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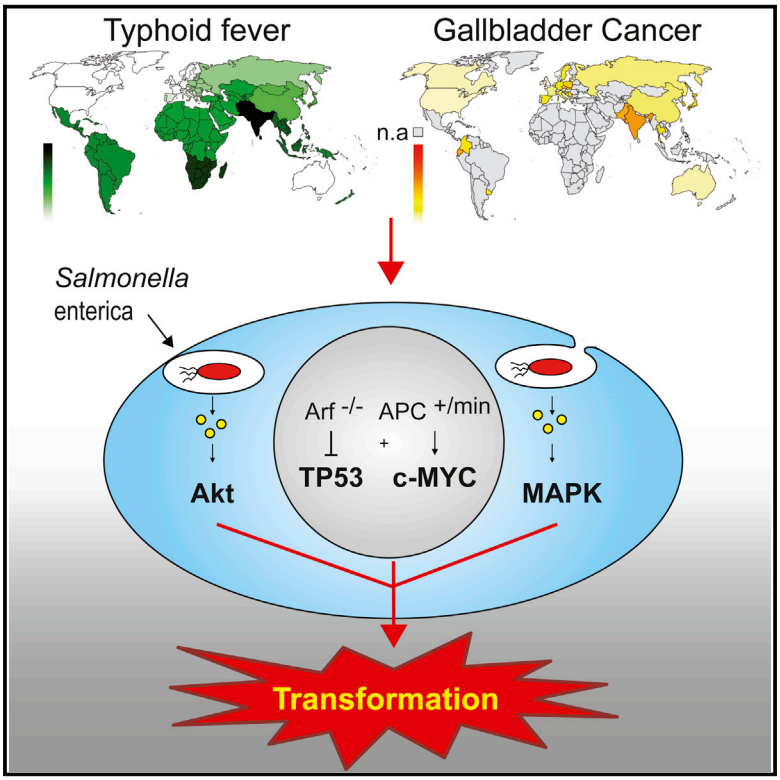
Cell Host & Microbe

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

Graphical Abstract



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In Brief

Salmonella Typhi infection is associated with gallbladder carcinoma (GBC), a frequent tumor in India and rare in the Western world. Scanu et al. show that by activating AKT and MAPK pathways during infection, *Salmonella* irreversibly transforms mice, gallbladder organoids, and MEFs with mutated TP53 and amplified c-MYC, as observed in GBC patients from India.

Highlights

- Gallbladder cancer (GBC) in India has unique mutations and is marked by *S.* Typhi DNA
- *Salmonella* is sufficient to transform genetically susceptible mice, organoids, and MEFs
- Transformation follows *Salmonella* manipulation of AKT and MAPK pathways during infection
- *Salmonella* infection imprints sustained transformation, as in GBC patients from India

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

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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

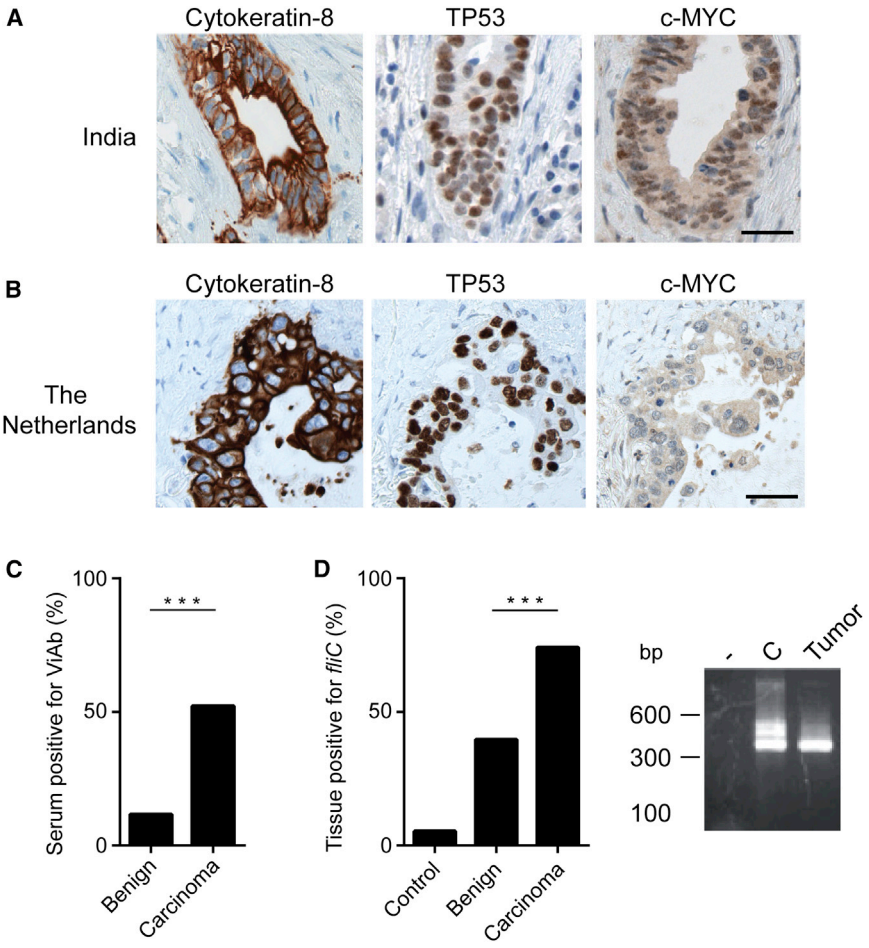

Figure 1. *Salmonella* Typhi Infection and GBC: India versus the Netherlands

(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 μm.

(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 μm.

(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 χ^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 χ^2 test. ***p < 0.001. See also Figure S1 and Table S1.

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

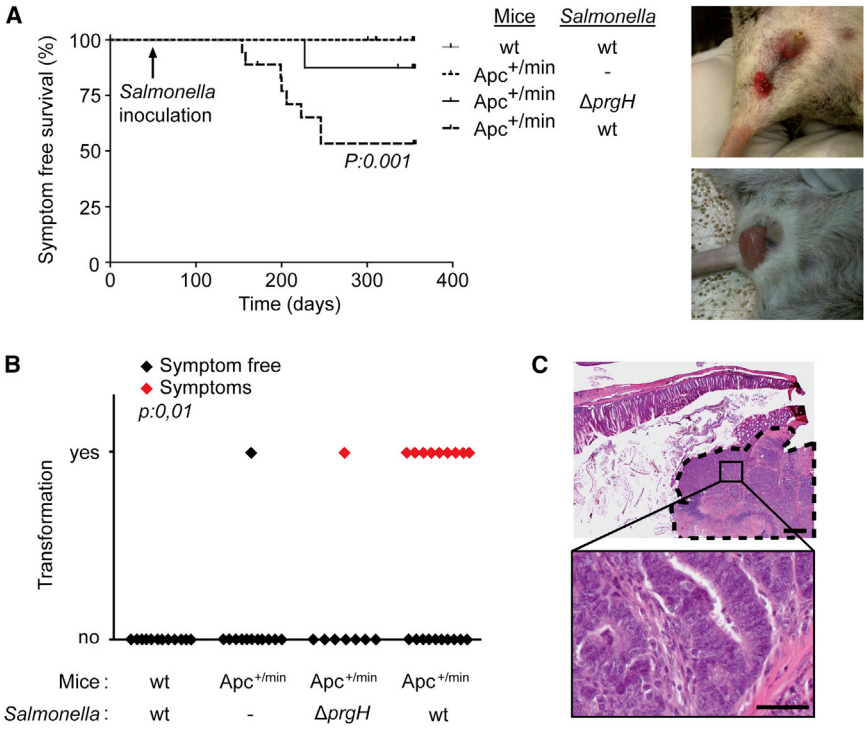

Figure 2. *Salmonella* Infection Drives Tumor Development in Predisposed Mice

(A) Kaplan-Meier curve of symptom-free survival of *Apc*$^{+/min}$ and WT mice infected or not (–) with WT or PrgH deletion mutant (Δ*prg*H) *S.* Typhimurium. $p < 0.001$ as defined by a log-rank (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*$^{+/min}$ (APC$^{+/min}$) mice infected with WT, Δ*prg*H *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*$^{+/min}$ 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 μm; bottom panel, 50 μm. See also Figure S2.

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*$^{+/min}$ 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 pre-transforming mutations in the host (as defined above) can cooperate with *Salmonella enterica* bacteria in cancer formation, we used mice heterozygous for Apc (*Apc*$^{+/min}$) that induces c-Myc overexpression (de Wind et al., 1998) to mimic the corresponding phenotype of GBC samples from India. The *Apc*$^{+/min}$ 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*$^{+/min}$ mice with *Salmonella*. As a further control, we infected *Apc*$^{+/min}$ and control mice with the *Salmonella* mutant *ΔprgH*, 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 *ΔprgH* mutant *Salmonella* can shed LPS and when eliminated expose factors including lipoteichoic acid and bacterial 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*$^{+/min}$ 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

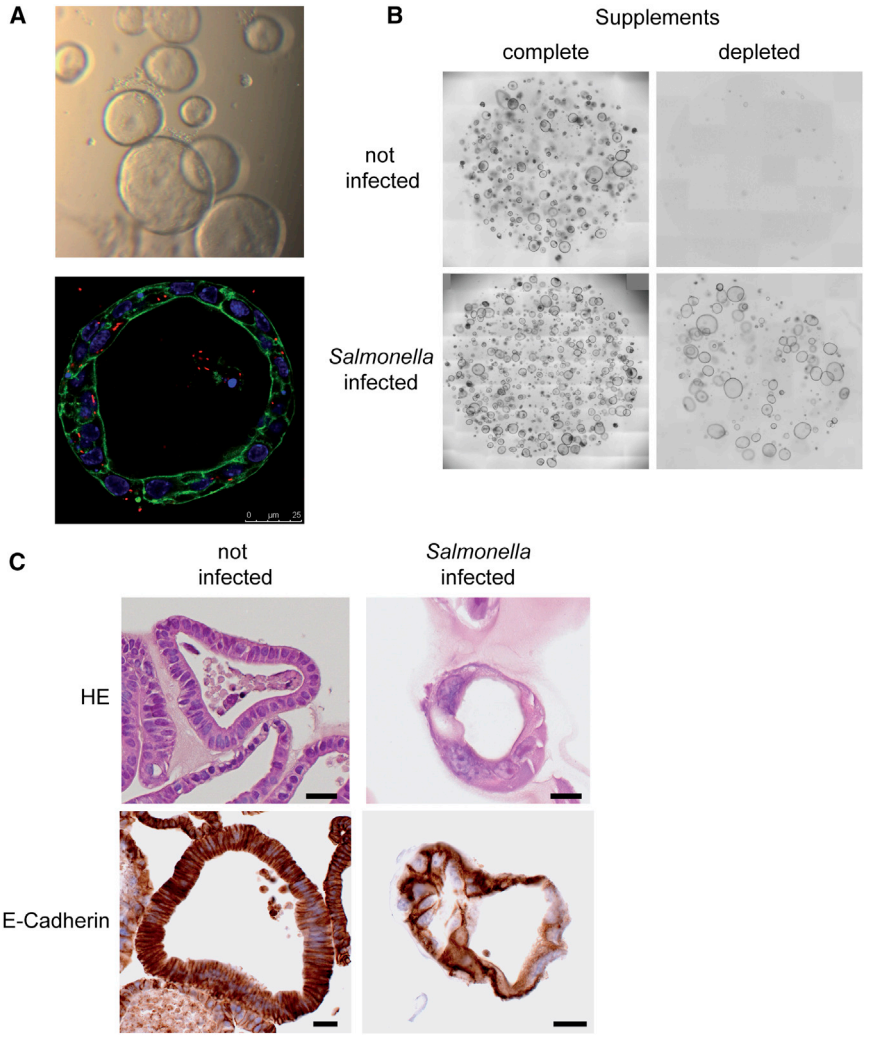

Figure 3. *Salmonella* Infection of Gallbladder Organoids Induces Cell Transformation

(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.

adeno(carcino)mas due to the $Apc^{+/min}$ background (Figures S2A–S2C), colorectal adenocarcinomas were observed in symptomatic $Apc^{+/min}$ 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-transformation by c-Myc oncogene overexpression resulting from loss of one copy of the APC gene. Since *Salmonella* lacking TTSS (*ΔprgH*) 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 pre-transformed 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 *ΔprgH* 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 *ΔprgH* 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

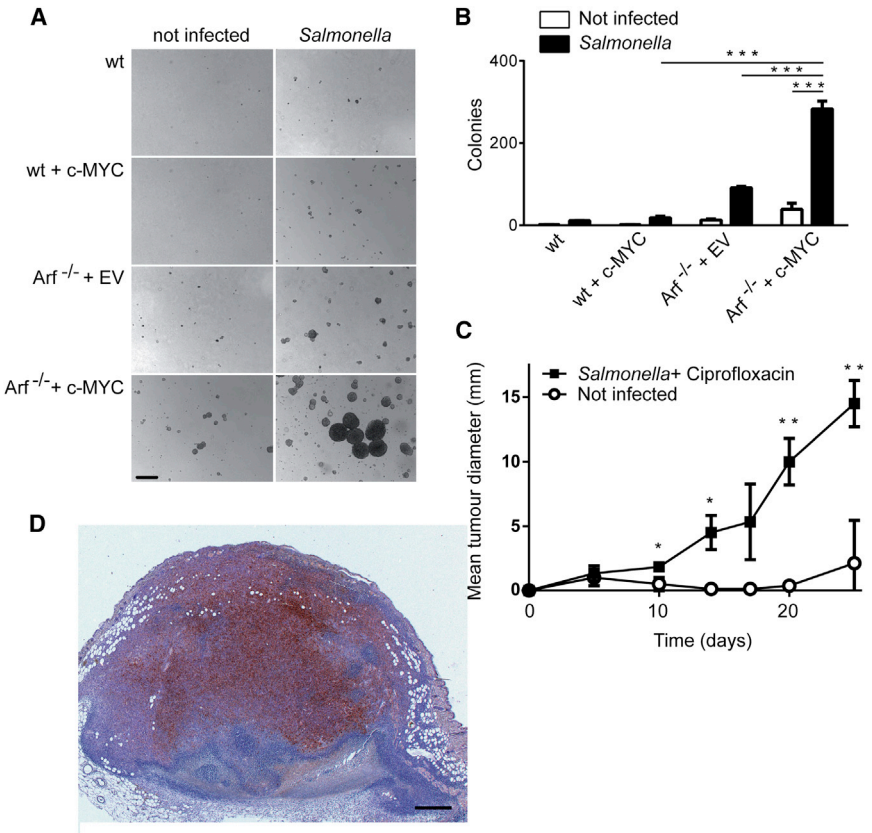

Figure 4. *Salmonella* Infection of Primary Pre-transformed Fibroblasts Induces Cell Transformation

(A) Representative images of the anchorage-independent assay of WT (top panels), WT overexpressing c-MYC (WT+cMyc; second panels), *Arf*-deficient ($Arf^{-/-}$+Empty vector [EV]; third panels), and *Arf*-deficient overexpressing c-MYC ($Arf^{-/-}$+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 μm.

(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 $Rag1^{-/-}\gamma c^{-/-}$ 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* $^{-/-}$ overexpressing c-Myc-IRES-GFP with a history of *S.* Typhimurium infection) transplanted in $Rag1^{-/-}\gamma c^{-/-}$ mice. The sections were counterstained by hematoxylin for detection of the nuclei (in blue). Scale bar, 500 μm. See also Figures S4A–S4C.

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 Pre-transformed 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* (resulting in TP53 inactivation) and overexpressing c-MYC ($Arf^{-/-}$ + 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 pre-transformed 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 $Rag1^{-/-}\gamma c^{-/-}$ 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 $Arf^{-/-}$ + 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).

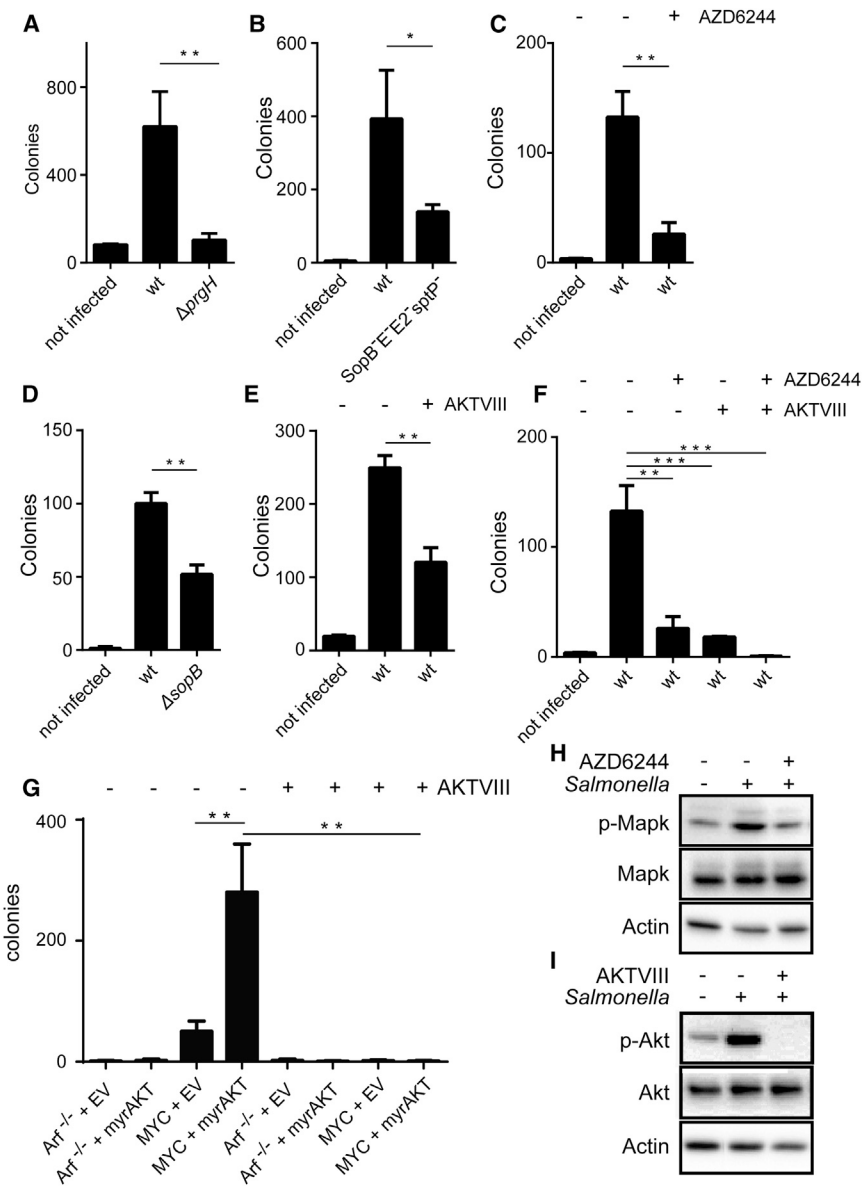

Figure 5. *Salmonella*-Mediated Activation of Akt and Map Kinase Pathways Drives Transformation in Pre-transformed Cells

(A) Number of soft agar colonies/well formed by $Arf^{-/-}$+cMyc MEFs either or not infected with WT or mutant *ΔprgH* (*ΔprgH*) *S.* Typhimurium. Results are representative of three independent experiments. Shown is mean ± SD of technical triplicate experiment. Statistical significance by Student's t test; $^{**}p < 0.01$.

(B) Number of soft agar colonies/well formed by $Arf^{-/-}$+cMyc MEFs infected with WT and *sopB*$^{-}$*sopE*$^{-}$*sopE2*$^{-}$*sptP*$^{-}$ *S.* Typhimurium. Results are representative of at least two independent experiments with technical triplicates. Shown are mean number of colonies ± 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 $Arf^{-/-}$+cMyc MEFs cultured in the presence (+) or absence (–) of MAPK inhibitor AZD6244. Shown are mean number of colonies ± SD from triplicate samples. Statistical significance by Student's t test, $^{**}p < 0.01$.

(D) Number of soft agar colonies/well from WT and Δ*sop*B *S.* Typhimurium-infected $Arf^{-/-}$+cMyc MEFs. Shown are mean number of colonies ± SD from triplicate experiments. Statistical significance by Student's t test; $^{**}p < 0.01$.

(E) Number of soft agar colonies/well from *S.* Typhimurium-infected (WT) $Arf^{-/-}$+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) $Arf^{-/-}$+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 ($Arf^{-/-}$) 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 $Arf^{-/-}$+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 $Arf^{-/-}$+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.

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 *ΔprgH 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$^{-/-}$ + 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–S4F). We then infected *Arf*$^{-/-}$ + 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*$^{-/-}$ + 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).

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* $^{-/-}$ + 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* $^{-/-}$ + 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 transcriptional alterations in the host as a result of infection. We then isolated two independent third-generation soft agar cultures of *Arf* $^{-/-}$ + cMyc MEFs with a history of *Salmonella* infection and compared their transcriptome with *Arf* $^{-/-}$ + 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

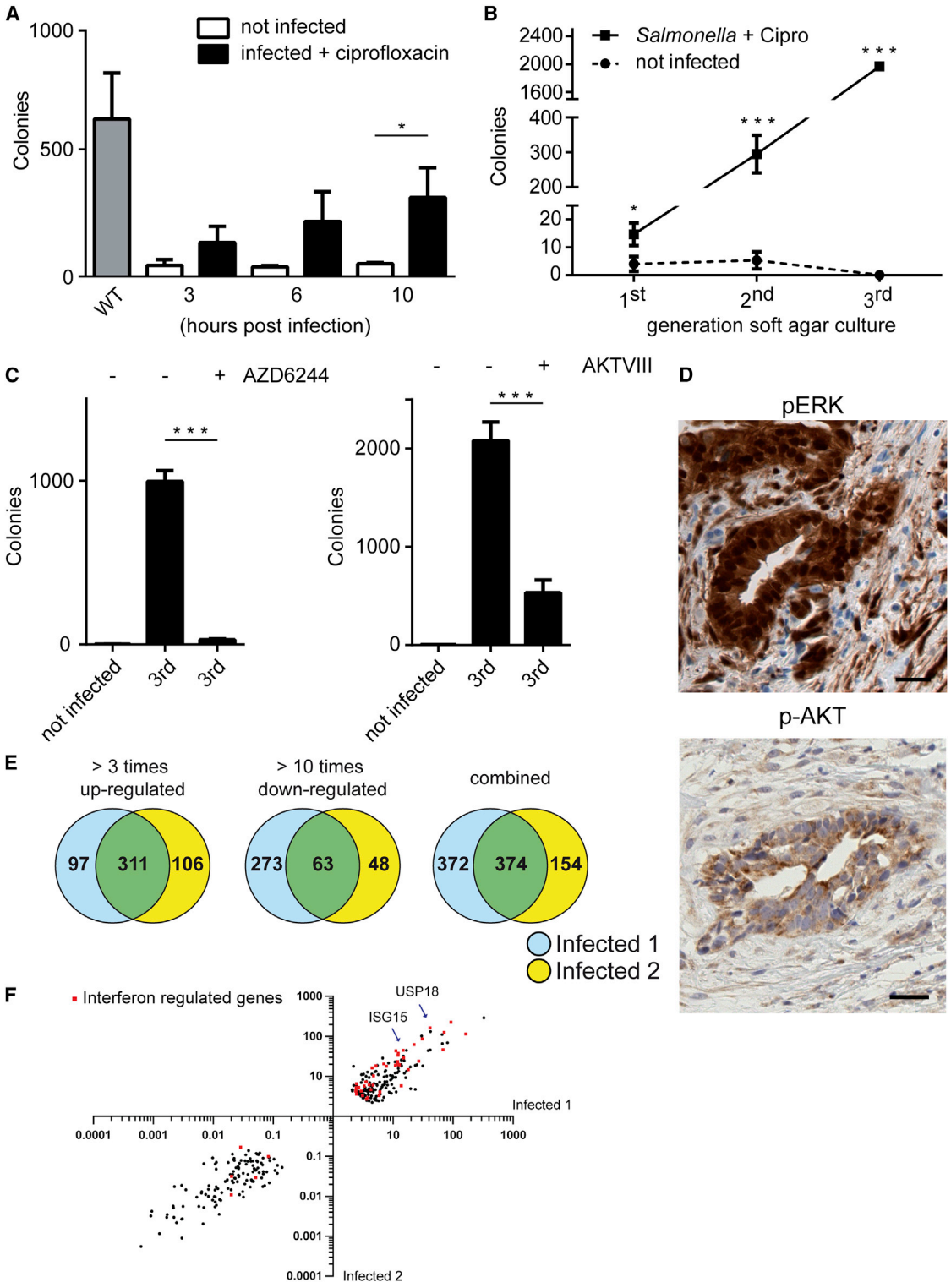

Figure 6. The Transformed State Induced by *Salmonella* Persists after Bacterial Eradication

(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 soft agar for a first (1st), second (2nd), and third (3rd) soft agar assay. Results are

(legend continued on next page)

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 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 pre-transforming 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

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 $Arf^{-/-}$+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 $Arf^{-/-}$+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 μm.

(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.

controlling the NF-kB 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 μg/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*$^{-/-}\gamma c^{-/-}$ mice (bred in-house) were implanted s.c. on the flank with 5×10^6 MEFs in 100 μl PBS. For WT and/or Δ*prg*H *S.* Typhimurium 14028 infection experiments, *Apc*$^{+/min}$ 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 μg/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 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.

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