



Universiteit  
Leiden  
The Netherlands

## **Manipulating endosomal systems: the molecular mechanisms of transport decisions and Salmonella-induced cancer**

Bakker, J.M.

### **Citation**

Bakker, J. M. (2019, December 12). *Manipulating endosomal systems: the molecular mechanisms of transport decisions and Salmonella-induced cancer*. Retrieved from <https://hdl.handle.net/1887/82070>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/82070>

**Note:** To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/82070> holds various files of this Leiden University dissertation.

**Author:** Bakker, J.M.

**Title:** Manipulating endosomal systems: the molecular mechanisms of transport decisions and Salmonella-induced cancer

**Issue Date:** 2019-12-12

## Chapter 5

### *Salmonella* Manipulation of Host Signaling Pathways Provokes Cellular Transformation Associated with Gallbladder Carcinoma

Tiziana Scanu<sup>1\*</sup>, Robbert M. Spaapen<sup>1\*</sup>, Jeroen M Bakker<sup>1\*</sup>, Chandra Bhan Pratap<sup>2\*</sup>, Lin-en Wu<sup>1</sup>, Ingrid Hofland<sup>3</sup>, Annegien Brooks<sup>3</sup>, Vijay Kumar Shukla<sup>4</sup>, Mohan Kumar<sup>5</sup>, Hans Janssen<sup>1</sup>, Ji-Ying Song<sup>6</sup>, E. Andra Neefjes-Borst<sup>7</sup>, Hein te Riele<sup>8</sup>, David W. Holden<sup>9</sup>, Gopal Nath<sup>2</sup> and Jacques Neefjes<sup>1</sup>

1Division of Cell Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, the Netherlands

2Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India

3Core Facility Molecular Pathology and Biobanking, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, the Netherlands

4Department of General Surgery

5Department of Pathology

Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh 221005, India

6Division of Experimental Animal Pathology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, the Netherlands

7Department of Pathology, Free University Medical Centre, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands

8Division of Biological Stress Response, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, the Netherlands

9Center for Molecular Bacteriology and Infection, Imperial College London, London SW72AZ, UK

\*These authors contributed equally to this work

## Summary

Cancer is fueled by deregulation of signaling pathways in control of cellular growth and proliferation. These pathways are also targeted by infectious pathogens en route to establishing infection. Gallbladder carcinoma (GBC) is frequent in the Indian subcontinent, with chronic *Salmonella enterica* serovar Typhi infection reported as a significant risk factor. However, direct association and causal mechanisms between *Salmonella* Typhi infection and GBC have not been established. Deconstructing the epidemiological association between GBC and *Salmonella* Typhi infection, we show that *Salmonella enterica* induces malignant transformation in predisposed mice, murine gallbladder organoids, and fibroblasts, with TP53 mutations and c-MYC amplification. Mechanistically, activation of MAPK and AKT pathways, mediated by *Salmonella enterica* effectors secreted during infection, is critical to both ignite and sustain transformation, consistent with observations in GBC patients from India. Collectively, our findings indicate that *Salmonella enterica* can promote transformation of genetically predisposed cells and is a causative agent of GBC.

## Introduction

Cancer is a major cause of death worldwide. Strikingly, some cancer types have a unique global distribution such as gallbladder carcinoma (GBC), which is frequent in India and Pakistan, but rare in the Western world (*Hundal and Shaffer, 2014*). In India, GBC represents the third most common gastrointestinal tumor with reported incidence rates of 22/100,000 persons (*Crump et al., 2004; Nath et al., 2008; Randi et al., 2006*). The prognosis of GBC is poor, and definition of causal factors underlying its unique global distribution may provide rationale for combatting this tumor (*Hundal and Shaffer, 2014; Lazcano-Ponce et al., 2001*).

Various factors are proposed to explain the peculiar global distribution of GBC cases. Especially chronic *Salmonella enterica* serovar Typhi (*S. Typhi*) infection, but also gallstones and chronic gallbladder inflammation (cholelithiasis) are reported risk factors for GBC (*Wistuba and Gazdar, 2004*). In the absence of causal molecular explanations such associations are insufficient for implementation in cancer prevention programs.

*S. Typhi* is endemic in India causing typhoid fever. A small percentage of the infected patients become chronic carriers when *S. Typhi* enters the gallbladder, where it propagates to shed new bacteria into the intestinal tract to spread infection (*Ruby et al., 2012*). Chronic typhoid carriers have a 9-fold greater risk for developing GBC (*Shukla et al., 2000*). Because molecular understanding of the mechanism(s) underlying this correlation is lacking, a contribution of chronic *S. Typhi* infection to cancer formation is not generally accepted (*Nagaraja and Eslick, 2014*).

While viruses are among the established causal factors for particular cancers (*Chang, 2014; Coghill and Hildesheim, 2014; Doorbar et al., 2012*), bacteria are largely ignored

as direct contributors for they fail to integrate their DNA into the host genome and do not leave a genetic imprint in the resulting tumors. Nevertheless, contribution of bacterial infection to cancer development has been suggested based on various epidemiological studies (*Mager, 2006; Samaras et al., 2010*). Most accepted is the link between *Helicobacter pylori* and gastric cancer (*Polk and Peek, 2010*). *Mycobacterium tuberculosis* has also been associated with lung cancer (*Kuo et al., 2013*), while *Chlamydia trachomatis* (*Arnheim Dahlström et al., 2011; Koskela et al., 2000; Shanmughapriya et al., 2012*) and *Chlamydia pneumonia* have been linked to cervical and lung cancer, respectively (*Chaturvedi et al., 2010; Zhan et al., 2011*).

How bacteria may contribute to cancer is poorly understood, and multiple mechanisms have been proposed. Sustained inflammation may contribute, for example, by continuous activation of MYD88 (*Elinav et al., 2013*). On the other side the toxin CagA derived from *H. pylori* activates signaling through the Met receptor, but it remains unclear whether this suffices to induce gastric carcinoma (*Hatakeyama, 2014; McCracken et al., 2014*). Since only a fraction of all individuals infected with *H. pylori* develop gastric cancer, additional factors are likely to influence cancer formation. Bacteria can also manipulate the host epigenome. For example, *Listeria*, *Shigella*, *Mycobacteria*, and *Chlamydia* may modify chromatin structure and histone modifications (*Schmeck et al., 2008*) of host cells and then alter the host transcriptome (*Chumduri et al., 2013; Hamon and Cossart, 2008*) with unknown consequences to tumorigenesis.

*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) secretes more than 40 different effector proteins into host cells through its type III secretion system (TTSS) in support of its infection cycle (*McGhie et al., 2009; Thomas et al., 2012*). These proteins are either directly endowed with enzymatic activities or function as critical modulators of host enzymes, all of which may in principle contribute to transformation. Although epidemiology suggests that infection by bacteria cannot be a single cause for tumor formation, it is possible that bacteria can clear one key hurdle in the multistep process toward transformation.

Here we show that *S. Typhimurium* bacteria can directly induce cellular transformation in gallbladder organoids and mouse embryonal fibroblasts (MEFs) adding to specific pretransforming mutations in the host. These predisposing mutations required for *S. Typhimurium* -induced transformation are also observed in GBC samples from India, along with the DNA of *S. Typhi*. We conclude that cellular transformation mediated by *Salmonella enterica* occurs through activation of host pathways that promote bacterial uptake and intracellular survival. Transformation of cells is then the result of collateral damage induced by *Salmonella enterica* during its infection cycle. Because other bacteria are also known to activate these host pathways in their infection cycle, our observations may have broader implications.

## Results

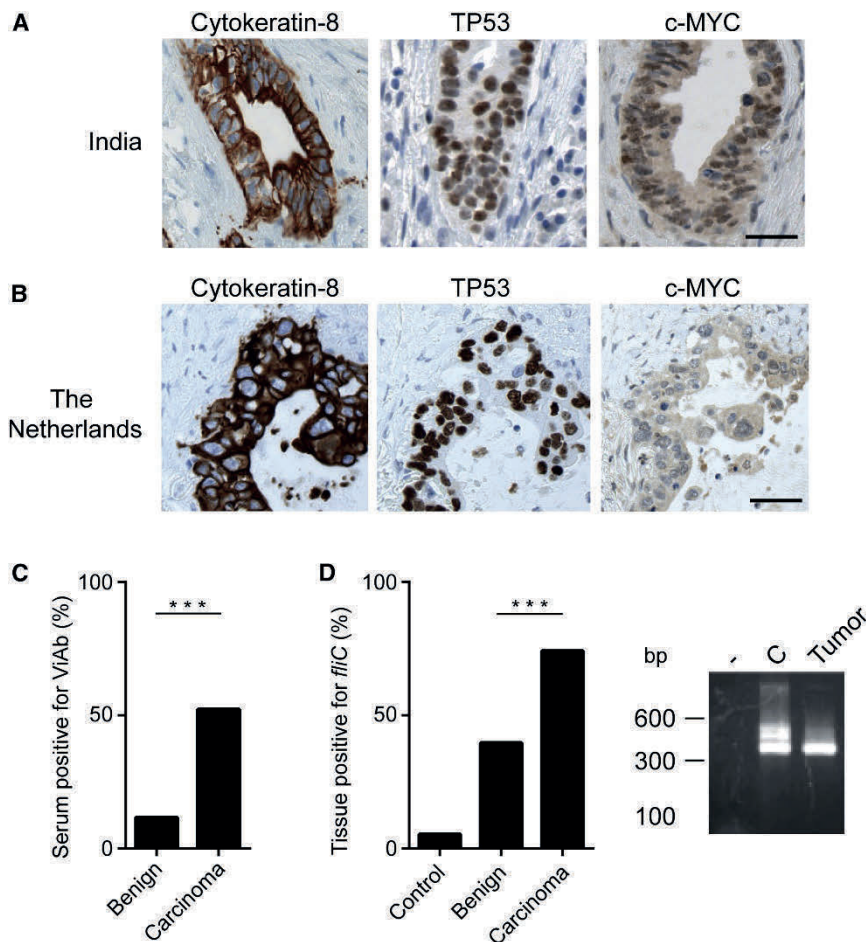
### Salmonella Typhi and GBC: India versus the Netherlands

GBC is rare in the Western world, unlike in India and Pakistan (*Hundal and Shaffer, 2014*). To assess whether GBC samples collected in India or the Netherlands are different in nature, we examined tissue sections for a series of markers by immunohistochemistry (IHC) (Figures 1A and 1B). Carcinomas from both groups stained positive for Cytokeratin-8 (a secretory epithelial differentiation marker) and TP53 (shown by a strong nuclear signal as mutated inactive TP53 aggregates), confirming earlier observations (*Wistuba and Gazdar, 2004*). By contrast, a marked geographical difference emerged for c-MYC, which presented strongly nuclear staining in 9/10 Indian samples, while only 1/6 patient samples from the Netherlands contained a small number of positive cells. The other five Dutch patients did not show c-MYC staining (Figures 1A and 1B; Table S1), suggesting that GBC tumors from India and the Netherlands are different in nature.

The high incidence of GBC reported in India correlates with higher prevalence of chronic *S. Typhi* carriers that can be defined by antibodies against *S. Typhi* (ViAb) in patient serum (*Nath et al., 2008*). Analysis of sera from 23 GBC and 60 control individuals (with benign gallbladder diseases) showed a clear correlation between serum antibodies detecting a history of chronic *S. Typhi* infection and GBC (Figure 1C), with 53% of GBC positive for ViAb, versus 11.6% of benign gallbladder samples. However, not all chronic patients may develop such antibodies. We therefore scored collected GBC samples for *S. Typhi* DNA as an indication of *S. Typhi* infection (*Nath et al., 2008; Pratap et al., 2013*). In tissues collected from Indian patients, DNA for *S. Typhi* *fliC* and *staA* (*Pratap et al., 2013*) was detected in ~74% of GBCs, as compared to ~40% of tissues from patients with benign gallbladder disease (including cholelithiasis) and ~6% of gallbladder tissues from healthy controls with no diagnosed gallbladder disease (control samples) (Figure 1D; Table S1; Figure S1). Importantly, DNA for *S. Typhi* was not detected in any of the GBC samples collected in the Netherlands (Table S1). On this basis, GBC samples from India differed markedly from those from the Netherlands.

### Salmonella enterica Infection in *Apc*<sup>+/*min*</sup> Mice Induces Colon Tumors

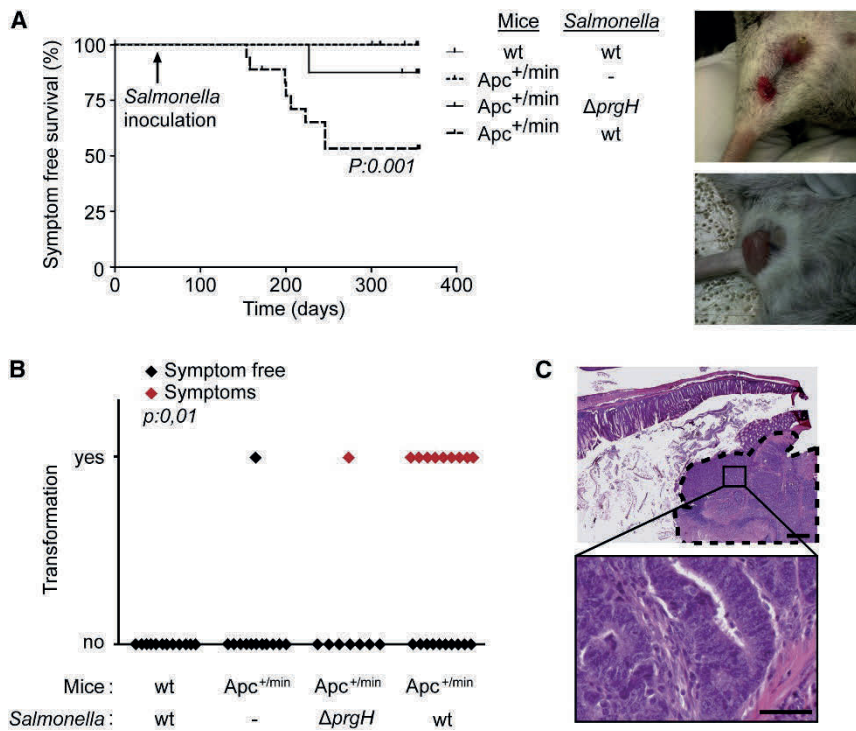
Because only a fraction of *S. Typhi*-infected persons in India develop GBC, *S. Typhi* cannot be a single causal factor of tumorigenesis. *S. Typhi* may, however, contribute one step in the multistep process leading up to cancer. To test whether pretransforming mutations in the host (as defined above) can cooperate with *Salmonella enterica* bacteria in cancer formation, we used mice heterozygous for *Apc* (*Apc*<sup>+/*min*</sup>) that induces c-Myc overexpression (*de Wind et al., 1998*) to mimic the corresponding phenotype of GBC samples from India. The *Apc*<sup>+/*min*</sup> mice were infected with *S. Typhimurium* (hereafter referred to as *Salmonella*) that causes typhoid-like disease in mice (*Santos et al., 2001*). To assess any contribution of a pre-transformed state to cancer formation, we infected either control or *Apc*<sup>+/*min*</sup> mice with *Salmonella*. As a further control, we infected *Apc*<sup>+/*min*</sup> and control mice with the *Salmonella* mutant



**Figure 1. Salmonella Typhi Infection and GBC: India versus the Netherlands**

(A) Representative images of a human GBC sample from India. IHC for detections of Cytokeratin-8 (left), TP53 (center), and c-MYC (right). The sections are counterstained by hematoxylin for detection of nuclei (in blue). Scale bar, 25 mm. (B) IHC of a representative GBC sample from the Netherlands stained for Cytokeratin-8 (left), TP53 (center), and c-MYC (right). The sections are counterstained by hematoxylin for detection of nuclei (in blue). Scale bar, 25 mm. (C) Detection of serum antibodies against *S. Typhi* (ViAb) in patients affected by benign gallbladder disease (benign, 11.6%;  $n = 60$ ) or GBC (carcinoma, 52.2%;  $n = 23$ ). Statistical significance determined by  $\chi^2$  test.  $***p < 0.001$ . (D) Percentage of gallbladder tissue containing *fliC* DNA of *S. Typhi* collected in India from control population (control, 5.33%;  $n = 150$ ), patients with benign gallbladder diseases (benign, 39.6%;  $n = 187$ ), or GBC (carcinoma, 74.1%;  $n = 54$ ). Right panel, PCR amplification of *S. Typhi* *fliC* DNA in GBC sample (indicated as "Tumor") compared with control DNA from *S. Typhi* reference strain (indicated as "C"). Statistical significance determined by  $\chi^2$  test.  $***p < 0.001$ . See also Figure S1 and Table S1.

$\Delta prgH$ , which is unable to inject effector proteins into the host cytosol due to lack of a functional TTSS (Behlau and Miller, 1993). Of note, the  $\Delta prgH$  mutant Salmonella can shed LPS and when eliminated expose factors including lipoteichoic acid and bacterial

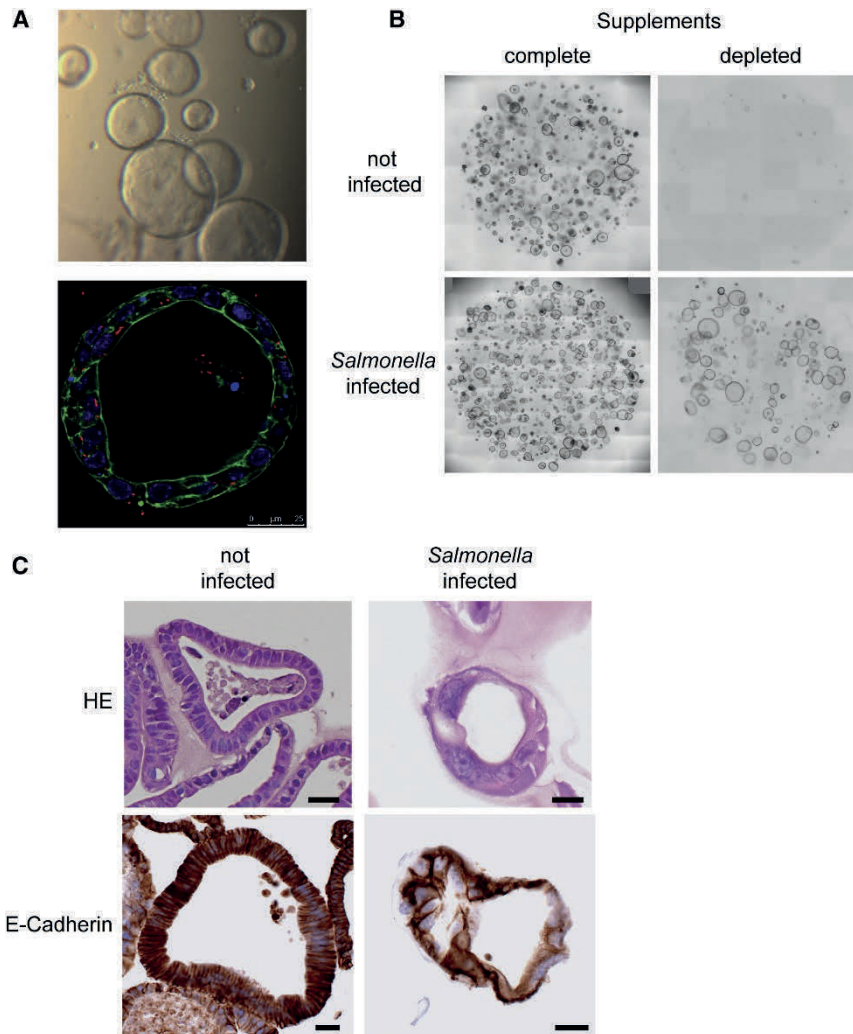


**Figure 2. Salmonella Infection Drives Tumor Development in Predisposed Mice**

(A) Kaplan-Meier curve of symptom-free survival of Apc<sup>+/min</sup> and WT mice infected or not (-) with WT or PrgH deletion mutant (ΔprgH) *S. Typhimurium*.  $p < 0.001$  as defined by a logrank (Mantel-Cox) test. Right, representative images of bleeding anus (top) and rectal prolapse (bottom). (B) Pathology assessment of colorectal transformation occurring in WT or Apc<sup>+/min</sup> (APC<sup>+/min</sup>) mice infected with WT, ΔprgH *S. Typhimurium*, or not infected (-). Red diamonds, mice with symptoms as indicated in Figure 2A. Black diamonds, mice with no symptoms.  $p = 0.0109$  by Fisher's exact test. (C) Representative case of H&E-stained adenocarcinoma sections from the distal colon of an Apc<sup>+/min</sup> mouse infected with WT *S. Typhimurium*. The tumor is moderately-to-poorly differentiated with invasion of submucosa, muscularis, and serosa of the colon. Scale bars: top panel, 500 mm; bottom panel, 50 mm. See also Figure S2.

DNA to mediate TLR-dependent inflammatory responses (Kaur and Jain, 2012). All mice were pretreated with streptomycin to eliminate the commensal gut flora and allow Salmonella infection (Barthel et al., 2003). The next day, the mice were orally infected with equal numbers of wild-type (WT) or ΔprgH Salmonella and then monitored over time. At 4–7 months post-infection, nearly half of the Apc<sup>+/min</sup> mice infected with WT Salmonella presented with bleeding anus and stool or rectal prolapse (Figure 2A; indicated as symptoms). At the age of 50 weeks, all mice were examined by pathology in a blinded fashion. In addition to the expected development of small intestinal adeno(carcino)mas due to the Apc<sup>+/min</sup> background (Figures S2A–S2C), colorectal adenocarcinomas were observed in symptomatic Apc<sup>+/min</sup> mice infected with WT Salmonella (Figures 2B and 2C). These tumors were observed only once in each of our control conditions (Figures 2A and S2C). These experiments demonstrate that Salmonella can promote carcinogenesis in vivo in the background of pre-





**Figure 3. Salmonella Infection of Gallbladder Organoids Induces Cell Transformation**

(A) Top panel, representative wide-field microscopy image of a gallbladder organoid culture. Bottom panel, confocal images of one representative gallbladder organoid infected with *S. Typhimurium* (in red). Nuclei are stained with DAPI (blue), and actin is shown in green (scale bar, 25 μm). Z stack projections of gallbladder organoids, see Movie S1. (B) Gallbladder organoid cells with a history of *S. Typhimurium* infection (*Salmonella* infected, bottom panels) or not (not infected, top panels) were cultured to reform organoids in complete medium (left panels) or supplements-depleted medium (right panels). Representative images of organoids culture from two independent experiments performed in triplicate are shown. (C) H&E staining (top) and E-cadherin IHC (bottom) of gallbladder organoids grown from cells infected (right panels) or not (left panels) with *S. Typhimurium*. The sections are counterstained by hematoxylin. Scale bar, 20 μm. See also Figure S3 and Movie S1.

transformation by c-Myc oncogene overexpression resulting from loss of one copy of the APC gene. Since *Salmonella* lacking TTSS ( $\Delta prgH$ ) failed to induce colon carcinomas, these experiments suggest that host cell manipulation by *Salmonella* is critical to bacterially mediated transformation.

### **Salmonella enterica Infection of Gallbladder Organoids Induces Cell Transformation**

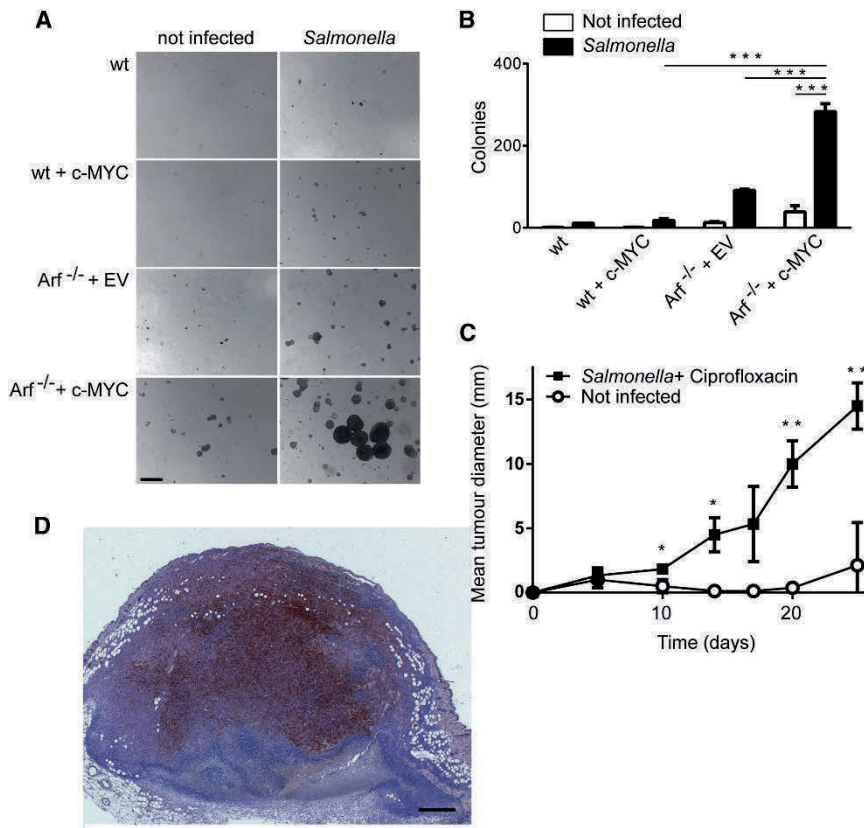
In order to test the potential primary contribution of Salmonella to transformation, we explored a minimal, yet still relevant, model system. We chose to test organoids derived from pretransformed murine gallbladders. Gallbladder organoids were cultured from gallbladders of mice deficient in the *Ink4b-Arf-Ink4a* locus, thereby presenting with inactive TP53 to resemble the analogous situation in GBC patients (*Krimpenfort et al., 2007*) (Figure 3A). These gallbladder organoids were generated under intestinal organoid culture conditions (*Koo et al., 2012; Sato et al., 2009*) lacking Noggin and R-spondin supplements, which in contrast to the intestinal organoids delayed the growth of these gallbladder organoids. The gallbladder organoids were characterized by staining for epithelial markers E-cadherin and Cytokeratin-8 (Figures 3C and S3A), showing an organized epithelial cell layer. Electron microscopy revealed polarized cells with microvilli on the apical surface facing the lumen (Figure S3B), similar to those reported in gallbladder organs (*Frierson, 1989*). Taken together, all tests showed that the organoids exhibit the epithelial layer characteristic of gallbladder organs.

To conduct Salmonella infections in the organoid system, single cells derived from gallbladder organoids were infected with WT or with  $\Delta$ *prgH* mutant Salmonella and subsequently cultured to allow de novo organoid formation (Figures 3A, 3B, and S3D; Movie S1). These new gallbladder organoids were once again dissociated into single cells for culturing under either normal or supplement-depleted growth conditions (*Koo et al., 2012; Sato et al., 2009*). Cells derived from gallbladder organoids with a history of WT Salmonella infection were able to form new organoids even under growth factor-restricted conditions, unlike the cells infected with  $\Delta$ *prgH* mutant Salmonella or never exposed to Salmonella, which required supplements for growth (Figures 3B and S3D).

Acquired growth factor independency is one of the hallmarks of transformation, as are altered cell morphology and polarity. To visualize this, we performed pathology of infected and control gallbladder organoids. H&E and epithelial marker staining revealed that the gallbladder organoids infected with Salmonella lost their cohesion and polarity and presented large irregular nuclei with prominent nucleoli (Figure 3C). These observations illustrate histopathological features of neoplastic transformation confirmed by electron microscopy analysis. Gallbladder organoids never exposed to Salmonella exhibited normal epithelial organization and showed no signs of transformation (Figures 3C, S3A, and S3C). These observations indicate that Salmonella bacteria can drive cellular transformation against a predisposed genetic background, even when immune cells and the resulting inflammatory responses are absent.

### **Salmonella enterica Infection of Primary Pretransformed Fibroblasts Induces Cell Transformation**

To understand the molecular basis of the interplay between Salmonella infection and genetic predisposition in the process of cell transformation, we used MEFs with different degrees of pre-transforming alterations. We selected MEFs lacking Arf



**Figure 4. Salmonella Infection of Primary Pre-transformed Fibroblasts Induces Cell Transformation**

(A) Representative images of the anchorage independent assay of WT (top panels), WT overexpressing c-MYC (WT+cMyc; second panels), Arf-deficient (Arf<sup>-/-</sup>+Empty vector [EV]; third panels), and Arf-deficient overexpressing c-MYC (Arf<sup>-/-</sup>+cMyc; bottom panels) MEFs either infected (right) or not (left) with *S. Typhimurium*. Images are representative of at least three independent experiments with technical triplicates. Scale bar, 200 mm. (B) Number of soft agar colonies/well formed by *S. Typhimurium*-infected (black bars) and not-infected (white bars) MEFs. The genetic status of the MEFs is indicated below the bars. Results are representative of at least three independent experiments with technical triplicates. One-way ANOVA with a post hoc Bonferroni test on selected sets was performed to test for significance of all three factors for transformation. \*\*\**p* < 0.001. (C) Tumor growth in immunodeficient Rag1<sup>-/-</sup>γc<sup>-/-</sup> mice subcutaneously transplanted with *S. Typhimurium*-infected MEFs after ciprofloxacin treatment (Salmonella + ciprofloxacin, *n* = 3 mice) or with not-infected MEFs (Not infected, *n* = 4 mice). Shown are mean tumor size ± SD. Unpaired two-sided Student's *t* test was applied. \**p* < 0.05; \*\**p* < 0.01. (D) GFP IHC (brown) of one representative solid tumor formed by the MEFs (Arf<sup>-/-</sup> overexpressing c-Myc-IRES-GFP with a history of *S. Typhimurium* infection) transplanted in Rag1<sup>-/-</sup> γc<sup>-/-</sup> mice. The sections were counterstained by hematoxylin for detection of the nuclei (in blue). Scale bar, 500 mm. See also Figures S4A–S4C.

(resulting in TP53 inactivation) and overexpressing c-MYC (Arf<sup>-/-</sup> + cMyc) to resemble conditions identified in human GBC from India (Figure 1A). WT MEFs, as well as Arf-deficient MEFs, were used as control. The MEFs harboring different mutations were infected with *Salmonella*, and transformation was assessed by colony formation in anchorage-independent (soft agar) assays. *Salmonella* only induced significant

numbers of colonies in MEFs harboring at least two pre-transforming mutations: Arf deficiency and overexpression of c-MYC (Figures 4A and 4B). Similar results were obtained when MEFs with the same pre-transforming mutations were infected with *S. Typhi*, suggesting that both serovars, *S. Typhi* and *S. Typhimurium*, induce transformation of pretransformed cells (Figure S4A). Since Arf deletion or c-MYC overexpression alone did not induce colony formation over background levels, this also defines the minimal pre-transforming requirements for Salmonella-induced transformation in this system and resembled the mutations observed in GBC patients from India.

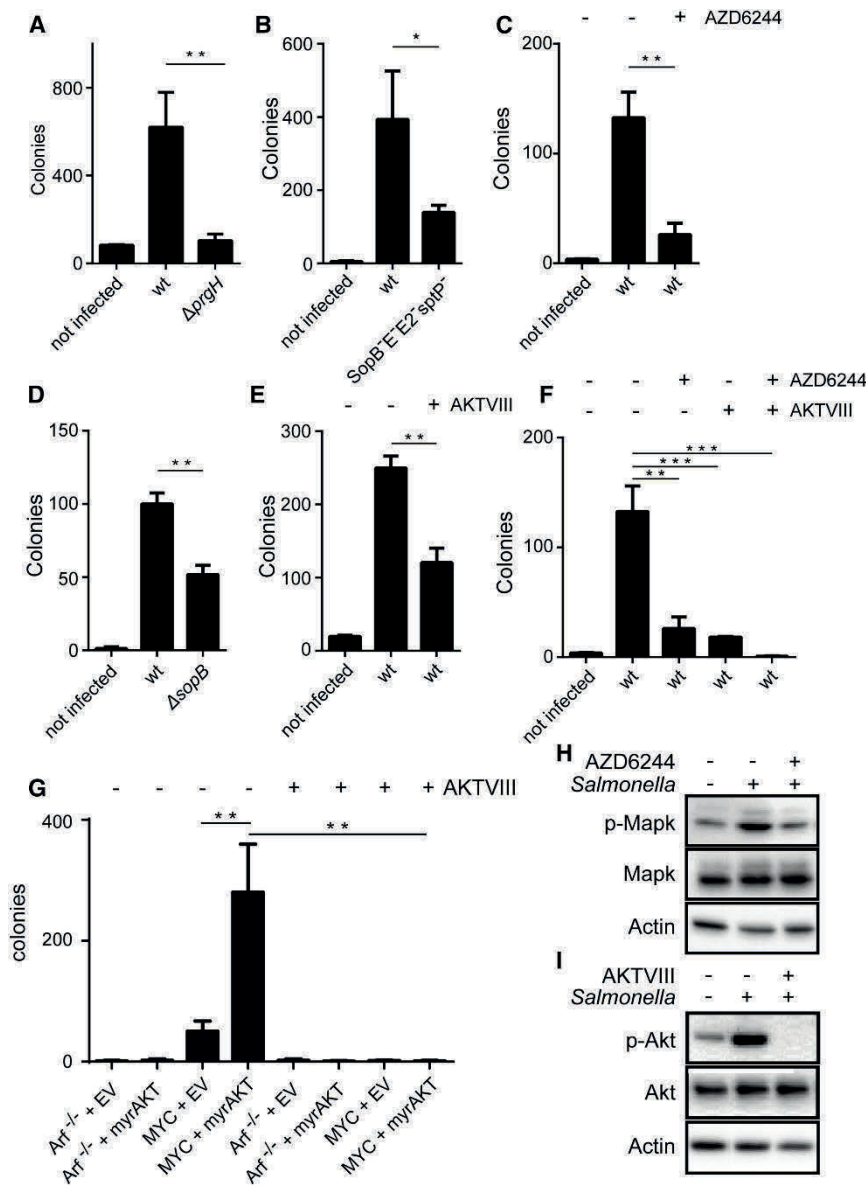
To confirm that the MEFs were transformed following Salmonella infection, we transplanted the cells isolated from soft agar colonies into immune-deficient Rag1<sup>-/-</sup> γc<sup>-/-</sup> mice (Rygaard and Povsen, 2007). First, Salmonella was eliminated from isolated MEFs with the antibiotic ciprofloxacin (Figure S4B), as immune-deficient mice are intolerant to bacterial infection. Subsequently, equal numbers of Arf<sup>-/-</sup> + cMyc MEFs with or without a history of Salmonella infection were implanted into the mice. The growth of solid tumors was followed in time (Figure 4C), revealing that only the MEFs isolated from soft agar colonies induced by Salmonella infection produced tumors in mice, and histopathology analysis showed high mitotic incidence of the tumors with necrotic areas (Figures 4D and S4C).

### **Salmonella enterica-Mediated Activation of Akt and Map Kinase Pathways Drives Transformation**

How then does Salmonella facilitate cellular transformation? When pre-transformed MEFs were exposed to Δ*prgH* Salmonella that lacks a functional TTSS, the MEFs failed in transformation (Figure 5A). This suggests that Salmonella injects effector proteins during the infection cycle to support cell transformation.

Many effector proteins are injected into host cells by Salmonella to invade, replicate, and survive in cells (Haraga *et al.*, 2008). SopB, SopE, and SopE2 are effector proteins that promote Salmonella uptake by host cells through the activation of host Rho GTPases and the MAPKinase pathway (Zhou *et al.*, 2001). Host kinase AKT is activated by the Salmonella effector SopB to inhibit fusion of the Salmonella-containing vacuole (SCV) with lysosomes, promoting intracellular survival of the bacterium (Kuijl *et al.*, 2007).

Since elevated AKT and MAP kinase activities are frequently observed in human cancers (Cseh *et al.*, 2014; Manning and Cantley, 2007), we tested whether Salmonella could employ the same host pathways to initiate transformation. The Arf<sup>-/-</sup> + cMyc MEFs were infected with either WT Salmonella, a mutant lacking four effectors (SopB, SopE, SopE2, and SptP) collectively rendering the bacterium incapable of activating Mapk and Akt (Zhou *et al.*, 2001), or a mutant deficient only in SopB (incapable of activating Akt) (Knodler *et al.*, 2005). Since the different effectors are also involved in bacterial uptake, we first determined the number of bacteria of each strain required to yield equal in vitro infection rates relative to WT Salmonella (Figures S4D–



**Figure 5. Salmonella-Mediated Activation of Akt and Map Kinase Pathways Drives Transformation in Pre-transformed Cells**

(A) Number of soft agar colonies/well formed by Arf<sup>-/-</sup>+cMyc MEFs either or not infected with WT or mutant  $\Delta prgH$  ( $\Delta prgH$ ) *S. Typhimurium*. Results are representative of three independent experiments. Shown is mean  $\pm$  SD of technical triplicate experiment. Statistical significance by Student's *t* test; \*\**p* < 0.01. (B) Number of soft agar colonies/well formed by Arf<sup>-/-</sup>+cMyc MEFs infected with WT and *sopB*  $\Delta sopE\Delta sopE2\Delta sptP\Delta$  *S. Typhimurium*. Results are representative of at least two independent experiments with technical triplicates. Shown are mean number of colonies  $\pm$  SD from duplicate experiments. Statistical significance by Student's *t* test; \**p* < 0.05. (C) Number of soft agar colonies/well from *S. Typhimurium*-infected Arf<sup>-/-</sup>+cMyc MEFs cultured in the presence (+) or absence (-) of MAPK inhibitor AZD6244. Shown are mean number of colonies  $\pm$  SD from triplicate samples. Statistical significance by Student's *t* test, \*\**p* < 0.01. (D) Number of soft agar colonies/well from WT and  $\Delta sopB$  *S. Typhimurium*-infected Arf<sup>-/-</sup>+cMyc MEFs. Shown are mean number of colonies  $\pm$  SD from triplicate experiments. Statistical significance by Student's *t* test; \*\**p* < 0.01. (E) Number of soft agar colonies/well from *S.* (legend continues on next page)

S4F). We then infected *Arf*<sup>-/-</sup> + cMyc MEFs with adequate numbers of bacteria from different *Salmonella* strains to yield equal infection and seeded these in a soft agar assay. Despite equal infection rates, both *Salmonella* mutants were considerably less efficient in inducing colony formation (Figures 5B and 5D).

To directly assess the role of these host Akt and Mapk kinases in transformation, *Arf*<sup>-/-</sup> + cMyc MEFs were infected with WT *Salmonella*, followed by a soft agar colony assay performed in the presence or absence of chemical inhibitors targeting these kinases alone or in combination (Figures 5C, 5E, 5F, 5H, and 5I). Chemical inhibitors were applied at concentrations not affecting growth of MEFs in monolayer conditions (Figure S5A). Soft agar colony formation was markedly reduced by chemical inactivation of either Akt or Map kinase and nearly abrogated by their combined inhibition (Figure 5F). Various other inhibitors targeting Mapk and Akt further confirmed their role in *Salmonella*-mediated transformation of predisposed MEFs (Figures S5B and S5D).

To directly test whether activation of the Mapk or Akt pathway alone suffices to induce transformation in our pre-transformed cell culture model, we selectively activated Mapk (by constitutively active Mek mutant MEKdd) or Akt (by silencing Pten or overexpressing a constitutively active Akt1 form) in *Arf*-deficient MEFs either overexpressing c-MYC or not. Only the *Arf*-deficient cell line overexpressing c-MYC efficiently generated colonies in soft agar following activation of either kinase (Figures 5G, S5C, and S5E).

**(continue from previous page)**

*Typhimurium*-infected (WT) *Arf*<sup>-/-</sup>+cMyc MEFs cultured in the presence (+) or absence (-) of Akt inhibitor AKTVIII. Shown are mean number of colonies ± SD from triplicate samples. Statistical significance by Student's *t* test; \*\**p* < 0.01. (F) Number of soft agar colonies/well from not-infected or *S. Typhimurium*-infected (WT) *Arf*<sup>-/-</sup>+ cMyc MEFs that were cultured in the presence (+) or absence (-) of MAPK inhibitor (AZD6244), Akt inhibitor (AKTVIII), or a combination of these inhibitors. Shown are mean number of colonies ± SD from triplicate samples. Statistical significance by Student's *t* test; \*\**p* < 0.01; \*\*\**p* < 0.001. (G) Number of soft agar colonies/well formed by *Arf*-deficient MEFs overexpressing a control empty vector containing GFP (*Arf*<sup>-/-</sup>) or *Arf*-deficient MEFs overexpressing c-MYC (MYC). These MEFs were either expressing a constitutively active form of Akt1 (*myrAKT1*) or empty control vector (EV). The MEFs were treated (+) or not treated (-) with Akt inhibitor AKTVIII. Results are representative of three independent experiments with technical triplicates. Shown are mean ± SD. Statistical significance by Student's *t* test; \*\**p* < 0.01; \*\*\**p* < 0.001. (H) Western blot detecting phosphorylated Mapk (p-Mapk, top panel), total Mapk (center panel), and Actin (loading control, bottom panel) in *Arf*<sup>-/-</sup>+cMyc MEFs that were either uninfected (*Salmonella* -) and not treated with MAPK inhibitor AZD6244 (left) or infected with *S. Typhimurium* (*Salmonella* +) in absence (-, center) or presence (+) of MAPK inhibitor AZD6244 (right). (I) Western blot for detection of phosphorylated Akt signal in *Arf*<sup>-/-</sup>+cMyc MEFs either uninfected (-) without Akt inhibitor AKTVIII (left) or infected with *S. Typhimurium* (*Salmonella*) in the absence (-, center) or presence of AKTVIII (+, right). See also Figures S4D–S4F and S5.

### **The Transformed State Induced by *Salmonella enterica* Persists even after Bacterial Eradication**

While activation of Mapk and Akt pathways by *Salmonella enterica* enables transformation in genetically predisposed host cells, LPS immunostaining of gallbladder tumor samples from India did not reveal the bacterium in the majority of cancer cells (Figures S1C and S1D). We then hypothesized that *Salmonella enterica* ignites the onset of transformation by leaving an imprint of infection in the host to allow the transformed state to persist even after bacterial eradication.

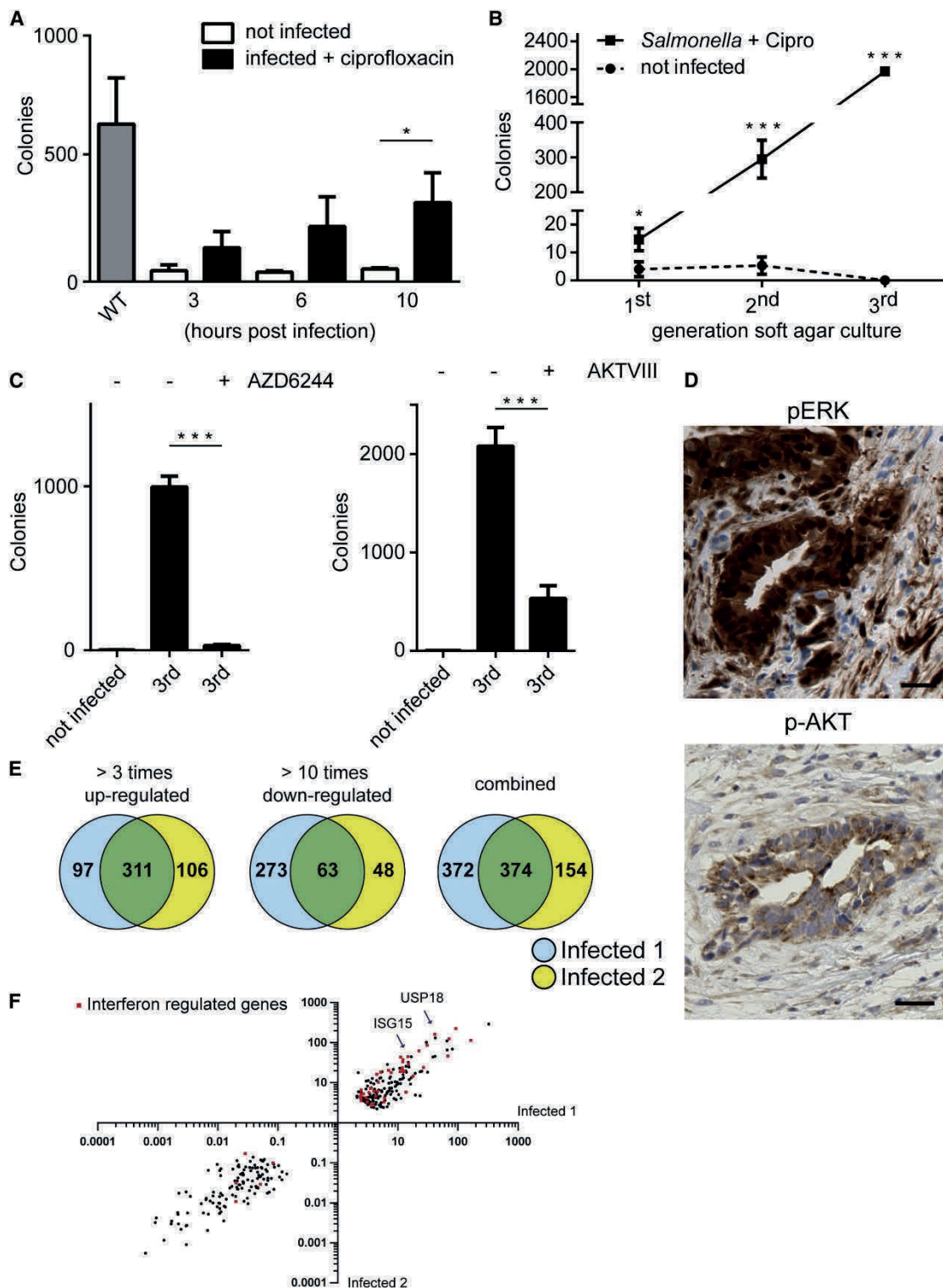
Infected MEFs derived from soft agar colonies, having been treated with ciprofloxacin prior to transplantation into immunodeficient mice, still produced tumors, suggesting that these cells no longer needed viable *Salmonella* to maintain the transformed state (Figure 4C). To define the minimal time of infection required for *Salmonella*-induced transformation, the pre-transformed MEFs were infected, and *Salmonella* was eliminated by ciprofloxacin treatment at different time points post infection. A total of 10 hr of *Salmonella* infection in Arf<sup>-/-</sup> + cMyc MEFs already sufficed to induce nearly 60% of colony growth in soft agar as compared to when the bacteria were left alive in the cells (Figure 6A). This suggests that imprinting of transformation in these MEFs occurs early during the *Salmonella* infection cycle.

### **Sustained Activated Host Signaling after *Salmonella enterica* Infection in Transformed Cells and GBC**

To understand the molecular basis of cellular transformation imprinted by *Salmonella* infection, we isolated single cells from soft agar colonies, eliminated the remaining *Salmonella* with ciprofloxacin (Figure S4B), and seeded equal numbers of these cells into a second-generation soft agar assay. This procedure was repeated to produce a third-generation soft agar assay. The number of anchorage-independent colonies increased per generation, reaching an efficiency of 5% (successful colonies per input cells) in the third iteration (Figure 6B). Western blot analyses of MEFs isolated from third-generation colonies and cultured under identical conditions as the control pre-transformed MEFs confirmed constitutive activation of the Akt pathway (Figure S6A). To test whether transformation still required an activated Akt and Map kinase pathway, we exposed third-generation colonies to chemical inhibitors of these signaling pathways. Inhibiting either Akt or Map kinase reduced the number of colonies (Figure 6C). Even in the absence of *Salmonella*, transformation of Arf<sup>-/-</sup> + cMyc MEFs remained dependent on activated Akt and Mapk pathways.

These same signaling pathways were also found activated in GBC patient tissues from India, with 5/10 and 7/10 staining positively for p-AKT and p-MAPK, respectively (Figure 6D; Table S2). Prevalence of these signaling markers along with the previously identified (Figure 1; Table S1) markers in GBC tissues from these patients underscore the importance of the features defined in the cell culture model in achieving cellular transformation.

*Salmonella enterica* bacteria may then act as a vehicle for imprinting a transformed state by virtue of sustained host cell signaling activation. This may be due to



**Figure 6. The Transformed State Induced by Salmonella Persists after Bacterial Eradication** (A) Number of soft agar colonies formed by  $Arf^{-/+}cMyc$  MEFs infected with WT *S. Typhimurium* (gray), not infected (white), or infected and then treated with antibiotic ciprofloxacin (black) at 3, 6, and 10 hr post infection, as indicated. Shown are mean and SD of triplicate samples. Statistical significance by Student's *t* test, \* $p < 0.05$ . (B) Number of soft agar colonies/well formed by *S. Typhimurium*-infected (bold line) or not infected (dotted line)  $Arf^{-/+}cMyc$  MEFs. *S. Typhimurium* was eliminated by ciprofloxacin, and equal numbers of MEFs were seeded in (legend continues on next page)



transcriptional alterations in the host as a result of infection. We then isolated two independent third-generation soft agar cultures of Arf<sup>-/-</sup> + cMyc MEFs with a history of Salmonella infection and compared their transcriptome with Arf<sup>-/-</sup> + cMyc MEFs that never encountered Salmonella. Both cell samples were expanded under identical monolayer culture conditions to ensure that differences were related to previous Salmonella infection. The samples were subjected to next-generation paired-end RNA sequencing allowing for evaluation of point mutations, rearrangements, and expression differences. We analyzed the RNA profile of MEFs from two independent cultures and observed a strong concordance in altered transcription as a function of Salmonella infection history (Figure 6E). We did not reveal any Salmonella-specific sequences, and a small number of point mutations found in host genes relative to the control samples had no apparent genetic relationship to transformation or deregulation of either Mapk or Akt pathways (Table S3). We then explored whether the transcriptome was altered post-infection to sustain transformation. Ingenuity network analysis of genes differentially expressed in MEFs that were transformed following Salmonella infection versus the uninfected control clustered these genes in various functional pathways (Figure S6B), with gastrointestinal disease and cancer as major constituents. When the differentially expressed genes were analyzed for potential networks, the network defined by Ingenuity centered on Akt and Map kinase pathways (Figure S6C). Salmonella infection may have altered the host transcriptome for sustained Akt and Mapk activation, as observed in MEFs as well as human GBC tissues from India. Previous studies reported that feedback mechanisms as a result of

**(Continue from previous page)**

soft agar for a first (1st), second (2nd), and third (3rd) soft agar assay. Results are representative of three independent experiments of technical triplicates. Shown are mean and SD of triplicate samples. Statistical significance by Student's *t* test; \**p* < 0.05; \*\*\**p* < 0.001. (C) Number of soft agar colonies/well formed by Arf<sup>-/-</sup>+cMyc MEFs with a history of *S. Typhimurium* infection (third-generation soft agar assay indicated as "3rd") after clearance of *S. Typhimurium* by ciprofloxacin treatment. Not-infected Arf<sup>-/-</sup>+cMyc MEFs were used as control for background transformation. The number of soft agar colonies decreases in the presence of MAPK inhibitor AZD6244 (left graph) and/or Akt inhibitor AKTVIII (right graph). Shown are mean and SD of triplicate samples. Statistical significance by Student's *t* test; \*\*\**p* < 0.01. (D) Representative image of IHC for phospho-ERK (dark brown, top panel) and for phospho-AKT (dark brown, bottom panel) of human GBC from the same patients as in Figure 1A. The sections are counterstained by hematoxylin for detection of the nuclei (in blue). Scale bar, 50 mm. (E) Venn diagrams representing the overlap in 3-fold increase (left) or 10-fold decrease (center) in fragments per kilobase of exon per million fragments mapped (FPKM) as detected by RNA sequencing of the third-generation MEFs isolated from two different soft agar cultures and then compared to the original MEFs never infected with *S. Typhimurium*. Right panel, combination of the up- and downregulated genes. (F) Dot plot graph comparing up- and downregulated genes relative to the non-infected cells from two independent samples of third-generation cells with history of *S. Typhimurium* infection, related to Figure 6E. *x* axis, infected sample 1; *y* axis, infected sample 2. Red squares, interferon-regulated genes. Fold increase values are plotted in log scale. See also Tables S2 and S3 and Figure S6.

cytokine production may cause sustained Akt and Mapk activation (*Cuenda and Rousseau, 2007; Zhang et al., 2011*). Indeed, almost 25% of transcripts upregulated in the transformed MEFs revealed a signature of interferon-response genes. These included the negative feedback molecules ISG15 and USP18 to downmodulate intrinsic inflammation (Figure 6F) (*Zhang et al., 2015*). The transformed MEFs thus presented an altered transcriptome with an imprint of a normal host cell response to bacterial infection.

Pathogens are able to epigenetically modify host cells, a phenomenon called patho-epigenetics (*Hamon and Cossart, 2008*). Expression changes for various epigenetic modulators were identified in the transformed MEFs, such as HDAC family member 9. How these different genes collectively generate a transformed state dependent on activated Akt and Map kinases is currently unclear.

## Discussion

Cancer arises through a multistep process involving various pretransforming hits. Given that only a fraction of chronic *S. Typhi* carriers develop GBC, and transformation of cells typically requires several independent steps, *S. Typhi* may simply contribute one such step. The chance of achieving this is considerably higher in chronically infected tissue, such as the gallbladder of a chronic typhoid fever carrier. We analyzed GBC patient samples from India and detected TP53 mutations, c-MYC overexpression, and *S. Typhi* DNA in the tumors. While GBC tissues obtained in the Netherlands also harbored TP53 mutations, they generally lacked c-MYC overexpression and were never found to contain *S. Typhi* DNA, suggesting that these tumors followed a different route to transformation.

We used pre-transformed mice, gallbladder organoids, and MEF cell cultures to show that inactivation of the Arf/TP53 pathway along with c-MYC overexpression was the minimal requirement for *S. Typhi* and Typhimurium to induce definitive transformation. Mechanistically, we established that *S. Typhimurium* activates host Mapk and Akt pathways to initiate transformation. These pathways are activated by *Salmonella enterica* derived effector proteins, secreted into host cells to promote bacterial uptake and its intracellular survival. Activation of host AKT and MAPK pathways is also employed by other intracellular bacterial pathogens, and such bacteria may thus be capable of inducing cancer of infected tissues. Next to *S. Typhi* (*Caygill et al., 1994; Nath et al., 2008; Shukla et al., 2000*), *C. trachomatis* (*Arnheim Dahlström et al., 2011*), *C. pneumoniae* (*Zhan et al., 2011*), and *M. tuberculosis* (*Kuo et al., 2013*) have reported relationships to cervical and lung cancer. Of note, these intracellular bacterial pathogens can all activate host AKT and MAPK pathways during their infection cycle (*Cho et al., 2010; Du et al., 2011; Roach and Schorey, 2002*). This suggests that a direct contribution of bacteria to tumor formation could be more common than previously anticipated and may potentially follow principles akin to those defined in the present study. Bacteria can also support transformation by different mechanisms, such

as secretion of toxins that influence host cell processes, modification of host chromatin structure with consequent manipulation of the host transcriptome (*Chumduri et al., 2013; Hamon and Cossart, 2008; Polk and Peek, 2010*), and post-translational histone modification, that may all contribute to genetic changes and transformation of the host (*Schmeck et al., 2008*). These and the host immune response to bacterial products may further contribute to cancer formation by bacterial infections. Of note, inflammation is not an a priori necessity to induce transformation by *Salmonella enterica*, as we show here using organoids and MEFs under conditions lacking all immune cell components.

Although *S. Typhi* DNA can be detected in most GBC tissues from India, the bacterium itself is only rarely detected in the tumor cells. This suggests that *S. Typhi* may ignite transformation, but viable bacteria are not required to sustain the transformed state. *S. Typhimurium* infection alters the host transcriptome and results in a sustained activated Akt and Mapk state to maintain transformation of cells with an inactivated Arf/TP53 axis and amplified c-MYC. These findings are phenocopied in GBC samples from India. Whether *Salmonella enterica* harbors effector proteins able to manipulate the host epigenome and how it then instills the stably transformed phenotype is unclear. It is possible that infection induces patho-epigenetic alterations that —when occurring in cells with some pre-transformed characteristics— can drive final transformation. The RNA profile of MEFs shows that a history of *S. Typhimurium* infection stably upregulates a series of interferon-response genes including ISG15 and USP18. While upregulation of interferon-responsive genes suggests some form of long-lasting intrinsic inflammation response, ISG15 and USP18 in fact act to limit inflammation by controlling the NF- $\kappa$ B pathway (*Liu et al., 2013; Zhang et al., 2015*). These transcriptome modifications suggest that *S. Typhimurium* infection has complex and stable effects on cells that contribute to transformation even after bacteria eradication. This study provides and explains a direct bacterial contribution to the formation of cancer. We define the minimal host cell requirements, i.e., mutations along the Arf/TP53 and c-MYC axes, which match with samples from GBC patients collected in India. GBC may therefore result as collateral damage of the normal *Salmonella enterica* infection cycle that drives definitive transformation when infecting pre-transformed cells. Improved control of (chronic) *S. Typhi* infection in areas where it is endemic may thus reduce the local incidence of GBC, a cancer type with poor prognosis at present.

## Experimental procedures

### Ethical Considerations and Specimens Collection

The human gallbladder specimens were collected between January 2009 and July 2013 at the University Hospital of Banaras Hindu University, Varanasi. The study design was approved by the Institute Ethics Committee, and a well-informed written consent was obtained from each of the patients. Dutch gallbladder tissue samples were obtained from the NKI-AVL biobank after approval of the Translational Research Board according to Dutch legislation and registered under number CFMPB67.

### IHC, Microscopy, Antibodies, and Antibiotics

Preparation of tissues and organoids for IHC was according to standard procedures, and details are reported in Supplemental Experimental Procedures.

Details of samples preparation for microscopy and antibodies used for IHC, western blot, and microscopy can be found in Supplemental Experimental Procedures. Antibiotic ciprofloxacin (Tokyo Chemical Industry) was added to infected cells 10 hr post-infection at a final concentration of 50 mg/ml.

### Animal Experiments

All mice were used according to protocols approved by the Institutional Animal Use Committee of the Netherlands Cancer Institute and maintained in pathogen-free isolators within the animal facility. For tumor growth experiments, Rag1<sup>-/-</sup> γc<sup>-/-</sup> mice (bred in-house) were implanted s.c. on the flank with 53106 MEFs in 100 μl PBS. For WT and/or ΔprgH *S. Typhimurium* 14028 infection experiments, Apc<sup>+/-</sup> FVB mice crossed with 129Ola mice were used (see also Supplemental Experimental Procedures).

### Gallbladder Organoids and Cell Lines

Gallbladder organs from Ink4b-Arf-Ink4a KO mice on FVB background (*Krimpenfort et al., 2007*) were handled for organoid growth as described (*Koo et al., 2012; Sato et al., 2009*). Noggin and RSpondin were excluded from the medium since they attenuated growth of gallbladder organoids. For details on organoids and MEF culture conditions, see Supplemental Experimental Procedures.

### Salmonella enterica Infection of MEFs and Gallbladder Organoids

*S. Typhi* and *S. Typhimurium* infection of the MEFs was performed as described (*Kuijl et al., 2007*). Details of *S. Typhimurium* infection of MEFs and gallbladder organoids are described in the Supplemental Experimental Procedures.

### Anchorage-Independent Assays

After *S. Typhimurium* infection at the different time points indicated, the MEFs diluted in DMEM with 10% FBS and 10 mg/ml gentamicin were seeded in a suspension layer of 0.4% soft agar (UltraPure Low Melting Point Agarose, Invitrogen) on top of 0.7%

soft agar bottom layer in six-well plates (50,000 cells seeded per well). Anchorage-independent cell growth and number of soft agar colonies were assessed after 2 weeks of culture and quantified and imaged as described in Supplemental Experimental Procedures.

### **Inhibitors**

Toxicity of the inhibitors was determined on MEFs growing under standard 2D tissue culture conditions using CellTiter-Blue Cell Viability Assays (Promega) and in *S. Typhimurium* cultures in LB medium by measuring growth at OD 600. For inhibitor names and concentrations see Supplemental Experimental Procedures.

### **ACCESSION NUMBERS**

The accession number for the deep sequencing data reported in this paper is GEO: GSE59623.

### **SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures, three tables, one movie, and Supplemental Experimental Procedures and can be found in the next chapter or with this article online at <http://dx.doi.org/10.1016/j.chom.2015.05.002>.

### **AUTHOR CONTRIBUTIONS**

The experiments were performed following a concept and under direction of J.N. T.S. designed and performed the initial soft agar experiments with *Salmonella Typhi* and *S. Typhimurium*, gallbladder organoids culture and their infection with *Salmonella*, microscopy imaging, and analysis. R.M.S. designed and performed the mouse experiments with J.M.B. J.M.B. performed the biochemistry and gallbladder organoids and soft agar cultures. H.J. performed electron microscopy. C.B.P., under the guidance of G.N., performed PCR on human material and obtained this from V.K.S. and M.K. Further pathology was performed by E.A.N.-B. with support from I.H. and A.B. Mouse genotyping and suggestions were from H.t.R. with pathology support from J.-Y.S. D.W.H. provided the *Salmonella* strains and information, and L.-e.W. performed bioinformatics.

## ACKNOWLEDGMENTS

This paper is dedicated to the memory of Els Borst-Eilers, mother and Minister of Health. We thank Ilana Berlin and Piet Borst for critical reading, G. Gargiuolo for the MEFs with Arf deletions, P. Krimpenfort for mutant mice, R. Arens for S. Typhi, G.L. Wilson for technical support with S. Typhi infection, Iris de Rink and the members of the Genomic Core Facility of the NKI, and the members of the Mouse Pathology and Molecular Pathology and Biobanking (CFMPB) facilities of the NKI for their support. This research was supported by an ERC Advanced Grant and a Dutch Cancer Society KWF Grant to J.N., an EMBO short-term fellowship and a grant from the Council of Scientific and Industrial Research India (CSIR) to C.B.P., and an NWO VENI grant to R.M.S. J.N. is a member of the Institute of Chemical Immunology (ICI).

## References

- Arnheim Dahlström, L., Andersson, K., Luostarinen, T., Thoresen, S., Ögmundsdóttir, H., Tryggvadóttir, L., Wiklund, F., Skare, G.B., Eklund, C., Sjölin, K., et al.** (2011). Prospective seroepidemiologic study of human papillomavirus and other risk factors in cervical cancer. *Cancer Epidemiol. Biomarkers Prev.* 20, 2541–2550.
- Barthel, M., Hapfelmeier, S., Quintanilla-Martínez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Rüschmann, H., and Hardt, W.D.** (2003). Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect. Immun.* 71, 2839–2858.
- Behlau, I., and Miller, S.I.** (1993). A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. *J. Bacteriol.* 175, 4475–4484.
- Caygill, C.P., Hill, M.J., Braddick, M., and Sharp, J.C.** (1994). Cancer mortality in chronic typhoid and paratyphoid carriers. *Lancet* 343, 83–84.
- Chang, M.H.** (2014). Prevention of hepatitis B virus infection and liver cancer. *Recent Results Cancer Res.* 193, 75–95.
- Chaturvedi, A.K., Gaydos, C.A., Agreda, P., Holden, J.P., Chatterjee, N., Goedert, J.J., Caporaso, N.E., and Engels, E.A.** (2010). *Chlamydia pneumoniae* infection and risk for lung cancer. *Cancer Epidemiol. Biomarkers Prev.* 19, 1498–1505.
- Cho, J.E., Kim, Y.S., Park, S., Cho, S.N., and Lee, H.** (2010). Mycobacterium tuberculosis-induced expression of Leukotactin-1 is mediated by the PI3-K/PDK1/Akt signaling pathway. *Mol. Cells* 29, 35–39.
- Chumduri, C., Gurusurthy, R.K., Zadora, P.K., Mi, Y., and Meyer, T.F.** (2013). *Chlamydia* infection promotes host DNA damage and proliferation but impairs the DNA damage response. *Cell Host Microbe* 13, 746–758.
- Coghill, A.E., and Hildesheim, A.** (2014). Epstein-Barr virus antibodies and the risk of associated malignancies: review of the literature. *Am. J. Epidemiol.* 180, 687–695.
- Crump, J.A., Luby, S.P., and Mintz, E.D.** (2004). The global burden of typhoid fever. *Bull. World Health Organ.* 82, 346–353.

- Cseh, B., Doma, E., and Baccarini, M.** (2014). “RAF” neighborhood: proteinprotein interaction in the Raf/Mek/Erk pathway. *FEBS Lett.* 588, 2398–2406.
- Cuenda, A., and Rousseau, S.** (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim. Biophys. Acta* 1773, 1358–1375.
- de Wind, N., Dekker, M., van Rossum, A., van der Valk, M., and te Riele, H.** (1998). Mouse models for hereditary nonpolyposis colorectal cancer. *Cancer Res.* 58, 248–255.
- Doorbar, J., Quint, W., Banks, L., Bravo, I.G., Stoler, M., Broker, T.R., and Stanley, M.A.** (2012). The biology and life-cycle of human papillomaviruses. *Vaccine* 30, F55–F70.
- Du, K., Zheng, Q., Zhou, M., Zhu, L., Ai, B., and Zhou, L.** (2011). Chlamydial antiapoptotic activity involves activation of the Raf/MEK/ERK survival pathway. *Curr. Microbiol.* 63, 341–346.
- Elinav, E., Nowarski, R., Thaiss, C.A., Hu, B., Jin, C., and Flavell, R.A.** (2013). Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat. Rev. Cancer* 13, 759–771.
- Frierson, H.F., Jr.** (1989). The gross anatomy and histology of the gallbladder, extrahepatic bile ducts, Vaterian system, and minor papilla. *Am. J. Surg. Pathol.* 13, 146–162.
- Hamon, M.A., and Cossart, P.** (2008). Histone modifications and chromatin remodeling during bacterial infections. *Cell Host Microbe* 4, 100–109.
- Haraga, A., Ohlson, M.B., and Miller, S.I.** (2008). Salmonellae interplay with host cells. *Nat. Rev. Microbiol.* 6, 53–66.
- Hatakeyama, M.** (2014). Helicobacter pylori CagA and gastric cancer: a paradigm for hit-and-run carcinogenesis. *Cell Host Microbe* 15, 306–316.
- Hundal, R., and Shaffer, E.A.** (2014). Gallbladder cancer: epidemiology and outcome. *Clin. Epidemiol.* 6, 99–109.
- Kaur, J., and Jain, S.K.** (2012). Role of antigens and virulence factors of Salmonella enterica serovar Typhi in its pathogenesis. *Microbiol. Res.* 167, 199–210.
- Knodler, L.A., Finlay, B.B., and Steele-Mortimer, O.** (2005). The Salmonella effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *J. Biol. Chem.* 280, 9058–9064.
- Koo, B.K., Stange, D.E., Sato, T., Karthaus, W., Farin, H.F., Huch, M., van Es, J.H., and Clevers, H.** (2012). Controlled gene expression in primary Lgr5 organoid cultures. *Nat. Methods* 9, 81–83.
- Koskela, P., Anttila, T., Bjørge, T., Brunsvig, A., Dillner, J., Hakama, M., Hakulinen, T., Jellum, E., Lehtinen, M., Lenner, P., et al.** (2000). Chlamydia trachomatis infection as a risk factor for invasive cervical cancer. *Int. J. Cancer* 85, 35–39.
- Krimpenfort, P., Ijpenberg, A., Song, J.Y., van der Valk, M., Nawijn, M., Zevenhoven, J., and Berns, A.** (2007). p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a. *Nature* 448, 943–946.
- Kuijl, C., Savage, N.D., Marsman, M., Tuin, A.W., Janssen, L., Egan, D.A., Ketema, M., van den Nieuwendijk, R., van den Eeden, S.J., Geluk, A., et al.** (2007).

Intracellular bacterial growth is controlled by a kinase network around PKB/AKT1. *Nature* 450, 725–730.

**Kuo, S.C., Hu, Y.W., Liu, C.J., Lee, Y.T., Chen, Y.T., Chen, T.L., Chen, T.J., and Fung, C.P.** (2013). Association between tuberculosis infections and non-pulmonary malignancies: a nationwide population-based study. *Br. J. Cancer* 109, 229–234.

**Lazcano-Ponce, E.C., Miquel, J.F., Muñoz, N., Herrero, R., Ferrecio, C., Wistuba, I.I., Alonso de Ruiz, P., Aristi Urista, G., and Nervi, F.** (2001). Epidemiology and molecular pathology of gallbladder cancer. *CA Cancer J. Clin.* 51, 349–364.

**Liu, X., Li, H., Zhong, B., Blonska, M., Gorjestani, S., Yan, M., Tian, Q., Zhang, D.E., Lin, X., and Dong, C.** (2013). USP18 inhibits NF- $\kappa$ B and NFAT activation during Th17 differentiation by deubiquitinating the TAK1-TAB1 complex. *J. Exp. Med.* 210, 1575–1590.

**Mager, D.L.** (2006). Bacteria and cancer: cause, coincidence or cure? A review. *J. Transl. Med.* 4, 14.

**Manning, B.D., and Cantley, L.C.** (2007). AKT/PKB signaling: navigating downstream. *Cell* 129, 1261–1274.

**McCracken, K.W., Catá, E.M., Crawford, C.M., Sinagoga, K.L., Schumacher, M., Rockich, B.E., Tsai, Y.H., Mayhew, C.N., Spence, J.R., Zavros, Y., and Wells, J.M.** (2014). Modelling human development and disease in pluripotent stem-cell-derived gastric organoids. *Nature* 516, 400–404.

**McGhie, E.J., Brawn, L.C., Hume, P.J., Humphreys, D., and Koronakis, V.** (2009). Salmonella takes control: effector-driven manipulation of the host. *Curr. Opin. Microbiol.* 12, 117–124.

**Nagaraja, V., and Eslick, G.D.** (2014). Systematic review with meta-analysis: the relationship between chronic Salmonella typhi carrier status and gallbladder cancer. *Aliment. Pharmacol. Ther.* 39, 745–750.

**Nath, G., Singh, Y.K., Kumar, K., Gulati, A.K., Shukla, V.K., Khanna, A.K., Tripathi, S.K., Jain, A.K., Kumar, M., and Singh, T.B.** (2008). Association of carcinoma of the gallbladder with typhoid carriage in a typhoid endemic area using nested PCR. *J. Infect. Dev. Ctries.* 2, 302–307.

**Polk, D.B., and Peek, R.M., Jr.** (2010). Helicobacter pylori: gastric cancer and beyond. *Nat. Rev. Cancer* 10, 403–414.

**Pratap, C.B., Kumar, G., Patel, S.K., Verma, A.K., Shukla, V.K., Kumar, K., and Nath, G.** (2013). Targeting of putative fimbrial gene for detection of *S. Typhi* in typhoid fever and chronic typhoid carriers by nested PCR. *J. Infect. Dev. Ctries.* 7, 520–527.

**Randi, G., Franceschi, S., and La Vecchia, C.** (2006). Gallbladder cancer worldwide: geographical distribution and risk factors. *Int. J. Cancer* 118, 1591–1602.

**Roach, S.K., and Schorey, J.S.** (2002). Differential regulation of the mitogen activated protein kinases by pathogenic and nonpathogenic mycobacteria. *Infect. Immun.* 70, 3040–3052.

**Ruby, T., McLaughlin, L., Gopinath, S., and Monack, D.** (2012). Salmonella's long-term relationship with its host. *FEMS Microbiol. Rev.* 36, 600–615.

**Rygaard, J., and Povsen, C.O.** (2007). Heterotransplantation of a human malignant tumour to “nude” mice. 1969. *APMIS* 115, 604–606.



- Samaras, V., Rafailidis, P.I., Mourtzoukou, E.G., Peppas, G., and Falagas, M.E.** (2010). Chronic bacterial and parasitic infections and cancer: a review. *J. Infect. Dev. Ctries.* 4, 267–281
- Santos, R.L., Zhang, S., Tsolis, R.M., Kingsley, R.A., Adams, L.G., and Baumler, A.J.** (2001). Animal models of *Salmonella* infections: enteritis versus typhoid fever. *Microbes Infect.* 3, 1335–1344.
- Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., and Clevers, H.** (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 459, 262–265.
- Schmeck, B., Beermann, W., N'Guessan, P.D., Hocke, A.C., Opitz, B., Eitel, J., Dinh, Q.T., Witzernath, M., Kru"ll, M., Suttorp, N., and Hippenstiel, S.** (2008). Simvastatin reduces *Chlamydia pneumoniae*-mediated histone modifications and gene expression in cultured human endothelial cells. *Circ. Res.* 102, 888–895.
- Shanmughapriya, S., Senthilkumar, G., Vinodhini, K., Das, B.C., Vasanthi, N., and Natarajaseenivasan, K.** (2012). Viral and bacterial aetiologies of epithelial ovarian cancer. *Eur. J. Clin. Microbiol. Infect. Dis.* 31, 2311–2317.
- Shukla, V.K., Singh, H., Pandey, M., Upadhyay, S.K., and Nath, G.** (2000). Carcinoma of the gallbladder—is it a sequel of typhoid? *Dig. Dis. Sci.* 45, 900–903.
- Thomas, M., Mesquita, F.S., and Holden, D.W.** (2012). The DUB-ious lack of ALIS in *Salmonella* infection: a *Salmonella* deubiquitinase regulates the autophagy of protein aggregates. *Autophagy* 8, 1824–1826.
- Wistuba, I.I., and Gazdar, A.F.** (2004). Gallbladder cancer: lessons from a rare tumour. *Nat. Rev. Cancer* 4, 695–706.
- Zhan, P., Suo, L.J., Qian, Q., Shen, X.K., Qiu, L.X., Yu, L.K., and Song, Y.** (2011). *Chlamydia pneumoniae* infection and lung cancer risk: a meta-analysis. *Eur. J. Cancer* 47, 742–747.
- Zhang, B., Li, S., and Harbrecht, B.G.** (2011). Akt-mediated signaling is induced by cytokines and cyclic adenosine monophosphate and suppresses hepatocyte inducible nitric oxide synthase expression independent of MAPK P44/42. *Biochim. Biophys. Acta* 1813, 73–79.
- Zhang, X., Bogunovic, D., Payelle-Brogard, B., Francois-Newton, V., Speer, S.D., Yuan, C., Volpi, S., Li, Z., Sanal, O., Mansouri, D., et al.** (2015). Human intracellular ISG15 prevents interferon- $\alpha/\beta$  over-amplification and auto-inflammation. *Nature* 517, 89–93.
- Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B., and Gala' n, J.E.** (2001). A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol. Microbiol.* 39, 248–259.

