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

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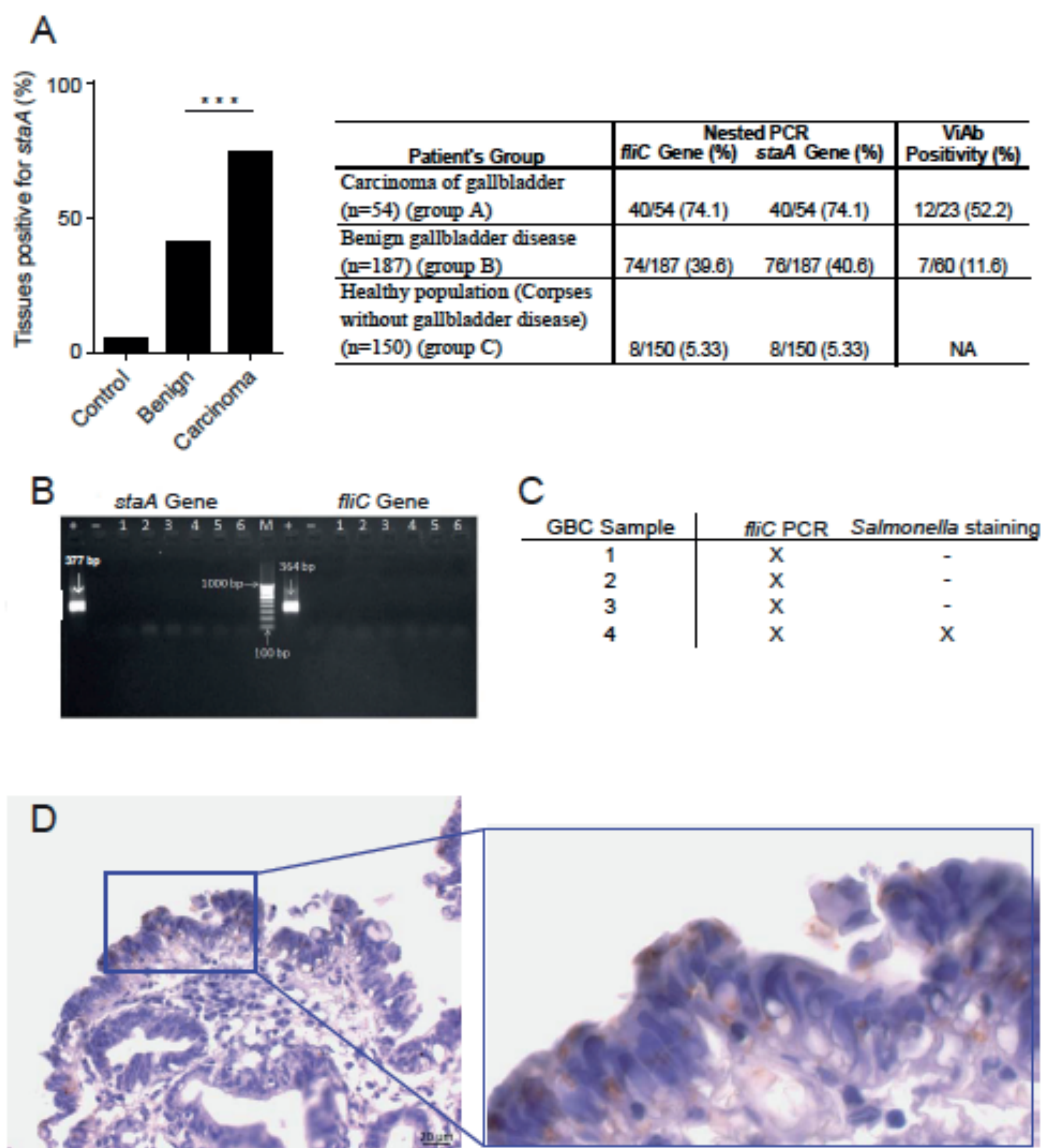
Chapter 6

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

SUPPLEMENTARY DATA

Figure S1



Patient's Group	Nested PCR *fliC* Gene (%)	Nested PCR *staA* Gene (%)	ViAb Positivity (%)
Carcinoma of gallbladder (n=54) (group A)	40/54 (74.1)	40/54 (74.1)	12/23 (52.2)
Benign gallbladder disease (n=187) (group B)	74/187 (39.6)	76/187 (40.6)	7/60 (11.6)
Healthy population (Corpses without gallbladder disease) (n=150) (group C)	8/150 (5.33)	8/150 (5.33)	NA

GBC Sample	*fliC* PCR	*Salmonella* staining
1	X	-
2	X	-
3	X	-
4	X	X

Figure S1: *Salmonella* detection in gallbladder carcinoma samples. Related to Figure 1.

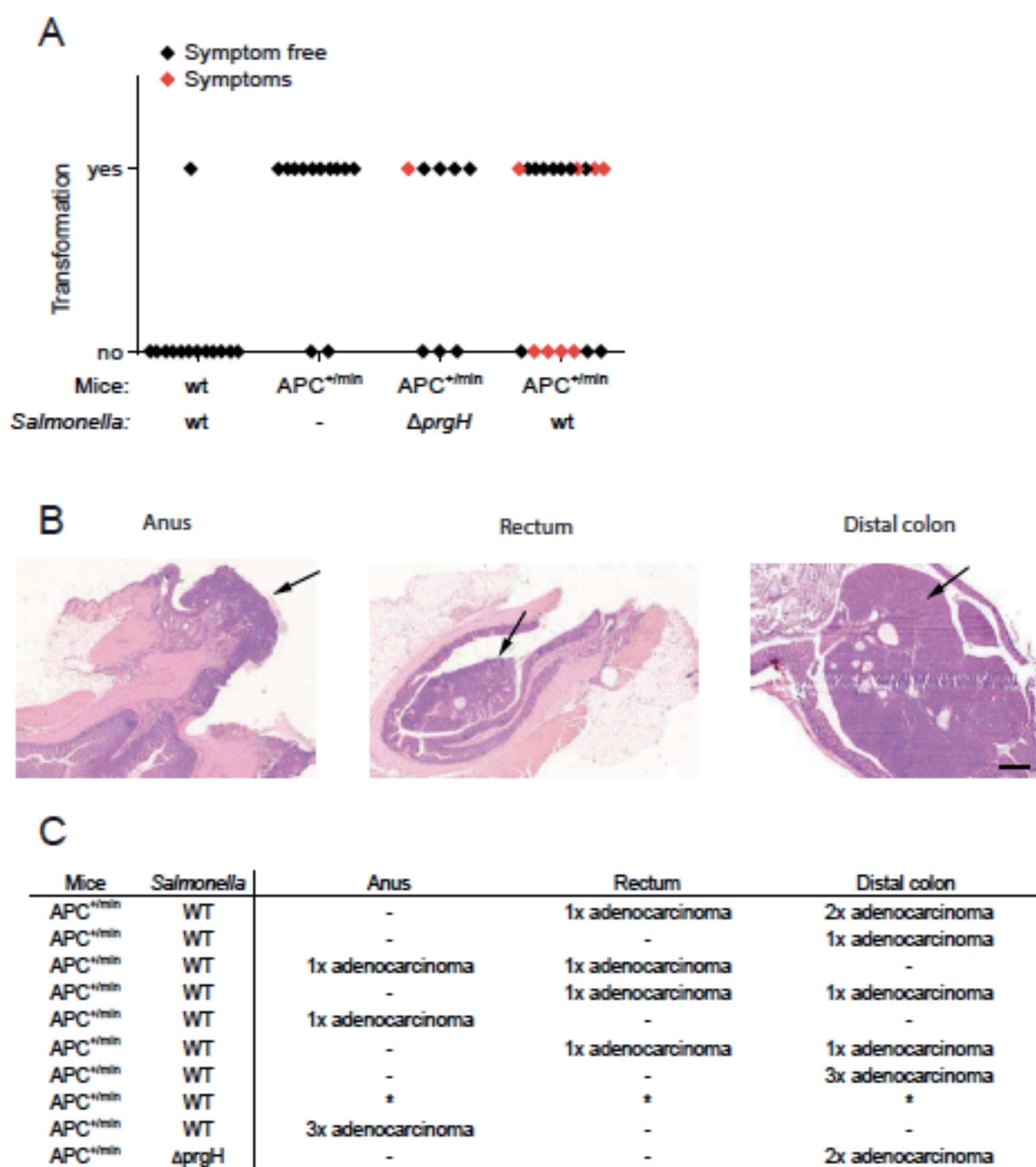
(A) Percentage of gallbladder tissue containing *staA* DNA of S. Typhi. Tissues were collected from healthy population (corpses without gallbladder diseases, healthy: 5.33%, n: 150); patients with benign gallbladder diseases (benign: 40.6%, n: 187), or gallbladder carcinoma samples (carcinoma: 74.1%, n: 54) that were collected in India. Statistical significance determined by chi-squared test. P-value: ***<0,01. Right: table summarizing the percentage of patients with gallbladder carcinoma positive for S. Typhi DNA (*fliC* and *staA*) and with serum positive for S. Typhi ViAb. NA: not analyzed.

(B) Nested PCR performed on the DNA extracted from gallbladder carcinoma samples collected in the Netherlands to detect the *staA* (left) and *fliC* (right) S. Typhi genes. All the collected samples were negative for *fliC* (364bp) and for *staA* (377 bp). M: DNA ladder. +: positive control (DNA from reference strain of S. Typhi); -: negative control (distilled water).

(C) Detection of S. Typhi in four representative gallbladder carcinomas collected in India. S. Typhi was detected in all samples by performing nested-PCR for the *fliC* gene, while the immunohistochemistry with anti-Salmonella LPS antibody detected S.Typhi in only one tumor sample.

(D) Representative image of the gallbladder carcinoma sample positive for S. Typhi as detected by immunohistochemistry with anti-*Salmonella* LPS antibody (brown). Scale bar: 20μm.

Figure S2



Mice	Salmonella	Anus	Rectum	Distal colon
APC+/min	WT	-	1x adenocarcinoma	2x adenocarcinoma
APC+/min	WT	-	-	1x adenocarcinoma
APC+/min	WT	1x adenocarcinoma	1x adenocarcinoma	-
APC+/min	WT	-	1x adenocarcinoma	1x adenocarcinoma
APC+/min	WT	1x adenocarcinoma	-	-
APC+/min	WT	-	1x adenocarcinoma	1x adenocarcinoma
APC+/min	WT	-	-	3x adenocarcinoma
APC+/min	WT	*	*	*
APC+/min	WT	3x adenocarcinoma	-	-
APC+/min	ΔprgH	-	-	2x adenocarcinoma

Figure S2: Tumor formation in S. Typhimurium infected *Apc* $^{+/min}$ mice. Related to Figure 2.

A) Pathology assessment of small intestine transformation occurring in wild type mice (wt) or *Apc*$^{+/min}$ (*APC*$^{+/min}$) mice infected with wild type (wt) or Δ*prg*H (Δ*prg*H) S. Typhimurium or not-infected (-). Red diamonds: fraction of mice with symptoms as indicated in Fig.2A. Black diamonds: fraction of mice with no symptoms. Wild type mice infected with wt *S.* Typhimurium did not present small intestine transformation. P value: 0.4013 as determined by Fisher Exact Test on *Apc*$^{+/min}$ mice groups.

(B) Representative HE staining of adenocarcinoma present in *Apc*$^{+/min}$ mice infected with *S*. Typhimurium localized in the anus (left), rectum (center) and distal colon (right). Arrows indicate the tumor. Scale bar: 500 µm.

(C) Summary of the geographical distribution of the tumors formed in Apc+/min mice infected with *S*. Typhimurium. The table displays only the mice that developed colorectal adenocarcinoma. *: The exact geographical location could not be determined due to neoplastic lesions of adenocarcinoma and large areas of necrosis that destroyed the colon present in the sections.

Figure S3

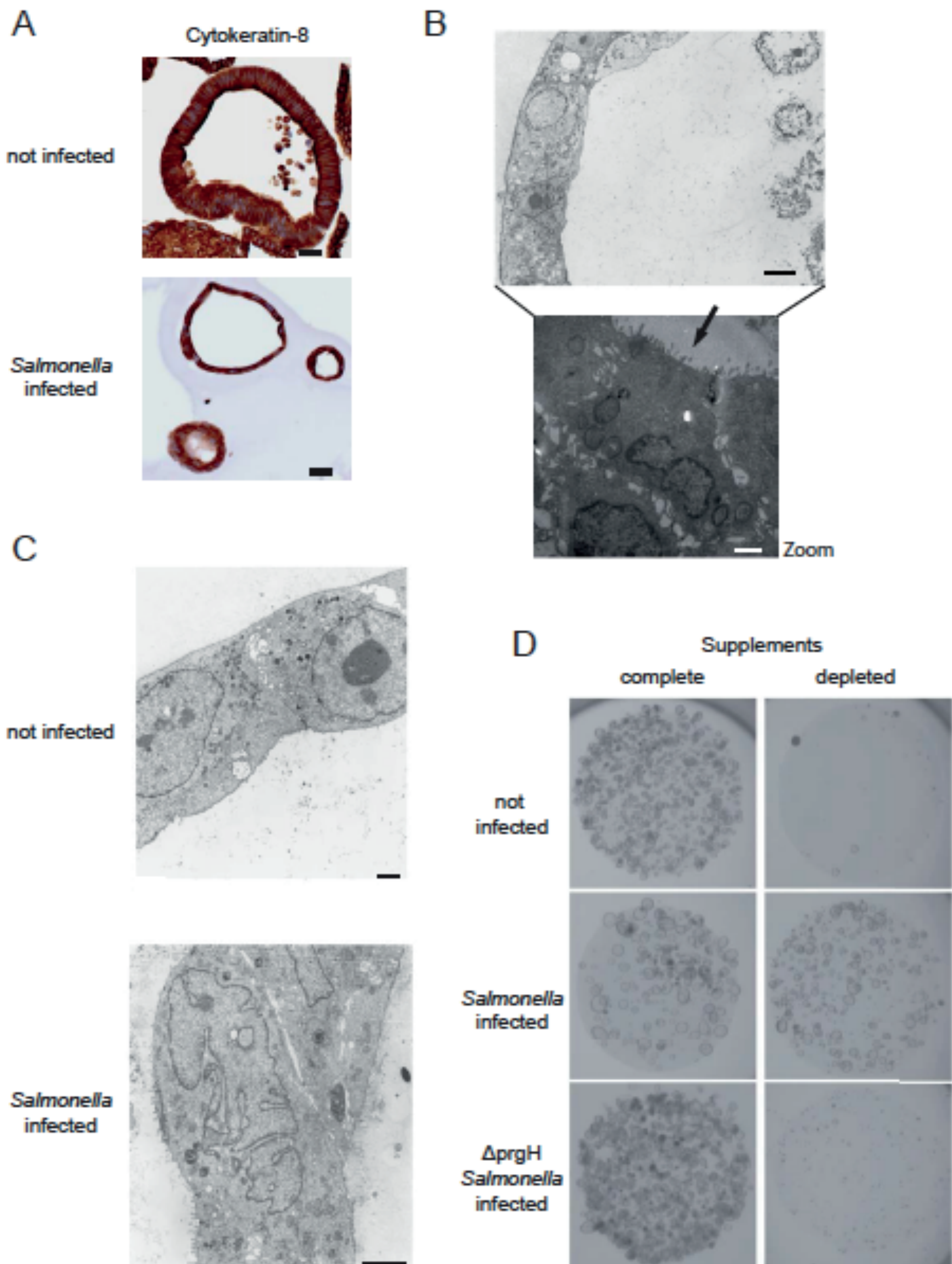


Figure S3: S.Typhimurium infection and characterization of gallbladder organoids. Related to Figure 3.

(A) Immunohistochemistry staining for cytokeratin-8 of gallbladder organoid that had been infected (Salmonella infected) or not (not infected) with *S*. Typhimurium. Gallbladder organoids that had been infected with S. Typhimurium lost their cohesion and polarity. Scale bar: 20 µm

(B) Electron Microscopy image of one representative gallbladder organoid (scale bar: 500 µm) and high magnification of gallbladder organoid epithelial cells (scale bar: 1 µm). The arrow indicates the microvilli present at the apical part of the cell in the lumen of the organoid.

(C) Representative electron micrographs of not-infected gallbladder organoids (top) or gallbladder organoids with a history of *S*. Typhimurium infection (bottom). The organoids grown from gallbladder cells originally infected with *S*. Typhimurium present irregular and fragmented nuclei unlike the organoids that were never infected with this bacterium. Scale bar: 100 µm.

(D) Gallbladder organoid cells with a history of wild type (Salmonella infected, center panels), *ΔprgH* mutant (*ΔprgH* Salmonella infected, bottom panel) S. Typhimurium infection, or not (not infected, top panels) were cultured to reform organoids in complete medium (complete, left panels) or supplements depleted medium (depleted, right panels). Representative images of organoids culture from two independent experiments performed in triplicate are shown.

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Figure S4

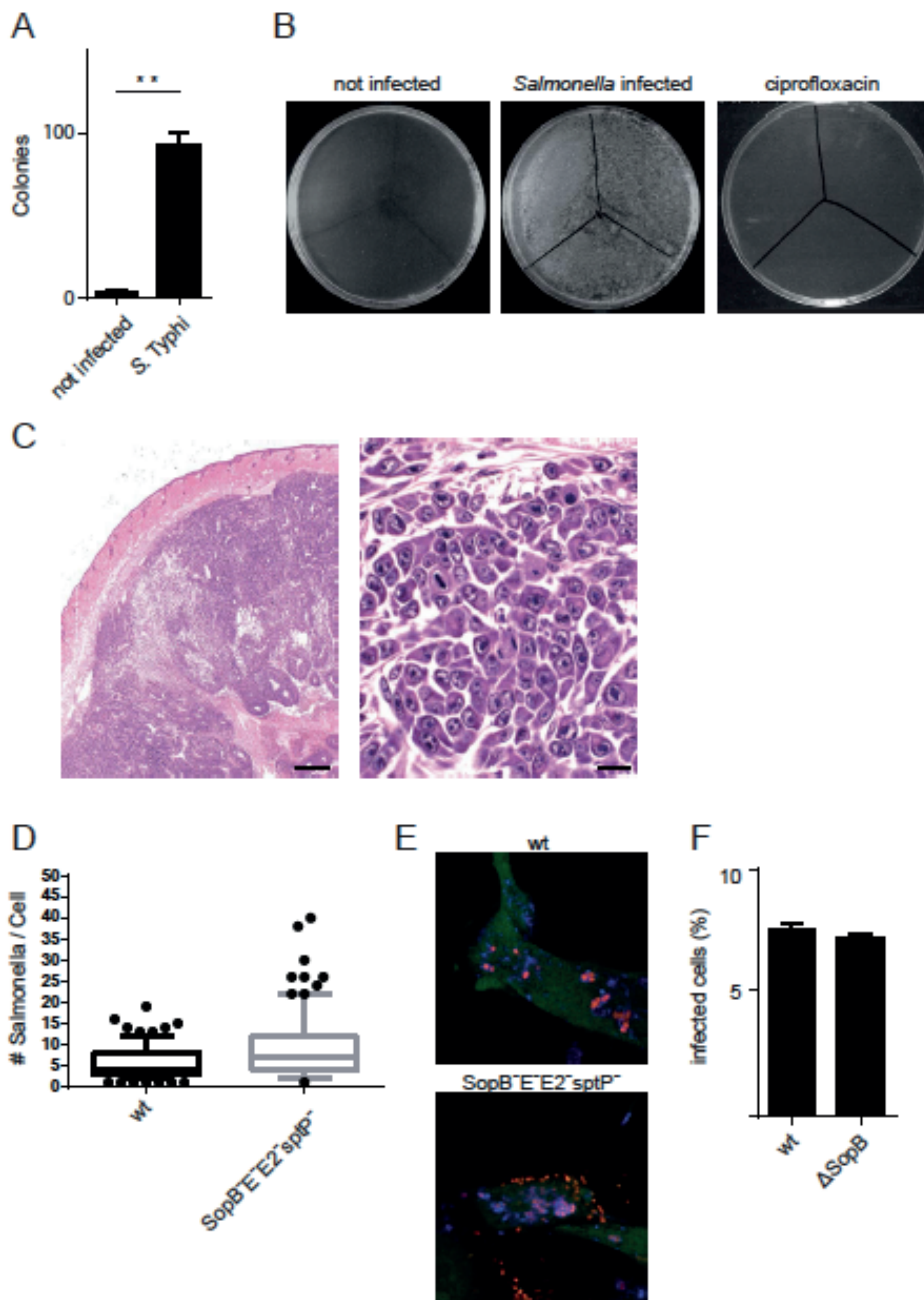


Figure S4: *Salmonella enterica* serovar Typhi and Typhimurium induce cell transformation and tumor formation even after their elimination and when able to activate Akt and Map kinases pathways. Related to Figure 4 and Figure 5.
(A) Quantification of the number of soft agar colonies of S. Typhi infected (S. Typhi) or not-infected (not infected) *Arf*-deficient MEFs overexpressing c-MYC. Shown is mean + S.D. of technical triplicate experiments. Statistical significance was determined by Student's T-test. P value: **<0,01.
(B) Elimination by ciprofloxacin of S. Typhimurium in infected MEFs after 10hpi. 50.000 MEFs per sample were lysed in milliQ water and the lysate was plated in LB-agar bacteria plates to detect the number of *Salmonella* residing in the cells (c.f.u). The experiment was performed in triplicate. Left panel: c.f.u of not-infected MEFs; Center panel: c.f.u of *Salmonella* infected MEFs; Right panel: c.f.u of MEFs treated with ciprofloxacin that are completely cleared of *Salmonella*.
(C) HE staining of a representative solid tumor formed by the MEFs ($Arf^{-/-}$ overexpressing c- Myc) transplanted in $Rag1^{-/-}\ \gamma c^{-/-}$ mice as in Figure 4D. The tumor shows high mitotic incidence and necrotic areas. Right: zoom-in. Scale bar: 500 μm (left), 50 μm (right).
(D) Number of *Salmonella*-containing vacuoles per cell positive for lysosomal marker Lamp1 after the infection of *Arf*-deficient MEFs overexpressing c-MYC with SopB-SopE-SopE-SptP deficient ($SopB^{-}E^{-}E2^{-}sptP^{-}$) or with wild type S. Typhimurium (wt) as quantified by fluorescence microscopy. The number of *Salmonella*-containing vacuoles per cell is similar when MEFs are infected for 15 minutes with 9.0 x 105 bacteria/ml of wt SL1344 and for 2h with 7.5x106 bacteria/ml of $sopB^{-}sopE^{-}sopE^{-}sptP^{-}$ SL1344.
(E) Representative confocal images of *Arf*-deficient MEFs overexpressing c-MYC infected with wild type (wt, top) and $sopB^{-}sopE^{-}sopE^{-}sptP^{-}$ (bottom) deficient S.Typhimurium. Blue: Lamp1, red: Salmonella, green: c-Myc-IRES-GFP.
(F) Percentage of MEFs (*Arf*-deficient and overexpressing c-MYC) infected with m-Cherry wild type (wt) or with m-Cherry-ΔSopB (ΔsopB) S. Typhimurium SL14028 as determined by flow cytometric analyses of mCherry-positive MEFs. Using 3.25 x 106 bacteria/ml for wt and 6.5 x 106 bacteria/ml for the *ΔsopB* mutant strain yielded similar numbers of infected cells.

Figure S5

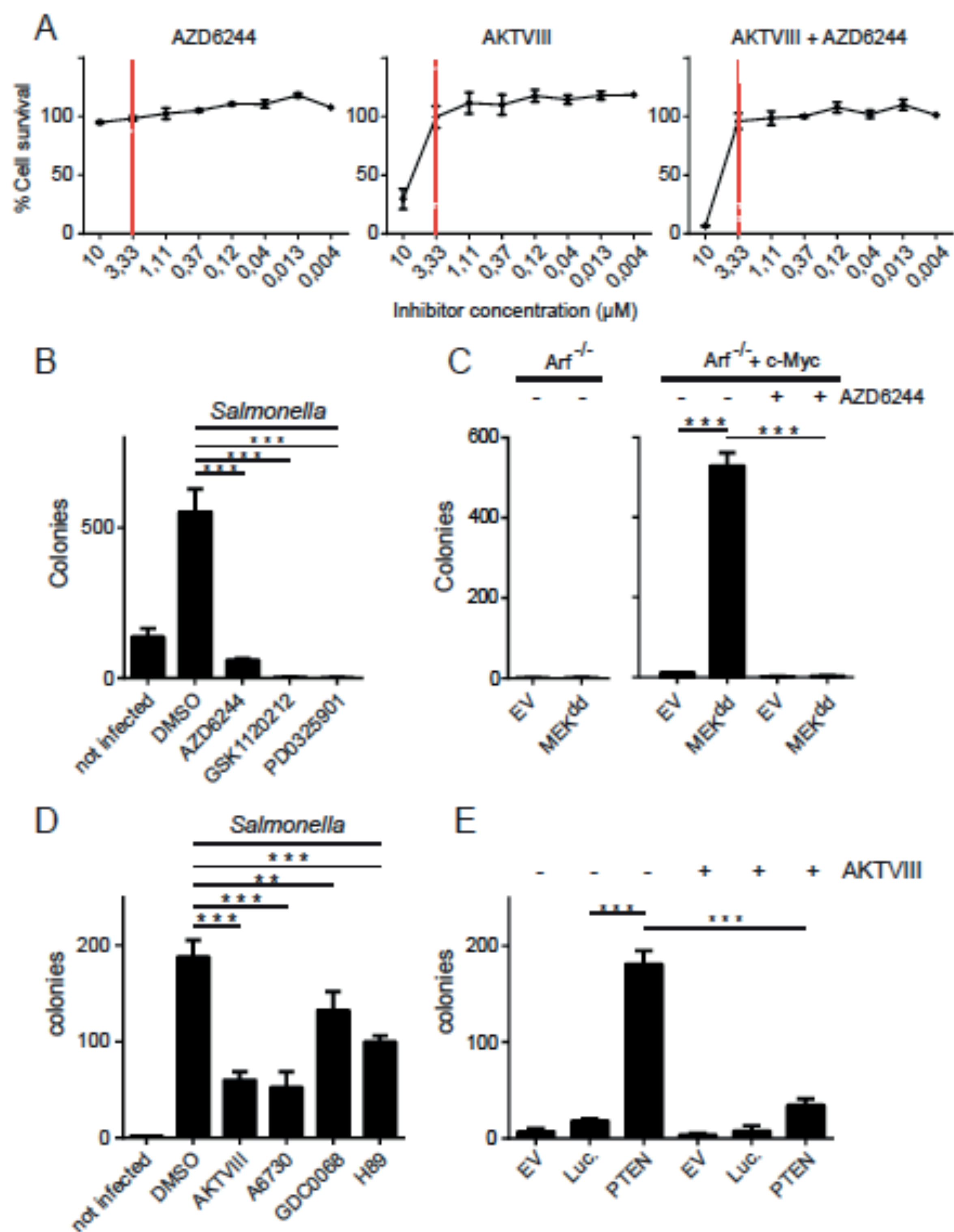


Figure S5: Salmonella enterica –mediated transformation requires activation of host's Akt and Map kinases pathways. Related to figure 5.

(A) Cell viability assay showing percentage of surviving cells after 3 days culturing in monolayer in the presence of indicated concentrations of AZD6244 (left graph), AKTVIII (middle graph) or a combination of the two inhibitors (right graph). Shown is mean +S.D of a technical triplicate experiment. Red dashed line: Used concentration of each inhibitor.

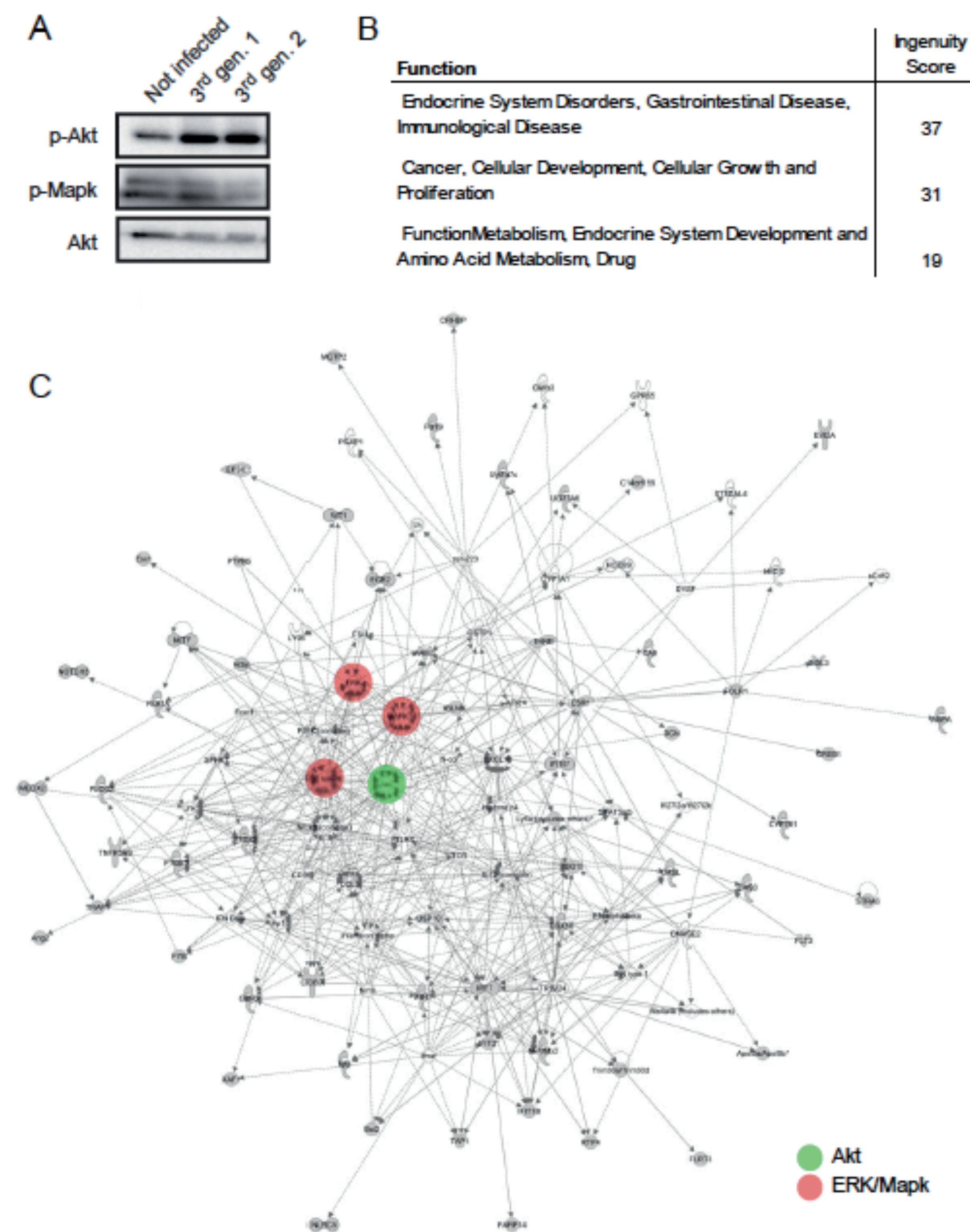
(B) Quantification of the number of soft agar colonies/well formed by *Arf*-deficient MEFs overexpressing c-MYC infected or not by S. Typhimurium while cultured in soft agar in presence of Mapk inhibitors AZD6244 (3.3μM), GSK1120212 (0,1μM), PD0325901 (0,1uM) and DMSO control at 0.03%, as indicated. The inhibitors were used at concentrations that did not affect growth of the MEFs in two-dimensional cultures. Shown is mean + S.D. of technical triplicate experiment. Statistical significance was calculated by Student's T-test. P-value: ***<0,001.

(C) Number of soft agar colonies/well induced by overexpression of constitutively activated Mek (MEKdd) or a control Empty vector (EV) in $Arf^{-/-}$ (Arf $^{-/-}$, left graph) and in $Arf^{-/}$- overexpressing c-Myc (Arf $^{-/-}$+cMyc, right graph) MEFs and in presence (+) or absence (-) of Mek inhibitor AZD6244. Shown is mean + S.D. of technical triplicate experiment. Statistical significance was calculated by Student's T-test. P-value: ***<0,001.

(D) Quantification of the number of soft agar colonies/well formed by *S*. Typhimurium infected or not infected Arf-deficient MEFs overexpressing c-MYC while cultured in soft agar in the presence of various Akt inhibitors, as indicated. 0.03% DMSO: control; 3.33μM AKTVII; 3.33μM A6730; 0.5 μM GDC0068; 1μM H89. The inhibitors were used at concentrations that did not affect growth of the MEFs in two-dimensional cultures. Shown is mean + S.D. of technical triplicate experiments. Statistical significance was calculated by Student's T-test. P-value: ** P<0.01, *** P<0.001.

(E) Quantification of the number of soft agar colonies/well formed by *Arf*-deficient MEFs overexpressing c-Myc and transduced with shRNA for PTEN (PTEN) or control shRNA for Luciferase (Luc.) or control shRNA of Empty vector (EV). This experiment was performed in the presence or absence of Akt inhibitor AKTVIII, as indicated. Shown are mean numbers of colonies +S.D. Statistical significance was calculated by Student's T-test. P-value: *** P<0.0001.

Figure S6



Function	Ingenuity Score
Endocrine System Disorders, Gastrointestinal Disease, Immunological Disease	37
Cancer, Cellular Development, Cellular Grow th and Proliferation	31
FunctionMetabolism, Endocrine System Development and Amino Acid Metabolism, Drug	19

Figure S6: *Salmonella*-mediated activation of Akt and Map kinase pathways drives transformation in pre-transformed cells. Related to Figure 6.

(A) Western blot of MEFs isolated from the 3rd generation soft agar colonies (MEFs from two independent cultures called 3rd generation 1, and 3rd generation 2) and compared to the original MEFs that were never exposed to S. Typhimurium. The MEFs were grown under identical conditions before analyses. The 3rd generation MEFs shows sustained Akt phosphorylation, and no detectable increase in Mapk phosphorylation. pAkt: phosphorylated Akt at Ser473; pMapk: phospho-p44/42 Mapk; Akt staining is used as loading control.

(B) Top 3 functional pathways identified by Ingenuity analysis of overlapping differently expressed genes of the 3rd generation MEFs with a history of *Salmonella* infection.

(C) Network of the top 3 scored functional clusters as analyzed by Ingenuity software on the 374 combined up and down regulated genes as depicted in Figure S7A. Grey: focus molecules. White: connecting molecules identified by Ingenuity. Green circle: Akt, Red circle: ERK/Mapk. Continuous arrows indicate direct interaction. Dashed arrows indicated indirect interaction.

Table S1

	Patient Code	Sex	Age(yr)	Cytokeratin-8	TP53	c-MYC	PCR fliC	PCR staA
India	T14-60099 A1	F	56	+++	+++	+/++	Positive	Positive
	T14-60100 A1	F	45	+++	+++	++	Positive	Positive
	T14-60102 A1	F	45	+++	+++	++	Positive	Positive
	T14-60103 A1	F	70	+++	ND**	-	Positive	Positive
	T14-60104 A1	F	65	-	-	+	Positive	Positive
	T14-60105 A1	F	50	+/++	+++	+	Positive	Positive
	T14-60106 A1	F	55	-	+/++	+	Positive	Positive
	T14-60107 A1	F	52	+++	+++	++	Positive	Positive
	T13-60082 A1	F	50	+++	+/+++	++	Positive	Positive
	T13-60081 A1	F	60	+++	+	++	Positive	Positive
The Netherlands	T94-04789	F	79	+++	+++	-	Negative	Negative
	T07-05804	F	57	+++	+/+++	-	Negative	Negative
	T89-02535	F	66	++	+++	-	Negative	Negative
	T96-03921	F	64	+++	++/+++	+	Negative	Negative
	T96-08672	F	44	+++	-	-	Negative	Negative
	T12-04617	F	57	+++	+++	-	Negative	Negative

Table S1: Pathology of gallbladder samples. Related to Figure 1.
Pathology diagnosis of gallbladder carcinoma samples collected in India (top) and in the Netherlands (bottom). The patient code (given by the NKI CFMPB facility), patient sex and age are given. A semi-quantitative score for the intensity of staining is given. -: negative staining; +/+++ indicates variable staining from detectable to high within on tissue; ND**: not detectable due to poor sample.

Table S2

	Patient Code	Sex	Age(yr)	P-AKT	P-ERK
India	T14-60099 A1	F	56	-	-
	T14-60100 A1	F	45	++/+++	++/+++
	T14-60102 A1	F	45	++/+++	+++
	T14-60103 A1	F	70	-	-
	T14-60104 A1	F	65	-	-
	T14-60105 A1	F	50	++	++
	T14-60106 A1	F	55	-	+++
	T14-60107 A1	F	52	ND*	+++
	T13-60082 A1	F	50	++/+++	+++
	T13-60081 A1	F	60	+++	++/+++
The Netherlands	T94-04789	F	79	++	-
	T07-05804	F	57	+/++	-
	T89-02535	F	66	+/++	-
	T96-03921	F	64	+++	+/+++
	T96-08672	F	44	-	-
	T12-04617	F	57	+	+/+++

Table S2: IHC for activated AKT and ERK in gallbladder carcinoma from patients from India and the Netherlands. Related to Figure 6.
Pathology diagnosis of gallbladder carcinoma samples collected in India (top) and in the Netherlands (bottom), as indicated. Patient code, sex and age are indicated. A semiquantitative score for the intensity of staining is given. -: negative staining; +/+++ indicates variable staining from detectable to high within on tissue; ND*: not detectable due to poor sample.

Table S3

gene name	bp position	from	to	aa change	function	
Ankrd44	54705179	G	C	A975G	Ankyrin Repeat Domain-Containing Prtoein 44	C1 vs Control
CDH11	105197795	A	T	V147D	Cadherin 11	C1 vs Control
	105197825	C	T	R137K		C1 vs Control
	105188571	T	G	D311A		C2 vs Control
	105188586	T	C	N306S		C2 vs Control
Fgd6	93506815	G	A	R262H	FYVE, RhoGEF and PH Domain Containing 6	C1 vs Control
Syne2	77063635	G	T	R2441L	Spectrin Repeat Containing Nuclear Envelope 2	C2 vs Control

Table S3: Genes with point mutation acquired after *Salmonella*-mediated cell transformation. Related to Figure 6.

Paired-end RNA-sequencing of two independent 3rd generation MEFs grown in two dimensions (MEFs Infected C1 and Infected C2) was compared to the original MEFs (MEFs control) cultured under identical conditions. The sequences were used to detect point mutations and gene expression differences. The table shows the genes that acquired point mutations during the transformation by Salmonella infection and culturing for three generations. The gene function and the effect on the amino acid sequence of the respective genes are indicated. Infected C1 or Infected C2: mutation in the culture from sample of infected MEFs 1 and 2, respectively. Only one gene (CDH11) is found mutated in both samples.

Supplemental Experimental Procedures

Specimens collection and extraction of genomic DNA

A total of 187 tissue samples from benign gallbladder diseases, 150 from corpses with no gallbladder diseases and 54 from gallbladder carcinoma tissues were collected by the Department of General Surgery, Pathology and Forensic Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. For processing of samples, two sets of samples were collected in 5 ml sterile tubes containing 3 ml normal saline (0.90% NaCl) and 3 ml of formalin solution (10% Neutral Buffered), for DNA isolation and immunohistological studies. Extraction of genomic DNA was performed by following the standard Phenol-chloroform method (Chomczynski and Sacchi, 1987). Hundred ng of total DNA was subjected to PCR using a primer set specific to the flagellin (fliC) of S. Typhi following the method described by (Song et al., 1993) which was further modified by (Frankel, 1994) and putative fimbrial (staA) gene of S. Typhi following the method described by (Pratap et al., 2013).

PCR assay

The reaction mixture for the first-round PCR contained 10 pmol of each primer: for fliC ST1 (5'-ACTGCTAAAACCACTACT-3') and ST2 (5'-TTAACGCAGTAAAGACAG-3'); for staA F1 (5'-TGG TTA CAT GAC CGG TAG TC-3') and R1 (5'-TAG CTG CCG CAA TGG TTA TG-3') (Pratap et al., 2013). The amplification reaction was performed in a thermal cycler (Biometra, Gottingen, Germany) with initial denaturation temperature at 94°C for 5 min followed by 35 cycles for 1 min and denaturation at 94°C, 1 min annealing at 57°C, and 1 min extension at 72°C, with a final extension at 72°C for 7 min. The nested PCR master mix contained 10 pmol of each primer for fliC ST3 (5'-AGATGGTACTGGCGTTGCTC-3') and ST4 (5'-TGGAGACTTCGGTCGCGTAG-3'); for staA F2 (5'-CAT CGG CAC GAA CGT AAG AC3') and R2 (5'-TC AAG CGA CTG ATG GTG ACG -3') and 2 µl of DNA template (amplified product of the primary cycle). Thermal cycling was carried out as described for first-round PCR, except that the annealing temperature was set to 65°C. The amplification was repeated 2-3 times to ensure that the amplification obtained with the primers was reproducible and consistent. Amplified DNA was loaded on agarose gels (1.5% w/v) and bands were visualized in a gel documentation system (Alfa Imager 2200, Alfa Innotech Corporation, California, USA). Although all essential precautions were taken to avoid laboratory contamination, known positive (DNA from reference strain of S. Typhi, MTCC 3216 and S. Typhi P-Stx-12) and negative controls (distilled water) were included to exclude contamination in each set up. The amplified products of fliC and staA were sent for partial sequencing to Bangalore Genei, India. Sequences of the above amplified product were analyzed using BLAST N (http://www.ncbi.nlm.nih.gov/BLAST/) to verify identity of the sequences as S. Typhi.

Immunohistochemistry, Microscopy and Antibodies

Immunohistochemistry of human gallbladder carcinoma samples was performed on a BenchMark Ultra autostainer (Ventana Medical Systems). Briefly, paraffin sections

were cut at 4 μm, heated at 75°C for 28 minutes and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 36 minutes at 95oC (p-Akt, Phospho-S6 R and CAM5.2) or 64 minutes at 95oC (p-Erk and c-Myc). Cytokeratin 8 was detected using clone CAM 5.2 (1:20 dilution, 32 minutes RT, BD BioSciences), Phospho-S6 R with clone D68F8 (1:500 dilution, 32 minutes RT, Cell Signalling), Phospho P-Akt (Ser473) using clone D9E (1:8000 in PBS/1% BSA/1,25% NGS, o/n at 4°C, Cell Signaling), Phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204) using cloneD13.14.4E (1/400 dilution, 1 hour RT, Cell Signaling), and c-MYC using clone 9E10 (1:50 dilution, 16 minutes incubation RT, NKI Protein Facility). Bound antibody (CAM5.2, Phopho-S6, P-AKT and P-ERK) was detected using the UltraView Universal DAB Detection Kit (Ventana Medical Systems), while c-MYC detection was visualized using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin.

Mouse tissues and gallbladder organoids were fixed in EAF fixatives (ethanol/acetic acid/formaldehyde/saline at 40:5:10:45 v/v) and embedded in paraffin. Sections at 2μm thickness were stained with hematoxylin and eosin (HE) according to standard procedures and were reviewed with a Zeiss Axioskop2 Plus microscope.

The immunohistochemistry of organoids was performed for cytokeratin 8 (clone: Throma-1, Uni. of Iowa, at dilution of 1:800), E-cadherin (clone: 610182, BD Bioscience, at dilution of 1:400), GFP (clone ab6556 from Abcam) and Salmonella (rabbit polyclonal anti- S. Typhimurium LPS, Difco, Detroit, MI, at dilution 1:1000).

For Electron Microscopy analysis the organoids were fixed in Karnovsky's fixative and successively treated with 1% Osmiumtetroxide in 0,1M cacodylate-buffer. The organoid pellets were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series, embedding in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, U.S.A), sectioned and stained with Ultrastain 2 (Leica, Vienna, Austria) and analyzed with a CM10 electron microscope (FEI, Eindhoven, the Netherlands).

For Confocal Microscopy analysis, the organoids were fixed with 4% formaldehyde and permeabilized with 0.1% Triton. The cell nuclei were stained with DAPI and actin with Alexa Fluor 488 Phalloidin (Life technologies). Acquisition of z-stacks was obtained by a Leica Confocal SP5 microscope.

Antibodies used for Western blot analyses were against phosphorylated AKT (Ser473) (clone D9E, Cell Signaling), total AKT (Cell Signaling), phosphorylated MAPK (Thr202/Tyr204) (Phospho-p44/42 MAPK (Erk1/2), Cell Signaling), total MAPK (p44/42 MAPK (Erk1/2), Cell Signaling) and ACTIN (β-Actin, Sigma).

Animal experiments

For tumor growth experiments, Rag1$^{-/-}$γc$^{-/-}$ mice (bred in-house) were implanted s.c. on the flank with 5x106 MEFs in 100 μl PBS. Mice with tumors larger than 1.5cm or ulcerating tumors were euthanized. For wild type and/or *ΔprgH* S. Typhimurium 14028 infection experiments, Apc$^{+/min}$ FVB mice were backcrossed to 129Ola mice to

generate a mixed F1 that is prone to develop small intestinal adenomas and survives S. Typhimurium infection. The Apc status was confirmed by tail DNA analyses (de Wind et al., 1998). At the age of 6-8 weeks, mice were starved for 4h before receiving an oral gavage of streptomycin (7.5mg in 100µl PBS per mouse). One day later, the mice orally received ~109 S. Typhimurium in 100µl PBS after 4h of starvation. The health status of infected mice was continuously monitored. After maximal 50 weeks, mice were sacrificed for histopathological analyses.

Gallbladder organoids, MEFs, Salmonella infection and anchorage-independent assay

Gallbladder organs from Ink4b-Arf-Ink4a KO-mice on FVB background (Krimpenfort et al., 2007) were passed through a cell strainer to generate single cells and embedded in Matrigel (growth factor reduced, phenol red free, BD Biosciences). The embedded cells were cultured in Advanced DMEM/F12 medium (Invitrogen) with 10mM HEPES, 2mM Glutamax, 1X B27 supplement, 1X N2 supplement (all from Invitrogen), 204.8 µg/ml of N-Acetylcystein (Sigma) and 50 ng/ml of mouse recombinant EGF (Biosource). Different from the previous published protocols (Koo et al., 2012; Sato et al., 2009), Noggin and RSpondin were not included in the medium since they attenuated the growth of organoids from gallbladder tissue.

Mouse Embryo Fibroblasts (MEFs) derived from wild type or Arf-deficient C57BL/6 mice were cultured at 37°C in 5%CO2 in DMEM medium (Invitrogen). MEFs overexpressing c-MYC were generated by retroviral transduction using a pLZRS-GFP (ires)-HA backbone. DsRed-S. Typhimurium SL1344 strain was a gift from S. Meresse (Meresse et al., 1999). S. Typhimurium SL14028 wt, PrgH and SopB-deficient and S. Typhimurium SL1344 SopBSopE-SopE2-SptP deficient strains (Zhou et al., 2001) were provided by D. Holden. Salmonella Typhi was a gift of R. Arens (LUMC, Leiden). Salmonella infection of the MEFs was performed as previously described (Kuijl et al., 2007). Briefly, the various Salmonella enterica strains were grown overnight at 37°C in LB medium and supplemented with 100µg/ml Ampicillin when required. The next day, the bacteria were subcultured at a dilution of 1:33 in fresh LB medium and incubated for 3.5h at 37°C while shaking. MEFs were infected in DMEM medium without antibiotics for 15min at 37°C in a tissue culture chamber, and then cultured in the presence of 100 µg/ml gentamicin (GIBCO) for 1 hour followed by 10µg/ml gentamicin for another 2 hours to eliminate extracellular bacteria. Infection rates of ∆sopB and wt SL14028 Salmonella were matched by using mCherry-positive strains and flow cytometric analyses of mCherry-positive MEFs. Using 3.25 x 106 bacteria/ml for wt and 6.5 x 106 bacteria/ml for the ∆sopB mutant strain yielded equal rates of infected cells.

The infection rates of SopB-SopE-SopE-SptP deficient and wt SL1344 were matched by infecting the MEFs for 2 h with the sopB-sopE-sopE-sptP- mutant and counting the number of Salmonella internalized and residing in phagosomes labelled with the marker Lamp1 as detected by confocal microscopy. Using 9.00x105 bacteria/ml for wt and 7.5x106 bacteria/ml for the sopB-sopE-sopE-sptP- mutant strain yielded equal

number of internalised Salmonella per cell. The infection with *ΔprgH* S. Typhimurium 14028 was performed with 5.8x106 bacteria/ml. The infection with S. Typhi was performed with 3.25 x 106 bacteria/ml.
For S. Typhimurium infection of gallbladder organoids, the organoids were dissociated into single cells by incubating for 15 minutes at 37°C in TrypLE express (Invitrogen). The single cells were pelleted by centrifugation at 1000rpm and infected in Advanced DMEM/F12 medium containing 3.0x105 wild type DsRed-SL1344 S. Typhimurium for 15 minutes at 37°C. After 3 washes with Advanced DMEM/F12 medium containing 100 μg/ml gentamicin, the single cells were embedded in Matrigel with complete or B27-, N2- and N-acetylcystein deprived medium to reform the organoids in the presence of 10 μg/ml gentamicin to prevent overgrow of released bacteria.
Anchorage-independent cell growth and number of soft agar colonies were assessed after 2 weeks of culture. Colony numbers were obtained with the GelCount (Oxford Optronix, UK). Colony images were obtained with Zeiss AxioObserver Z1 inverted microscope equipped with a cooled Hamamatsu ORCA AG Black and White CCD-camera and/or with Leica Confocal SP5 microscope.

Constructs and plasmids
Expression of myristoylated AKT (Kohn et al., 1998) and of MEK mutant MEKdd (*Irie et al., 2005*) was obtained by transduction of MEFs with pBabePuro-MyrAkt and pBabePuro-MEKdd respectively. The shRNA-pRS-puro-PTEN vector to silence PTEN by transduction of MEFs was a gift from Dr. K. Nacerddine (*Nacerddine et al., 2012*). Control shRNA used were shRNA-pRS-puro-Luciferase and shRNA-pRS-puro-Empty. When required, the cells were selected in DMEM medium (Invitrogen) supplemented with 10% Fetal Calf Serum and 2 μg/ml of puromycin.

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. Before infection, MEFs were pretreated with inhibitors for 1 hour. S. Typhimurium infection occurred in presence of inhibitors. After infection, MEFs were seeded into soft agar containing inhibitors at the final concentrations indicated. Inhibitors used for anchorage independent assays were AKTVIII (Merck Millipore), A6730 (Sigma-Aldrich), H89 (Merck Millipore), GDC0068 (Selleckchem) for AKT inhibition and AZD6244 (Selleckchem), PD0325901 (Selleckchem) and GSK1120212 (Selleckchem) for MEK inhibition. Inhibitors were used at 3.3μM (AKTVIII, A6730, AZD6244), 1μM (H89), 0.5μM (GDC0068) and 0,1uM (PD0325901, GSK1120212) where no effects on MEF proliferation under normal tissue culture conditions and on Salmonella growth were detected.

Deep sequencing

MEFs cells isolated from third generation soft agar colonies were cultured in monolayer under identical conditions as the non-infected control MEFs. The MEFs with identical morphology to the original non-infected MEFs were selected by light microscopy and successively lysed in TRIzol (Invitrogen). The extracted total RNA samples were sequenced on Illumina Genome Analyzer IIx or HiSeq 2000 platforms. The paired-end sequence reads (+/- 25 million paired end reads per sample) were mapped against the mouse genome (NCBI build 37) using Tophat 2.0.6 software. The insert size was determined using Bowtie (version 0.12.9) software. Cufflinks (version 2.1.1) software was used to determine the gene expressions. Cufflinks normalizes for the total number of reads per sample as well as the length of a gene. Ratios were calculated by dividing the infected samples against the MEFs control. Only point mutations unique to the respective cultures were considered. Genes with at least a 3-fold increase and 10-fold decrease in Fragments Per Kilobase of exon per Million fragments mapped (FPKM), compared with non-infected control cells, were considered differentially expressed and selected for subsequent network analysis by Ingenuity Software (http://www.ingenuity.com).

Statistics

Student's T-test values were calculated by Microsoft Excel program. $\chi 2$ test values were calculated by Quantpsy (http://www.quantpsy.org/chisq/chisq.htm). Fisher Exact tests were calculated by Vassarstats (http://vassarstats.net). ANOVA and post-hoc Bonferroni test values were calculated by using GraphPad Prism 6.01 software.

Supplemental References

6

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