

## ORIGINAL ARTICLE

## Enteric bacterial protein AvrA promotes colonic tumorigenesis and activates colonic beta-catenin signaling pathway

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*Salmonella* infections can become chronic and increase the risk of cancer. The mechanisms by which specific *Salmonella* organisms contribute to cancer, however, are still unknown. Live and attenuated *Salmonella* are used as vectors to target cancer cells, but there have been no systematic studies of the oncogenic potential of chronic *Salmonella* infections in cancer models. AvrA, a pathogenic product of *Salmonella*, is inserted into host cells during infection and influences eukaryotic cell pathways. In the current study, we colonized mice with *Salmonella* AvrA-sufficient or AvrA-deficient *Salmonella* typhimurium strains and induced inflammation-associated colon cancer by azoxymethane/dextran sulfate sodium (AOM/DSS). We confirmed *Salmonella* persisted in the colon for up to 45 weeks. *Salmonella* was identified not only in epithelial cells on the colonic luminal surface and base of the crypts but also in invading tumors. Tumor incidence in the AvrA + infected group was 100% compared with 51.4% in the AOM/DSS group without bacterial gavage and 56.3% in mice infected with the AvrA- strain. Infection with AvrA + strain also altered tumor distribution from the distal to proximal colon that might reflect changes in the microbiome. AvrA-expressing bacteria also upregulated beta-catenin signaling as assessed by decreased beta-catenin ubiquitination, increased nuclear beta-catenin and increased phosphorylated-beta-catenin (Ser552), a marker of proliferating stem-progenitor cells. Other  $\beta$ -catenin targets increased by AvrA included Bmi1, a cancer stem cell marker, matrix metalloproteinase-7, and cyclin D1. In summary, AvrA-expressing *Salmonella* infection activates  $\beta$ -catenin signals and enhances colonic tumorigenesis. Our findings provide important new mechanistic insights into how a bacterial protein targets proliferating stem-progenitor cells and contributes to cancer development. Our observations also raise a note of caution regarding the use of mutant *Salmonella* organisms as vectors for anti-cancer therapy. Finally, these studies could suggest biomarkers (such as AvrA level in gut) to assess cancer risk in susceptible individuals and infection-related dysregulation of  $\beta$ -catenin signaling in cancer.

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## INTRODUCTION

Colon cancer is a major cause of cancer-related deaths in the Western world. Increasing evidence has implicated chronic inflammation in its causation, and recently attention has focused on the role of the microbiome. Chronic bacterial infection and the ensuing colonic inflammation contribute to tumor initiation and tumor progression.<sup>1,2</sup> The number of microbial cells comprising the commensal microflora is 10 times larger than the number of eukaryotic cells in the human body. More than 1000 species of bacteria inhabit the human colon. Because of the complexity of the gut flora, identification of the specific microbial organisms or bacterial products contributing to colon cancer remains challenging. Deciphering the roles of specific bacterial products is in its infancy. Progress has been made, however. For example, a recent study demonstrated that a human colonic commensal bacterium promoted tumorigenesis via induction of interleukin-17 T helper cell responses.<sup>3</sup> In mice, colitis can promote tumorigenesis by altering microbial composition and inducing the expansion of microorganisms with genotoxic capabilities.<sup>4</sup> Recent studies suggested that polyketide synthase is an oncogenic factor in that genetic deletion of this element in *Escherichia coli* NC101 decreased azoxymethane (AOM)-induced tumor multiplicity and invasion in interleukin-10 knockout mice.<sup>4</sup>

*Salmonella* infection and colonization in humans can be chronic<sup>2</sup> and increase the risk of other gastrointestinal diseases, including chronic cholecystitis and gallbladder cancer.<sup>5,6</sup> Whereas increasing evidence links some *Salmonella* species to carcinogenesis, other non-invasive *Salmonella* strains appear promising as vectors to specifically target cancer cells.<sup>7</sup> *Salmonella* and its derivatives invade tumor cells more efficiently than normal cells in animal models.<sup>8,9</sup> The safety of *Salmonella* as an anti-cancer vector is uncertain, however, because there have been no systematic studies of the effects of chronic infections of non-pathogenic or attenuated *Salmonella* strains in cancer models.

Bacteria can modulate their eukaryotic host by secreting microbial proteins that modulate host cell function. AvrA is a multifunctional pathogenic gene of enteric bacteria whose encoded protein is inserted into host cells. Our own studies, as well as those of other investigators, have demonstrated that AvrA influences eukaryotic cell pathways by altering ubiquitination and acetylation of target proteins to modulate inflammation, epithelial apoptosis and proliferation.<sup>10–16</sup> For example, AvrA acts as a deubiquitinase to suppress  $\beta$ -catenin ubiquitination thereby stabilizing  $\beta$ -catenin and inhibiting its degradation.<sup>15</sup> This results in increased  $\beta$ -catenin signaling, which enhances intestinal epithelial cell proliferation.<sup>15</sup>

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In the current study, our goal was to elucidate the role of AvrA on colon cancer development. We hypothesized that the bacterial effector AvrA would activate the  $\beta$ -catenin pathways to promote colonic tumorigenesis. Other investigators have taken advantage of the colitis induced by *Salmonella* to study early acute infections or late chronic infections and associated inflammation.<sup>17</sup> In previous studies, we have established a mouse model with persistent of *Salmonella* AvrA<sup>+</sup> or AvrA<sup>-</sup> infection in the gastrointestinal tract.<sup>18</sup> In the current study, using this model, we examined the effects of chronic infection with AvrA-expressing bacteria on inflammation-associated colon cancer. We initiated colonic epithelial cell mutations with AOM and accelerated tumorigenesis with dextran sulfate sodium (DSS) treatment. To determine the effects of AvrA, we infected mice with *Salmonella* strains that differed in AvrA expression level. We used AvrA (parental PhoP<sup>C</sup>) or AvrA deficient strains (AvrA<sup>-</sup>) or PhoP<sup>C</sup> AvrA<sup>-</sup> strain complemented with a plasmid encoding AvrA (PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup>), as we previously described.<sup>13–15,19,20</sup> We used the mutant bacterial strain, PhoP<sup>C</sup>, for this chronic infection because wild-type (WT) *Salmonella* induced death of infected mice within a week.<sup>18</sup> We therefore focused on mutated bacterial strains for these chronic infection and cancer studies. Previous studies have indicated that PhoP<sup>C</sup> strain expressing AvrA activates the  $\beta$ -catenin pathway in acute and chronic infection.<sup>14,21</sup> PhoP-PhoQ is a regulatory system that controls the expression of *Salmonella* virulence and survival. PhoP<sup>C</sup> is a PhoP-PhoQ constitutive mutation.<sup>22</sup> We demonstrate for the first time that bacterial effector AvrA has an essential role in the effects of chronic *Salmonella* infection on colonic tumorigenesis. Our findings provide important new insights into mechanisms by which the bacterial protein AvrA contributes to colon cancer development.

## RESULTS

### Persistence of *Salmonella* colonization in the colon

Mice were infected with the indicated *Salmonella* strains by one oral gavage and then treated with an AOM-DSS protocol to induce colon cancer. The treatment protocol is summarized in Figure 1a. Colonic *Salmonella* persistence was confirmed by *Salmonella* culture (pink colonies) and 16S RNA PCR of fecal bacteria DNA (Figures 1b and c). AvrA persistence was also confirmed by western blotting of colon tissue from mice infected with AvrA-expressing bacteria 45 weeks postinfection (Figure 1d). Furthermore, we confirmed persistent colonization of *Salmonella* in the colon by immunostaining (Figure 1e). Note that *Salmonella* was identified at the base and surface of the crypts in the infected colon (indicated by arrows) (Figure 1e). We found no significant changes in body weight associated with infection (Supplementary Figure S1). *Salmonella* levels in the intestine, gallbladder, spleen and liver also correlated with expression of AvrA in infected mice (Supplementary Figure S2).

### Persistent infection with *Salmonella* expressing AvrA increases tumor incidence and multiplicity and shifts tumor distribution to proximal colon

We collected tissue samples 45 weeks postinfection. Representative colons with tumors are shown in Figure 2a. There was a striking difference in tumor incidence in mice with AvrA<sup>+</sup> and AvrA<sup>-</sup> bacterial colonization (Table 1). The tumor incidence was 51.4% in the AOM/DSS control group (no infection) and 60.6% in the parental PhoP<sup>C</sup>-infected mice, 56.3% in the AvrA<sup>-</sup> infected mice and 100% in the PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup>.

The average number of tumors in colon (tumor multiplicity) was also significantly higher in the PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup> compared with the AvrA<sup>-</sup> group or the AOM/DSS group without infection (Figure 2b). We also analyzed the distribution of tumors. AOM/DSS-treated mice that were not gavaged with *Salmonella*

were located in the distal colon (Figure 2c, AOM/DSS). In contrast, in mice infected with PhoP<sup>C</sup> or PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup> *Salmonella*, the tumors were located more proximally (Figure 2c). We investigated the pathological changes associated with chronic *Salmonella* infection in AOM/DSS mice using Swiss rolls (Figure 2d). We also found greater number of cancers in AvrA<sup>+</sup> compared with AvrA<sup>-</sup> mice treated with AOM/DSS (Table 1, 40% versus 12.5%, respectively). However, we did not see significant differences in tumor size among the various experimental groups (Supplementary Figure S3).

We detected *Salmonella* by immunofluorescence invading the colonic mucosa 45 weeks postinfection. *Salmonella* organisms were present not only on the colonic luminal surface and within the crypt as shown in Figure 1e but also in tumors from infected mice (Figure 2e). Furthermore, the load of invading *Salmonella* appeared to be higher in tumors, especially in mice colonized with AvrA(+)-expressing *Salmonella*. Thus, these data demonstrate that a single gavage of *Salmonella* was sufficient to induce persistent *Salmonella* colonization, and upregulation of AvrA enhanced tumorigenesis and tumor burden in the AOM/DSS model and altered tumor distribution in the colon.

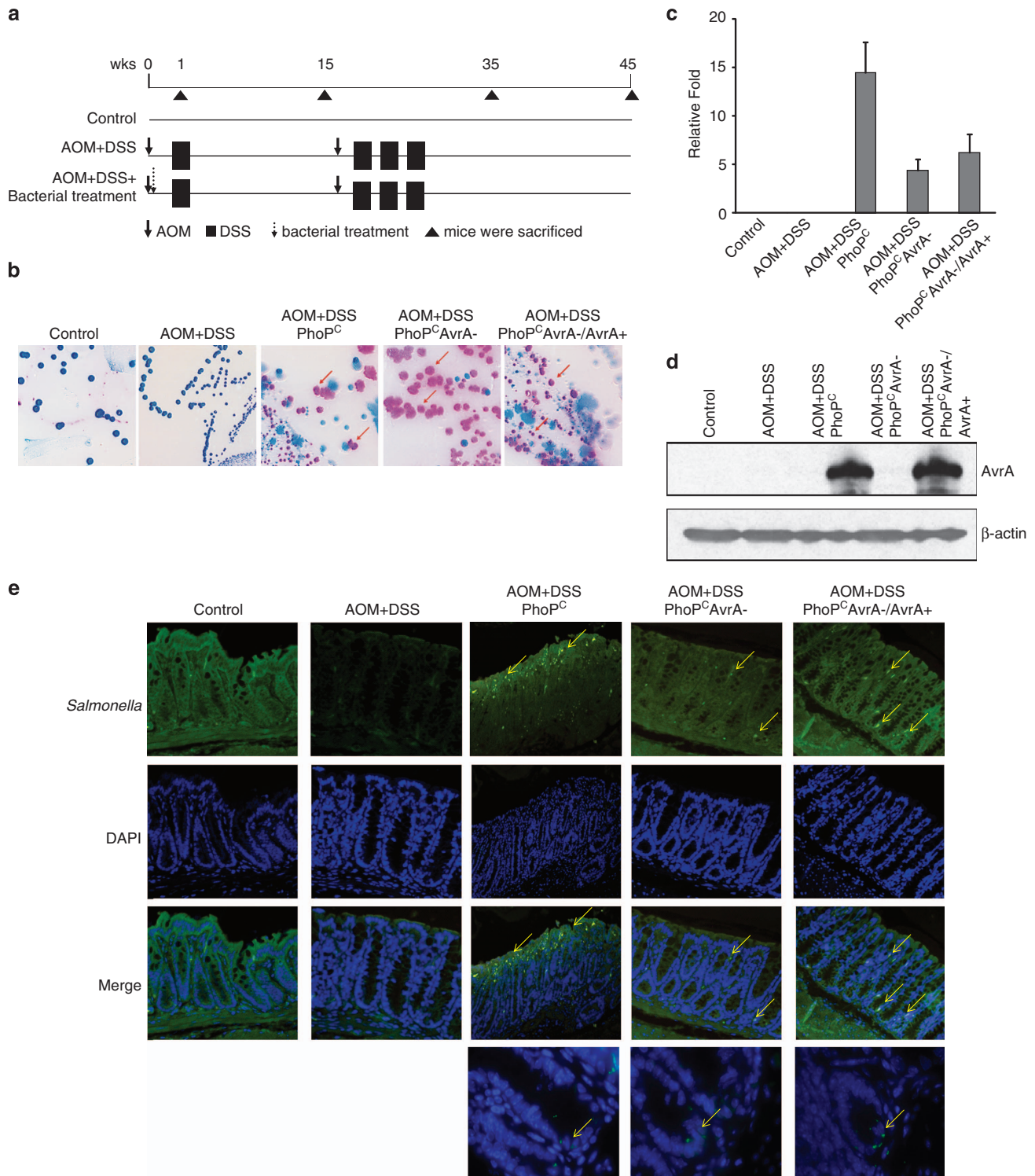
### Activation of the $\beta$ -catenin signaling in the *Salmonella* chronically infected colon.

It is known that  $\beta$ -catenin signaling is activated in colonic tumorigenesis.<sup>23</sup> In agreement with previous studies, we observed that nuclear  $\beta$ -catenin was upregulated in AOM/DSS-induced tumors (Figure 3a). Nuclear localization suggested that  $\beta$ -catenin is also activated (Figure 3a).

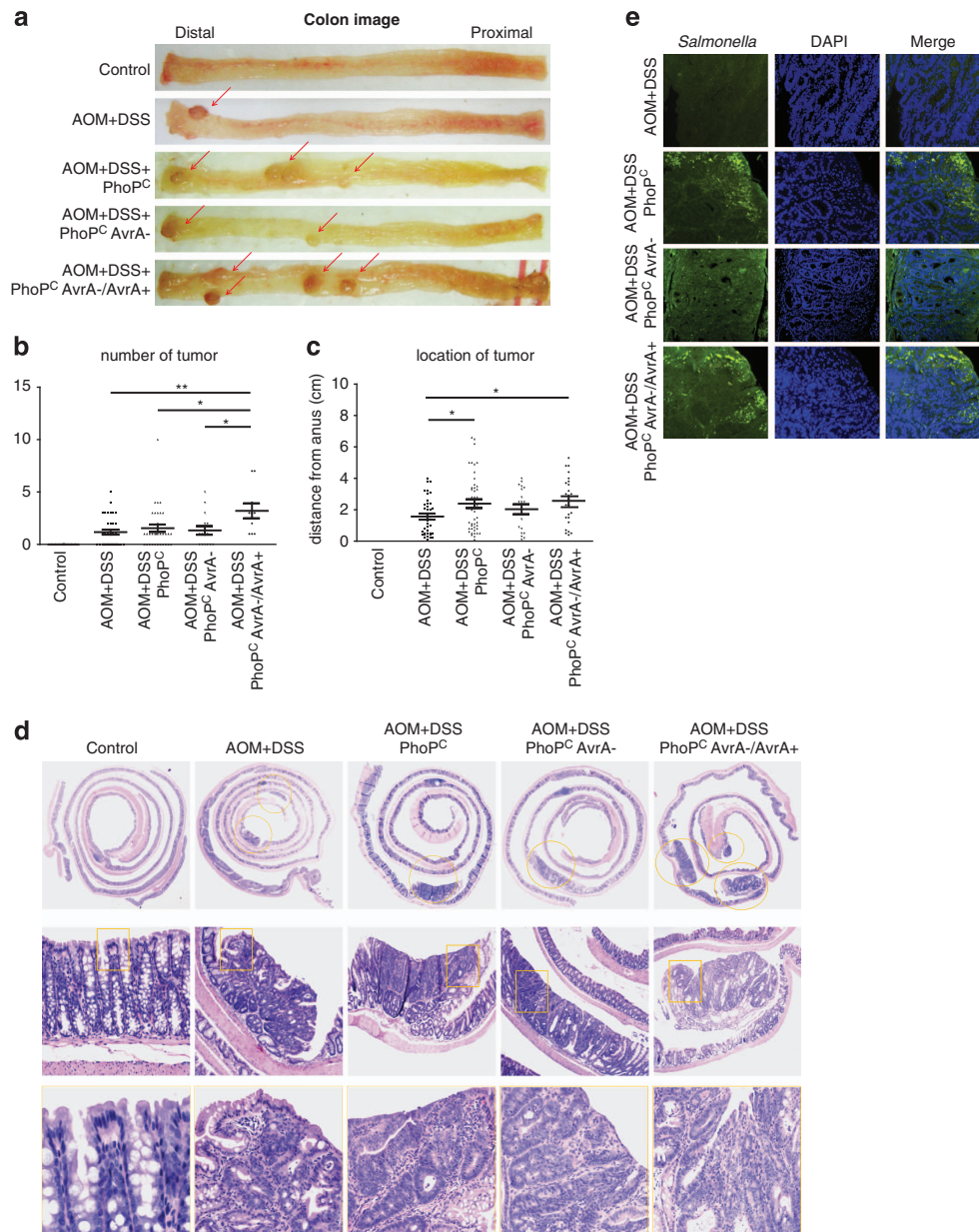
Residue specific phosphorylations control activation or destabilization of  $\beta$ -catenin and are regulated by several upstream kinases. Inflammation increases phosphorylation of  $\beta$ -catenin on Ser552 in stem progenitor cells inducing proliferation.<sup>24</sup> In tumors from mice infected with *Salmonella*, AvrA expression increased phosphorylated  $\beta$ -catenin Ser552 whereas tumors from mice infected with mutant *Salmonella* deficient in AvrA had significantly lower levels of phosphorylated  $\beta$ -catenin Ser552 (Figures 3b and c). By western blotting, we confirmed that protein levels of phosphorylated  $\beta$ -catenin Ser552 were higher in tumors from mice infected with AvrA-expressing bacteria, compared with control mice treated only with AOM/DSS alone or also colonized with *Salmonella* deleted of AvrA (Figure 3d). In contrast, we found no significant change in phosphorylated Ser33/Ser37 or Thr41 associated with AvrA expression in tumors from mice infected with *Salmonella* (Figure 3d), whereas it was increased slightly in control mice treated with AOM/DSS alone.  $\beta$ -Catenin phosphorylations on residues Ser33/Ser37 and Thr41 are associated with destabilization and degradation of  $\beta$ -catenin. This might explain why we did not find significant AvrA-related differences in total  $\beta$ -catenin in tumors from chronically infected mice.

Akt is an upstream regulator of the  $\beta$ -catenin pathway and phosphorylates  $\beta$ -catenin on Ser552.<sup>25</sup> This phosphorylation is implicated in nuclear  $\beta$ -catenin localization.<sup>25</sup> Because there was increased  $\beta$ -catenin Ser552 phosphorylation with bacterial AvrA, we investigated Akt activation in tumors from infected mice. We observed that phospho-active Akt was also increased in tumors, especially in the PhoP<sup>C</sup>AvrA<sup>-</sup>/AvrA<sup>+</sup> group, whereas total Akt levels were not changed in tumors from the PhoP<sup>C</sup>- and PhoP<sup>C</sup>AvrA<sup>-</sup>/AvrA<sup>+</sup>-infected mice (Figure 3d). The phospho active- $\beta$ -catenin phosphorylated on Ser552 was also increased in colonocytes 15 and 35 weeks after *Salmonella* gavage in the PhoP<sup>C</sup> and PhoP<sup>C</sup>AvrA<sup>-</sup>/AvrA<sup>+</sup> groups (data not shown). Thus colonic phospho active- $\beta$ -catenin (phospho-Ser552  $\beta$ -catenin) is persistently elevated in mice colonized by *Salmonella* expressing AvrA.

The Bmi1 (B-cell-specific Moloney murine leukemia virus integration site 1) gene was first isolated as an oncogene



**Figure 1.** Persistent *Salmonella* infection in mice. Mice were infected with the indicated *Salmonella* strains by oral gavage and treated with AOM-DSS to induce colon cancer. **(a)** Schema of experimental design. Day-1: Mice were infected by *Salmonella* gavage 24 h after streptomycin pretreatment (7.5 mg/mouse), ↓ azoxymethane (AOM), 10 mg/kg body weight, intraperitoneal injection; ■ 1% dextran sodium sulfate (DSS) in drinking water, ↓ *Salmonella* gavage; ▲ mice were killed. Experimental groups: Control, normal WT mice (no *Salmonella*, no AOM/DSS); AOM/DSS, only (no *Salmonella*), PhoP<sup>C</sup>, AOM/DSS-treated mice infected with *Salmonella* with WT AvrA; PhoP<sup>C</sup>AvrA<sup>-</sup>, AOM/DSS-treated mice infected with AvrA<sup>-</sup> mutant strain derived from PhoP<sup>C</sup>; PhoP<sup>C</sup>AvrA<sup>-</sup>/AvrA<sup>+</sup>, AOM/DSS-treated mice infected with PhoP<sup>C</sup>AvrA<sup>-</sup> transfected with plasmid encoding AvrA. **(b)** Chronic *Salmonella* infection was assessed by fecal cultures at week 45. On a BBL CHROMagar plate, intestinal *Salmonella* species appear mauve (rose to purple) in color, due to metabolic differences in the presence of selected chromogens. Other bacteria are either inhibited or produce blue–green or colorless colonies. **(c)** *Salmonella* detected in fecal microbial DNA by 16S rRNA PCR 45 weeks postinfection. Mice were infected with PhoP<sup>C</sup> expressing AvrA protein or PhoP<sup>C</sup>AvrA<sup>-</sup> (AvrA deficient) or AvrA-deficient bacteria transfected with AvrA-expressing plasmid. **(d)** AvrA western blotting. Protein lysates from colonic tissue were probed for AvrA levels. **(e)** Immunofluorescence confocal microscopy. *Salmonella* invading the colonic epithelium 45 weeks postinfection appear green.



**Figure 2.** AvrA-expressing bacteria significantly increase colonic tumor incidence. **(a)** Colonic tumors *in situ*. Representative colons from the indicated groups 45 weeks after bacterial infection. Tumors are indicated by red arrows. **(b)** Tumor multiplicity. Average number of tumors in each experimental group. \* $P < 0.05$ , \*\* $P < 0.01$ . **(c)** Tumor location. The distance of each tumor from the anus was measured. \* $P < 0.05$ . **(d)** Swiss rolls of representative colons from the indicated groups. Rectangles in the middle panel are shown at higher magnification in the lower panel. **(e)** *Salmonella* invasion. Localization of *Salmonella* in tumor tissue was assessed by immunofluorescence staining.

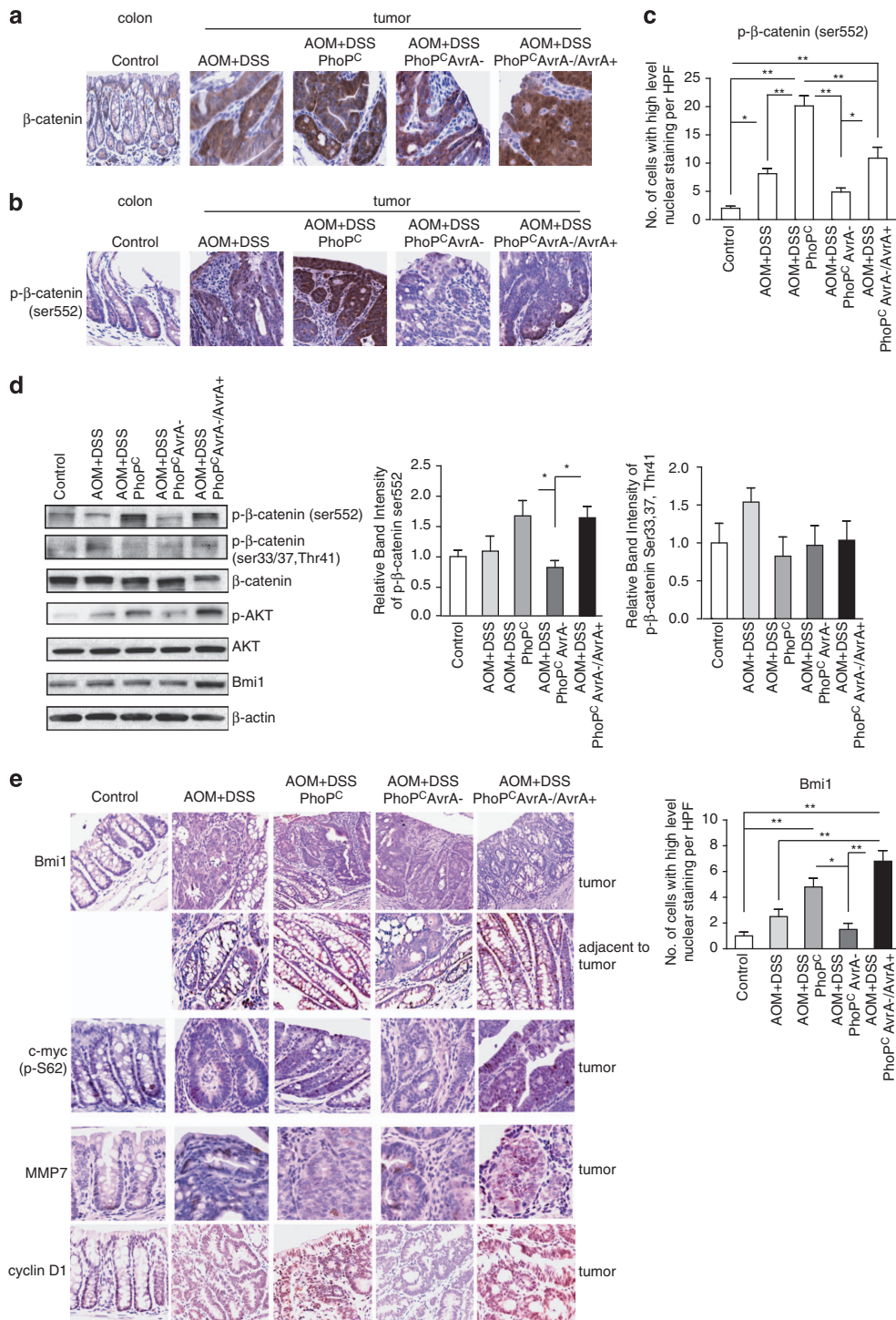
**Table 1.** Tumor incidence of mice infected with *Salmonella*

Group	Total no. of mice	No. of mice to develop tumor		
		Total	Adenoma	Carcinoma
Control	25	0	0	0
AOM + DSS	35	18 (51.4%)	13 (37.1%)	5 (14.3%)
AOM + DSS + PhoP <sup>C</sup>	33	20 (60.6%)	13 (39.4%)	7 (21.2%)
AOM + DSS + PhoP <sup>C</sup> AvrA <sup>-</sup>	16	9 (56.3%)	7 (43.8)	2 (12.5%)
AOM + DSS + PhoP <sup>C</sup> AvrA <sup>-</sup> /AvrA <sup>+</sup>	10	10 (100%) <sup>a</sup>	6 (60.0%)	4 (40.0%)

<sup>a</sup> $P < 0.05$  compared with other groups.

that cooperates with c-myc in oncogenesis.<sup>26</sup> Bmi1 is also implicated as a stem cell marker.<sup>27</sup> We found that colonic Bmi1 was significantly increased in AvrA colonized mice (Figure 3e). Although there was no detectable Bmi1 in the tumors by staining, enhanced Bmi1 staining was found in crypts adjacent to tumor of the PhoP<sup>C</sup>- and PhoP<sup>C</sup>AvrA<sup>-</sup>/AvrA<sup>+</sup>-infected mice (Figure 3e).

We next assessed Akt and  $\beta$ -catenin target genes. Although we did not see significant changes in total c-Myc expression, phospho-c-Myc (P-S62) was upregulated in tumors from mice infected with AvrA-expressing bacteria. Colonic levels of MMP-7 (matrix metalloproteinase 7) and cyclin D1 were also increased in mice colonized with *Salmonella* expressing AvrA (Figure 3e).

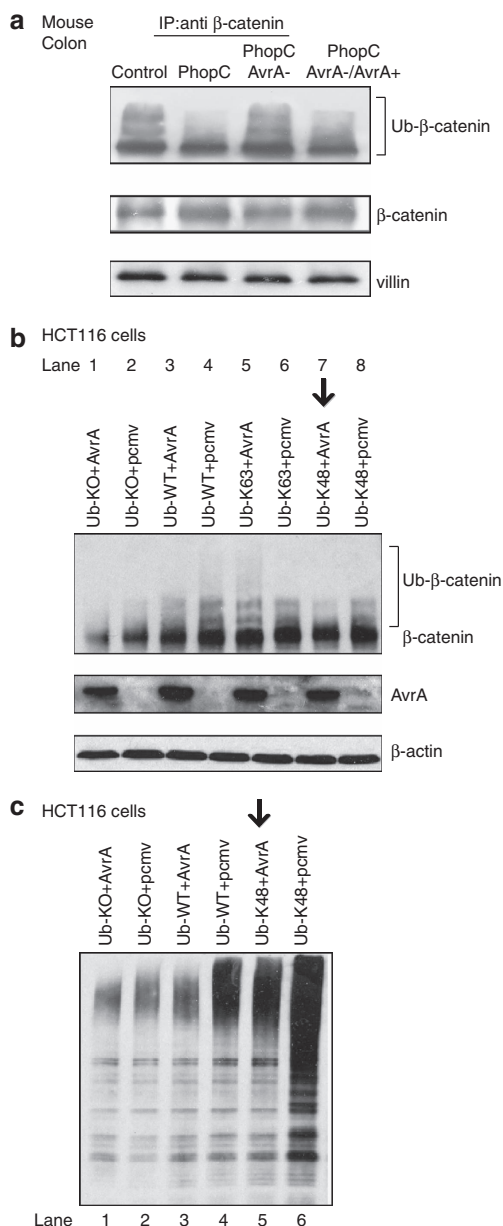


**Figure 3.** AvrA regulates  $\beta$ -catenin activation as assessed by phospho (Ser552)  $\beta$ -catenin in AOM/DSS-induced tumors. **(a)**  $\beta$ -Catenin localization in control mucosa and colonic tumors. **(b)**  $P$ - $\beta$ -catenin Ser552 localization in control mucosa and colonic tumors. **(c)** Quantitation of nuclear  $P$ - $\beta$ -catenin Ser552 in control mucosa and colonic tumors. Data are means  $\pm$  s.d. of  $n = 3$  control mice or AOM/DSS tumors in each group.  $*P < 0.05$ ,  $**P < 0.01$ . **(d)** Expression levels of  $p$ - $\beta$ -catenin, total  $\beta$ -catenin and Akt and  $P$ -Akt as assessed by western blotting in control and AOM/DSS-induced tumors. Shown are control mucosa and tumors representative of three mice in each group. **(e)** Immunostaining of  $p$ -Myc, MMP-7, Cyclin D1 and Bmi1 in control colons and AOM/DSS-induced tumors (left panel). Quantitative imaging of nuclear Bmi1 in control mucosa and mucosa adjacent to tumors (right panel). Data are means  $\pm$  s.d. of  $n = 3$  control mice or AOM/DSS mice in each group.  $*P < 0.05$ ,  $**P < 0.01$ .

*Salmonella* effector protein AvrA modulates  $\beta$ -catenin ubiquitination *in vitro* and *in vivo*

Our previous study showed that AvrA protein acts as a deubiquitinase to stabilize  $\beta$ -catenin.<sup>14</sup> To determine whether

AvrA is responsible for the activation of  $\beta$ -catenin signaling through ubiquitination, we investigated the ubiquitinated  $\beta$ -catenin levels in the AOM/DSS mice with or without *Salmonella* (Figure 4a). The ubiquitinated proteins results in the appearance of



**Figure 4.** Bacterial effector protein AvrA modulates  $\beta$ -catenin ubiquitination in colonic epithelial cells. **(a)** AvrA expression decreased the ub- $\beta$ -catenin in the colonic epithelial cells. At 6 weeks after *Salmonella* infection, colonic epithelial cells were collected and immunoprecipitated with anti- $\beta$ -catenin antibody. Membranes were probed with anti- $\beta$ -catenin and anti-ubiquitin antibody and visualized using enhanced chemiluminescence. Protein input was normalized by the colonic epithelial marker villin. Ubiquitinated (ub)  $\beta$ -catenin is shown as smear. Colon infected by the PhoP<sup>C</sup> lacked the ub- $\beta$ -catenin bands. **(b)** AvrA expression decreased the ub- $\beta$ -catenin through ubiquitination in the human colonic epithelial HCT116 cells. Cells were transiently cotransfected with AvrA and the indicated ubiquitin plasmids; 24 h later, the cells were treated with MG262 for 3 h. The ubiquitin plasmids included pRK5-HA-Ub-KO (all lysines mutated), -Ub WT, -Ub-K48 (K48 only, other lysines mutated) and -Ub-K63 (K63 only, other lysines mutated). Equal amounts of total cell lysates were analyzed for protein levels by immunoblot with anti- $\beta$ -catenin antibody. **(c)** AvrA expression mostly removes k48-link ubiquitins in the human colonic epithelial HCT116 cells. Cells were transiently cotransfected with AvrA and the indicated ubiquitin plasmids. Equal amounts of total cell lysates were analyzed for protein levels by immunoblot with anti-ub-48 antibody.

multiple higher molecular weight bands above the regular protein band in western blotting. We found that mice colonized with *Salmonella* strain PhoP<sup>C</sup> and PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup> lacked detectable ubiquitinated  $\beta$ -catenin bands (Figure 4a). In contrast, in the PhoP<sup>C</sup> AvrA<sup>-</sup> group, ubiquitinated  $\beta$ -catenin levels were similar to the control AOM/DSS mice without *Salmonella* (Figure 4a).

The protein modifications can be either a single ubiquitin protein or chains of ubiquitin. Ubiquitin has seven lysines (K6, K11, K27, K29, K33, K48 and K63), all of which can be conjugated to another ubiquitin to form a polyubiquitin chain.<sup>28</sup> K63 and K48 linkage-specific ubiquitinations are the most predominant forms of ubiquitination.<sup>29</sup> To explore the molecular mechanism of AvrA on ubiquitination, we then transiently cotransfected AvrA and different ubiquitin plasmids in the human colonic epithelial HCT116 cells. The ubiquitin plasmids included pRK5-HA-Ub-KO (all lysines mutated to arginines), -Ub WT, -Ub-K48 (K48 only, other lysines mutated to arginines) and -Ub-K63 (K63 only, other lysines mutated to arginines). As expected, AvrA expression decreased the ubiquitinated  $\beta$ -catenin (ub- $\beta$ -catenin) in the ub-WT group (Figure 4b, lanes 3 and 4). In the presence of AvrA expression and ub-K48, the ubiquitinated  $\beta$ -catenin (ub- $\beta$ -catenin) was decreased (Figure 4b, lane 7, indicated by an arrow), whereas ub- $\beta$ -catenin levels were more in the absence of AvrA (Figure 4b, lane 8). In contrast, there is no difference in the ub-K63 group with or without AvrA (Figure 4b, lanes 5 and 6). As a negative control, the ub-KO group with or without AvrA (Figure 4b, lanes 1 and 2) did not show any difference of ubiquitinated  $\beta$ -catenin due to the mutation of the all lysines to arginines. Immunoblotting with the anti-Ub-K48 further showed that AvrA protein mostly decreased UbK48 linkage-specific ubiquitination (Figure 4c, lane 5). However, we did not find significant change of Ub K6, K11, K27, K29 or K33 (data not shown). These *in vivo* and *in vitro* data indicate that bacterial effector AvrA prevents  $\beta$ -catenin ubiquitination in epithelial cells.

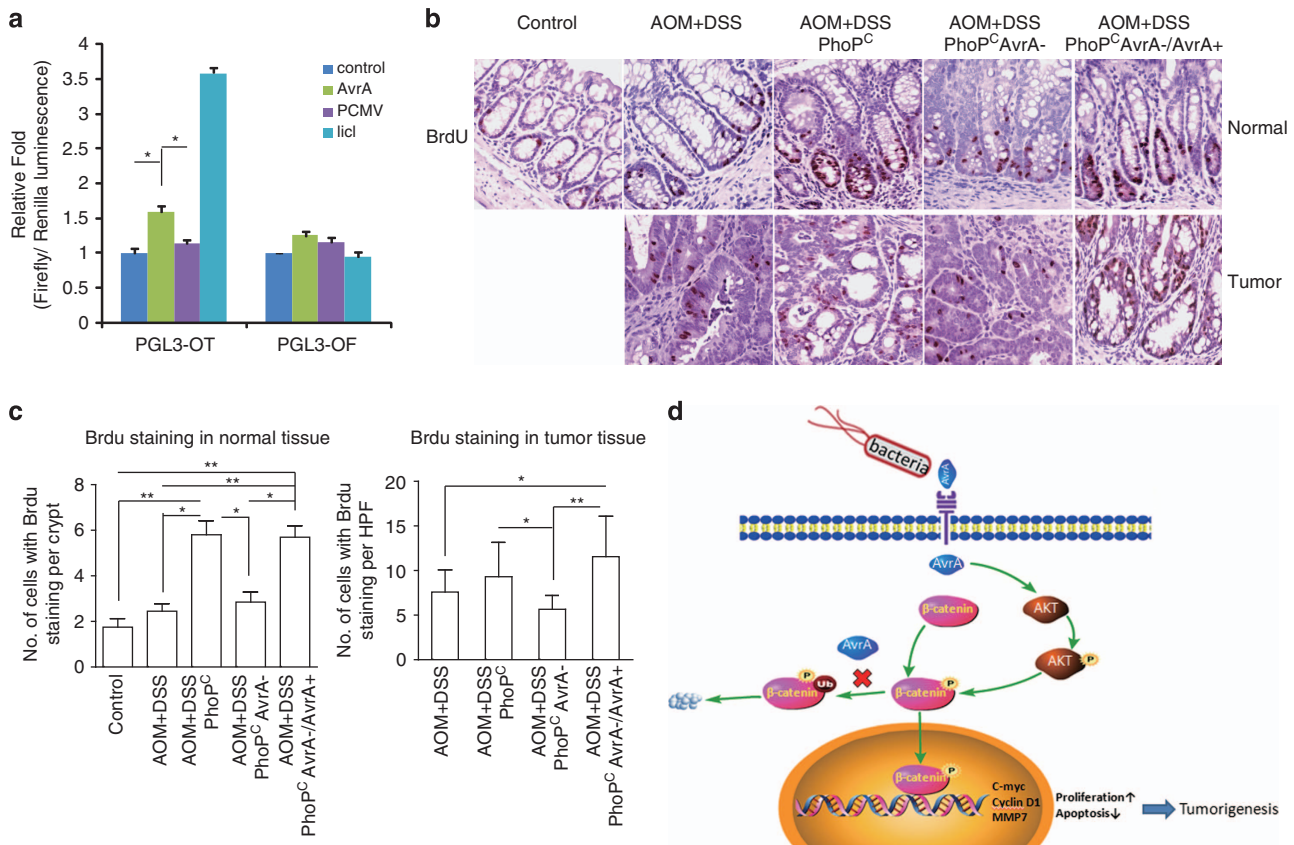
#### AvrA activates $\beta$ -catenin signaling pathways in the *Salmonella*-infected cells *in vitro*

To further investigate the role of bacterial AvrA in modulating the host signaling pathways *in vitro*, we transfected human HEK293 cells with AvrA and TCF luciferase reporter plasmid. We found that AvrA could increase the  $\beta$ -catenin activity as assessed by increased luciferase activity in cells transfected with a PGL3-OT plasmid with WT TCF-binding site (Figure 5a). In contrast, AvrA did not increase activation of mutated PGL3-OF that is unresponsive to  $\beta$ -catenin–TCF transactivation.

$\beta$ -Catenin signals drive proliferation that is mediated by several target genes, including *c-myc* and *cyclin D1*.<sup>30–32</sup> AvrA upregulated colonic Myc and cyclin D1 expression in *Salmonella*-colonized mice (Figure 3e). In the 'normal' tissue close to tumors, we also found proliferation marker BrdU (bromodeoxyuridine) incorporation was increased in control colon and tumors from mice infected with AvrA-expressing bacteria (Figure 5b). Taken together, our data indicate that AvrA activates the  $\beta$ -catenin signals *in vitro* and *in vivo* in *Salmonella*-infected murine colonocytes and tumor cells.

#### DISCUSSION

In the current study, we investigated the effects of chronic *Salmonella* infection on tumor development in the AOM/DSS model. We used a genetic approach to dissect the effects of AvrA on tumorigenesis, comparing *Salmonella* sufficient in AvrA to microbes deficient in AvrA or AvrA-deficient microbes stably transfected to express AvrA. We showed for the first time that AvrA enhanced proliferation and promoted colonic tumorigenesis and tumor progression and concomitantly activated proto-oncogene  $\beta$ -catenin and its down-stream targets c-Myc and cyclin D1. In part, upregulated  $\beta$ -catenin signals may be mediated by



**Figure 5.** Bacterial AvrA activates  $\beta$ -catenin signaling. **(a)** AvrA induces  $\beta$ -catenin signaling in HEK293 cells. HEK293 cells were transiently co-transfected with a pcmv-myc-AvrA plasmid and a pGL3-OT plasmid (TCF-responsive reporter with WT TCF binding site) or pGL3-OF (mutant TCF binding site), using lipofectin reagent according to the manufacturer's instructions. LiCl treatment (20 mmol/l) was used as the positive control. Each experiment was assayed in triplicate.  $N=3$  independent platings  $*P<0.05$ . **(b)** Colonic BrdU was detected in normal mucosa and tumors in control mice (AOM/DSS alone) or mice also gavaged with the indicated bacteria 45 weeks postinfection. **(c)** Quantitation of BrdU staining in normal tissue and tumors from the indicated groups.  $*P<0.05$ ,  $**P<0.01$ . **(d)** Model of AvrA regulation of  $\beta$ -catenin signaling in colonic tumorigenesis. In mice chronically infected with *Salmonella*, AvrA activates  $\beta$ -catenin, which upregulates its target genes to promote colonic tumorigenesis.

increased phosphorylation of Ser552 and decreased  $\beta$ -catenin ubiquitination. AvrA expression also increased the abundance of *Salmonella* organisms in tumors, confirming a direct effect of AvrA on *Salmonella* invasiveness (data in Figure 2e, summarized in Figure 5d). This study provides novel and potentially important insights into bacterial mechanisms promoting colonic tumorigenesis and tumor promotion. Specifically, our studies identify for the first time a novel tumor-promoting role for AvrA in promoting tumorigenesis.

WT mice rarely develop spontaneous colon cancers but require a carcinogen such as AOM to induce tumorigenesis. As C57B/6 mice are relatively resistant to AOM alone, we used the AOM/DSS model to promote robust colonic tumorigenesis. Because the AOM/DSS model is driven by  $\beta$ -catenin signals and AvrA was shown to activate the  $\beta$ -catenin pathway in colonocytes, we speculated that AvrA might enhance tumorigenesis. Infections with *Salmonella* expressing AvrA, however, were insufficient to induce colonic tumors in the absence of AOM or AOM/DSS. When combined with AOM and DSS, however, AvrA enhanced tumor initiation and progression, suggesting AvrA possesses co-carcinogen activity.

#### *Salmonella* AvrA altered tumor distribution

The PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup> *Salmonella* strain is known to express high levels of AvrA.<sup>25</sup> Supporting the oncogenic role of AvrA, the PhoP<sup>C</sup> AvrA<sup>-</sup>/AvrA<sup>+</sup> group had the highest tumor incidence and

cancer burden. In contrast, chronic infection with AvrA-negative *Salmonella* was not sufficient to increase tumor incidence. These data indicate that bacterial protein AvrA promotes oncogenesis in this model.

In addition to AvrA-enhanced tumor multiplicity and tumor progression, another novel finding in the current study was that this pathogenicity factor altered tumor distribution. In agreement with previous studies,<sup>33,34</sup> uninfected mice treated with AOM/DSS developed tumors in the distal colon (Figure 2c, AOM/DSS). In contrast, in mice infected with AvrA-expressing bacteria, tumors were found more in the proximal colon. We conclude that AvrA alters the colonic milieu so as to enhance tumorigenesis in the right colon. Compared with the left colon, the cecum has a greater bacterial load and increased bacterial fermentation that we speculate contributes to this rightward shift in tumors (Figure 1e). Increasing incidence in right-sided tumors has also been reported in the Western world. While increased endoscopic screening that probably clears distal colonic lesions more effectively than proximal colonic lesions, based on our studies, this shift might also reflect changes in the microbiome.

AvrA-expressing *Salmonella* strains enhance  $\beta$ -catenin signaling and upregulate BMI1 stem cell marker.

A growing body of literature suggests that chronic inflammation leads to tissue damage, stem cell expansion and a predisposition to transforming mutations.<sup>35</sup> In this regard, colonic stem cells are

known to have a critical role in the pathogenesis of colon cancer.<sup>36</sup> We found that Bmi1, a marker of stem cells, was increased in an AvrA-dependent manner. AvrA also increased levels of phosphorylated Ser552  $\beta$ -catenin and colonic crypt cell proliferation.  $\beta$ -Catenin is involved in inflammation-induced stem progenitor cell expansion.<sup>24,25</sup> In previous publications, we demonstrated that AvrA inhibited  $\beta$ -catenin degradation and increased  $\beta$ -catenin transcriptional activity through the Wnt pathway.<sup>15,16,21</sup> As  $\beta$ -catenin activation is a critical step in stem cell proliferation and colonic tumorigenesis, our current findings regarding AvrA induction of  $\beta$ -catenin activation place our previous observations in a pathophysiological context relevant to cancer and highlights the potential synergistic role of aberrant Wnt signaling (driven by APC or  $\beta$ -catenin mutations) and AvrA to increase  $\beta$ -catenin activation. Thus AvrA upregulation of  $\beta$ -catenin Ser552 phosphorylation provides new insights into the mechanism by which AvrA can expand stem cells by activating the  $\beta$ -catenin pathway *in vivo*.

#### AvrA modulates $\beta$ -catenin ubiquitination

Ubiquitination is a key posttranslational protein modification that controls cellular protein levels. In our previous study, we found that AvrA can regulate colonic epithelial cell inflammation by deubiquitination.<sup>15</sup> Here we found that AvrA deubiquitinates  $\beta$ -catenin and thus blocks degradation of this key oncogenic Wnt effector protein during colonic tumorigenesis. We also investigated which ubiquitin lysine residues are controlled by AvrA. We demonstrated that AvrA stabilized  $\beta$ -catenin at the ubiquitination level by blocking lysine 48 ubiquitination. Without degradation,  $\beta$ -catenin can accumulate in the cytoplasm. And in the presence of activated AKT, phospho-active  $\beta$ -catenin translocates into the nucleus. Nuclear  $\beta$ -catenin heterodimerizes with TCF to stimulate transcription of target genes, including c-Myc and cyclin D1 (Figure 5d).

In summary, we have uncovered novel effector functions of *Salmonella*-associated AvrA that activates  $\beta$ -catenin pathways to promote tumorigenesis. *Salmonella* expressing AvrA significantly increased tumor multiplicity and tumor progression and shifted tumor distribution more proximally. This study increases our understanding of the role of bacteria in colon cancer. Our observations of *Salmonella* effects on tumorigenesis also raise a note of caution regarding the use of mutant *Salmonella* organisms as vectors for anti-cancer therapy. Further studies are needed to confirm whether AvrA is expressed in these strains and the safety of such approaches. Finally, our results bring us one step closer to unraveling infection-related dysregulation of  $\beta$ -catenin signaling in cancer. These studies, moreover, could suggest biomarkers (such as AvrA level in gut) to assess colon cancer risk in susceptible individuals.

## MATERIALS AND METHODS

### Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the University of Rochester University Committee on Animal Resources (UCAR) committee (UCAR 2007-065) and Rush University Institutional Animal Care and Use Committee (IACUC Number 12-018). All efforts were made to minimize suffering.

### Bacterial strains and growth condition

*Salmonella typhimurium* strains used in this study included *Salmonella* mutant strains PhoP<sup>C</sup>,<sup>22</sup> PhoP<sup>C</sup>AvrA<sup>-</sup>, and PhoP<sup>C</sup>AvrA<sup>-</sup>/AvrA<sup>+</sup>.<sup>14</sup> Non-agitated microaerophilic bacterial cultures were prepared by inoculating 10 ml of Luria-Bertani broth with 0.01 ml of a stationary-phase culture followed by overnight incubation (~18 h) at 37 °C, as previously described.<sup>14</sup>

### *Salmonella*-infected colon cancer mouse model

Animal experiments were performed using specific pathogen-free female C57BL/6 mice (Taconic, Albany, NY, USA) that were 6–7-weeks old as previously described.<sup>20</sup> Water and food were withdrawn 4 h before oral gavage with 7.5 mg/mouse of streptomycin (100  $\mu$ l of sterile solution). Afterwards, animals were supplied with water and food *ad libitum*. Twenty hours after streptomycin treatment, water and food were withdrawn again for 4 h before the mice were infected with  $1 \times 10^6$  colony-forming units of *Salmonella* (100- $\mu$ l suspension in HBSS (Hank's Balanced Salt Solution)) or treated with sterile HBSS (control) by oral gavage, as previously described.<sup>37</sup> After *Salmonella* gavage, the mice were treated with 10 mg/kg of AOM (Sigma-Aldrich, Milwaukee, WI, USA) by intraperitoneal injection as described by Greten and Karin.<sup>38</sup> After a 7-day recovery period, the animals received 1% DSS *ad libitum* (see schematic in Figure 1a). The initial sample size was  $n = 40$  mice in the control group with no treatment and  $n = 50$  in each experimental group. Sixteen weeks later, mice received second AOM injection and another three cycles of 1% DSS in the drinking water. Tissue samples were collected at 1, 15, 25, 35 and 45 weeks after *Salmonella* gavage. Tumor counts and measurements were performed in a blinded fashion under a stereo-dissecting microscope (Nikon SMZ1000, Melville, NY, USA). Microscopic analysis was performed for severity of inflammation and dysplasia<sup>39</sup> on hematoxylin and eosin-stained 'Swiss rolled' colons by a gastrointestinal pathologist blinded to treatment conditions.

### Detection of *Salmonella* in intestine

Mouse feces (about 100 mg) were collected, vortexed in phosphate-buffered saline (PBS), and centrifuged for 10 min at 800 r.p.m. The sample was transferred to a clean microfuge tube and centrifuged again at 6000 r.p.m. for 5 min. The supernatant was discarded, and 200  $\mu$ l PBS was added to the pellet, which was then vortexed. Colonic *Salmonella* was detected by culturing fecal content at 37 °C overnight on a BBL CHROMagar plate (BD Biosciences, San Jose, CA, USA). *Salmonella* species appeared mauve (rose to purple).

### Microbial analysis

After euthanasia, 1-ml samples from the cecal contents of colonized mice were serially diluted in PBS. Total bacterial concentrations were determined using counting chambers. Colonic *Salmonella* distribution was determined by immunostaining colon sections using anti-*Salmonella* lipopolysaccharide antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).<sup>16,18,25</sup> The presence of *Salmonella* existence was also confirmed by real-time PCR measurements of *Salmonella* 16S rRNA. DNA was extracted from colonic tissues after washing to remove residual feces. Primers specific to 18S rRNA from intestinal cells in fecal samples were used as an endogenous control to normalize loading between samples. For the quantification of *Salmonella*, a pair of primers specific for *Salmonella* species of 16S rRNA were used to amplify this bacterial gene: 16S rRNA Fw 5'-TATAGCCCCATCGTGTAGTCAGAAC-3', Rv 5'-TGCGGCTGGATCACCTC CTT-3'; and 18S rRNA Fw 5'-AGGGGAGAGCGGTAAGAGA-3', Rv 5'-GGAC AGGACTAGCGGAACA-3'. The relative amount of 16S rRNA in each sample was estimated using the  $\Delta\Delta C_T$  method.

### *Salmonella* burden in the intestine, gallbladder, spleen and liver

Intestine, gallbladder, spleen and liver were harvested from each mouse, placed into 14-ml tubes with 5 ml sterile PBS, minced with scissors and homogenized using a Polytron PT2100 (Luzern, Switzerland). Tissue homogenates were diluted 1000x to 10 000x with LB, plated (100  $\mu$ l) on MacConkey agar plates, and incubated overnight at 37 °C. Colony-forming units were quantified.

### Histological testing

Tissues were fixed in 10% neutral buffered formaldehyde for 4–12 h, transferred into 70% ethanol and processed by standard techniques. Sections (5  $\mu$ m) were stained with hematoxylin and eosin. For immunostaining, antigens were retrieved by 10-min boiling in 10 mM citrate (pH 6.0). Blinded histological inflammatory scores were performed by a validated scoring system.<sup>40</sup> Histological assessment of intestinal tumors in the rodent was performed using the recommended standards.<sup>41</sup> Immunostaining, including BrdU staining, was performed as previously described.<sup>15,42</sup> The slides were stained with anti-BrdU, anti-Bmi1, anti-phospho-c-Myc, anti-cyclin D1, anti-MMP-7 (Abcam, Cambridge, MA, USA), anti-phospho- $\beta$ -



catenin (Ser552), anti- $\beta$ -catenin (BD, Transduction Laboratories, Lexington, KY, USA).

### Immunoblotting

Mouse colonic mucosa was collected by scraping proximal and distal regions as previously described.<sup>20</sup> Tumors were collected separately. Mouse epithelial cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM sodium orthovanadate, protease inhibitor cocktail). Immunoblotting was performed with primary antibodies: anti-AvrA (custom-made in Sub lab<sup>13</sup>), anti- $\beta$ -catenin (BD, Transduction Laboratories), anti-Bmi 1 (Abcam), and anti-villin (Santa Cruz Biotechnology Inc.), anti-GSK-3 $\beta$  (BD), anti-phospho-GSK-3 $\beta$  (Ser9), anti-phospho- $\beta$ -catenin (Ser552), anti-phospho- $\beta$ -catenin (Ser33, Ser37, Thr41) and anti-phospho-Akt (T308) (Cell Signaling, Beverly, MA, USA), or anti- $\beta$ -actin (Sigma-Aldrich) antibodies, and visualized by enhanced chemiluminescence.<sup>43,44</sup>

### Immunofluorescence

Colonic tissue and colonic tumors were freshly isolated fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections (5  $\mu$ m) of mouse colons. After tissue preparation, as described previously,<sup>15</sup> sections were stained with anti-*Salmonella* lipopolysaccharide antibody (Santa Cruz Biotechnology).<sup>16,18,25</sup> Specimens were examined with a Leica SP5 Laser Scanning confocal microscope (Buffalo Grove, IL, USA).

### Transfection and luciferase assay for $\beta$ -catenin transcriptional activity

The transcriptional activity of  $\beta$ -catenin was measured using the TCF Reporter Assay.<sup>14</sup> Briefly, HEK293 cell line was co-transfected with a WT TCF/LEF-responsive firefly luciferase reporter (pGL3-OT) or a mutant firefly luciferase reporter (pGL3-OF) as a negative control, as well as specific pCMV-AvrA or pCMV. LiCl treatment (20 mM for 24 h after transfection) was used as the positive control. PRL-TK vector was used to control for transfection efficiency. Luciferase activity was monitored using the dual luciferase assay system (Promega, Madison, WI, USA). Experiments were done in triplicate with three independent platings.

### AvrA and ubiquitin assay *in vitro*

HCT116 cells were transiently cotransfected with AvrA and the indicated ubiquitin plasmids, including pRK5-HA-Ub WT, -Ub K48, -UbK63 (K48/63/33 only, other lysines mutated to arginines) and -Ub KO (all lysines mutated to arginines). Plasmids were purchased from Addgene (Cambridge, MA, USA). After 24 h, cells were treated with MG262 for 3 h. Total cell lysates were analyzed by immunoblotting.<sup>14,15</sup>

### Coimmunoprecipitation assay

Animal experiments were performed using 6–7-weeks-old specific pathogen-free female C57BL/6 mice (Taconic). Mice were infected with the indicated *Salmonella* strains by oral gavage. At 6 weeks after *Salmonella* infection, colonic mucosal scrapings were rinsed twice in ice-cold HBSS and lysed in cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM sodium orthovanadate and protease inhibitor cocktail (Boehringer, Mannheim, Germany)). Samples were immunoprecipitated with anti- $\beta$ -catenin antibody. Membranes were probed with anti- $\beta$ -catenin and anti-ubiquitin antibody and visualized using enhanced chemiluminescence. Protein input was normalized by villin, a colonic epithelial cell marker.

### Statistical analysis

Data were expressed as mean  $\pm$  s.d. All statistical tests were two-sided. *P*-values < 0.05 were considered to be statistically significant. For cecum data, four areas of mouse body were checked to obtain the average infection score. Then, multiple comparisons of mean infection scores were performed using analysis of variance. Following analysis of overall group differences on the mean scores at *P* < 0.05,  $\alpha$  levels were adjusted using the Tukey's correction for multiple comparisons. As the study involved a longitudinal design, the generalized estimating equations were utilized to provide statistical inference.<sup>45</sup> The analysis was conducted using the Genmod procedure in SAS. The overall treatment effects were modeled using the time by treat-

ment interaction term through intake ( week 0 ) to week 45.<sup>18</sup> Statistical analyses were performed using the SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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