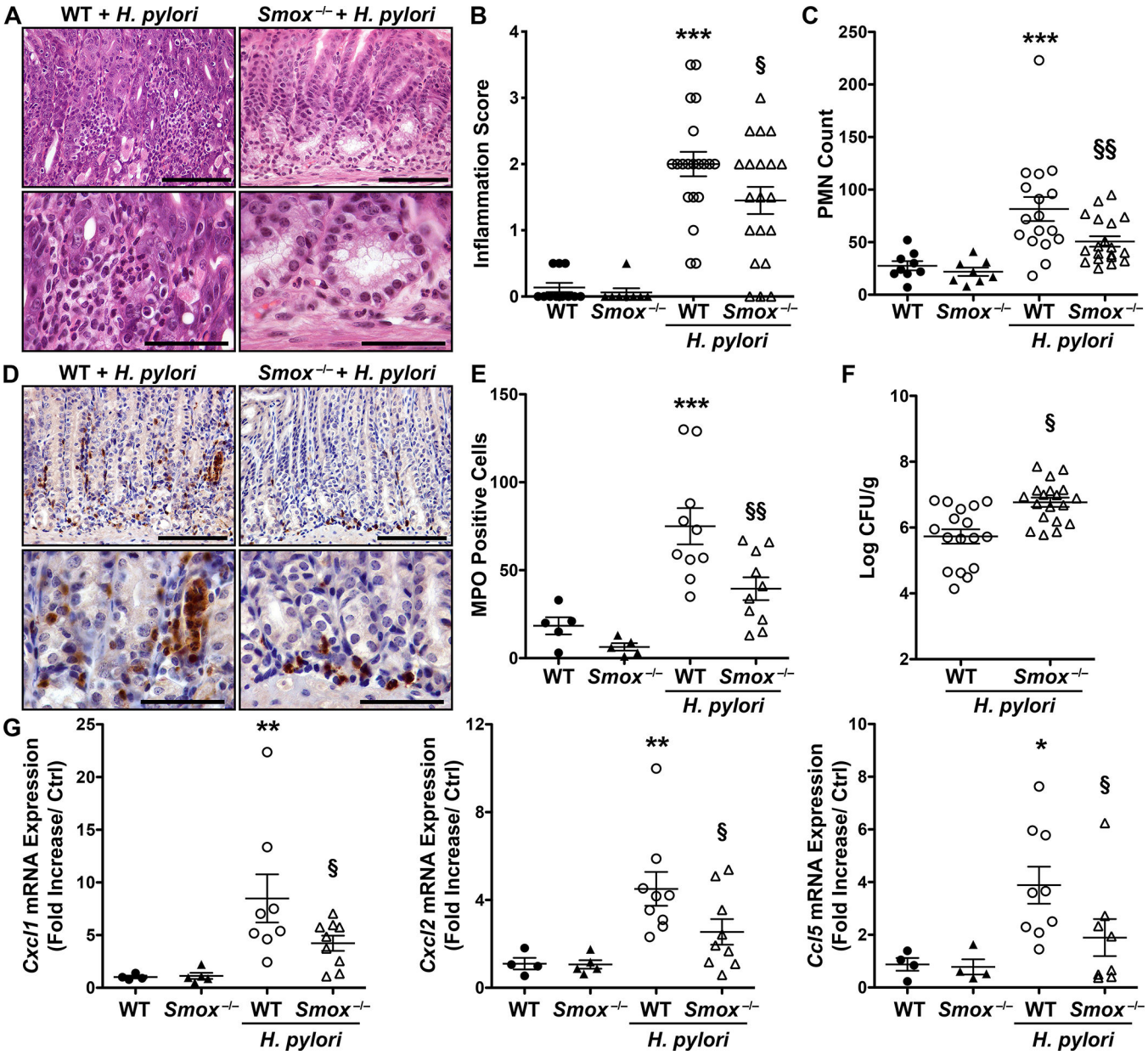

Figure 2.

H. pylori-induced gastritis in C57BL/6 WT and *Smox*$^{-/-}$ mice. Mice were infected or not with *H. pylori* strain PMSS1. (**A**) H&E staining of WT and *Smox*$^{-/-}$ mouse stomachs after 4 weeks of infection. (**B**) Inflammation score and (**C**) number of PMNs per tissue assessed in the antrum from WT and *Smox*$^{-/-}$ animals at the 4-week time point. (**D**-**E**) Gastric tissues from mice infected for 8 weeks were immunostained for MPO (**D**) and the number of MPO^{+} cells in the antrum-corpus transition zone was determined (**E**). (**F**) Colonization of the stomach determined by serial dilution and culture after 4 weeks of infection. (**G**) The expression of *Cxcl1*, *Cxcl2*, and *Ccl5* mRNA in gastric tissues was assessed by RT-real-time PCR in the 4-week infected mice. In (**B**-**C**) and (**E**-**G**), **P*<0.05, ***P*<0.01, and ****P*<0.001 versus control WT mice; §*P*<0.05 and §§*P*<0.01 versus WT animals infected with *H. pylori*.

In all panels, each symbol represents a different mouse. In (**A**) and (**D**), scale bars are 50 μm (top images) and 100 μm (bottom images).

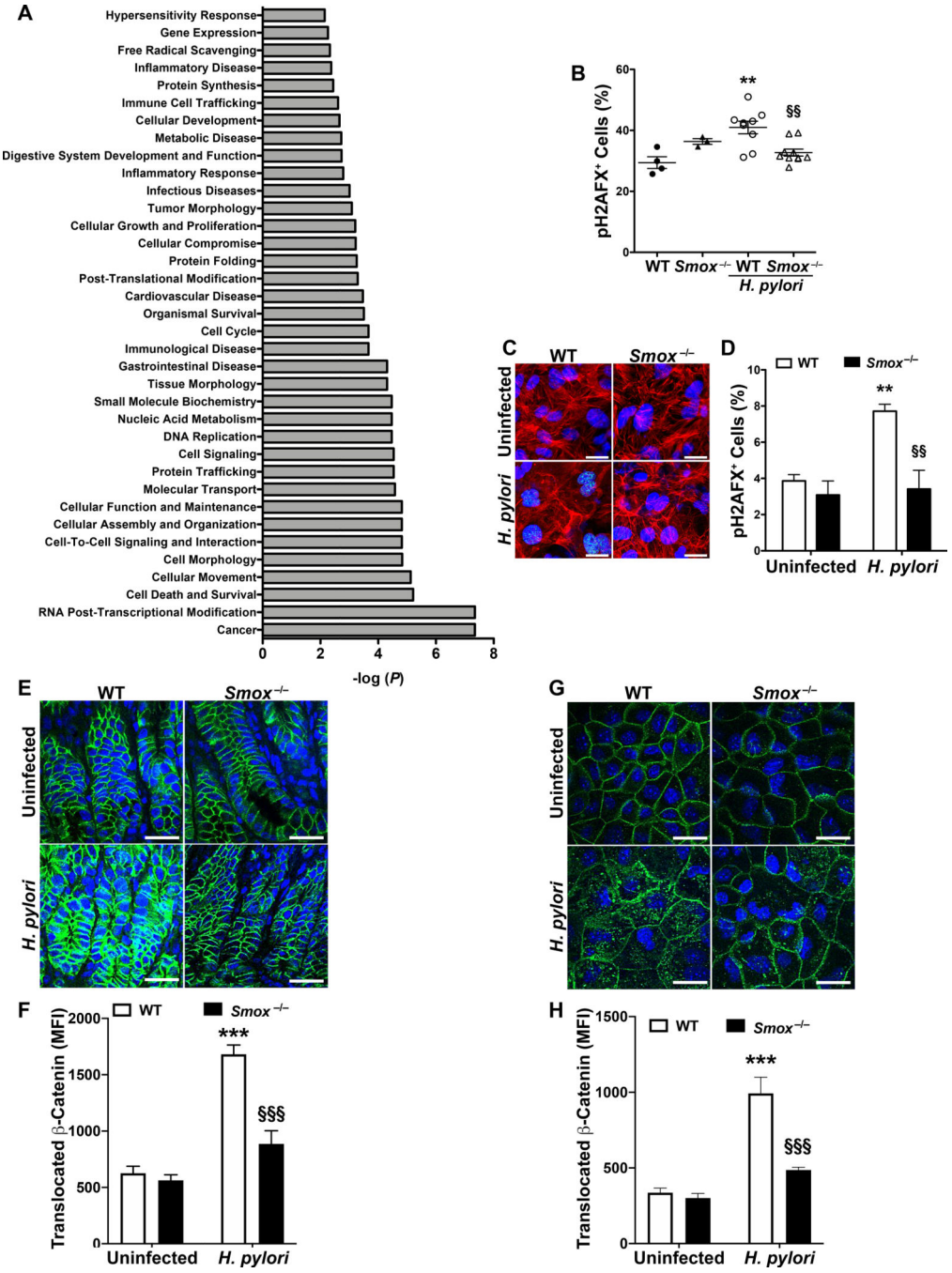

Figure 3.

Effect of *Smox* deletion on *H. pylori*-stimulated GECs derived from gastric organoids. Cells were infected with *H. pylori* PMSS1 for 16 h. (**A**) Ingenuity Pathway Analysis, performed from the iTRAQ data, was used to categorize the pathways related to disease and function in *H. pylori*-infected monolayers of gastric organoids from WT and *Smox*$^{-/-}$ mice. (**B**) The percentage of pH2AFX^{+} GECs in WT and *Smox*$^{-/-}$ mice, infected or not with *H. pylori* for 4 weeks, was determined by flow cytometry. Immunofluorescence (**C**) and flow cytometry (**D**) for pH2AFX were performed on the GECs from WT and *Smox*$^{-/-}$ mice, infected or not

with *H. pylori* for 16 h. In (**C**), only the merged images are shown; actin is in red, nuclei in blue, and pH2AFX in green; scale bar, 25 μm. (**E** and **G**) β-catenin activation was assessed by immunofluorescence in WT and *Smox*$^{-/-}$ mice infected or not with *H. pylori* for 4 weeks (**E**) and in monolayers of primary GECs infected or not for 16 h with *H. pylori* (**G**); β-catenin in green and nuclei in blue; scale bar, 100 μm. Mean ± SEM of three experiments. (**C** and **G**) Representative image from 3 independent experiments. (**F** and **H**) The fluorescence of images in **E** and **G** was quantified by measuring the translocation of β-catenin to the cytoplasm and nucleus using ImageJ. ***P*<0.01, ****P*<0.001 versus uninfected WT mice/cells; §§ *P*<0.01, §§§ *P*<0.001 compared to infected WT mice/cells.

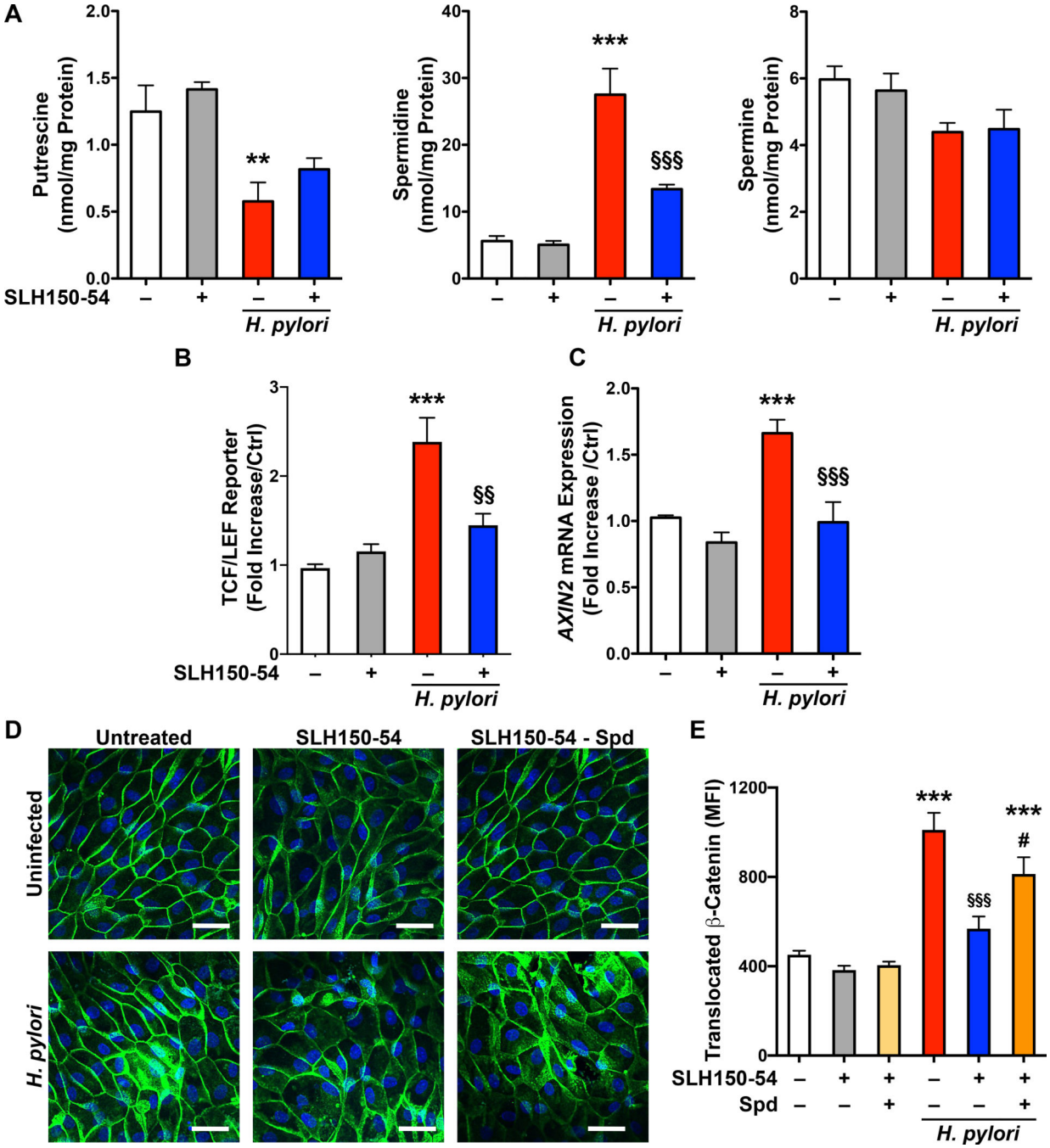

Figure 4.

Effect of SMOX activity on β-catenin activation in human-derived gastric organoids. (**A**) Polyamine levels measured by mass spectrometry in monolayers of human gastric organoids pre-treated with SLH150–54 (100 μM) for 2 h and then infected with *H. pylori* PMSS1 for 16 h. (**B**) TCF/LEF reporter assay in human GECs pre-treated with with SLH150–54 and then infected with *H. pylori* PMSS1 for 6 h. (**C**) *AXIN2* mRNA expression in human GECs pre-treated with SLH 150–54 and/or infected for 3 h with *H. pylori*. (**D**) Immunofluorescence staining for β-catenin was performed on human organoid GEC

monolayers pre-treated with SLH150–54 and supplemented or not with spermidine (10 μM), then infected with *H. pylori* PMSS1 for 16 h; representative image from three experiments, performed with two human organoid lines. The merged images are shown, β-catenin is green, and nuclei are blue; scale bar, 100 μm. (**E**) The fluorescence of images shown in (**D**) was quantified by measuring the translocation of β-catenin to the cytoplasm and nucleus using ImageJ. Mean ± SEM of three experiments with two organoid lines. ** $P<0.01$, *** $P<0.001$ compared to control cells; §§§ $P<0.001$ versus *H. pylori*-infected cells; # $P<0.05$ versus *H. pylori*-infected cells pre-treated with SLH150–54.