

**Inhibition of NF- κ B signalling by a family of type III
secretion system effector proteins during *Salmonella*
infection**

Elliott James Jennings

Medical Research Council Centre for Molecular Bacteriology and Infection
Department of Medicine
Imperial College London

Submitted for the degree of
Doctor of Philosophy

Supervised by Dr. Teresa L. M. Thurston and Prof. David W. Holden

September 2018

Abstract

During *Salmonella* infection, bacterial proteins called 'effectors' are translocated into host cells by two type III secretion system apparatuses encoded by *Salmonella*-pathogenicity island 1 and 2. These effectors manipulate host cell processes to facilitate the formation of an intracellular replicative niche, to prevent bacterial clearance, and ultimately promote bacterial transmission to another susceptible host. A subset of SPI-2 T3SS effector proteins manipulate innate immune signalling pathways thereby preventing formation of an appropriate immune response. In this thesis, I identify three related effector proteins - GtgA, GogA, and PipA, as sufficient to inhibit NF- κ B signalling when expressed ectopically. Furthermore, I demonstrate that GtgA, GogA, and PipA are zinc metalloproteases that inhibit NF- κ B signalling by cleaving the NF- κ B transcription factor subunits p65, cRel, and RelB, but not NF- κ B1 (p105/p50) or NF- κ B2 (p100/p52). Accordingly, in *Salmonella*-infected cells, p65 was cleaved dependent on *gtgA*, *gogA*, and *pipA* leading to inhibition of NF- κ B signalling.

To investigate the molecular basis for substrate recognition, mutational analysis of residues in close proximity to the p65 cleavage site (G40/R41) was done and showed that the P1' residue (R41 in p65) is a critical determinant of substrate specificity. In NF- κ B1 and NF- κ B2, a proline residue is present at the corresponding site and this residue prevents cleavage by GtgA, GogA, and PipA. I also present the crystal structure of GtgA alone and in complex with the N-terminal domain of p65. The crystal structure demonstrates the importance of the P1' residue in substrate specificity and supports a model of DNA mimicry as the mechanism of substrate recognition. This thesis therefore provides detailed insight into the functions and mechanism of substrate recognition, for a family of previously uncharacterised *Salmonella* virulence proteins.

Acknowledgements

First, I would like to thank my two supervisors - Teresa and David, for their advice, guidance, and mentorship, as well as for affording me opportunities to grow as a person and a scientist. I would also like to thank you both for your patience and understanding when I developed RSI in my first year. I know of others who were in a similar position with less understanding supervisors, so your support was very much appreciated.

I would also like to thank Diego for taking me under his wing at the Crick and for teaching me all things crystallographic. Working alongside you has definitely been a highlight! Thanks also to Katrin without whom the collaboration would not have been possible.

To all members of the Holden and Thurston lab, past and present – Regina, GG, Eric, Luciano, Charlotte, Camilla, Sophie M., Ondrej, Xin, Mei, Xiujun, Ethel, Alan, Hai Xia, and Julian, I say thank you! Thank you for all the help you all gave me, as well as for being a fantastic bunch of colleagues to work with. I'd also like to thank Megan, for keeping the lab running smoothly, as well as all other members of CMBI2.

To all my friends outside the lab, thanks for your willingness to grab a beer - spending time with you guys was always a welcome escape from the lab. A huge thanks also to Mairi, who shared much of this adventure with me. I am glad that you were a part of it and am incredibly appreciative of the support you gave. The last few years would not have been the same without you.

Lastly to my parents and to my sister Elise. Thanks for your continued support and encouragement, even though I know that most of the time it probably seemed as though I was speaking gibberish! At least you all now know what a Western blot is!

Declaration of originality

I hereby declare that the work presented in this thesis is my own original work. Any contribution to this thesis by others, published or otherwise, is acknowledged throughout the text with all references listed in the bibliography.

This thesis contains material, including figures and intellectual content, which is published in:

JENNINGS, E., THURSTON, T. L. M. & HOLDEN, D. W. 2017. *Salmonella* SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences. *Cell Host Microbe*, 22, 217-231.

JENNINGS, E., ESPOSITO, D., RITTINGER, K. & THURSTON, T. L. M. 2018. Structure–function analyses of the bacterial zinc metalloprotease effector protein GtgA uncovers key residues required for deactivating NF- κ B. *Journal of Biological Chemistry*.

Copyright declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose. When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Table of Contents

Abstract	2
Acknowledgements	3
Declaration of originality	4
Copyright declaration	5
List of Figures	10
List of Tables	12
Abbreviations	13
1 Introduction	16
1.1 <i>Salmonella</i> pathogenesis	16
1.1.1 Human diseases caused by <i>Salmonella</i>	16
1.1.1.1 Non-typhoidal <i>Salmonella</i> : gastroenteritis	17
1.1.1.2 Invasive non-typhoidal <i>Salmonella</i>	18
1.1.1.3 Typhoid and paratyphoid fever	18
1.1.2 Experimental models of Salmonellosis	19
1.1.2.1 Animal models of typhoidal <i>Salmonella</i>	19
1.1.2.2 Animal models of human gastroenteritis	20
1.1.2.3 <i>In vitro</i> tissue culture models	20
1.1.3 Molecular determinants of <i>Salmonella</i> virulence	21
1.2 Cell autonomous immunity	22
1.2.1 Autophagy	24
1.2.2 Innate immune signalling	24
1.2.2.1 The NF- κ B signalling pathway	25
1.2.2.2 The MAPK signalling pathway	27
1.2.3 Host cell death	28
1.2.3.1 Apoptosis	28
1.2.3.2 Necroptosis	29
1.2.3.3 Pyroptosis	30
1.3 <i>Salmonella</i> T3SS effector proteins	30
1.3.1 SPI-1 T3SS effector proteins	32
1.3.1.1 Epithelial cell invasion	32
1.3.1.2 Induction of intestinal inflammation	33
1.3.2 SPI-2 T3SS effector proteins	35
1.3.2.1 SCV membrane dynamics	35

1.3.2.2	SCV intracellular positioning	39
1.3.2.3	Lipid droplets and cytoplasmic aggregates	39
1.3.2.4	Cytoskeletal remodelling	40
1.3.2.5	Modulation of innate immune signalling pathways	40
1.3.2.6	Manipulation of the adaptive immune system	43
1.3.2.7	Uncharacterised SPI-2 T3SS effectors	43
1.4	Zinc metalloprotease type III secretion system effector proteins	44
1.4.1	GtgA, GogA, and PipA	44
1.4.2	NleC – a bacterial zinc metalloprotease T3SS effector	46
1.4.3	Zinc metalloproteases	48
1.5	Aims of the project	49
2	Materials and methods	52
2.1	Materials	52
2.1.1	Bacterial strains	52
2.1.2	Plasmids	52
2.1.3	Antibodies	52
2.1.4	Mammalian cell lines	52
2.2	Methods	58
2.2.1	Bacterial growth conditions	58
2.2.2	Plasmid DNA purification	58
2.2.3	Isolation of bacterial genomic DNA	58
2.2.4	RNA extraction and complementary DNA synthesis	58
2.2.5	Construction of expression vectors	59
2.2.6	Preparation and transformation of electrocompetent bacteria	60
2.2.7	One-step PCR mutagenesis	60
2.2.8	P22 phage transduction	61
2.2.9	Mammalian cell culture	61
2.2.10	DNA transfections	61
2.2.11	Mammalian cell transduction	61
2.2.12	<i>Salmonella</i> Typhimurium infection of HeLa cells	62
2.2.13	<i>Salmonella</i> Typhimurium infection of RAW264.7 macrophages	62
2.2.14	Immunoblot analysis	63
2.2.15	Coomassie Blue staining	63
2.2.16	Flow cytometry	64
2.2.17	Luciferase assays	64

2.2.18 Immunofluorescence microscopy	65
2.2.19 Protein purification	65
2.2.20 Cleavage of expression tags	66
2.2.21 Size-exclusion chromatography	67
2.2.22 Measurement of protein concentration	67
2.2.23 <i>In vitro</i> cleavage assays	67
2.2.24 LUMIER binding assay	68
2.2.25 Protein crystallisation	68
2.2.26 Structural determination	69
2.2.27 Statistical analysis	70
3 NF-κB inhibition by <i>Salmonella</i> SPI-2 T3SS effector proteins	71
3.1 Results	72
3.1.1 SPI-2 T3SS-dependent NF- κ B inhibition in <i>Salmonella</i> -infected macrophages	72
3.1.2 GtgA is sufficient to inhibit TNF- α -induced NF- κ B activation in 293ET cells	76
3.1.3 GtgA, GogA, and PipA inhibit NF- κ B signalling dependent on a functional zinc metalloprotease motif	79
3.1.4 NleC residues 281-330 are required for inhibition of IFN- β -induced ISRE signalling	85
3.1.5 GtgA, GogA, and PipA cleave the N-terminal domain of p65	85
3.1.6 GtgA, GogA, and PipA cleave p65, cRel, and RelB, but not NF- κ B1 (p50/p105), NF- κ B2 (p52/p100) or NFAT transcription factor subunits	89
3.1.7 GtgA, GogA, and PipA do not cleave NFAT transcription factor subunits	91
4 NF-κB inhibition by GtgA, GogA, and PipA in <i>Salmonella</i>-infected cells	93
4.1 Results	94
4.1.1 Construction of <i>Salmonella</i> Typhimurium <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> deletion mutant strains	94
4.1.2 Characterisation of <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> mutant strains: intracellular replication	94
4.1.3 p65 cleavage in <i>Salmonella</i> -infected cells is dependent on <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i>	97
4.1.4 Characterisation of <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> mutant strains: NF- κ B reporter activation	100
4.1.5 Translocation of GtgA and GogA into RAW264.7 macrophages is dependent on the SPI-2 T3SS	106

5 Structural insights into the substrate specificity of GtgA	110
5.1 Results	111
5.1.1 The P1' residue R41 in p65 is critical for substrate specificity of GtgA, GogA, and PipA	111
5.1.2 Crystal structure of GtgA	113
5.1.3 Crystal structure of GtgA in complex with the N-terminal domain of p65	120
5.1.4 Mutagenic analysis of GtgA-p65 interacting residues	123
6 Discussion	128
6.1 NF- κ B inhibition by SPI-2 T3SS effectors	128
6.2 Catalytically independent functions of NleC	131
6.3 GtgA, GogA, and PipA are zinc metalloproteases that cleave NF- κ B subunits	132
6.4 Physiological relevance of NF- κ B cleavage	134
6.5 Nuclear localisation of GtgA, GogA, and PipA	138
6.6 Structural characterisation of GtgA	140
7 References	147

List of Figures

Figure 1.1: Maturation of the <i>Salmonella</i> -containing vacuole in <i>Salmonella enterica</i> -infected epithelial cells.	23
Figure 1.2: The NF- κ B signalling pathway.	26
Figure 1.3: SPI-2 T3SS effector distribution across different <i>Salmonella enterica</i> serovars.	32
Figure 1.4: Functions of SPI-2 T3SS effectors.	36
Figure 1.5: Amino acid sequence alignment of GtgA, GogA, and PipA.	45
Figure 1.6: Amino acid alignment of GtgA and NleC.	47
Figure 1.7: Structural characteristics of the Zincin superfamily of metalloproteases.	50
Figure 1.8: Catalytic mechanism of zinc metalloproteases.	51
Figure 3.1: Modulation of NF- κ B signalling by SPI-2 T3SS effector mutant strains.	73
Figure 3.2: The SseK family of effector proteins inhibit the NF- κ B signalling pathway redundantly in <i>Salmonella</i> -infected macrophages.	74
Figure 3.3: GtgA, SseK1, SseK2, SseK3, SteB, and SteD are sufficient to inhibit TNF- α -induced NF- κ B activation.	78
Figure 3.4: GtgA, GogA, and PipA inhibit the NF- κ B signalling pathway.	82
Figure 3.5: NleC inhibits IFN- β -induced ISRE signalling independently of its zinc metalloprotease activity.	84
Figure 3.6: NleC residues 281 to 330 are required for ISRE reporter inhibition but not NF- κ B reporter inhibition.	86
Figure 3.7: GtgA, GogA, and PipA directly cleave the p65 N-terminal domain.	88
Figure 3.8: Substrate specificity of GtgA, GogA, PipA, and NleC.	90
Figure 4.1: Construction of <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> mutant strains.	95
Figure 4.2: <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> are not required for <i>Salmonella</i> replication in HeLa cells.	97
Figure 4.3: <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> are not required for <i>Salmonella</i> replication in RAW264.7 macrophages.	98
Figure 4.4: p65 is cleaved and degraded by the proteasome, dependent on <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> in HeLa cells and RAW264.7 macrophages.	99
Figure 4.5: Analysis of NF- κ B reporter activation by <i>gtgA</i> , <i>gogA</i> , and <i>pipA</i> mutant strains in <i>Salmonella</i> -infected cells	102
Figure 4.6: GtgA and GogA expressed from a low copy-number plasmid inhibit NF- κ B reporter activation in <i>Salmonella</i> -infected HeLa cells.	104
Figure 4.7: GtgA and GogA are translocated into RAW264.7 macrophages dependent on the SPI-2 T3SS and localise to the nucleus.	107

Figure 4.8: GFP-tagged GtgA, GogA, and PipA localise to the nucleus independent of their catalytic activity.	109
Figure 5.1: Substrate specificity of the GtgA family of effector proteins is governed by the P1' site residue.	112
Figure 5.2: The first 19 amino acids of GtgA are dispensable for GtgA's catalytic activity.	115
Figure 5.3: Size exclusion chromatography of GtgA ^{20-228 E183Q} and p65 ²⁰⁻¹⁸⁸ .	116
Figure 5.4: Crystal structure of Zn ²⁺ -free GtgA ^{20-228 E183Q} in complex with p65 ²⁰⁻¹⁸⁸ .	117
Figure 5.5: Crystal structure of Zn ²⁺ -bound GtgA ^{20-228 E183Q} .	119
Figure 5.6: The GtgA-p65 interaction interface.	121
Figure 5.7: Residues in helix α F clash in the GtgA apo structure sterically with the p65 N-terminal domain.	122
Figure 5.8: Identification of GtgA residues important for GtgA-p65 stable complex formation and <i>in vitro</i> catalytic activity.	124
Figure 5.9: Identification of GtgA residues required for NF- κ B reporter inhibition.	126
Figure 6.1: Structural comparison of GtgA and NleC.	142
Figure 6.2: Amino acid alignment of NleC and AIP56.	143
Figure 6.3: GtgA recognises NF- κ B transcription factor subunits by mimicking DNA.	144
Figure 6.4: Differential surface charge of the active site cleft of PipA.	146

List of Tables

Table 1.1: Functions of immunomodulatory <i>Salmonella</i> T3SS effector proteins	34
Table 2.1: Bacterial strains	54
Table 2.2: Plasmids	55
Table 2.3: Primary antibodies	57
Table 4.1: Expected sizes of PCR products (base pairs)	96
Table 5.1: Crystallographic data collection and refinement statistics	118

Abbreviations

ANOVA	Analysis of variance
AP-1	Activator protein-1
ATR	Acid Tolerance Response
A.u.	Arbitrary units
BAFF	B-cell activating factor
BMDM	Bone marrow-derived macrophages
cDNA	Complementary DNA
CDS	Coding DNA sequence
CIP	Calf intestinal alkaline phosphatase
CSD	C-terminal subdomain
DAMP	Damage associated molecular pattern
DAPI	4',6-Diamindino-2-Phenylindole, Dihydrochloride
DC	Dendritic cell
DD	Dimerisation domain
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FRT	FLP recombinase target
GAP	GTPase-activating protein
gDNA	Genomic DNA
GEF	Guanine exchange factor
GlcNAc	N-acetylglucosamine
H p.i.	Hours post invasion
H p.u.	Hours post uptake
HDC	Hypothetically disrupted CDS
HGT	Horizontal gene transfer
HRP	Horse radish peroxidase
I κ B	Inhibitor of κ B

IKK	Inhibitor of κ B kinase
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRF	Interferon regulatory factor
ISRE	Interferon-stimulated response element
LB	Luria Bertani
LPS	Lipopolysaccharide
LRO	Lysosomal-related organelle
LUMIER	Luminescence-based mammalian interactome mapping
M cells	Microfold cells
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MGE	Mobile genetic element
MHCII	Major histocompatibility class II
MPR	Mannose-6-phosphate receptor
MWCO	Molecular weight cut-off
NEMO	NF- κ B essential modifier
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells
NIK	NF- κ B-inducing kinase
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerisation domain
N.s.	Non-significant
NSD	N-terminal subdomain
NTD	N-terminal domain
NTS	Non-typhoidal <i>Salmonella</i>
ORF	Open reading frame
p	Phospho
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PNS	Post-nuclear supernatant
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
RHR	Rel homology region

RIG	Retinoic acid-inducible gene
RLR	RIG-I like helicases
RMSD	Root mean square deviation
ROS	Reactive oxygen species
RPS3	Ribosomal protein S3
SCF	Skp-Cullin-F box
SCV	<i>Salmonella</i> -containing vacuole
SDS	Sodium dodecyl sulphate
SEC	Size-exclusion chromatography
SEM	Standard error of the mean
SIT	<i>Salmonella</i> -induced tubule
SPI	<i>Salmonella</i> -pathogenicity island
T3SS	Type III secretion system
TAD	Transactivation domain
TCEP	Tris(2-carboxyethyl)phosphine
TEV	Tobacco etch virus
TLR	Toll-like receptor
Ub	Ubiquitin
VR	Variable region
WT	Wild-type

1 Introduction

1.1 *Salmonella* pathogenesis

Bacteria belonging to the genus *Salmonella* are Gram-negative, flagellated, rod-shaped, facultative anaerobes that cause significant morbidity and mortality in a range of vertebrate hosts. The genus comprises two species - *Salmonella bongori* and *Salmonella enterica*, with the latter divided into 6 subspecies: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV), and indica (VI). Members of each subspecies are further classified into serovars based on the immunogenicity of surface antigens including lipopolysaccharide (LPS) and flagellin (Desai et al., 2013, Grimont and Weill, 2007). Although approximately 2500 different serovars have been identified, only a small percentage of these cause disease in humans and livestock. Disease-causing serovars, most of which belong to *Salmonella enterica* subspecies enterica, vary greatly in their host range, clinical manifestation, and virulence factor repertoire. For example, typhoidal serovars including *S. Typhi* and *S. Paratyphi* are restricted to a single host and cause systemic disease. Non-typhoidal serovars such as *S. Typhimurium* and *S. Enteritidis*, are frequently transmitted between different hosts including humans, sheep, cattle, horses, pigs, and birds, and cause gastrointestinal disease characterised by gastroenteritis. A third category of serovars including porcine-adapted *S. Choleraesuis*, preferentially infect a single host, however they do still occasionally cause disease in other species.

1.1.1 Human diseases caused by *Salmonella*

Infection of humans with *Salmonella* begins following ingestion of contaminated food and water. The bacteria pass through the acidic environment of the stomach and enter the small intestine, where environmental conditions induce the expression of specific virulence factors that are required for *Salmonella*-induced invasion of intestinal epithelial cells including enterocytes and Microfold cells (M cells) (Galan and Curtiss, 1989, Clark et al., 1994). Bacteria in the lumen are also phagocytosed by CD18-positive phagocytes, which transmigrate across

the intestinal epithelium (Vazquez-Torres et al., 1999). Epithelial cell invasion and phagocytosis by transmigratory phagocytes leads to colonisation of underlying tissues including the lamina propria and Peyer's patches (Vazquez-Torres et al., 1999). At these sites, bacteria are engulfed by mononuclear phagocytes such as macrophages. The expression of another set of virulence factors by intracellular *Salmonella*, facilitates the survival and replication of bacteria within these cells (Cirillo et al., 1998).

1.1.1.1 Non-typhoidal *Salmonella*: gastroenteritis

There are estimated to be 93.8 million infections caused by non-typhoidal *Salmonella* (NTS) serovars such as *S. Typhimurium* and *S. Enteritidis*, leading to approximately 155,000 deaths, per annum (Majowicz et al., 2010). Invasion of the intestinal epithelium by NTS is a highly inflammatory process, categorised by the expression of proinflammatory cytokines, and the infiltration of large numbers of neutrophils into the intestinal lumen (Harris et al., 1972). Reactive oxygen species (ROS) produced by these neutrophils oxidises thiosulfate in the gut, generating tetrathionate, which can then be used by *Salmonella* as a terminal electron acceptor (Winter et al., 2010). Inflammation therefore allows NTS to outcompete the gut microbiota, which rely on fermentation for energy production and growth (Thiennimitr et al., 2011). Additionally, inflammation leads to disruption of the intestinal barrier, and symptoms of diarrhoea including abdominal cramps, loose and watery stools, and vomiting. Bacteria replicating in the intestinal lumen are shed in the stools of infected individuals facilitating transmission to another susceptible host.

Generally, NTS infection leading to gastroenteritis will resolve without antimicrobial treatment in approximately 1 week, however rehydration therapy might be required to replace fluids lost through diarrhoea (Chen et al., 2013). In addition, cross-reactivity between *Salmonella* antigens and those of an infected individual can lead to the development of reactive arthritis after the infection has cleared (Ajene et al., 2013).

1.1.1.2 Invasive non-typhoidal *Salmonella*

Infection with NTS serovars can also cause a more severe invasive disease in young children, the elderly, and immunocompromised individuals. Malnourished or malaria-infected children, and HIV-infected adults are particularly susceptible (Uche et al., 2017). Symptoms of gastroenteritis are usually absent and instead replaced by fever, septicaemia, hepatosplenomegaly, and respiratory problems (Feasey et al., 2012). There are 3.4 million cases of invasive NTS each year with a mortality rate of 20% (Crump and Heyderman, 2015). Invasive NTS infections are mostly constrained to the developing world including sub-Saharan Africa, and genomic analysis of clinical isolates has revealed that invasive NTS is generally caused by specific lineages of *S. Enteritidis* (Feasey et al., 2016) or *S. Typhimurium* (Kingsley et al., 2009), that have undergone significant genome degradation.

1.1.1.3 Typhoid and paratyphoid fever

In contrast to NTS, invasion of the intestinal epithelium by typhoidal *Salmonella* serovars including *S. Typhi* and *S. Paratyphi* generates only a mild inflammatory response. Instead of replicating in the intestinal lumen, bacteria enter the blood and disseminate to secondary sites of infection such as the spleen, the liver, the bone marrow, and the gall bladder. Secretion of bile from the gall bladder, periodically reseeds the intestine with *Salmonella* bacteria which are then shed in the faeces (Raffatellu et al., 2008).

Although rare in developed countries, typhoidal *Salmonella* serovars are prominent in the developing world; it has been estimated that there are approximately 27 million cases of typhoid and paratyphoid fever per annum (Buckle et al., 2012, Crump et al., 2004). Symptoms include fever, hepatosplenomegaly, and abdominal pain and the mortality rate is approximately 10 to 30%. Appropriate antimicrobial therapy, reduces the mortality rate to between 1 to 4% (Buckle et al., 2012), however like many other globally important Gram-negative bacterial pathogens, multi-drug resistance is a growing problem (Klemm et al., 2018).

1.1.2 Experimental models of Salmonellosis

The majority of our understanding of *Salmonella* pathogenesis has been derived using a small number of laboratory-adapted *S. Typhimurium* isolates, animal models that partially replicate specific stages of Salmonellosis in humans, and a selection of human and murine tumorigenic cell lines.

1.1.2.1 Animal models of typhoidal *Salmonella*

Infection of mice with *S. Typhimurium* leads to the development of systemic disease characterised by minimal intestinal inflammation and dissemination of bacteria from the intestine to secondary sites of infection including the mesenteric lymph nodes, spleen, and liver. In wild-type mice, the murine immune system prevents overwhelming bacterial replication in these organs and symptoms of acute typhoid fever do not develop. However, bacteria are able to persist within macrophages in the mesenteric lymph nodes. Infection of wild-type mice with *S. Typhimurium* has therefore been used to model the chronic carriage of *S. Typhi* in humans (Monack et al., 2004). In addition, acute typhoid fever can be modelled with mice lacking the gene encoding the natural resistance-associated macrophage protein-1 (Nramp1 [also called Slc11a1]). Nramp1 is an integral membrane protein that localises to lysosomal compartments in monocytes and macrophages. It is a divalent cation transporter that limits the availability of metal ions in the lysosomal lumen, which is important in the clearance of vacuolated Gram-negative bacteria including *Salmonella* and *Mycobacterium* (Vidal et al., 1996, Wessling-Resnick, 2015). Infection of *Nramp1*^{-/-} mice with *S. Typhimurium* proceeds as in wild-type mice, however the mutant mice are unable to control bacterial replication at secondary sites of infection and quickly develop acute systemic disease (Watson and Holden, 2010). Unfortunately, these models are imperfect due to differences in the repertoire of virulence factors between *S. Typhimurium* and *S. Typhi*. One manner in which this limitation might be overcome in the future is through the use of humanised mouse models. Immunocompromised mice inoculated with human haematopoietic cells can be infected with

S. Typhi, leading to colonisation of the liver, spleen, blood, and bone marrow (Firoz Mian et al., 2011).

1.1.2.2 Animal models of human gastroenteritis

Several animal models with similarities to NTS-induced gastroenteritis in humans have also been developed. Infection of non-human primates and cattle with NTS most closely resembles the pathology and symptoms of human NTS infection, however these models are used less frequently than a murine model of colitis. In this model, mice are pre-treated with the antibiotic streptomycin to severely deplete the intestinal microbiota, and are then infected orally with NTS. The reduced abundance of microbes in the gut allows *Salmonella* to invade the small intestine and replicate to high densities in the colon. This leads to localised inflammation prior to dissemination of the bacteria to secondary organs (Barthel et al., 2003). Unfortunately, this model does not resemble *Salmonella*-induced gastroenteritis in humans as closely as the primate and bovine models, as much of the microbiota has been removed and bacterial replication occurs in the lumen of the colon and not the small intestine (Higginson et al., 2016). However, the streptomycin pre-treated mouse model is lower in cost and benefits from the ability to utilise transgenic and knockout mice models to investigate host factors important for Salmonellosis.

1.1.2.3 In vitro tissue culture models

Salmonella infection of tumorigenic cell lines and primary cells, has also delivered many insights into the virulence factors and mechanisms used by *Salmonella* to replicate within host cells. Epithelial cell lines such as HeLa, Caco-2, and Intestine-407 cells have been used to investigate *Salmonella*-induced bacterial invasion as well as the formation and maturation of the *Salmonella*-containing vacuole and *Salmonella*-induced tubules. In addition, human and murine macrophage cell lines such as THP1, J774, and RAW264.7 cells, as well as primary bone marrow-derived macrophages (BMDM) have been used to identify bacterial virulence factors required for survival and growth within macrophages.

1.1.3 Molecular determinants of *Salmonella* virulence

The *Salmonella* genome encodes a suite of virulence factors including flagella, adhesins, the *phoP/phoQ* regulon, and two type III secretion systems (T3SS) that are important in the colonisation of the gastrointestinal tract, and the dissemination of bacteria to secondary sites of infection. Many virulence factors are encoded on mobile genetic elements (MGEs) including pathogenicity islands, prophages, and plasmids that were acquired by horizontal gene transfer (HGT). The repertoire of virulence factors that are present and functional in different serovars varies greatly. Generally, serovars which are host-adapted or host-restricted, have a reduced number of functional virulence factors, suggesting that those that are absent or non-functional are important only in specific hosts or during *Salmonella* colonisation of specific host tissues (Nuccio and Baumler, 2014). In addition, some serovars including *S. Typhi* and *S. Paratyphi*, have acquired distinct virulence factors such as the Vi polysaccharide capsule and typhoid toxin that are important for pathogenesis (Johnson et al., 2018). Different *Salmonella* serovars have up to 21 pathogenicity islands, the most well-known of these are SPI-1 and SPI-2, which each encode a distinct T3SS apparatus. T3SSs are needle-like apparatuses that assemble across bacterial and host membranes, and inject bacterial proteins called 'effectors', through a pore that is assumed to form in the host membrane, into host cells (Portaliou et al., 2016). In addition to a T3SS apparatus, SPI-2 also contains genes required for tetrathionate respiration, however these genes were likely acquired in a separate horizontal gene transfer event (Hensel et al., 1999).

Following ingestion, bacteria enter the stomach where they initiate the Acid Tolerance Response (ATR), which induces resistance to low pH environments (Álvarez-Ordóñez et al., 2012). Bacteria that successfully pass through the stomach enter the small intestine and induce the expression of long appendages called flagella that sense extracellular stimuli and rotate to provide motility (Berg and Anderson, 1973), hair-like structures called fimbriae that facilitate the attachment of bacteria to intestinal epithelial cells (van der Velden et al., 1998), and the SPI-1 T3SS. Non-flagellated *Salmonella* mutants are defective in their ability to

penetrate the mucus layer and migrate towards the intestinal epithelium in the streptomycin pre-treated mouse model of colitis (Stecher et al., 2004), whereas *Salmonella* mutants deleted of fimbrial operons are attenuated in their ability to colonise *Nramp1*^{+/+} mice (Weening et al., 2005). These data suggest that these virulence factors are important in the colonisation of the intestinal epithelium.

The SPI-1 T3SS translocates effector proteins across the host cell plasma membrane, where they induce remodelling of the host cell actin cytoskeleton leading to engulfment of bacteria into the *Salmonella*-containing vacuole (SCV) (**Figure 1.1**) (Patel and Galan, 2005). SPI-1 T3SS effectors also induce intestinal inflammation (Bruno et al., 2009) leading to neutrophil recruitment and tetrathionate production, which is beneficial for replication of NTS in the intestinal lumen (Winter et al., 2010). Studies using SPI-1 T3SS-null mutant strains demonstrate that the SPI-1 T3SS is important for colonisation of the small intestine, but it is dispensable for survival and replication of *Salmonella* at extraintestinal sites (Galan and Curtiss, 1989).

As the SCV matures, the low pH and low nutrient conditions induces the expression of the SPI-2 T3SS and its associated effector proteins (**Figure 1.1**) (Cirillo et al., 1998). The SPI-2 T3SS translocates effector proteins across the vacuole membrane into the host cell where they manipulate endosomal and lysosomal trafficking as well as the actin cytoskeleton, and modulate innate and adaptive immunity (Jennings et al., 2017). The functions of SPI-2 T3SS effector proteins are required for the intracellular survival and replication of *Salmonella* phagocytosed by macrophages (Bispham et al., 2001, Carnell et al., 2007), and for colonisation of the spleen in murine models of acute typhoid fever (Hensel et al., 1995, Shea et al., 1999).

1.2 Cell autonomous immunity

The innate immune system mounts an immediate, non-specific defence against invading pathogens prior to the initiation of a slower, tailored, adaptive immune response. Although

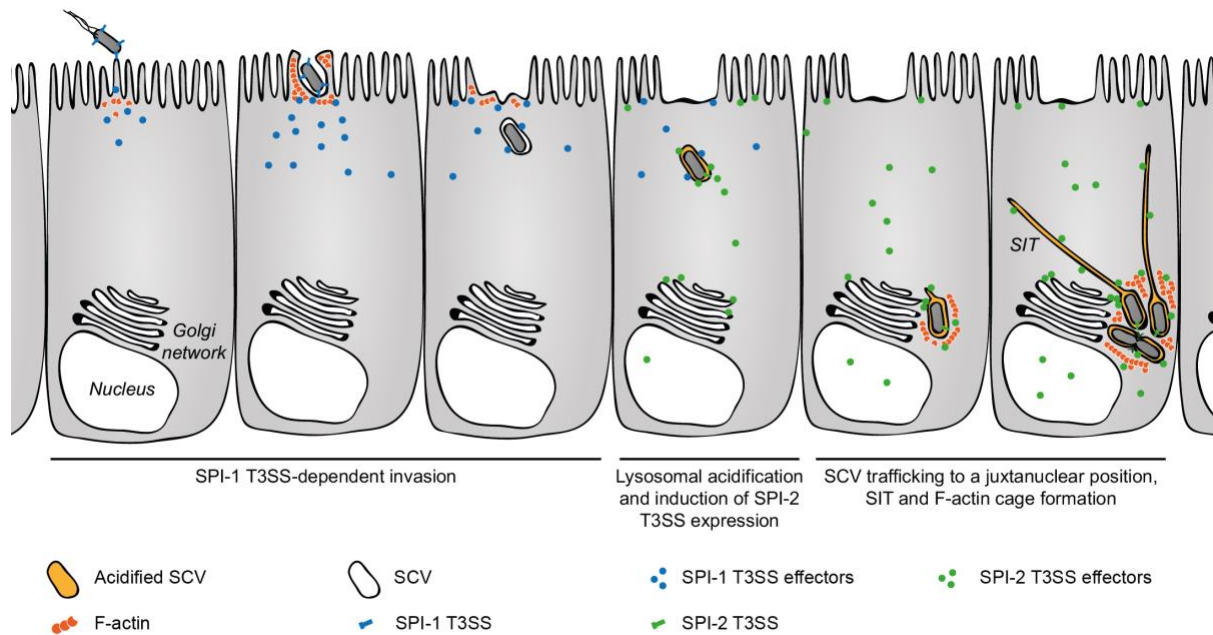


Figure 1.1: Maturation of the *Salmonella*-containing vacuole in *Salmonella enterica*-infected epithelial cells.

Translocation of SPI-1 T3SS effector proteins induces actin polymerisation at the site of translocon insertion leading to membrane ruffling and macropinocytosis. Following internalisation into the SCV, membrane ruffling is reversed and expression of the SPI-1 T3SS downregulated. SCV acidification leads to expression of the SPI-2 T3SS and the translocation of a second set of effector proteins into the host cell. SPI-2 T3SS effectors are required for formation of an F-actin cage surrounding SCVs in a juxtanuclear position, elongation of SITs, and dampening of innate immune responses. Abbreviations: SCV, *Salmonella*-containing vacuole; SIT, *Salmonella*-induced tubules; SPI, *Salmonella*-pathogenicity island; T3SS, type III secretion system.

specific immune cell types such as macrophages and neutrophils are integral to a strong innate immune response and pathogen clearance, all cell types possess intrinsic defence mechanisms that protect nutrient-rich intracellular compartments from pathogen exploitation. Vacuoles containing phagocytosed pathogens ultimately fuse with lysosomes subjecting the contents to an acidic pH, nutrient starvation, and lysosomal hydrolases, whereas replication of cytosolic pathogens can be restricted by several cell autonomous mechanisms including autophagy. Autophagy is a process whereby cytosolic material is enclosed in a double-membraned compartment called an autophagosome. Autophagosomes then fuse with

lysosomes and the contents are degraded. In addition, cell surface, endosomal, and cytosolic PRRs which recognise PAMPs and DAMPs generated by invading pathogens activate intracellular signalling cascades which might alter the transcriptional landscape of infected host cells and or induce one of several forms of programmed cell death.

1.2.1 Autophagy

Autophagy is a process whereby cytosolic material - either defunct organelles, misfolded proteins, or pathogens, are first enclosed in a double-membraned compartment and then targeted for lysosomal degradation. Upon initiation of the pathway, a membranous structure called a phagophore forms from the endoplasmic reticulum, and microtubule-associated light chain 3 (LC3) and gamma-aminobutyric acid receptor associated proteins (GABARAP) are lipidated and inserted into the phagophore membrane. Lipidated LC3 (also called LC3-II) and GABARAP, interact with a family of adaptor proteins including p62/SQSTM1, NDP52, optineurin, NBR1, and TAXBP1 to link the phagophore with material that is destined for autophagic clearance (Randow et al., 2013). Cytosolic viruses and bacteria can be detected by these adaptor proteins, via a ubiquitin-binding domain that detects a ubiquitin “coat” around the pathogen. However cellular damage including ruptured pathogen-containing vacuoles can also be sensed and targeted for autophagic clearance: host glycans usually constrained to the inner leaflet of vacuoles, are exposed to the cytosol following vacuolar rupture. Galectin-3, Galectin-8, and Galectin-9, three cytosolic glycan-binding proteins, interact with these exposed glycans, with Galectin-8 recruiting the autophagic adaptor protein NDP52 to the damaged vacuole directly (Thurston et al., 2012). In the absence of autophagy receptors or Galectin-8, *Salmonella* that enter the cytosol of epithelial-like cells, undergo unrestrained replication.

1.2.2 Innate immune signalling

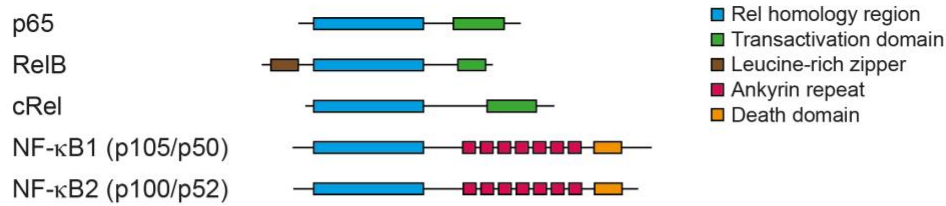
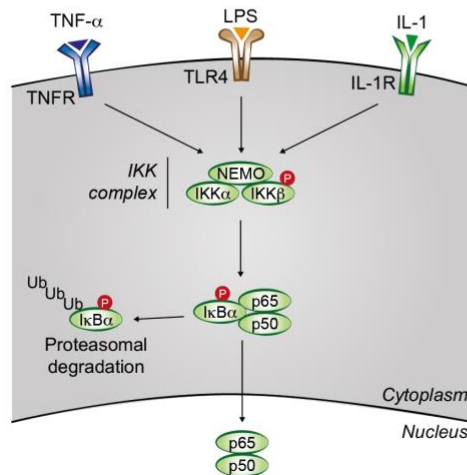
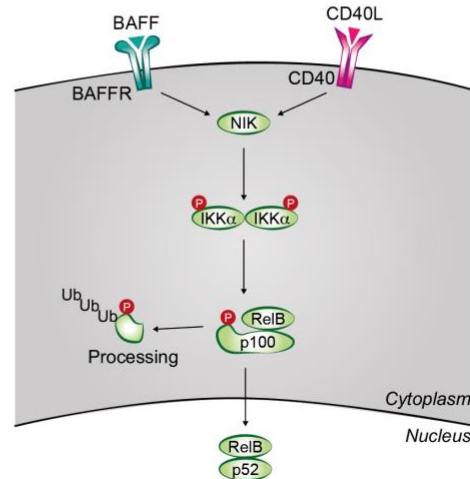
PRRs activated during bacterial infections include cell surface and endosomal Toll-like receptors (TLRs), as well as cytosolic nucleotide-binding oligomerisation domain (NOD)-like

receptors (NLRs) and retinoic acid-inducible gene (RIG)-I like helicases (RLRs). These detect PAMPs including LPS, flagellin, peptidoglycan, and bacterial RNA and DNA (Akira et al., 2006) leading to activation of intracellular signalling cascades including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and mitogen-activated protein kinase (MAPK) signalling pathways.

1.2.2.1 The NF- κ B signalling pathway

The NF- κ B signalling pathway regulates the expression of a broad range of genes involved in inflammation and immunity (Hayden and Ghosh, 2012). During Gram-negative bacterial infections, recognition of PAMPs (primarily LPS recognition by TLR4), induces a cascade of post-translational modifications leading to the accumulation of NF- κ B transcription factors in the cell nucleus. Subsequently, these transcription factors bind to palindromic nucleotide sequences near to NF- κ B-responsive genes termed ' κ B-sites', the consensus sequence of which is 5'-GGGRNWYYC-3' (N = any base, R = purine, W = adenine or thymine, and Y = pyrimidine) (Zhang et al., 2017).

NF- κ B transcription factors are homo- and hetero-dimers of five subunits: p65, RelB, cRel, NF- κ B1 (p105/p50), and NF- κ B2 (p100/p52) (**Figure 1.2A**). Although p65, RelB, and cRel are translated as mature proteins, p50 and p52 are translated as immature precursors – p105 and p100, respectively. p105 is constitutively processed to p50, whereas p100 is generally only processed to p52 following cellular stimulation. Each NF- κ B subunit contains a structurally conserved protein domain called the Rel homology region (RHR) towards their N-terminus. The RHR is required for NF- κ B binding to κ B-sites, subunit dimerisation, as well as for interactions with regulatory proteins. Structural analyses have revealed that each RHR consists of two immunoglobulin-like domains called the N-terminal domain (NTD) and the dimerisation domain (DD) separated by a short flexible linker. Specific DNA base contacts are made by residues in the N-terminal domain and the flexible linker, and positively charged residues in each of the immunoglobulin-like domains interact electrostatically with the

A NF- κ B subunits**B Canonical activation****C Non-canonical activation****Figure 1.2: The NF- κ B signalling pathway.**

(A) Domain architecture of NF- κ B transcription factor subunits. (B) Canonical activation of NF- κ B signalling. Engagement of PRRs including TNFR, TLR4, and IL-1R leads to phosphorylation of IKK β and activation of the kinase activity of the IKK complex. The IKK complex phosphorylates I κ B α , which then dissociates from NF- κ B dimers (e.g. p65-p50). Phosphorylated I κ B α is ubiquitinated and subsequently proteasomally degraded, whereas NF- κ B transcription factors translocate into the nucleus and bind to target DNA. (C) Non-canonical activation of NF- κ B signalling. Engagement of receptors including B-cell activating factor receptor and CD40 leads to phosphorylation of IKK α homodimers by NIK. IKK α then phosphorylates p100, which is subsequently processed to form p52. p52-RelB heterodimers translocate into the nucleus and bind to target DNA.

negatively charged DNA phosphate backbone (Chen et al., 1998b, Muller et al., 1995, Ghosh et al., 1995). p65, RelB, and cRel also contain transactivation domains (TAD) towards their C-terminus, which recruit transcriptional co-factors to κ B-sites. In contrast, p105 and p100 contain multiple copies of ankyrin repeats, which prevent nuclear import of these unprocessed

subunits. Generally, TAD-containing NF- κ B transcription factor dimers induce gene expression, whereas homodimers of p50 and p52, repress gene expression in the absence of additional co-factors (Zhang et al., 2017).

In unstimulated cells, the nuclear localisation signals (NLS) of NF- κ B transcription factor subunits is masked by a member of the inhibitor of κ B (I κ B) family of proteins preventing nuclear accumulation (**Figure 1.2B**). Activation of the NF- κ B signalling pathway leading to unmasking of the NLS can occur via either the canonical or non-canonical pathway. In the canonical pathway, upstream signalling events lead to phosphorylation and activation of a kinase complex called the I κ B kinase (IKK) complex. This complex comprises a regulatory protein called NF- κ B essential modifier (NEMO), and two kinases – IKK α and IKK β . The activated IKK complex phosphorylates I κ B proteins, leading to their dissociation from NF- κ B transcription factors, and subsequent K48-linked ubiquitination and degradation by the proteasome (**Figure 1.2B**). In the non-canonical pathway, NF- κ B-inducing kinase (NIK) phosphorylates and activates the kinase activity of IKK α homodimers, which subsequently phosphorylate p100 in complex with RelB. Phosphorylated p100 is cleaved to form p52 and the p52-RelB dimer then translocates to the nucleus (**Figure 1.2C**). The canonical pathway is stimulated by an array of PAMPs and DAMPs and leads to the rapid expression of proinflammatory genes, whereas the non-canonical pathway is stimulated by cytokines such as B-cell activating factor (BAFF) and CD40 ligand, and leads to the prolonged induction of genes involved in host processes such as organogenesis (Zhang et al., 2017).

1.2.2.2 The MAPK signalling pathway

Similar to the NF- κ B signalling pathway, MAPK signalling is activated following engagement of a PRR by its ligand. Various protein kinases recruited to the ligand-bound PRR phosphorylate and activate one of several serine/threonine-specific MAP kinase kinase kinases, thereby initiating a three-tiered cascade of sequential kinase phosphorylation and activation. Activated MAPKKKs phosphorylate downstream MAPKKs, which in turn phosphorylate MAPKs such as ERK, JNK, and p38 MAPKs. MAPKs then phosphorylate

members of the activator protein-1 (AP-1) transcription factor family including c-Jun, and c-Fos. Phosphorylation of AP-1 family members increases the transactivation activity of these transcription factors thereby inducing expression of genes involved in cellular processes including cell growth and proliferation, cellular differentiation, and apoptosis (Keshet and Seger, 2010).

1.2.3 Host cell death

As well as tackling invading pathogens directly, host cells also control pathogen replication by undergoing one of several forms of programmed cell death. This releases the pathogen into the extracellular milieu, sometimes with a proinflammatory signal, leading to the recruitment of professional immune cells such as neutrophils which kill or neutralise the pathogen (Jorgensen and Miao, 2015). PAMP recognition during infection can lead to several forms of programmed cell death including apoptosis, necroptosis, and pyroptosis.

1.2.3.1 Apoptosis

Apoptotic cell death plays an important function in various cellular processes including normal cell turnover, embryogenesis, and the development of the immune system. It is characterised by several morphological changes including cell shrinkage, DNA condensation, and blebbing of the plasma membrane. As the cell contents remain enclosed in membrane-bound blebs, apoptosis is not a proinflammatory process (Elmore 2007).

Apoptosis can be induced by cell stresses including ER stress, DNA damage, and reactive oxygen species (ROS) overload, or following engagement of cell surface receptors by extracellular cytokines. These two forms of apoptosis, termed intrinsic and extrinsic apoptosis, respectively, are dependent on the activation of two cysteine proteases – caspase-3 and -7. However, the mechanism by which these two proteases are activated during intrinsic and extrinsic apoptosis differs (Galluzzi et al., 2018).

In intrinsic apoptosis, cell stress causes irreversible permeabilisation of the outer membrane of mitochondria, thereby releasing mitochondrial proteins including cytochrome C into the host cell cytosol. Cytosolic cytochrome C leads to activation of caspase-9, which then

cleaves and activates caspase-3 and -7. In contrast, during extrinsic apoptosis, interaction of death receptors including FAS cell surface death receptor, TNF receptor 1, and TRAIL receptor 1 with their respective ligands, leads to formation of a multimeric protein complex called the death-inducing signalling complex (DISC). The DISC activates caspase-8, which in turn cleaves and activates caspase-3 and -7 (Galluzzi et al., 2018). In tissue culture models of *Salmonella* infection, *Salmonella* induces apoptosis at late time points post infection. Although the mechanism by which this is induced has not yet been elucidated, it is dependent on the two-component system *phoP/Q*, the SPI-2 T3SS, and the SPI-2 T3SS effector protein SseL (Rytönen et al., 2007).

1.2.3.2 Necroptosis

Death receptors, in addition to interferon receptors, and TLRs such TLR3 and TLR4, can also initiate a second form of cell death called necroptosis. Necroptosis, which requires the kinase RIPK3 and the pseudokinase MLKL, is an inflammatory form of cell death. In the most well studied pathway leading to necroptotic cell death, binding of death receptors to their respective ligands, induces the formation of a multimeric signalling complex called the ripoptosome. Ripoptosomes comprise the proteins TRADD, TRAF2, RIPK1, and RIPK3, which phosphorylates MLKL directly. Following its phosphorylation MLKL oligomerises and associates with the inner leaflet of the plasma membrane leading to permeabilization of the plasma membrane and subsequent cell death (Sun et al., 2012). The mechanism by which MLKL induces plasma membrane permeabilization is not clear, however two models have been proposed. In the first model, MLKL is thought to function as a scaffold for the recruitment of calcium and sodium ion channels to the plasma membrane and in the second model, MLKL oligomers have been hypothesised to form a pore in the plasma membrane, thereby inducing membrane permeabilisation directly (Galluzzi et al., 2018). In the context of *Salmonella*-infected macrophages, the SseK family of T3SS effector proteins inhibit TNF- α -induced phosphorylation of MLKL and subsequent necroptotic cell death (see Chapter 1.3.2.5) (Gunster et al., 2017).

1.2.3.3 Pyroptosis

Another form of programmed cell death that is highly inflammatory and most frequently induced following sensing of intracellular pathogens is pyroptosis. First, PAMPs including flagellin and T3SS needle apparatus proteins, as well as non-host double stranded DNA is recognised by intracellular PRRs such as NLRC4, NLRP3, and AIM2. Ligand-bound PRRs, then recruit an adaptor protein called ASC and inactive pro-caspase-1 to form an oligomeric complex called an inflammasome (Galluzzi et al., 2018). Subsequently, pro-caspase-1 undergoes autoproteolysis leading to the production of the active cysteine protease caspase-1. Known substrates of caspase-1 include precursors of two proinflammatory cytokines - pro-IL-1 β and pro-IL-18, as well as gasdermin D, which is critical for pyroptosis (Shi et al., 2015; Kayagaki et al., 2015). Gasdermin D consists of an N-terminal domain and a C-terminal domain separated by a flexible linker that contains a caspase-1 cleavage site. In full length gasdermin D, the C-terminal domain autoinhibits the activity of the N-terminal domain. However, following cleavage of the flexible linker by caspase-1, the N-terminal domain oligomerises, inserts into the plasma membrane to form a pore, leading to pyroptosis (Liu et al., 2016). Caspase-11, which is activated following binding to intracellular LPS, can also cleave gasdermin D leading to pyroptosis (Shi et al., 2015; Kayagaki et al., 2015). In mouse models of *Salmonella* infection both caspase-1 and -11 are important in controlling *Salmonella* replication. *Casp1/11*^{-/-} double knockout mice are more susceptible to *Salmonella* infection, with increased bacterial loads measured at secondary sites of infection such as mesenteric lymph nodes and the spleen, and lower rates of survival relative to wild-type mice (Lara-Tejero et al., 2006).

1.3 ***Salmonella* T3SS effector proteins**

Effector proteins translocated by the SPI-1 and SPI-2 T3SS subvert, avoid, or reprogramme host cellular processes to facilitate the formation of a replicative niche. Therefore, an appreciation of *Salmonella* pathogenesis requires mechanistic insight into the functions of individual effectors, as well as an understanding of how the functions of one

effector might affect another. Much work has been done to unravel effector functions and several general insights into effector biology can be drawn from the SPI-1 and SPI-2 T3SS effector literature. These are summarised below:

- 1) The effector repertoire of different *Salmonella* serovars and isolates of the same serovar varies greatly (**Figure 1.3**) (Nuccio and Baumler, 2014, Jennings et al., 2017). All serovars appear to contain functional copies of a 'core' set of effector proteins, suggesting that they are essential for *Salmonella* pathogenesis. A second set of effectors are present, and presumably functional, in serovars that cause gastroenteritis in a wide range of hosts, but are absent or non-functional, in host-adapted or host-restricted serovars that cause systemic disease. This suggests that these effectors are important for the colonisation of a particular host species or tissue. A third group of 'accessory' effectors, mostly encoded on MGEs, are distributed amongst serovars intermittently.
- 2) The majority of *Salmonella* effector proteins described to date are enzymes. Although many catalyse reactions that occur naturally in eukaryotic cells, some catalyse novel reactions that cannot be reversed by the host cell machinery. However, there are other effectors that have no demonstrable enzymatic activity, and instead they appear to function as adaptors. These might allosterically modulate the activity of host proteins or facilitate interactions between host proteins that would otherwise not occur.
- 3) Multiple effectors target the same host processes in a co-operative or antagonistic manner.
- 4) A single effector might have more than one biological function or host interaction partner.

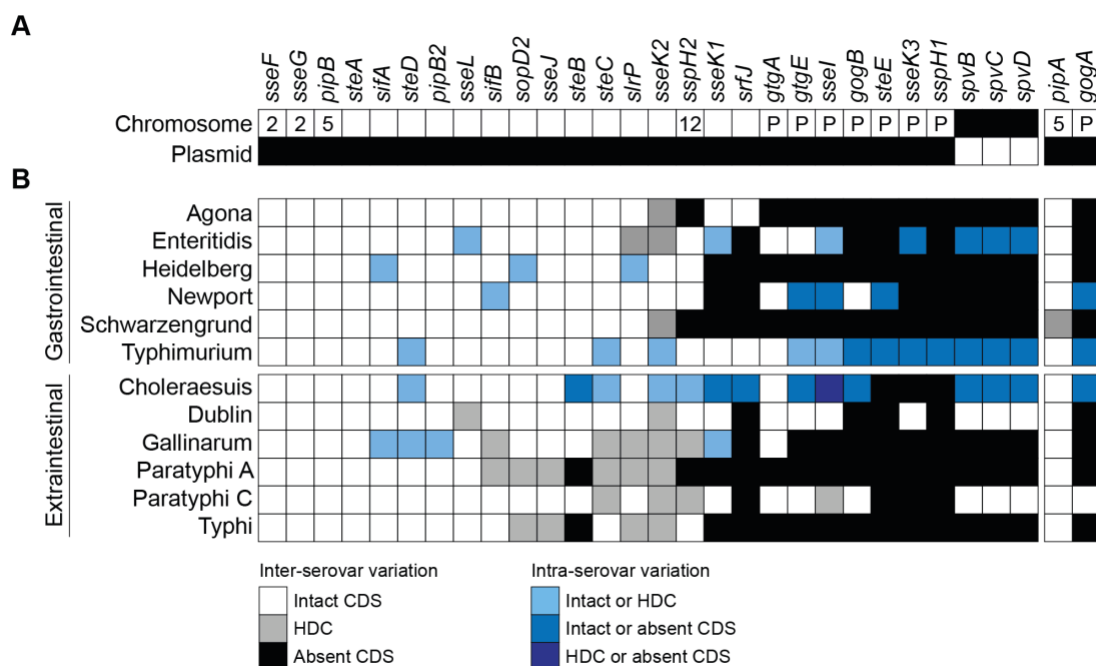


Figure 1.3: SPI-2 T3SS effector distribution across different *Salmonella enterica* serovars.

(A) Location of effector genes in *Salmonella* Typhimurium ATCC strain 14028s. Where genes are encoded in a pathogenicity island, the number of the SPI is stated. 'P' denotes that the gene is in a prophage. (B) Distribution of SPI-2 T3SS effector proteins in various *Salmonella* serovars. Abbreviations: *CDS*, coding DNA sequence; *HDC*, hypothetically disrupted CDS. Figure is adapted from Nuccio and Baumler (2014) and reproduced from Jennings et al. (2017).

- 5) Following translocation, post-translational modifications, interactions with host cell proteins, and changes to the effectors subcellular localisation can all modulate effector activity.

1.3.1 SPI-1 T3SS effector proteins

1.3.1.1 Epithelial cell invasion

SPI-1 T3SS-dependent invasion of epithelial cells requires the co-ordinated activities of at least 4 effector proteins - SopB, SopE, SopE2, and SipA, and the SPI-1 T3SS translocon protein SipC. SopE and SopE2 possess guanine exchange factor (GEF) activity that activates the small Rho GTPases Rac1 and Cdc42 by catalysing the exchange of GDP for GTP (Hardt

et al., 1998). Once activated, these GTPases facilitate remodelling of the actin cytoskeleton leading to membrane ruffling and macropinocytosis. This is enhanced by SopB - an effector with phosphoinositide phosphatase activity (Norris et al., 1998), in a Cdc42-dependent manner (Zhou et al., 2001). SopB is also required for the recruitment of protein complexes involved in actin remodelling to the site of invasion (Liebl et al., 2017, Jolly et al., 2014), and increases the rate of membrane fission at the site of membrane invagination (Terebiznik et al., 2002). SipA and SipC bind directly to actin, leading to actin nucleation at the site of insertion of the translocon pore (Hayward and Koronakis, 1999, McGhie et al., 2001) and to the inhibition of actin depolymerisation (Lilic et al., 2003, Galkin et al., 2002, Zhou et al., 1999).

Following bacterial entry into the SCV, effector-induced cytoskeletal rearrangement is reversed. The GTPase-activating protein (GAP) SptP inactivates Cdc42 and Rac1 by catalysing the hydrolysis of GTP bound to Cdc42 and Rac1, to GDP (Fu and Galan, 1999). SPI-1 T3SS effector proteins then contribute towards the maturation of the early SCV, regulating the interactions of the SCV with early endosomes, and in epithelial cells, promoting the movement of the SCV to a juxtanuclear position (LaRock et al., 2015).

1.3.1.2 Induction of intestinal inflammation

SopE, SopE2, and SopB-dependent activation of Rac1 and Cdc42, not only induces cytoskeletal rearrangement, but also stimulates activation of NF- κ B and MAPK signalling pathways, leading to the expression of proinflammatory cytokines (**Table 1.1**) (Bruno et al., 2009). Proinflammatory cytokine expression is also stimulated by the HECT E3 ubiquitin ligase SopA as well as SipA. SopA ubiquitinates two host E3 ubiquitin ligases - TRIM56 and TRIM65, thereby boosting activation of innate immune signalling pathways downstream of the DNA and RNA sensing PRRs, RIG-I and MDA-5 (Kamanova et al., 2016), whereas SipA induces NF- κ B signalling dependent on NOD1 and NOD2 by an unknown mechanism (Keestra et al., 2011). Additionally, caspase-1 is activated in epithelial cells dependent on SopE via Rac-1 and Cdc42 (Muller et al., 2009), and in macrophages following intracellular sensing of flagellin,

Table 1.1: Functions of immunomodulatory *Salmonella* T3SS effector proteins

Effector	Biochemical Activity	Interaction Partner(s)	Function(s)
SopE	Guanine exchange factor	Rac1, Cdc42	Induces membrane ruffling and intestinal inflammation; activates caspase-1
SopE2	Guanine exchange factor	Rac1, Cdc42	Induces membrane ruffling and intestinal inflammation
SopB	Inositol phosphatase	Cdc42	Manipulates surface charge of the SCV membrane
SptP	GTPase activating protein; tyrosine phosphatase	Rac1, Cdc42, VCP	Restores epithelial cell morphology after invasion; inhibits MAPK signalling
AvrA	Acetyltransferase	p53, MKK4/7	Inhibits MAPK signalling
SopA	HECT E3 ubiquitin ligase	TRIM56, TRIM65	Activates RIG-I and MDA5 signalling
SipA	?	Actin	Induces actin polymerisation at site of invasion; activates caspase-3
SpvC	Phosphothreonine lyase	p-ERK, p-p38, p-JNK	Inhibits MAPK signalling
SseK1	Glycosyltransferase	FADD, TRADD	Inhibits TNF- α -induced NF- κ B signalling and necroptosis
SseK2	Putative glycosyltransferase	?	Inhibits TNF- α -induced NF- κ B signalling
SseK3	Glycosyltransferase	TRIM32, TRADD	Inhibits TNF- α -induced NF- κ B signalling and necroptosis
SspH1	E3 ubiquitin ligase	PKN1	Inhibits Androgen Receptor signalling
SspH2	E3 ubiquitin ligase	UbchH5-Ubiquitin, SGT1, NOD1	Activates NOD1 signalling
SlrP	E3 ubiquitin ligase	Erdj3, TRX1	Inhibits inflammasome activation
GogB	?	SKP1, FBXO22	Inhibits NF- κ B signalling
SpvD	Cysteine hydrolase	XPO2	Inhibits NF- κ B signalling
SteE	?	STAT3	Induces IL-10 expression
SseI (SrfH)	Deamidase	IQGAP1, Gα_i proteins	Inhibits macrophage and dendritic cell chemotaxis
SteD	?	mMHCII, MARCH8	Inhibits antigen presentation by dendritic cells

Interaction partners that are substrates are shown in **bold**. Abbreviations: *p*, phospho; *mMHCII*, mature major histocompatibility complex II.

the T3SS rod protein PrgJ, or the translocon protein SipB (Miao et al., 2010b). Caspase-1 activation leads to caspase-1-dependent proinflammatory cell death called pyroptosis (Miao et al., 2010a).

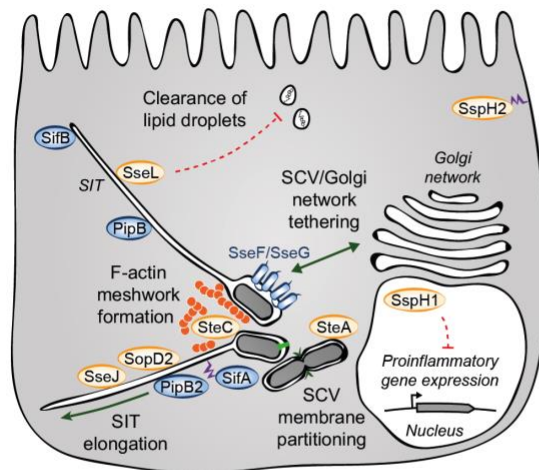
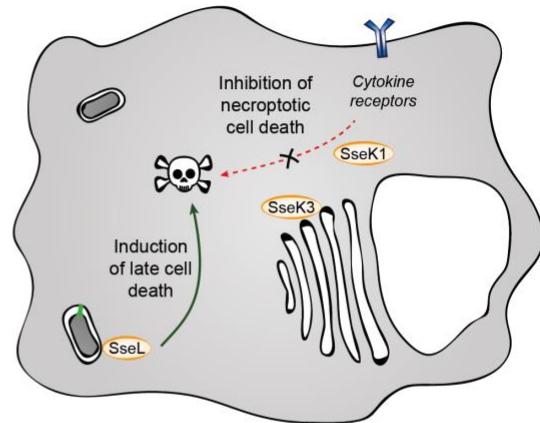
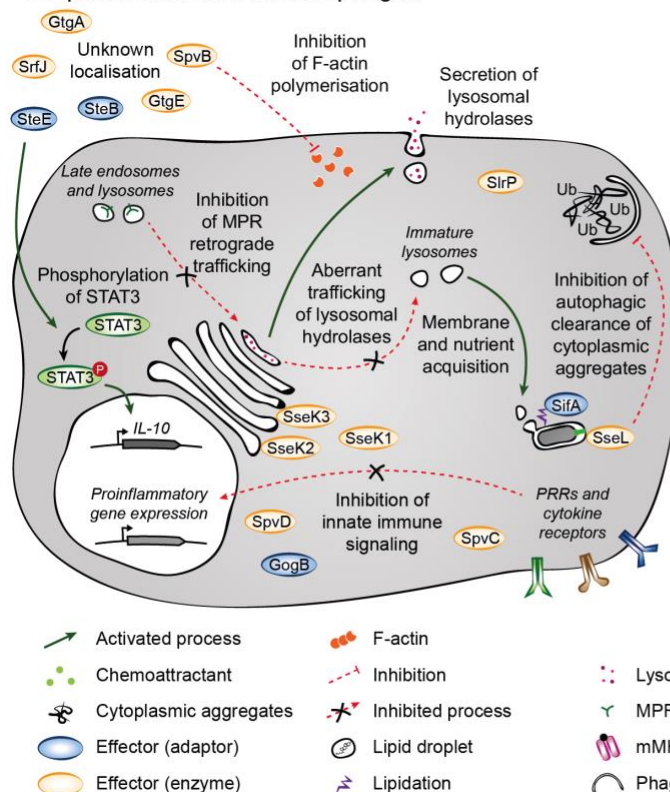
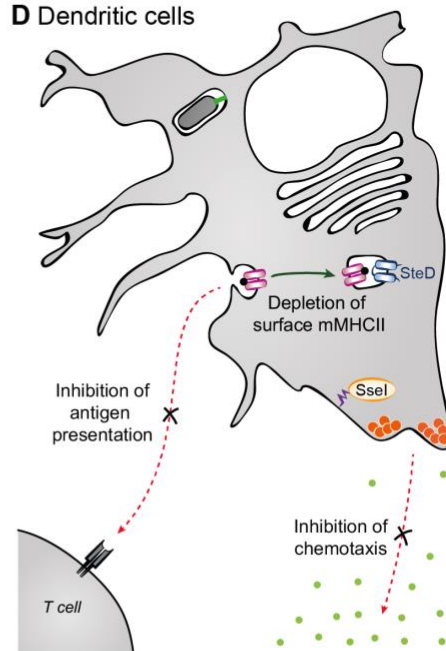
Although intestinal inflammation is beneficial for luminal replication of NTS, activation of the innate immune system can lead to pathogen clearance or death of the host, thereby limiting bacterial transmission. It is therefore unsurprising that SPI-1 T3SS effectors also function to dampen intracellular signalling. SptP inhibits MAPK signalling by inhibiting Raf activation dependent on both its GAP and tyrosine phosphatase activities (Lin et al., 2003), whereas AvrA is an acetyltransferase that acetylates a subset of MAPK kinases (MAPKKs) to prevent JNK-dependent gene expression and epithelial cell apoptosis (Jones et al., 2008).

1.3.2 SPI-2 T3SS effector proteins

The expression of the SPI-2 T3SS and its repertoire of associated effector proteins is induced following acidification of the SCV. To date, 28 effector proteins have been demonstrated to be translocated into host cells dependent on the SPI-2 T3SS. These function to regulate SCV membrane dynamics and intracellular positioning, to manipulate the host cell actin cytoskeleton, to modulate activation of innate immune signalling pathways, and to inhibit the development of adaptive immunity (**Table 1.1; Figure 1.4**) (Jennings et al., 2017). *Salmonella* mutant strains unable to translocate any SPI-2 T3SS effector proteins are severely attenuated both *in vitro* and *in vivo*, demonstrating the importance of the SPI-2 T3SS in *Salmonella* virulence.

1.3.2.1 SCV membrane dynamics

After bacterial engulfment a small percentage of SCVs rupture, releasing bacteria into the nutrient rich environment of the host cell cytosol. However, the cytosol of both epithelial cells and macrophages contain cell-autonomous defence mechanisms that restrict cytosolic bacterial replication. Growth of cytosolic bacteria is restricted in epithelial cells by autophagy (Birmingham et al., 2006), and polarised epithelial cells containing cytosolic *Salmonella*

A Epithelial cells**B Macrophages****C Epithelial cells and macrophages****D Dendritic cells****Figure 1.4: Functions of SPI-2 T3SS effectors.**

(A) In *Salmonella*-infected epithelial cells, the SPI-2 T3SS effectors SseF and SseG function to tether *Salmonella*-containing vacuoles (SCVs), surrounded by an F-actin meshwork, to the Golgi network. The coordinated activities of SifA, SseJ, SopD2, and PipB2 function to form and elongate *Salmonella*-induced tubules (SITs). (B) In *Salmonella*-infected macrophages, SseK1 and SseK3 inhibit necroptotic cell death, whereas SseL induces cell death at late timepoints post bacterial uptake. (C) SifA prevents lysosomal maturation by interfering with retrograde trafficking of mannose-6-phosphate receptors (MPR). SifA might also recruit

immature lysosomes to the SCV in order to acquire nutrients for bacterial growth, and membrane for SCV expansion. GogB, SpvC, SpvD, SseK1, and SseK3 inhibit activation of innate immune signalling pathways, whereas SteE induces STAT3 phosphorylation leading to expression of the anti-inflammatory cytokine IL-10. (D) SseI inhibits dendritic cell chemotaxis, whereas SteD prevents activation of CD4⁺ T cells by depleting surface levels of mMHCI. Abbreviations: *SIT*, *Salmonella*-induced tubule; *SCV*, *Salmonella*-containing vacuole; *MPR*, mannose-6-phosphate receptor; *Ub*, ubiquitin; *mMHCI*, mature major histocompatibility complex; *PRR*, pattern recognition receptor. Figure is reproduced from Jennings et al. (2017).

undergo pyroptosis, leading to the extrusion of infected cells from the intestinal epithelium (Knodler et al., 2014, Knodler et al., 2010). Growth of cytosolic *Salmonella* in macrophages is restricted in a caspase-1 and caspase-11-dependent manner through an undefined mechanism (Thurston et al., 2016) prior to pyroptotic cell death and neutrophil-mediated bacterial killing (Miao et al., 2010a). As vacuoles generally fuse with lysosomes, exposing vacuole contents to degradative hydrolases, both the host cell cytosol and vacuole represent niches that are usually refractory to bacterial replication.

Approximately 50% of vacuole membranes surrounding *sifA* mutant bacteria rupture (Beuzon et al., 2000), showing that the SPI-2 T3SS effector SifA maintains the integrity of the vacuole membrane and thus prevents bacteria from inducing immune responses that occur upon exposure to the host cell cytosol. Indeed, a $\Delta sifA$ mutant strain is severely attenuated for growth in macrophages and *in vivo* (Beuzon et al., 2002). SifA also prevents degradation of vacuolated *Salmonella* by lysosomal hydrolases: SifA recruits PLEKHM2 to the SCV membrane (Boucrot et al., 2005), preventing it from facilitating Rab9-mediated retrograde trafficking of mannose-6-phosphate receptors. This leads to aberrant trafficking of lysosomal hydrolases through the secretory pathway, reducing the potency of lysosomes (McGourty et al., 2012). In addition, SifA might actively facilitate fusion of late endosomes and immature lysosomes with the SCV through an interaction with PLEKHM1 (McEwan et al., 2015). The SCV membrane surrounding wild-type *Salmonella* in PLEKHM1^{-/-} cells does not partition around individual bacteria and instead bacteria replicate in a ‘bag-like’ vacuole. Additionally, long tubular

extensions of the SCV called *Salmonella*-induced tubules (SITs) do not form. These data suggest that the function of the SifA-PLEKHM1 complex might be to provide a source of membrane required for SCV membrane partitioning and SIT formation. A similar 'bag-like' vacuole is observed in cells infected with a $\Delta steA$ mutant strain suggesting that SteA also contributes to these processes (Domingues et al., 2014).

SITs are long extensions of the SCV that extend along microtubules and contain proteins usually found in late endosomes (Garcia-del Portillo et al., 1993, Rathman et al., 1997) and the secretory pathway (Mota et al., 2009). The functional significance of SITs is not fully understood. However, it was demonstrated recently that the lumen of SITs and the SCV is contiguous, and that vacuoles containing endocytosed material regularly fuse with SITs. This suggests that SITs facilitate acquisition of endocytosed nutrients (Liss et al., 2017). At least 4 SPI-2 T3SS effectors - SifA, PipB2, SopD2, and SseJ contribute towards SIT formation. PipB2 localises to the SCV membrane and recruits inactive forms of the microtubule motor protein kinesin-1 (Henry et al., 2006). An interaction between kinesin-1 and the SifA-PLEKHM2 complex then activates kinesin-1 (Yip et al., 2016) leading to elongation of the SCV along microtubules to form SITs (Schroeder et al., 2010). The function that SopD2 and SseJ plays in SIT formation is less clear, however deletion of either *sseJ* or *sopD2* in a $\Delta sifA$ mutant strain restores vacuole membrane stability suggesting that these two effectors function to destabilise the vacuole membrane surrounding $\Delta sifA$ mutant bacteria. SseJ is a RhoA-activated acyltransferase, which transfers acyl chains from glycerophospholipids, in order to esterify cholesterol (Kolodziejek and Miller, 2015, Lossi et al., 2008, Ohlson et al., 2008). Esterified cholesterol dissociates from the SCV membrane and accumulates in the host cell cytosol in the form of lipid droplets (Fujimoto and Parton, 2011). The significance of SseJ's activity is not clear, however cholesterol content can affect membrane fluidity, signalling, sorting and vesicular transport.

SopD2 regulates SCV membrane dynamics in several ways. First, SopD2 interacts with Rab7, uncoupling Rab7 from two Rab7 effectors – RILP and FYCO1, which each regulate the activity of microtubule motor proteins (D'Costa et al., 2015). SopD2 also inactivates the small

GTPase Rab32 dependent on its GAP activity (Spano et al., 2016). Rab32 is also inactivated by the cysteine protease GtgE, which cleaves Rab32 and two related Rabs – Rab29 and Rab38 (Spano et al., 2011). These Rab proteins are important in the maturation of lysosomal-related organelles (LROs) and likely contribute towards the restriction of *Salmonella* replication by promoting fusion of LROs with the SCV. Accordingly, a *S. Typhimurium* $\Delta sopD2 \Delta gtgE$ double mutant strain is attenuated in murine macrophages, as well as in *in vivo* mouse models (Spano and Galan, 2012, Spano et al., 2016).

1.3.2.2 SCV intracellular positioning

In epithelial cells, vacuolated *Salmonella* replicates in tight clusters termed microcolonies, in a juxtanuclear position. Juxtanuclear positioning requires two effector proteins – SseF and SseG, that are encoded in SPI-2. SseF and SseG are integral membrane proteins that localise to the SCV membrane, where they both interact with the Golgi-network associated protein ACBD3 (Yu et al., 2016). The SseF-SseG-ACBD3 complex functions to tether SCVs to the Golgi network, possibly facilitating the acquisition of membrane and nutrients.

1.3.2.3 Lipid droplets and cytoplasmic aggregates

The esterification of cholesterol by SseJ, leads to the dissociation of cholesterol from the SCV membrane, and the subsequent accumulation in lipid droplets in the cytosol of infected cells (Nawabi et al., 2008). The abundance of lipid droplets is increased in gallbladder epithelial cells in mice infected with an $\Delta sseL$ mutant strain suggesting that the effector SseL functions to reduce lipid droplet accumulation. This is dependent on SseL's deubiquitinase activity as the phenotype could be complemented with wild-type SseL but not a catalytically inactive point mutant (Arena et al., 2011). Although the substrate of SseL's catalytic activity is not currently clear, SseL is known to interact with OSBP1 (Auweter et al., 2012). In uninfected cells, OSBP1 functions to transport cholesterol and the lipid PI(4)P between the endoplasmic reticulum and the Golgi apparatus (Mesmin et al., 2013). As SseL localises to the SCV

membrane (Pruneda et al., 2016), it is possible that SseL coordinates the activities of OSBP1 to manipulate the lipid content of the SCV.

Salmonella infection also induces formation of ubiquitinated aggregates in the cytosol of infected cells. SseL prevents the autophagic clearance of these aggregates by inhibiting autophagic flux (Mesquita et al., 2012). Aggregates of RNA and post-transcriptional regulatory proteins called P-bodies also form in *Salmonella*-infected cells and these are disassembled by SpvB, an effector with ADP-ribosyltransferase activity that is encoded on the *Salmonella* virulence plasmid (Eulalio et al., 2011).

1.3.2.4 Cytoskeletal remodelling

SCVs clustered in a juxtanuclear position are surrounded by an F-actin cage (M eresse et al., 2001, Miao et al., 2003), the formation of which is dependent on the kinase activity of SteC (Poh et al., 2008). Known substrates include the kinase MEK1 (Odendall et al., 2012) and HSP27 (Imami et al., 2013). Phosphorylation of MEK1 by SteC, activates a signalling pathway involving ERK, MLCK, and Myosin IIB, leading to localised F-actin polymerisation (Odendall et al., 2012). HSP27 is a known regulator of actin polymerisation therefore it is possible that phosphorylation of HSP27 by SteC modulates its actin regulating activity. Intracellular replication of an $\Delta steC$ mutant strain is greater than wild-type *Salmonella* suggesting that the F-actin cage suppresses bacterial replication (Odendall et al., 2012).

At later time points following bacterial internalisation, SpvB ADP-ribosylates actin leading to destabilisation of the host cell cytoskeleton (Lesnick et al., 2001, Hochmann et al., 2006), and in macrophages, to the induction of apoptotic cell death (Browne et al., 2008). Although the mechanism by which SpvB induces apoptotic cell death is unclear, it is conceivable that SpvB disassembles the F-actin cages generated by SteC.

1.3.2.5 Modulation of innate immune signalling pathways

Intestinal inflammation is induced during *Salmonella* infection following both PAMP recognition and by several SPI-1 T3SS effectors (Bruno et al., 2009). However, activation of the innate immune system can clear the infection and uncontrolled inflammation might kill the

host. Therefore, it is unsurprising that a subset of SPI-2 T3SS effectors function to dampen innate immune signalling. SpvC, SseK1, SseK2, SseK3, SspH1, GogB, and SpvD all inhibit expression of proinflammatory cytokines (**Table 1.1**) (Mazurkiewicz et al., 2008, Yang et al., 2015, Gunster et al., 2017, Keszei et al., 2014, Pilar et al., 2012, Rolhion et al., 2016).

SpvC is a phosphothreonine lyase that irreversibly dephosphorylates the MAPKs ERK, p38, and JNK (Mazurkiewicz et al., 2008, Haneda et al., 2012). SpvC translocation is dependent on both the SPI-1 and SPI-2 T3SSs, and suppresses innate immune activation in the intestine (Haneda et al., 2012) and at systemic sites (Mazurkiewicz et al., 2008).

The SseK family of effector proteins (comprising SseK1, SseK2, and SseK3) is a family of N-acetylglucosamine (GlcNAc)-transferases that irreversibly transfer a GlcNAc moiety to an arginine residue. This is in contrast to mammalian GlcNAc-transferases that transfer a GlcNAc moiety to either a serine or threonine residue. SseK1, SseK2, and SseK3 are homologous to the effector protein NleB1 from enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC/EHEC) which GlcNAcylates the death domain containing proteins FADD, TRADD, RIPK1, and TNFR1 (Pearson et al., 2013, Li et al., 2013). SseK1 and SseK3 both arginine-GlcNAcylate TRADD, and SseK1 additionally modifies FADD (Gunster et al., 2017). Using an antibody that specifically recognises GlcNAcylated arginine residues, immunoblot analysis of macrophages infected with various *sseK1*, *sseK2*, and *sseK3* mutant strains revealed that additional proteins are GlcNAcylated dependent on *sseK1* and *sseK3*, however the identity of these are currently unknown (Gunster et al., 2017). SseK3 also interacts with TRIM32 (Yang et al., 2015), however the physiological relevance of this interaction is unknown as TRIM32 is not required for SseK3-dependent arginine-GlcNAcylation of host proteins, nor for SseK-dependent inhibition of NF- κ B signalling and necroptosis (Gunster et al., 2017).

Salmonella also encodes three E3 ubiquitin ligases - SspH1, SspH2, and SlrP, which interfere with activation of the innate immune system by catalysing K48-linked ubiquitin chains and transferring these onto host cell proteins. SspH1 ubiquitinates PKN1 (Haraga and Miller, 2006), leading to its proteasomal degradation and inhibition of androgen receptor signalling

(Keszei et al., 2014), likely suppressing macrophage activation (Lai et al., 2009). The direct host cell substrates of SspH2 and SlrP are currently unknown, however SspH2 interacts with the NOD-like receptor co-chaperone protein SGT1 (Auweter et al., 2011), and appears to induce IL-8 secretion dependent on NOD1 (Bhavsar et al., 2013). In contrast, SlrP inhibits inflammasome activation and IL-1 β secretion in the intestine of infected mice (Rao et al., 2017).

GogB also interferes with the host ubiquitin system, however GogB does not appear to function as an E3 ubiquitin ligase. Instead, GogB interacts with two components of the Skp-Cullin-F box containing multi-protein ubiquitin ligase complex (SCF complex): Skp1 and FBXO22. The SCF complex targets host proteins for proteasomal degradation. It is not clear if GogB is a substrate of this complex, or if GogB modulates its activity, but through Skp1 GogB appears to prevent I κ B α ubiquitination and NF- κ B-dependent gene transcription. Accordingly, increased cecal inflammation is induced in mice infected with a Δ *gogB* mutant strain relative to wild-type *Salmonella* (Pilar et al., 2012).

Another immunomodulatory effector whose mechanism of action is not fully understood is SpvD. The crystal structure of SpvD demonstrates that it is a cysteine hydrolase, but the exact enzymatic activity and host cell substrate is unknown (Grabe et al., 2016). SpvD interacts with Exportin-2, which transports importins in the nucleus to the cytosol. SpvD is thought to inhibit the nuclear export of an importin important for nuclear translocation of p65 and in *Salmonella*-infected cells, SpvD appears to modulate the nuclear import/export cycle of p65 thereby dampening NF- κ B-dependent gene transcription (Rolhion et al., 2016), however Exportin-2 does not appear to be modified by SpvD.

As well as inhibiting the expression of proinflammatory cytokines, SteE, an effector translocated by both the SPI-1 and SPI-2 T3SSs, functions to induce the expression of the anti-inflammatory cytokine IL-10. SteE interacts with the transcription factor STAT3, and somehow induces phosphorylation of residue Y705 (Jaslow et al., 2018). It is not yet clear

how the interaction between SteE and STAT3 leads to STAT3 phosphorylation, however SteE-induced IL-10 expression was abrogated in cells treated with STAT3 inhibitors.

1.3.2.6 Manipulation of the adaptive immune system

Dendritic cells (DCs) represent one of the first cell types that *Salmonella* encounters in the intestinal lumen (Farache et al., 2013). DCs present antigens to CD4⁺ T cells using major histocompatibility class II (MHCII) molecules, thereby stimulating an adaptive immune response to pathogens. CD4⁺ T cells are important in clearing *Salmonella* infections (Hess et al., 1996); therefore, it is unsurprising that *Salmonella* subverts CD4⁺ T cell function by inhibiting DC migration and antigen presentation.

Ssel (also called SrfH) inhibits DC chemotaxis and specifically inhibits the dissemination of *Salmonella*-infected DCs from the gut to other organs (McLaughlin et al., 2009). Ssel is a deamidase that deamidates G α_i family G proteins leading to persistent activation of the G protein (Brink et al., 2018). Although it is well established that G proteins are important in chemotaxis (Thelen and Stein, 2008), it is not clear how persistent G protein activation might prevent DC chemotaxis. Another effector, SteD promotes ubiquitination of mature peptide-loaded MHCII molecules (Bayer-Santos et al., 2016). MHCII molecules on the surface of antigen-presenting cells are constantly internalised and then sorted back to the plasma membrane (Roche and Furuta, 2015). However, in *Salmonella*-infected DCs, SteD-dependent ubiquitination of MHCII, leads to MHCII internalisation and a reduction in the surface levels of peptide-loaded MHCII. This subsequently inhibits the presentation of *Salmonella* antigens to CD4⁺ T cells and T cell activation *in vivo*, preventing the induction of an adaptive immune response (Bayer-Santos et al., 2016).

1.3.2.7 Uncharacterised SPI-2 T3SS effectors

Although much work has been done to elucidate the biochemical activities, host cell interaction partners, and physiologically relevant functions of many SPI-2 T3SS effectors, the functions of PipB, GtgA, SifB, SrfJ, and SteB are unclear. PipB and SifB are homologues of the effectors PipB2 and SifA, respectively, however they do not appear to function in regulating

SCV membrane dynamics. The crystal structure of SrfJ suggests that it is a glycoside hydrolase, however the physiological relevance of SrfJ's putative catalytic activity is not clear (Kim et al., 2009).

1.4 Zinc metalloprotease type III secretion system effector proteins

1.4.1 GtgA, GogA, and PipA

Proteomic analysis demonstrated previously that GtgA was secreted into the culture medium when *S. Typhimurium* was cultured in SPI-2-inducing conditions (Niemann et al., 2011). SPI-2 T3SS-dependent translocation of GtgA into J774 cells, was then confirmed using a CyaA' translocation assay. In this assay, GtgA was fused via its C-terminus to the calmodulin-dependent adenylate cyclase domain of CyaA. Calmodulin is present in eukaryotic but not bacterial cells, therefore conversion of ATP to cAMP only occurs following translocation of an effector into the host cell. The genome of *S. Typhimurium* strain ATCC 14028s, which was used in this study, contains two genes – *gogA* and *pipA*, the protein products of which share 97% and 67% sequence identity with GtgA (**Figure 1.5**). However, unlike GtgA, neither GogA nor PipA have been described previously to be T3SS effector proteins. The genes encoding GtgA (STM1026), GogA (STM2614), and PipA (STM1087) are located in the Gifsy-2 prophage, the Gifsy-1 prophage, and SPI-5, respectively. PipA is encoded in an operon with the SPI-2 T3SS effector protein PipB, and is widely distributed throughout different *Salmonella* serovars (Jennings et al., 2017, Nuccio and Baumler, 2014). In contrast, *gtgA* and *gogA* are found sporadically throughout different *Salmonella* serovars, which likely reflects the fact that they are encoded on bacteriophages.

An analysis of the abundance of *gtgA*, *gogA*, and *pipA* mRNA in 22 different environmental conditions and in macrophages 8 hours post-bacterial uptake demonstrated that *gtgA* is not well expressed in any condition. In contrast, *gogA* is weakly expressed in late-exponential phase and early stationary phase bacteria, whereas expression of *pipA* is induced in SPI-2-inducing conditions and in macrophages (Kroger et al., 2013, Srikumar et al., 2015).

```

GtgA  -MPTGIKPIFINNMSTYGLSHPHDSKVFPDLPEHQDNPSQLRLQHDGLATDDKARLEPM 59
GogA  -MPAGIKPIFINNMMSIYGLSHPHDSKVFPDLPEHQDNPSQLRLQHDGLATDDKARLEPM 59
PipA  MLPVTTYR-LIPQSGVSTYRL-NTADTPVFPDIPEHAPNPSRLRLAHDSLAINSEFRLEPE 58
      :*. : :. :. :* * * : * : ****:*** ***:*** **.*. :. : ****

GtgA  CLAEYLISGPGGMDPDIEIDDDTYDECREVLSRILEDAYTQSGTFRRLMNYAYDQELHDV 119
GogA  CLAEYLISGPGGMDPDIEIDDDTYDECREVLSRILEDAYTQSGTFRRLMNYAYDQELHDV 119
PipA  CVVEYLISGAGGIDPDTEIDDDTYDECYDELSSVLQNAYTQSETFRRLMNYAYEKELHDV 118
      *: .***** ***:*** ***** : ** :*: .***** *****: :*****

GtgA  EQRWLLGAGENFGTTVTDEDLESSEGRKVIALNLDDTDDDSIPEYYESNDGPPQFDTTTRS 179
GogA  EQRWLLGAGENFGTTVTDEDLESSEGRKVIALNLDDTDDDSIPECYESNDGPPQFDTTTRS 179
PipA  EQRWLLGAGEAFETTVAQEHFKLSEGRKVICNLDDSDS-SYTEHYESNEGRQLFDTKRS 177
      ***** * ***: :*. :. : ***** .*****: ** * * ****: * * ***.**

GtgA  FIHEVVHALTHLQDKEDSNPRGPVVEYTNIIILKEMGHTSPPRIAYEFSN 228
GogA  FIHEVVHALTHLQDKEDNNPRGPVVEYTNIIILKEMGHTSPPRIAYESSN 228
PipA  FIHEVVHALTHLQDKEENHPRGPVVEYTNIIILKEMGHPSPRMVYIFNK 226
      *****: :. :***** ***** ***: . * . :

```

Figure 1.5: Amino acid sequence alignment of GtgA, GogA, and PipA.

Sequence alignment of GtgA, GogA, and PipA from *Salmonella* Typhimurium strain ATCC 14028s (Uniprot: A0A0F6AZI6, A0A0F6B537, and A0A0F6AZQ0). The 'HEXXH' zinc metalloprotease motif is highlighted in grey.

These data suggest that if GogA and PipA are T3SS effector proteins, GogA might be translocated through the SPI-1 T3SS, whereas PipA might be a SPI-2 T3SS effector protein.

At the beginning of this study, little was known about the functions of GtgA, GogA, and PipA. However, a Δ gtgA mutant strain had no replication defect in bone marrow-derived macrophages (Figueira et al., 2013), suggesting that GtgA is not important for *Salmonella* intra-macrophage replication. Replication of a Δ gtgA Δ gogA Δ pipA triple mutant strain was not analysed so the possibility that GtgA, GogA, and PipA might function redundantly to promote intra-macrophage replication cannot be ruled out. However, Δ gtgA and Δ gogA single deletion mutant strains were negatively selected in an *Nramp1*^{+/+} chronic mouse model, suggesting that *gtgA* and *gogA* are important in establishing long-term *Salmonella* persistence *in vivo* (Kidwai et al., 2013, Lawley et al., 2006). Additionally, a Δ pipA mutant strain induced less inflammation in an ileal bovine loop model relative to *Salmonella* suggesting that PipA functions to induce inflammation (Wood et al., 1998).

GtgA, GogA, and PipA each have a predicted molecular weight of approximately 26 kDa and contain the short zinc-binding motif 'HEXXH' (where X is any residue) (Cerdà-Costa and Xavier Gomis-Rüth, 2014) towards their carboxy termini, suggesting that they are zinc metalloproteases. Additionally, the C-terminal regions of GtgA, GogA, and PipA are predicted to be structurally similar to the zinc metalloprotease effector protein NleC from EPEC/EHEC, despite the fact that the amino acid sequence identity shared between members of the GtgA family and NleC is low (<20%) (**Figure 1.6**).

1.4.2 NleC – a bacterial zinc metalloprotease T3SS effector

Similar to *Salmonella*, the pathogenesis of EPEC and EHEC requires the activities of effector proteins. EPEC/EHEC effector translocation depends on the locus of enterocyte effacement encoded T3SS. A subset of these effectors, including the zinc metalloprotease effector protein NleC, dampen activation of innate immune signalling pathways and suppress expression of proinflammatory genes. NleC cleaves NF- κ B transcription factor subunits including p65, cRel, RelB, and NF- κ B1 (p105/p50) (Yen et al., 2010, Baruch et al., 2011, Muhlen et al., 2011, Pearson et al., 2011, Sham et al., 2011, Turco and Sousa, 2014, Li et al., 2014). It has not been determined if NleC also cleaves NF- κ B2 (p100/p52). NleC cleaves the N-terminal domain of p65 directly (Giogha et al., 2015) between residues C38 and E39, rendering p65 transcriptionally inactive and thereby inhibiting NF- κ B-dependent gene transcription (Baruch et al., 2011). The larger C-terminal cleavage fragment (p65³⁹⁻⁵⁴⁹) is degraded by the proteasome (Yen et al., 2010), whereas the smaller N-terminal fragment (p65¹⁻³⁸) interacts with the transcriptional co-activator Rps3, preventing Rps3 from interacting with full length p65 and being imported into the nucleus. Consequently, expression of Rps3-dependent NF- κ B-responsive genes is also inhibited (Hodgson et al., 2015). NleC has also been reported to cleave p65 between residue P10/A11 (Yen et al., 2010), however the efficiency of cleavage at this site is reduced relative to C38/E39, and the functional significance of the P10/A11 cleavage site is unclear (Hodgson et al., 2015).

GtgA	MPTGIKPIFINNMMSTYGLSHPHDSKVFPDLPEHQDNPSQLRLQHDGLATDDKARLEPMC	60
NleC	-----MKIPSLQSNFNFSAPAGYS-APIAPNRAENAYADYV----LDIGKRIPLSAAD	48
	: * .: *.:*: * . . * *: *: : * : * .: *	
GtgA	LA---EYLISGPGGMDPDIEIDDDTYDECREVLSRILEDAYTQSGTFRRLMNYAYDQELH	117
NleC	LSNVYESVIRAVHDS-RSRLIDQH---TVDMIGNTVLDALSRSTFRDAVSYGHIHNEKV	103
	*: * *: * . . . *: . : : : : : * *: *: * *: .*. .: *	
GtgA	DVEQR-WLLGAGENFGTTVTDEDLES-----SEGRKVIALNLDDTDDDSIPEYYES	167
NleC	HIGCIKYRNEYELNEESSVKIDDIQSLTCNELYEYDVGQEPFIPICEAGENDNEEPYVSF	163
	.: : * :*: *: *: . *: * : :*: * .	
	Predicted to be structurally similar	
GtgA	NDGP-----QQFDTTSRFIHEVVHALTHLQDKED--SNPRGPVVEYTNIIKEMGHTSPP	220
NleC	SVAPDSDSYEMPSWQEGLIHEIIHHVTGSSDSPSGDSNIELGPTTEILARRVAQELGWSVPD	223
	. . * : . . :*: *: *: * . * . . * . : . :*: * : *	
GtgA	RIAYEFSN-----	228
NleC	FKGYAEPEREHLRLRLNLALRQAAMRHEENERAFFERLGTISDRYEASPDFTEYSAVSN	283
	. * :	
GtgA	-----	228
NleC	IGYGFIQQHDFPGLAINDNLQDANQIQLYHGAPYIFTFGDVKHNQR	330

Figure 1.6: Amino acid alignment of GtgA and NleC.

Sequence alignment of GtgA from *Salmonella* Typhimurium strain ATCC 14028s and NleC from *E. coli* strain O127:H6 E2348/69 (Uniprot: A0A0F6AZI6 and B7UNX4). Zinc-coordinating residues in NleC, and the corresponding residues in GtgA are highlighted in grey. The region of GtgA predicted to be structurally similar to NleC using the Phyre2 structural prediction server (Kelley et al., 2015) is annotated.

Apart from its homologue AIP56 from *Photobacterium damsela piscicidia* (Silva et al., 2013), NleC does not share significant sequence identity to any other zinc metalloproteases. However, the crystal structure of NleC demonstrates that the catalytic core of NleC retains many of the structural characteristics found in the Zincin superfamily of metalloproteases (see below). Furthermore, the NleC active site cleft is highly negatively charged and is similar in shape to the DNA major groove (Turco and Sousa, 2014, Li et al., 2014). These observations led to the hypothesis that NleC recognises NF- κ B transcription factor subunits by mimicking the shape and charge of DNA. Accordingly, mutational analysis of negatively charged glutamate and aspartate residues in the negatively charged active site cleft of NleC, were found to be important for cleavage of p65 *in vitro* (Turco and Sousa, 2014, Li et al., 2014).

In addition to cleaving NF- κ B transcription factor subunits, NleC has also been reported to interact with the TAZ1 domain of the histone acetyltransferase p300 (Shames et al., 2011). The p300 TAZ1 domain usually interacts with the TAD of host transcription factors including p65 (Mukherjee et al., 2013) and p53 (Krois et al., 2016a). Immunoblot analysis of cells infected with NleC-expressing EPEC demonstrated that NleC might facilitate the degradation of p300, however direct cleavage of p300 by NleC has not been demonstrated convincingly (Shames et al., 2011), therefore the physiological relevance of NleC's interaction with p300 is not clear.

EPEC and EHEC do not cause pathogenesis in mice, a commonly used model organism, therefore infection of mice with the closely related, natural mouse pathogen *Citrobacter rodentium* is used to model EPEC/EHEC infection in humans. Mice infected with a *Citrobacter rodentium* $\Delta nleC$ mutant strain induced increased expression of NF- κ B-dependent cytokines demonstrating that NleC functions to dampen the innate immune response *in vivo* (Hodgson et al., 2015).

1.4.3 Zinc metalloproteases

Proteolytic cleavage is an irreversible post-translational modification that is important in a wide range of cellular processes. Proteases are generally classified into seven groups dependent on the identity of their catalytic residue. These include serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases, metalloproteases, and asparagine peptide lyases. Metalloproteases such as NleC, are proteases that require a metal ion for catalysis. The metal ion, frequently zinc, is usually coordinated by three ligands and a water molecule that once activated, performs nucleophilic attack on the carbonyl group of a substrate peptide. Approximately half of all described metalloproteases belong to the Zincin superfamily and contain the short metal-binding motif HEXXH. In this motif, the two histidine residues coordinate the active site zinc ion, whereas the catalytically important glutamate residue activates the zinc-bound water molecule (Cerdà-Costa and Xavier Gomis-Rüth, 2014).

The structurally conserved catalytic domain of Zincin superfamily members consists of between 130 to 270 amino acids. The active site helix which contains the HEXXH motif, lies at the bottom of a cleft formed by two subdomains – an upper, N-terminal subdomain (NSD) and a lower, C-terminal subdomain (CSD) (**Figure 1.7A**). The NSD comprises the active site helix, a backing helix which stacks against the active site helix, as well as a β -sheet containing 3 to 5 β -strands. The three N-terminal β -strands form a ψ -loop motif in which the N-terminal strand is threaded through the middle two outer anti-parallel β -strands (**Figure 1.7B**). The CSD includes all residues following the active site helix and varies greatly between different proteases. Enzyme-substrate complexes have revealed that substrate peptides bind to the active site cleft in an extended conformation so that the scissile bond is positioned directly above the active site zinc (Cerdà-Costa and Xavier Gomis-Rüth, 2014).

It is generally accepted that following binding of the substrate to the active site cleft, the substrate carbonyl oxygen atom binds to the active site zinc. This leads to nucleophilic attack of the carbonyl carbon atom by the activated zinc-bound water molecule. This reaction generates a *gem*-diolate intermediate, which is stabilised by the metal and residues in close proximity to active site. The scissile bond then breaks as protons are transferred to the new α -amino terminus, and the two products then dissociate from the enzyme (**Figure 1.8**).

1.5 Aims of the project

The aim of this project is to identify and characterise SPI-2 T3SS effector proteins that modulate innate immune signalling pathways. This will be achieved by:

- Screening a library of *Salmonella* effector mutant strains to identify strains that induce increased activation of an NF- κ B luciferase reporter.
- Testing sufficiency of GFP-tagged effector proteins to inhibit NF- κ B signalling.
- Identifying biochemical activities and host cell interaction partners/substrates of effector proteins identified to be important.

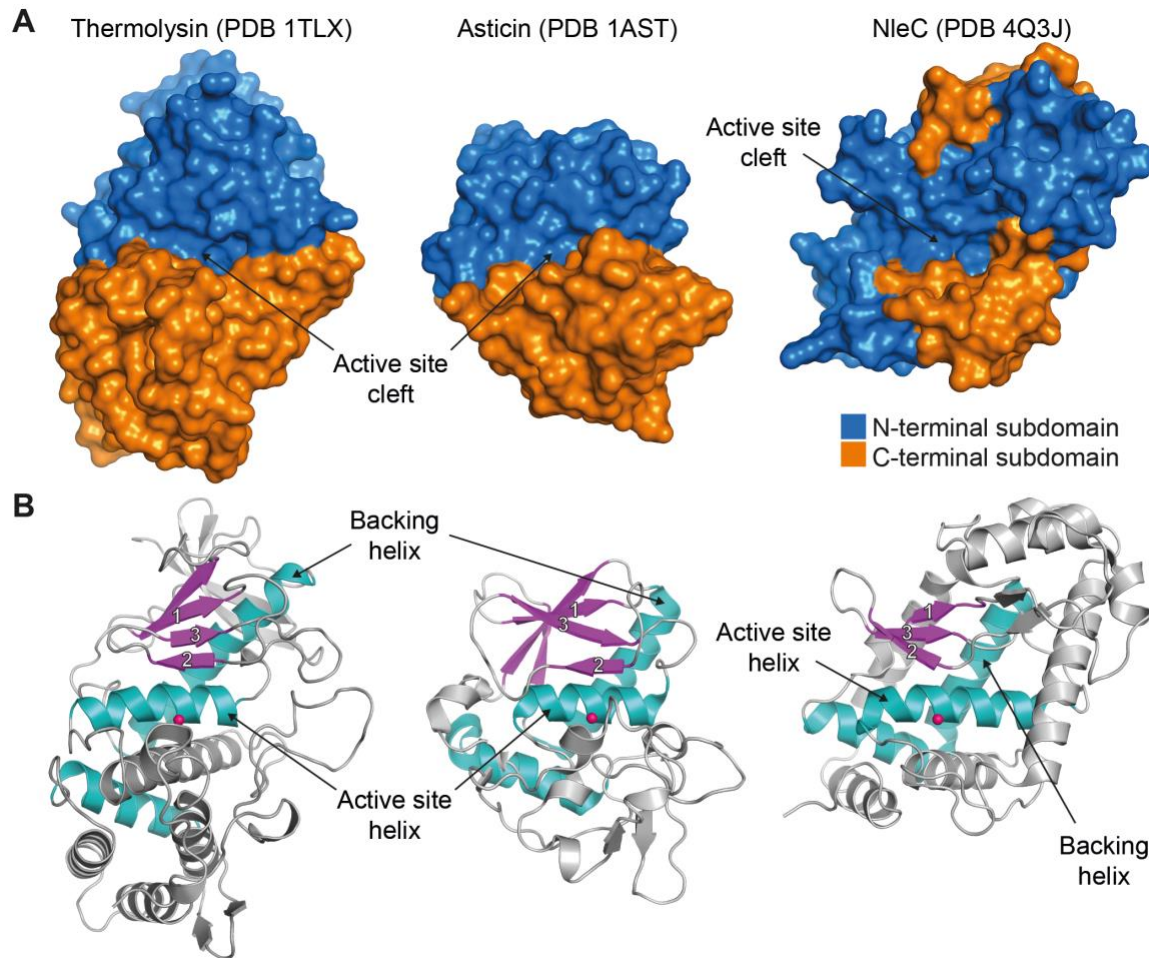


Figure 1.7: Structural characteristics of the Zincin superfamily of metalloproteases.

(A) Surface and (B) cartoon representation of Zincin superfamily members. In panel B, the Zincin catalytic core is coloured as in Cerdà-Costa and Xavier Gomis-Rüth (2014). α -helices are coloured teal, whereas the N-terminal subdomain β -sheet is coloured purple. β -strands that form the ψ -loop motif are numbered 1 to 3. The catalytic zinc ion is represented as a pink sphere.

- Determining the physiological relevance of innate immune inhibition using *in vivo* models.

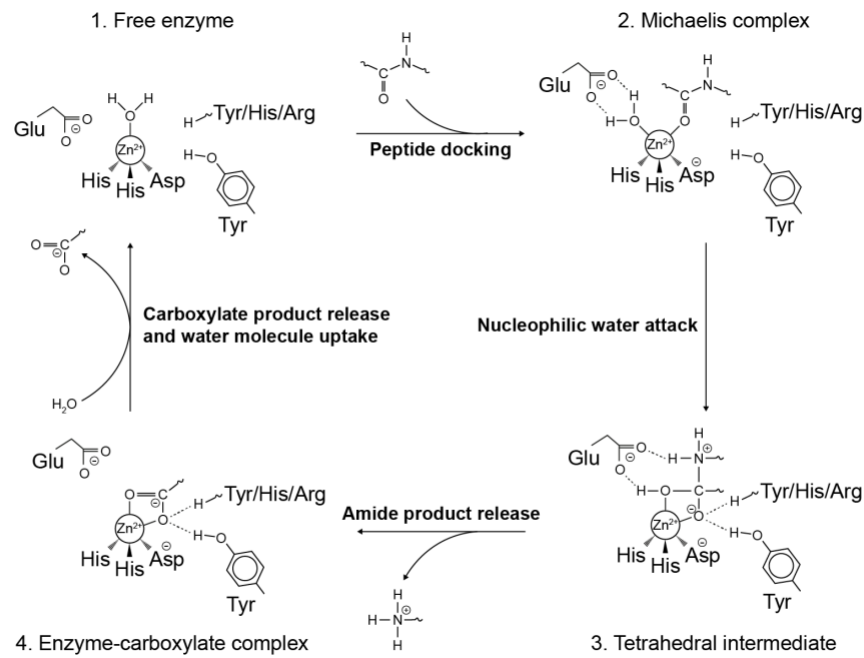


Figure 1.8: Catalytic mechanism of zinc metalloproteases.

Following peptide docking, the glutamate residue in the HEXXH motif activates a zinc-bound water molecule. The polarised water then performs nucleophilic attack on the carbon atom of the peptide carbonyl group. This leads to the formation of an unstable *gem*-diolate tetrahedral intermediate that is stabilised by residues in close proximity to the active site zinc. The peptide bond then breaks, the amide and carboxylate products are released, and a new water molecule is taken up by the enzyme. Figure adapted from Pelmeshnikov et al. (2002).

2 Materials and methods

2.1 Materials

2.1.1 Bacterial strains

Salmonella enterica subspecies *enterica* serovar Typhimurium strain ATCC 14028s, *Escherichia coli* strain O127:H6 E2348/69 (EPEC) and their isogenic mutant derivatives used in this study are listed in **Table 2.1**. The *S. Typhimurium* $\Delta sifA \Delta sopD2$ and $\Delta sifA \Delta sopD2 \Delta pipB2$ mutant strains were a gift from Dr. Stéphane Méresse (Aix-Marseille University), whereas the $\Delta slrP$ mutant strain was a gift from Dr. Andreas Bäumlér (University of California, Davis). *E. coli* strain DH5 α was used for the amplification of plasmid DNA, whereas *E. coli* BL21-Gold (DE3) (Agilent Technologies) and BL21 (DE3) PC2 (Cherepanov, 2007) were used for the expression of recombinant proteins.

2.1.2 Plasmids

Plasmids used in this study are listed in **Table 2.2**.

2.1.3 Antibodies

Antibodies used in this study are listed in **Table 2.3**. Alexa 488-conjugated donkey α -rat and Alexa 555-conjugated donkey α -goat (Life Technologies) secondary antibodies were used at a dilution of 1:1000 for immunofluorescence labelling. Horseradish peroxidase (HRP)-conjugated goat α -rabbit, goat α -mouse (Dako, Aligent Technologies), and donkey α -goat (Sigma) were used at a dilution of 1:5000 for immunoblot analysis.

2.1.4 Mammalian cell lines

293ET cells were a gift from Felix Randow (University of Cambridge). RAW264.7 cells and HeLa cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). RAW264.7 macrophages stably transduced with an NF- κ B-dependent *Firefly* luciferase

reporter and constitutively expressed *Renilla* luciferase were described previously (Gunster et al., 2017).

Table 2.1: Bacterial strains

Name	Strain	Reference/source
<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium strain ATCC 14028s		
Wild-type	Wild-type	
$\Delta ssaV$	$\Delta ssaV::aphT$	(Beuzon et al., 1999)
$\Delta avrA$	$\Delta avrA::kan$	(Figueira et al., 2013)
$\Delta cigR$	$\Delta cigR::kan$	(Figueira et al., 2013)
$\Delta gogB$	$\Delta gogB::kan$	(Figueira et al., 2013)
$\Delta gtgA$	$\Delta gtgA::kan$	This study
$\Delta gtgE$	$\Delta gtgE::kan$	(Figueira et al., 2013)
$\Delta pipB$	$\Delta pipB::kan$	(Figueira et al., 2013)
$\Delta pipB2$	$\Delta pipB2::kan$	(Figueira et al., 2013)
$\Delta sifA$	$\Delta sifA::mTn5$	(Beuzon et al., 2000)
$\Delta sifA \Delta sopD2$	$\Delta sifA \Delta sopD2::kan$	(Schroeder et al., 2010)
$\Delta sifA \Delta sopD2 \Delta pipB2$	$\Delta sifA::kan \Delta sopD2::cm \Delta pipB2$	(Schroeder et al., 2010)
$\Delta sifB$	$\Delta sifB::kan$	(Figueira et al., 2013)
$\Delta slrP$	$\Delta slrP::kan$	Andreas Bäumler
$\Delta sopD$	$\Delta sopD::kan$	(Figueira et al., 2013)
$\Delta sopD2$	$\Delta sopD2::cm$	(Figueira et al., 2013)
$\Delta spvB$	$\Delta spvB$	(Figueira et al., 2013)
$\Delta spvC$	$\Delta spvC$	(Mazurkiewicz et al., 2008)
$\Delta spvD$	$\Delta spvD$	(Figueira et al., 2013)
$\Delta srfH$	$\Delta srfH::kan$	(Figueira et al., 2013)
$\Delta srfJ$	$\Delta srfJ::kan$	(Figueira et al., 2013)
$\Delta sseF$	$\Delta sseF::aphT$	(Hensel et al., 1998)
$\Delta sseG$	$\Delta sseG::aphT$	(Hensel et al., 1998)
$\Delta sseJ$	$\Delta sseJ::cm$	(Lossi et al., 2008)
$\Delta sseK1$	$\Delta sseK1::kan$	(Figueira et al., 2013)
$\Delta sseK2$	$\Delta sseK2::kan$	(Figueira et al., 2013)
$\Delta sseK3$	$\Delta sseK3::kan$	(Figueira et al., 2013)
$\Delta sseK1/2/3$	$\Delta sseK1 \Delta sseK2 \Delta sseK3::kan$	(Figueira et al., 2013)
$\Delta sseL$	$\Delta sseL::kan$	(Rytönen et al., 2007)
$\Delta sspH1$	$\Delta sspH1::kan$	(Mesquita et al., 2012)
$\Delta sspH2$	$\Delta sspH2::kan$	(Mesquita et al., 2012)
$\Delta sspH1/2$	$\Delta sspH1 \Delta sspH2::kan$	(Mesquita et al., 2012)
$\Delta steA$	$\Delta steA::kan$	(Figueira et al., 2013)
$\Delta steB$	$\Delta steB::kan$	(Figueira et al., 2013)
$\Delta steC$	$\Delta steC::kan$	(Figueira et al., 2013)
$\Delta steD$	$\Delta steD::kan$	(Bayer-Santos et al., 2016)
$\Delta steE$	$\Delta steE::kan$	Charlotte Durkin
$\Delta gogA$	$\Delta gogA::kan$	This study
$\Delta pipA$	$\Delta pipA::kan$	This study
$\Delta gtgA \Delta gogA$	$\Delta gtgA::kan \Delta gogA::cm$	This study
$\Delta gtgA \Delta gogA \Delta pipA$	$\Delta gtgA \Delta gogA \Delta pipA::kan$	This study
<i>Escherichia coli</i>		
DH5 α		Lab stock
BL21-Gold (DE3)		Invitrogen
BL21 (DE3) PC2		(Cherepanov, 2007)
EPEC	O127:H6 E2348/69	Gad Frankel

Table 2.2: Plasmids

Name	Reference/source
Mammalian expression vectors	
pCMV-GFP (pEGFP-N1)	
pCMV.GFP-AvrA	Teresa Thurston
pCMV.GFP-CigR	Teresa Thurston
pCMV.GFP-GogB	Teresa Thurston
pCMV.GFP-GtgA	This study
pCMV.GFP-GtgE	Teresa Thurston
pCMV.GFP-PipB	Teresa Thurston
pCMV.GFP-PipB2	Teresa Thurston
pCMV.GFP-SifA	Teresa Thurston
pCMV.GFP-SifB	Teresa Thurston
pCMV.GFP-SlrP	Teresa Thurston
pCMV.GFP-SopD	Teresa Thurston
pCMV.GFP-SopD2	Teresa Thurston
pCMV.GFP-SpvB	This study
pCMV.GFP-SpvC	This study
pCMV.GFP-SpvD	Teresa Thurston
pCMV.GFP-SrfH (SseI)	Teresa Thurston
pCMV.GFP-SrfJ	This study
pCMV.GFP-SseF	Teresa Thurston
pCMV.GFP-SseG	Teresa Thurston
pCMV.GFP-SseK1	(Gunster et al., 2017)
pCMV.GFP-SseK2	(Gunster et al., 2017)
pCMV.GFP-SseK3	(Gunster et al., 2017)
pCMV.GFP-SseL	Teresa Thurston
pCMV.GFP-SspH1	This study
pCMV.GFP-SspH2	Teresa Thurston
pCMV.GFP-SteA	Teresa Thurston
pCMV.GFP-SteB	Teresa Thurston
pCMV.GFP-SteC	This study
pCMV.GFP-SteD	(Bayer-Santos et al., 2016)
pCMV.GFP-SteE	This study
pCMV.GFP-GogA	This study
pCMV.GFP-PipA	This study
pCMV.GFP-GtgA ^{E183A}	This study
pCMV.GFP-GogA ^{E183A}	This study
pCMV.GFP-PipA ^{E181A}	This study
pCMV.GFP-NleC	This study
pCMV.GFP-NleC ^{E184A}	This study
pCMV.FLAG-p100	This study
pCMV.FLAG-p100 ^{P60R}	This study
pCMV.FLAG-NFATc1	This study
pCMV.FLAG-NFATc2	This study
pCMV.FLAG-NFATc3	This study
pCMV.FLAG-NFATc4	This study
pCMV.FLAG-NFAT5	This study
pcDNA3.RelB-cFlag	Stephen Smale (Addgene #20017)
pcDNA3.FLAG-Rel	Thomas Gilmore (Addgene #27253)
pcDNA3.p50-cFLAG	This study
pcDNA3.p50 ^{V61K} -cFLAG	This study
pcDNA3.p50 ^{P65R} -cFLAG	This study
pcDNA3.p50 ^{H67A} -cFLAG	This study
pcDNA3.p65-cFLAG	This study

Table 2.2: Plasmids continued...

Name	Reference/source
pcDNA3.p65 ^{K37V} -cFLAG	This study
pcDNA3.p65 ^{R41P} -cFLAG	This study
pcDNA3.p65 ^{A43H} -cFLAG	This study
pcDNA3.p65-Renilla	This study
Protein purification vectors	
pESUMO	LifeSensors
pESUMO-p65 ²⁰⁻²⁹¹	This study
pESUMO-p65 ²⁰⁻¹⁸⁸	This study
pESUMO-NFATc2 ³⁹²⁻⁵⁸³	This study
pQLinkG2	(Scheich et al., 2007)
pETM30	(Dummler et al., 2005)
pETM30-GtgA	This study
pETM30-GogA	This study
pETM30-PipA	This study
pETM30-GtgA ^{E183A}	This study
pETM30-GogA ^{E183A}	This study
pETM30-PipA ^{E181A}	This study
pETM30-GtgA ^{E183Q}	This study
pETM30-GtgA ²⁰⁻²²⁸	This study
pETM30-GtgA ^{20-228 E183Q}	This study
pETM30-NleC	This study
pETM30-NleC ^{E184A}	This study
Bacterial expression vectors	
pWSK29	(Wang and Kushner, 1991)
pWSK29. <i>gtgA</i> -2HA	This study
pWSK29. <i>gogA</i> -2HA	This study
pWSK29. <i>pipB</i> -2HA	This study
pWSK29. <i>pipB</i> -2HA	This study
pWSK29. <i>ssaG</i> -2HA	This study
pWSK29. <i>ssaG</i> -2HA	This study
pWSK29. <i>ssaG</i> -2HA	This study
pFPV25.1	(Valdivia and Falkow, 1996)
Retroviral transduction vectors	
m6p.PAC.FLAG-GFP	Teresa Thurston
m4p.GFP-GtgA	This study
m4p.GFP-GogA	This study
m4p.GFP-PipA	This study
pMD-VSVG	(Randow and Sale, 2006)
pMD-OGP	(Randow and Sale, 2006)
One-step PCR mutagenesis	
pKD3	(Datsenko and Wanner, 2000)
pKD4	(Datsenko and Wanner, 2000)
pKD46	(Datsenko and Wanner, 2000)
pCP20	(Datsenko and Wanner, 2000)
Luciferase reporter plasmids	
pPRDII-luc	Felix Randow
pAP1-luc	Felix Randow
pISRE-luc	Felix Randow
pRL-TK	Felix Randow

Table 2.3: Primary antibodies

Antibody	Species	Manufacturer	Dilution
Immunofluorescent labelling			
CSA-1	Goat	BacTrace	1:200
HA 3F10	Rat	Roche	1:200
Immunoblot analysis			
GFP Abfinity	Rabbit	Life Technologies	1:2500
Tubulin E7	Mouse	DSHB	1:2500
GST	Rabbit	Sigma	1:2500
p65 sc-372-G	Goat	Santa Cruz	1:1000
NF- κ B1 p50	Mouse	Biolegend	1:1000
FLAG M2	Mouse	Sigma	1:2000
HA.11	Mouse	Biolegend	1:1000
DnaK	Mouse	Enzo Life Sciences	1:5000

2.2 Methods

2.2.1 Bacterial growth conditions

All strains were grown in Luria Bertani (LB) broth overnight with agitation or on LB agar plates at 37°C. LB broth and agar plates were supplemented with carbenicillin (50 µg/ml), kanamycin (50 µg/ml), tetracycline (25 µg/ml) or chloramphenicol (17 µg/ml) as required. For long-term storage, bacteria were grown in LB broth to stationary phase and stored at -80°C in 22% glycerol.

2.2.2 Plasmid DNA purification

Purification of plasmid DNA from stationary phase cultures of *E. coli* DH5α grown in LB broth was done using either the GenElute™ Plasmid Miniprep Kit (Sigma Aldrich) or NucleoBond® Xtra Midiprep kit (Macherey-Nagel) according to the manufacturer's instructions. Following elution in sterile water, DNA concentrations were measured using a NanoDrop™ Lite spectrophotometer (Thermo Scientific).

2.2.3 Isolation of bacterial genomic DNA

Isolation of genomic DNA (gDNA) from stationary phase cultures of *S. Typhimurium* and EPEC grown in LB broth was done using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions.

2.2.4 RNA extraction and complementary DNA synthesis

RNA was extracted from approximately 1×10^6 293ET cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. To avoid repeated freeze-thaw cycles, eluted RNA was aliquoted and stored at -80°C. To synthesise complementary DNA (cDNA), an aliquot of isolated RNA (400 ng) was thawed and then reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

2.2.5 Construction of expression vectors

All expression vectors were constructed using traditional restriction enzyme-mediated cleavage, followed by ligation with T4 DNA ligase (NEB). DNA fragments were first amplified from template DNA (plasmid DNA, *S. Typhimurium* gDNA, or 293ET cDNA) using the Expand High Fidelity PCR system (Roche). Overlap-extension PCR (Heckman and Pease, 2007) was used for site-directed mutagenesis. Resulting PCR amplicons were purified using the PCR Purification Kit (Qiagen), eluted in 32 µl sterile tissue-culture grade water (Sigma) and digested for 3 h at 37°C with the appropriate restriction enzymes (NEB) in a 40 µl reaction. If the amplified DNA fragment contained an internal restriction enzyme cleavage site, the desired overhang was achieved using the Type IIS restriction enzymes BsaI or BsmBI, which cleave DNA outside of their recognition sequences. To prepare the vector backbone, 0.5 to 4 µg of plasmid DNA was linearised using restriction enzymes as outlined above, with the exception that 1 µl of Calf Intestinal Alkaline Phosphatase (CIP, NEB) was added to the reaction for the final 30 min to dephosphorylate the 5'-ends of the linearised plasmid DNA.

Agarose gel electrophoresis was used to separate digested PCR amplicons and linearised plasmid DNA. DNA bands of the appropriate size were then excised and purified using the QIAQuick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The purified, digested DNA fragments were ligated into the vector backbone using 0.25 µl T4 DNA ligase (NEB) in a 10 µl reaction. The reaction was allowed to proceed at room temperature for 15 min prior to heat-shock transformation into chemically competent *E. coli* DH5α. Transformed bacteria were then incubated at 37°C for 1 h in SOC medium (0.5% Yeast extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM Glucose) to allow time for the expression of antibiotic-resistance genes before plating onto LB agar plates supplemented with the appropriate antibiotic. Following overnight growth at 37°C, plasmid DNA was purified from single clones and the plasmid DNA sequenced (GATC Biotech). Geneious v5.6.7 was used to interpret sequencing reads.

2.2.6 Preparation and transformation of electrocompetent bacteria

Bacteria grown to stationary phase in LB were sub-cultured 1:75 into 15 ml of fresh LB media and grown to an OD₆₀₀ between 0.4 and 0.6. With the exception of strains carrying the temperature sensitive plasmid pKD46, which were grown at 30°C, bacteria were grown at 37°C. Next, bacteria were cooled on ice for 15 min prior to centrifugation at 2000 x *g* for 2 min. Bacteria were then washed three times with sterile, ice-cold MilliQ water. A fourth wash using 10% glycerol was then performed before the bacteria were suspended in 400 µl of 10% glycerol. 50 µl aliquots were then mixed with approximately 100 ng of DNA (purified plasmid or PCR product) in a 2 mm electroporation cuvette and electroporated at 2.5 V, 25 µF, 200 ohms (Gene Pulser 2, Bio-Rad). Following electroporation, bacteria were transferred to a sterile 1.5 ml eppendorf tube containing 450 µl of SOC media and incubated for 1 h at either 30 or 37°C to allow time for the expression of antibiotic-resistance genes. The selection of single colonies was achieved following growth on LB agar plates supplemented with the appropriate antibiotic.

2.2.7 One-step PCR mutagenesis

Bacterial deletion mutants were constructed using one-step PCR mutagenesis (Datsenko and Wanner, 2000). First, DNA primers with 5'-overhangs homologous to 40 nucleotides outside of the gene of interest were used to amplify by PCR either the kanamycin or chloramphenicol resistance cassettes (*kan* and *cm*) from the plasmids pKD4 and pKD3, respectively. PCR products were purified using the PCR Purification Kit (Qiagen) and stored at -20°C until later use. The desired bacterial strain transformed with the temperature sensitive plasmid pKD46 were prepared and electroporated with 100 ng of purified PCR product as described above, with the exception that the expression of the λ-red recombinase encoded on pKD46 was induced with 0.1 M L-arabinose when the OD₆₀₀ of the bacterial subculture was 0.2. Transformants were screened for the presence of the antibiotic resistance cassette at the appropriate gene locus by colony PCR and the temperature sensitive plasmid pKD46 cured from positive clones by overnight growth at 42°C in LB broth.

Where required, the resistance cassettes were removed by transforming the bacteria with the plasmid pCP20, which encodes the FLP recombinase. Transformants were screened by colony PCR and the temperature sensitive plasmid pCP20 cured from positive clones by overnight growth at 42°C in LB broth.

2.2.8 P22 phage transduction

To prepare P22 phage lysate, 1 ml LB broth supplemented with 0.2% glucose, 1 x E salts was inoculated with 250 µl of stationary phase donor strain, and 10 µl wild-type P22 lysate, then incubated overnight at 37°C with agitation. Bacteria were then pelleted by centrifugation and the supernatant harvested. Chloroform (50 µl) was then added to the supernatant, vortexed for 30 sec, then allowed to settle for 2 h at room temperature.

P22 phage transduction was then performed by inoculating 200 µl of stationary phase recipient strain with between 0.1 to 10 µl of P22 phage lysate. 15 min post inoculation, 1 ml of LB broth was added and the culture was incubated at 37°C for 1 h prior to plating on agar plates supplemented with the appropriate antibiotic(s). To select lysogens, colonies were then passaged on Evans Blue Uranine plates, until the colonies were light coloured.

2.2.9 Mammalian cell culture

All mammalian cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich) supplemented with 10% fetal calf serum (FCS, Sigma Aldrich) at 37°C in 5% CO₂.

2.2.10 DNA transfections

Transfection of mammalian cell lines was done using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

2.2.11 Mammalian cell transduction

293ET cells seeded into 24 well plates at a density of 1.5×10^5 per well, were transfected with 250 ng of the required m4p retroviral vector, 75 ng pMD-VSVG and 175 ng pMD-MLV-

OGP. 24 h post transfection, the cells were washed with PBS and incubated with 1 ml of fresh culture media for a further 24 h. The culture supernatant containing retroviral particles was then harvested and centrifuged at 16000 x g to remove contaminating 293ET cells. Culture supernatants were aliquoted and stored at -20°C for later use.

For retroviral transduction, 293ET cells were seeded into 6-well plates at a density of 6×10^5 per well. 24 h later, the culture media was replaced with fresh cell culture media supplemented with 8 µg/ml Polybrene and a 1:10 dilution of a thawed retroviral stock. Cells were then centrifuged for 2 h at 2700 x g and 37°C before incubation for 24 h at 37°C in 5% CO₂. The culture medium was then removed, cells washed twice with PBS and fresh culture medium applied. At 48 h post-transduction fluorescence-activated cell sorting (FACS) was used to enrich GFP-expressing cells.

2.2.12 Salmonella Typhimurium infection of HeLa cells

HeLa cells were seeded in 24-well plates at a density of 1×10^5 cells per well 24 h prior to infection. Stationary phase cultures of *Salmonella* grown in LB broth were sub-cultured into fresh LB broth at a dilution of 1:33 and grown at 37°C for 3.5 h. Sub-cultured *Salmonella* was then inoculated onto HeLa cells at a MOI of 10 before being incubated at 37°C in 5% CO₂ for 15 min. The medium was then removed and the cells washed twice with PBS. To kill extracellular bacteria, the cells were incubated for 1 h in fresh culture medium supplemented with 100 µg/ml gentamicin. The culture medium was then aspirated and replaced with fresh culture medium supplemented with 20 µg/ml gentamicin for the remaining time of the infection.

2.2.13 Salmonella Typhimurium infection of RAW264.7 macrophages

RAW264.7 macrophages were seeded in 24-well plates at a density of 1×10^5 per well 24 h prior to infection. Stationary phase cultures of *Salmonella* grown in LB broth were diluted 1:40 in cell culture medium supplemented with 2.5% mouse serum (Sigma) and opsonised for 20 min at room temperature. Opsonised *Salmonella* were then inoculated onto RAW264.7 macrophages at a MOI of 10. To synchronise the infection, inoculated cells were then

centrifuged for 5 min at 110 x *g* and then incubated for 25 min at 37°C in 5% CO₂. The cells were then incubated at 37°C in 5% CO₂ for 1 h in fresh culture medium supplemented with 100 µg/ml gentamicin, before this was replaced with fresh culture medium supplemented with 20 µg/ml gentamicin for the remaining time of the infection.

2.2.14 Immunoblot analysis

Whole cell lysates diluted in Laemmli buffer were boiled for 5 min and then loaded onto sodium dodecyl sulphate (SDS), polyacrylamide gels of varying percentages (8-14%). Following electrophoresis in 25 mM Tris, 192 mM glycine and 0.1% SDS, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Transblot Turbo Transfer system (Bio-Rad). PVDF membranes were then incubated at room temperature in blocking buffer comprising 5% (w/v) skimmed milk powder in TBS-T (100 mM Tris pH 7.4, 150 mM NaCl, 1% Tween 20) for at least 1 h before incubation with the desired primary antibody (**Table 2.3**) diluted in blocking buffer overnight at 4°C. Next, membranes were washed at least 5 times with TBS-T and incubated with the required HRP-conjugated secondary antibody diluted in blocking buffer for 2 h at room temperature. HRP-conjugated secondary antibodies were detected using enhanced chemiluminescent (ECL) reagents (Amersham or Pierce) and either X-ray hyperfilm (Amersham) or a ChemiDoc gel imaging system (Bio-Rad). Images were processed using both ImageJ v1.48 and Image Lab v5.2.1.

2.2.15 Coomassie Blue staining

Samples diluted with Laemmli buffer were boiled for 5 min and loaded onto polyacrylamide gels of varying percentages (8-14%). Following electrophoresis in 25 mM Tris, 192 mM glycine, 0.1% SDS running buffer for 100 min at 120 V, polyacrylamide gels were washed with MilliQ water for 10 min and then stained overnight with either Coomassie Brilliant Blue R-250, Quick Coomassie (Generon), or PageBlue protein staining solution (Thermo Scientific). Gels were destained as required in 40% methanol and 10% glacial acetic acid, or

MilliQ water. Images of stained gels were acquired using a ChemiDoc gel imaging system (Bio-Rad) and then processed using Image Lab v5.2.1.

2.2.16 Flow cytometry

HeLa cells and RAW264.7 macrophages infected in 12-well plates with GFP-expressing *Salmonella* for the indicated amounts of time, were washed once with PBS then detached by trypsin/ethylenediaminetetraacetic acid (EDTA) treatment or cell scraping. Harvested cells were then pelleted by centrifugation at 700 x *g* for 2 min at 4°C, washed with 1 ml of PBS and then suspended in 200 µl of PBS. The GFP-geometric mean of at least 10,000 infected cells was then measured using a FACSCalibur flow cytometer (BD Biosciences) and these data analysed using FlowJo v10. The percentage of infected cells was calculated by gating on GFP-positive cells, whereas fold replication was calculated by dividing the GFP-geometric mean of GFP-positive cells at a late time point by the GFP-geometric mean of GFP-positive cells at 1 or 2 h post inoculation.

2.2.17 Luciferase assays

For infection assays, HeLa cells were seeded into 24-well plates at a density of 1.5×10^5 cells per well were transfected with 200 ng pPRDII-luc and 80 ng pRL-TK. 8 h post transfection cells were then washed once with PBS and infected with *S. Typhimurium* as described above. Alternatively, RAW264.7 NF-κB reporter macrophages were infected with *S. Typhimurium* as described above. At the appropriate time post inoculation, HeLa cells and RAW264.7 macrophages were lysed by the addition of 50 µl of passive lysis buffer (Promega) and stored at -80°C for future analysis.

For transfection assays, 2×10^5 293ET cells seeded in 24-well plates were transfected with 50 ng pPRDII-luc, 10 ng pRL-TK, and 300 ng pCMV.GFP-[effector]. 24 h post transfection cells were washed once with PBS and stimulated with fresh culture medium supplemented with either TNF-α (R & D Systems), IFN-β (R & D Systems) or phorbol 12-myristate 13-acetate

(PMA, Sigma) for 8 h. Cells were then lysed by the addition of 50 µl of passive lysis buffer (Promega) and stored at -80°C for future analysis.

To measure luciferase activity, collected lysates were first thawed and then 15 µl of lysate was transferred into a white 96-well plates (Greiner). The Dual Luciferase reporter assay system (Promega) and a Tecan Infinite 200 PRO plate reader was then used to measure luciferase activity in cell lysates. The activity of the *Renilla* luciferase was used to normalise *Firefly* luciferase activity for differences in transfection efficiency.

2.2.18 Immunofluorescence microscopy

Cells seeded on glass coverslips (VWR) in 24-well plates were washed once with PBS and then fixed with 3% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Fixed cells were washed a further 3 times with PBS and stored in 50 mM NH₄Cl at 4°C to quench PFA autofluorescence until immunolabelling was performed.

For immunolabelling, cells were permeabilised for 10 min in permeabilisation buffer (10% horse serum [Sigma] and 0.1% Triton X-100 in PBS) prior to incubation in the dark for 1 h with permeabilisation buffer supplemented with the desired primary antibodies. Cells were then washed twice with PBS and incubated for 1 h with permeabilisation buffer supplemented with an Alexa Fluor 488- or 555-conjugated secondary antibody and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Coverslips were then washed twice with PBS, once with sterile water then mounted onto glass slides using Aqua-Poly/Mount (Polysciences, Inc.). Quantification of immunofluorescently labelled cells was done using an Olympus BX50 light microscope. Images were acquired using an LSM 710 inverted confocal microscope (Zeiss GmbH) and then processed using ImageJ v.1.48.

2.2.19 Protein purification

E. coli BL21 transformed with a plasmid encoding the desired protein were grown in LB broth to an OD₆₀₀ of 0.6. For the expression of zinc metalloproteases, LB broth was supplemented with 100 µM ZnCl₂. Bacteria were then incubated on ice for 30 min before

protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for approximately 16 h at 18°C with agitation. Bacteria were harvested by centrifugation at 4000 x g, the supernatant discarded and the pellet suspended in 50-100 ml lysis buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 2 μ g/ml DNase I, 10 μ g/ml lysozyme, 2 mM MgCl₂, 0.5 mM tris(2-carboxyethyl)phosphine [TCEP], 10% [v/v] glycerol). Suspended pellets were then either frozen at -80°C until later use or lysed immediately by sonication using a Bandelin Sonoplus sonicator. For the purification of non-proteolytic proteins, the lysis buffer was supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche). The sonicated bacterial lysate was then clarified by centrifugation at 38000 x g for 1 h at 4°C.

Gravity flow affinity chromatography was then done at 4°C with ice-cold buffers. For the purification of His₆-GST-tagged proteins, Glutathione Sepharose 4B (GE Healthcare) was placed into a gravity flow chromatography column and equilibrated with 20 ml of wash buffer (50 mM Tris pH 8.0, 300 mM NaCl). The bacterial lysate soluble fraction was flowed over the resin and the resin then washed with at least 250 ml of wash buffer. Bound proteins were eluted in 10 ml buffer containing 50 mM Tris pH 8, 300 mM NaCl, 25 mM reduced glutathione. His₆-SUMO-fusion proteins were purified in a similar manner except proteins were bound to HisPur™ Ni-NTA resin (Thermo Fisher) and the wash buffer was supplemented with 20 mM Imidazole pH 8.0. Proteins were eluted from Ni-NTA resin in 10 ml buffer containing 50 mM Tris pH 8.0, 300 mM NaCl, 500 mM Imidazole pH 8.0.

Eluted proteins were then dialysed overnight into 25 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM dithiothreitol (DTT) and concentrated to 250-500 μ M using Amicon Ultra-15 (MWCO 10 kDa) centrifugation filters (Merck). Aliquots were frozen in liquid nitrogen and stored at -80°C for later use.

2.2.20 Cleavage of expression tags

When removal of the cleavable His₆-GST and His₆-SUMO expression tags was required, eluted proteins were incubated with 1 mg of purified His₆-TEV (Tobacco etch virus) protease, or the SUMO protease His₆-ULP1 during overnight dialysis into 50 mM Tris pH 8.0, 300 mM

NaCl, 1 mM DTT. Removal of the His₆-GST tag with the TEV protease left the amino acids GAM before the first residue of GtgA, whereas removal of the His₆-SUMO tag with ULP1, left a single alanine residue before the first residue of p65. Gravity flow affinity chromatography using either His-PurTM Ni-NTA or Glutathione Sepharose 4B was then carried out. Uncleaved protein, the cleaved expression tags and the protease bound to the resin, whereas cleaved purified proteins, flowed through the resin without binding and were then concentrated to a volume less than 5 ml in preparation for size exclusion chromatography (SEC).

2.2.21 Size-exclusion chromatography

To further increase protein purity and remove protein aggregates, proteins were subjected to SEC. Either a Hi Load 16/60 Superdex 75 or Superdex 200 coupled to an ÄKTA purifier 10 UPC (GE Healthcare Life Sciences) was equilibrated with 1 column volume of running buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP) at a flow rate of 0.1 ml/min. Proteins samples concentrated to a volume less than 5 ml were then injected into the column, and the column washed with running buffer at a flow rate of 1 ml/min. 2 ml fractions were collected following passage of the void volume and fractions corresponding to the peak UV absorbance at 280 nm were analysed by SDS-PAGE and Commassie Blue staining. Fractions of the appropriate purity were pooled and concentrated to between 10 to 25 mg/ml using Amicon Ultra-15 (MWCO 10 KDa) centrifugation filters (Merck). Aliquots were frozen in liquid nitrogen and stored at -80°C for later use.

2.2.22 Measurement of protein concentration

Protein concentrations were measured by dividing the UV absorption at 280 nm by the proteins theoretical absorbance co-efficients calculated using ProtParam (Gasteiger et al., 2005).

2.2.23 In vitro cleavage assays

His₆-SUMO-fusion proteins at a concentration of 5 µM were mixed with 0.1 µM His₆-GST-fusion proteins in a 40 µl reaction buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl,

1 mM DTT. The reaction was incubated at 37°C for 5 h until it was quenched with the addition of 40 µl 2 x Laemmli buffer. Substrate cleavage was analysed by SDS-PAGE and Coomassie Blue staining.

2.2.24 LUMIER binding assay

293ET cells were seeded in 24-well plates at a density of 1.5×10^5 per well and then 24 h later cells were transfected with 300 ng of pcDNA3.p65-Renilla. Cells were lysed in luminescence-based mammalian interactome mapping (LUMIER) lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 0.1% Triton-X100, and cOmplete EDTA-free protease inhibitor cocktail [Roche]) 24 h post-transfection and then post-nuclear supernatants (PNS) harvested following centrifugation. The PNS was then incubated with 5 µg recombinant purified proteins and 5 µl of Glutathione Sepharose 4B resin (GE Healthcare) for 2 h at 4°C. The resin was then washed 4 times with LUMIER lysis buffer and bound GST-tagged proteins eluted using *Renilla* luciferase assay lysis buffer (Promega) containing 10 mM Glutathione. *Renilla* luciferase activity was then measured using the *Renilla* luciferase assay system (Promega) and a Tecan Infinite 200 PRO plate reader.

2.2.25 Protein crystallisation

Crystallisation trials were undertaken at the Francis Crick Structural Biology Science Technology Platform. Approximately 1000 commercially available crystallisation conditions were screened in a 96-well plate, sitting-drop vapour diffusion experiment. A Mosquito crystallisation robot (TTP Labtech) was used to dispense 100 nl of protein solution and 100 nl of reservoir solution into each drop. Crystal growth following incubation at 20°C was then monitored using a Rock Imager 1000 (Formulatrix), with bright field images taken at 1 day, 2 days, 3 days, 5 days, 8 days, 13 days, 21 days, 34 days and 55 days post set-up. The proteinaceous content of crystals was then verified by ultra-violet (UV) fluorescence microscopy. Crystallisation conditions were then optimised by systematic alterations to the buffer pH, precipitant concentration and crystallisation temperature.

GtgA^{20-228 E183Q} was crystallised at a concentration of 24 mg/ml in a buffer containing 0.1 M Tris pH 8.5, 25% (v/v) 2-propanol, and 20% (w/v) polyethylene glycol (PEG) 6000. The GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸ complex was prepared by mixing 175 μ M of each protein immediately prior to crystallisation, and then crystallised in a buffer containing 0.1 M Tris pH 8.4, 0.5 M LiCl and 32.5% (w/v) PEG 3350. Crystals were flash-frozen in liquid N₂ without cryoprotectant and data collected at Diamond Light Source (Oxford, UK) at beamlines i24 and i04. Data for GtgA^{20-228 E183Q} were integrated with DIALS (<http://dials.diamond.ac.uk/>) and scaled and merged with Aimless (Evans and Murshudov, 2013). Data for GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸ were processed with DIALS (<http://dials.diamond.ac.uk/>).

2.2.26 Structural determination

Molecular replacement using the structure of p65¹⁹⁻¹⁸⁸ (PDB 2RAM) (Chen et al., 1998b) as a search model in PHASER was done to calculate initial phases of GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸. The final model of GtgA^{20-228 E183Q} in complex with p65²⁰⁻¹⁸⁸ was built using a combination of manual chain building in Coot (Emsley et al., 2010) and automated chain building using Phenix AutoBuild wizard (Terwilliger et al., 2008). Structural refinement was performed using both Phenix (Adams et al., 2010) and REFMAC (Murshudov et al., 1997). A model of GtgA^{20-228 E183Q} was built in a similar manner except that molecular replacement was done using the structure of GtgA from the complex structure as a search model. PDBsum (Laskowski, 2001) was used to analyse the interaction interface between GtgA and p65 in the complex structure, whereas SWISS-MODEL (Biasini et al., 2014) was used to generate structural homology models of GogA and PipA. PyMOL was used to prepare all structural figures and to calculate the electrostatic surface potential using Adaptive Poisson-Boltzmann Solver (APBS) (Baker et al., 2001).

2.2.27 Statistical analysis

Statistical significances were computed between multiple groups using an ordinary one-way analysis of variance (ANOVA) and post-hoc Dunnett's test in GraphPad Prism v7 (*, $P < 0.5$; **, $P < 0.01$).

3 NF- κ B inhibition by *Salmonella* SPI-2 T3SS effector proteins

The SPI-2 T3SS has been shown previously to modulate the activation of innate immune signalling pathways including the MAPK and NF- κ B signalling pathways. For example, infection of RAW264.7 murine macrophages stably transduced with an NF- κ B-dependent luciferase reporter, with a SPI-2 T3SS-null mutant strain (Δ ssaV) induced increased reporter activation relative to infection with wild-type *Salmonella* Typhimurium (Gunster et al., 2017). In addition, infection of *Tlr4*^{-/-} bone marrow-derived macrophages (BMDM) with an Δ ssaV mutant strain leads to increased expression and secretion of the NF- κ B regulated, pro-inflammatory cytokines TNF- α and IL-1 β (Rolhion et al., 2016).

Characterisation of individual SPI-2 T3SS effector proteins and mutant strains has attributed these phenotypes to specific SPI-2 T3SS effectors including GogB (Pilar et al., 2012), SpvD (Rolhion et al., 2016), SseL (Le Negrate et al., 2008), SseK1, SseK2, SseK3 (Yang et al., 2015, Gunster et al., 2017) and SspH1 (Haraga and Miller, 2003). However, experiments have been done by multiple laboratories using different techniques and cell types leading to data that are not always consistent (Le Negrate et al., 2008, Mesquita et al., 2013). Additionally, a systematic and unbiased review of NF- κ B inhibition by individual SPI-2 T3SS effector proteins has not been done, raising the possibility that other effector proteins might function to inhibit NF- κ B signalling.

To systematically investigate NF- κ B inhibition by SPI-2 T3SS effector proteins, I conducted two unbiased NF- κ B luciferase reporter screens. I first analysed the contribution of individual effector proteins to NF- κ B inhibition in *Salmonella*-infected macrophages using a panel of *Salmonella* mutant strains, then I tested the sufficiency of effector proteins expressed ectopically in 293ET cells, to inhibit NF- κ B luciferase reporter activation. This chapter describes the results of these screens and begins to characterise a family of three highly related effector proteins - GtgA, GogA, and PipA, that were found to be sufficient to inhibit NF-

κ B signalling in 293ET cells, a function that had not been attributed to them at the time that these experiments were conducted.

3.1 Results

3.1.1 SPI-2 T3SS-dependent NF- κ B inhibition in *Salmonella*-infected macrophages

To identify SPI-2 T3SS effectors that modulate NF- κ B signalling in *Salmonella*-infected macrophages, I infected RAW264.7 macrophage NF- κ B reporter cells stably transduced with an NF- κ B dependent *Firefly* luciferase reporter and constitutive *Renilla* luciferase as an internal control (Gunster et al., 2017), with a panel of *Salmonella* mutant strains deleted for at least one gene encoding a SPI-2 T3SS effector protein. These strains were available from the Holden laboratory strain collection and have been described previously (see Chapter 2.1.1). 16 hours post-uptake (h p.u.) cells were lysed and the luciferase activity in cellular lysates analysed. As a positive control, cells were treated with LPS for 16 h. LPS stimulation and infection with wild-type *Salmonella* led to a 25.3-fold (± 1.5) and 10.6-fold (± 0.7) increase in reporter activation respectively, whereas infection with a SPI-2 T3SS deficient mutant strain (Δ ssaV) induced a 13.5-fold (± 0.8) increase in NF- κ B reporter activation (**Figure 3.1**). Although reproducible across 3 independent experiments, the difference in NF- κ B reporter activation induced by wild-type *Salmonella* and the SPI-2 T3SS-null mutant strain was non-significant. NF- κ B inhibition by the SPI-2 T3SS is probably underestimated as the replication of the Δ ssaV mutant strain is impaired at this time point in RAW264.7 macrophages (**Figure 3.2**; see below) (Gunster et al., 2017).

Of the 31 single deletion mutant strains analysed, infection with only a single strain – the Δ sifA mutant, induced a significant increase in activation of NF- κ B relative to wild-type *Salmonella*. Relative to uninfected cells, there was a 16.0-fold (± 1.8) increase in NF- κ B reporter activation in Δ sifA mutant infected cells, a 1.51-fold increase relative to wild-type infected cells. The vacuole membrane surrounding Δ sifA mutant bacteria is unstable and

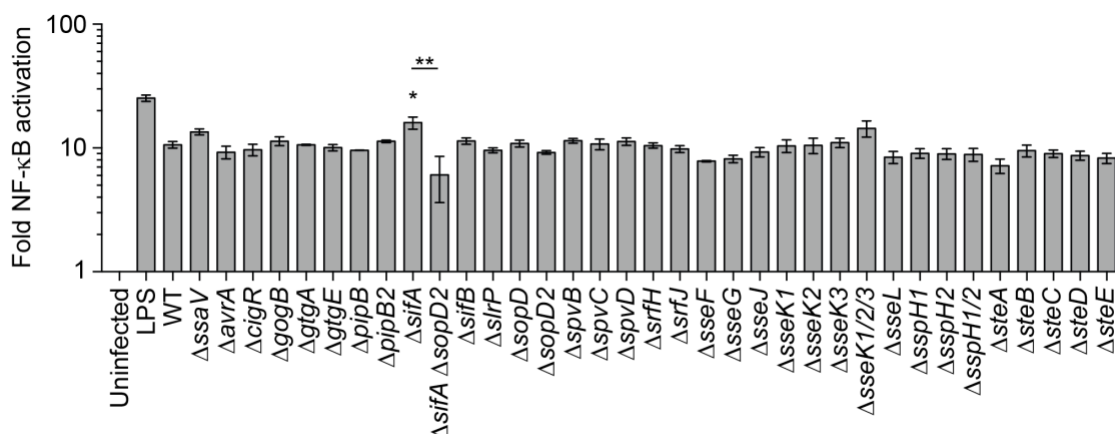


Figure 3.1: Modulation of NF-κB signalling by SPI-2 T3SS effector mutant strains.

RAW264.7 NF-κB reporter macrophages were inoculated with wild-type (WT) *Salmonella* or the indicated isogenic deletion mutant strain. 16 h p.u. luciferase activity was then measured in cellular lysates. Data are presented relative to NF-κB activity in uninfected cells and represent the mean $\pm$ SEM of 3 independent experiments. As a positive control, cells were treated for 16 h with LPS (1 μ g/ml). Statistical significances were computed between WT and each isogenic mutant strain, as well as between Δ sifA and Δ sifA Δ sopD2 mutant strains (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$).

frequently ruptures, releasing the bacteria and other contents of the vacuole into the host cell cytosol (Beuzon et al., 2000). Therefore, it is possible that increased reporter activation in Δ sifA mutant-infected cells is a consequence of vacuole rupture rather than because SifA functions to directly inhibit activation of the NF-κB signalling pathway. To distinguish between these possibilities, I simultaneously infected the RAW264.7 macrophage reporter cell line with a Δ sifA Δ sopD2 double mutant strain; deletion of *sopD2* in a Δ sifA mutant restores vacuole stability (Schroeder et al., 2010). There was no difference in NF-κB luciferase reporter activation between wild-type and Δ sifA Δ sopD2 double mutant-infected cells (**Figure 3.1**) suggesting that the increase in NF-κB reporter activation measured in Δ sifA-infected cells is due to vacuole rupture.

The SPI-2 T3SS effectors GogB (Pilar et al., 2012), SpvD (Rolhion et al., 2016), SseL (Le Negrate et al., 2008), SseK1, SseK2, SseK3 (Yang et al., 2015, Gunster et al., 2017), and

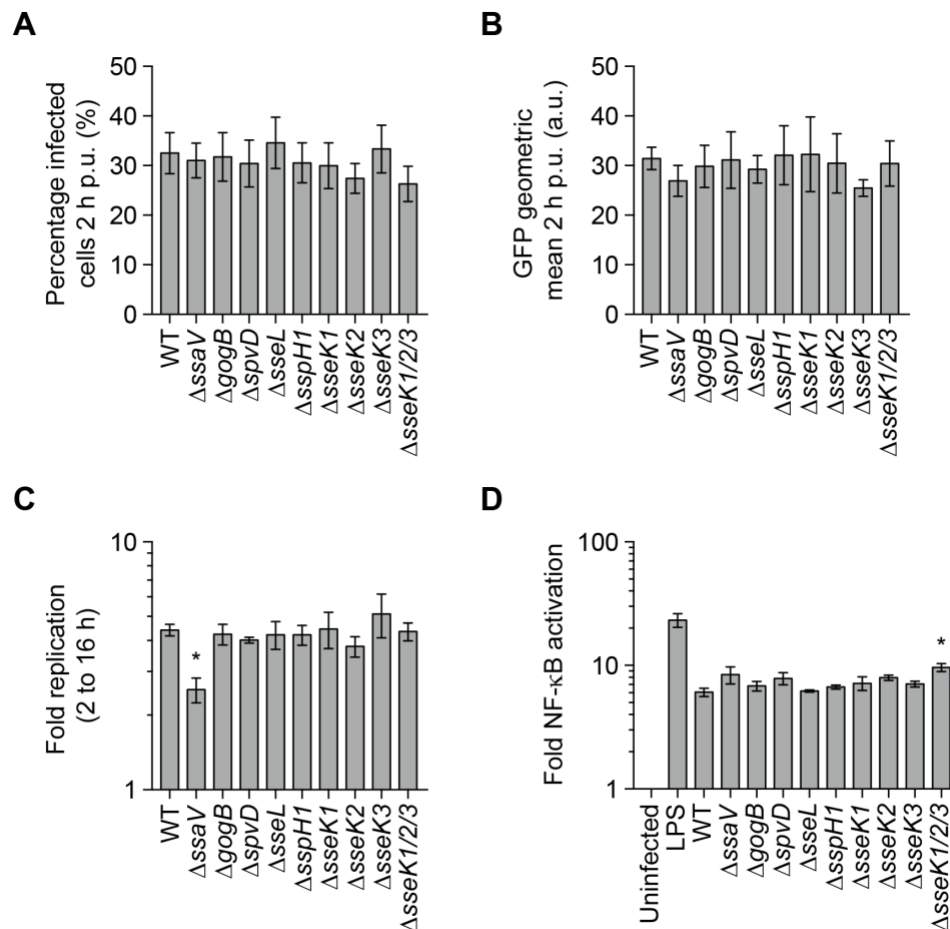


Figure 3.2: The SseK family of effector proteins inhibit the NF- κ B signalling pathway redundantly in *Salmonella*-infected macrophages.

RAW264.7 NF- κ B reporter macrophages were inoculated with GFP-expressing wild-type *Salmonella* or the indicated isogenic deletion mutant strain. At 2 and 16 h p.u., flow cytometry was done to identify the percentage of infected cells (A), the relative bacterial load of infected cells at 2 h p.u. (B) and fold replication (C). Gating on GFP-positive events identified infected cells and fold replication was calculated as the fold change in the GFP-geometric mean of infected cells from 2 to 16 h p.u. (D) Luciferase activity was measured in the lysates of RAW264.7 NF- κ B reporter macrophages infected for 16 h with the indicated GFP-expressing *Salmonella* strains. Data are presented relative to NF- κ B activity in uninfected cells and represent the mean $\pm$ SEM of 3 independent experiments. As a positive control, cells were treated for 16 h with LPS (1 μ g/ml). Statistical significances were computed between WT and each isogenic mutant strain (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$). Experiments in panels A to D were done simultaneously; RAW264.7 NF- κ B reporter macrophages were seeded at the same time and density, and infected at the same time with the same stationary phase bacterial cultures. Abbreviations: a.u., arbitrary units.

SspH1 (Haraga and Miller, 2003) have been reported previously to dampen NF- κ B-dependent gene expression. However, there was no significant difference in NF- κ B reporter activation between wild-type *Salmonella*-infected cells and cells infected with $\Delta gogB$, $\Delta spvD$, $\Delta sseL$, $\Delta sseK1$, $\Delta sseK2$, $\Delta sseK3$, and $\Delta sspH1$ single deletion mutant strains (**Figure 3.1**). SseK1 and SseK3 inhibit NF- κ B signalling redundantly (Gunster et al., 2017), and in accordance with this there was a modest 1.36-fold increase in NF- κ B reporter activation in cells infected with a $\Delta sseK1 \Delta sseK2 \Delta sseK3$ ($\Delta sseK1/2/3$) triple mutant strain relative to wild-type infected cells. This difference was however non-significant.

Next, I focussed on a subset of mutant strains in which an effector described previously to inhibit NF- κ B signalling was deleted. These strains were transformed with the plasmid pFPV25.1, which encodes GFP from the constitutively active *rpsM* promoter (Valdivia and Falkow, 1996), enabling analysis of bacterial uptake and replication. RAW264.7 reporter macrophages were inoculated with the mutant strains and then at 2 h p.u. cells were harvested and analysed by flow cytometry. The percentage of infected cells was calculated by gating on GFP-positive events and bacterial load per cell analysed by calculating the GFP-geometric mean of GFP-positive cells. Bacterial uptake and bacterial load were similar in RAW264.7 reporter macrophages infected with wild-type *Salmonella* and the indicated mutant strains, with bacteria taken up by approximately 30% of cells (**Figure 3.2A and B**).

Simultaneously infected cells, were also harvested at 16 h p.u. and analysed by flow cytometry. Bacterial replication was then calculated as the fold change in the GFP-geometric mean of GFP-positive cells from 2 to 16 h p.u. By 16 h p.u. the bacterial load in wild-type infected cells had increased 4.4-fold (± 0.2), whereas the fold increase in bacterial load in $\Delta ssaV$ mutant-infected cells (2.5-fold [± 0.3]) was significantly reduced in line with studies reporting that the SPI-2 T3SS is required for intra-macrophage replication (**Figure 3.2C**) (Cirillo et al., 1998, Figueira et al., 2013). However, the fold change in bacterial load in $\Delta gogB$, $\Delta spvD$, $\Delta sseL$, $\Delta sspH1$, $\Delta sseK1$, $\Delta sseK2$, $\Delta sseK3$, or $\Delta sseK1/2/3$ mutant strains infected cells was similar to that observed for wild-type *Salmonella*. There was a 6.1-fold (± 0.5) and 8.4-fold

(± 1.3) increase in NF- κ B reporter activity in cells infected with wild-type *Salmonella* or a Δ ssaV mutant strain respectively. Although the difference was non-significant this represents a 1.38-fold increase in NF- κ B activation in Δ ssaV mutant-infected cells relative to wild-type-infected cells. In agreement with previous observations (Gunster et al., 2017), there was a significant increase in NF- κ B reporter activation in Δ sseK1/2/3 triple mutant infected cells, however, there was no difference in NF- κ B reporter activation induced by the other deletion mutants compared to wild-type *Salmonella*.

Together these data show that the SseK family redundantly inhibit NF- κ B signalling in *Salmonella*-infected macrophages whereas GogB, SpvD, SseL, and SspH1, which have been described previously to inhibit NF- κ B activation, are not important for NF- κ B inhibition in our experimental set-up.

3.1.2 GtgA is sufficient to inhibit TNF- α -induced NF- κ B activation in 293ET cells

T3SS effectors have frequently been described to either function redundantly or target the same cellular process at different levels (Galan, 2009). This could explain why, with the exception of the SseK family, I did not identify any SPI-2 T3SS effectors that inhibit NF- κ B activity in *Salmonella*-infected macrophages. Therefore, I next analysed if any effectors were sufficient to inhibit TNF- α -induced NF- κ B reporter activation when expressed ectopically in 293ET cells. I constructed a panel of mammalian expression vectors, each member of which encodes a known SPI-2 T3SS effector fused via its N-terminus to GFP. These plasmids were transiently transfected into 293ET cells along with an NF- κ B-dependent luciferase reporter plasmid (pPRDII-luc) and the plasmid pRL-TK. pRL-TK, which encodes the *Renilla* luciferase constitutively expressed from the herpes simplex virus thymidine kinase promoter, served as an internal control to normalise for transfection efficiency.

First, expression of GFP-tagged effector proteins was confirmed by Western blotting. Of 31 GFP-effector fusions, 24 were detectable. GFP-effector fusions that were not detected

included GFP-tagged GogB, SopD2, SpvB, SrfJ, SspH2, SteB, and SteE (**Figure 3.3A**). In addition, high levels of cell death were observed by light microscopy in cells expressing GFP-tagged SopD2, SseF, SseG, and SseJ (data not shown). GFP-effector fusions were then analysed for their ability to inhibit basal activation of the NF- κ B reporter in unstimulated cells relative to GFP-expressing cells. In cells expressing GFP-tagged GtgA, SseL, and SspH1 there was a significant reduction in basal NF- κ B reporter activation (**Figure 3.3B**). In contrast, basal NF- κ B reporter activation was significantly increased in cells expressing GFP-tagged SpvC, SpvD, SrfH, SseK2, SteB, SteC, and SteD. Although GFP-tagged SspH2 was not detectable by immunoblot analysis (**Figure 3.3A**), basal NF- κ B reporter activation was decreased in cells transfected with a plasmid encoding GFP-SspH2 (**Figure 3.3B**).

Sufficiency to inhibit TNF- α -induced-NF- κ B reporter activity was analysed next. Transfected cells were treated with TNF- α for 18 h and the fold change in NF- κ B reporter activity following TNF- α treatment calculated. 18 h TNF- α treatment of GFP-expressing 293ET cells induced a 48.1-fold (± 9.6) increase in NF- κ B reporter activity (**Figure 3.3C**). The SseK family has been shown previously to be sufficient to inhibit TNF- α -induced NF- κ B activation (Gunster et al., 2017, Yang et al., 2015). I confirmed these results: TNF- α -induced NF- κ B reporter activation was inhibited in cells expressing GFP-tagged SseK1, SseK2 or SseK3. TNF- α -induced NF- κ B reporter activation was also significantly inhibited in cells expressing GFP-tagged GtgA, SteB, and SteD (**Figure 3.3C**). Ectopic expression of SspH1 in CHO-K1 cells has been shown previously to inhibit LPS-stimulated activation of an NF- κ B luciferase reporter in which the expression of the *Firefly* luciferase expression is controlled by the *ELAM1* promoter (Haraga and Miller, 2003). We failed to detect inhibition of TNF- α -induced NF- κ B reporter activation in cells expressing SspH1; instead relative to GFP-expressing cells, TNF- α -induced activation of the PRDII reporter was significantly increased in SspH1-expressing 293ET cells (**Figure 3.3C**).

At the time that these experiments were conducted GtgA, SteB, and SteD were poorly

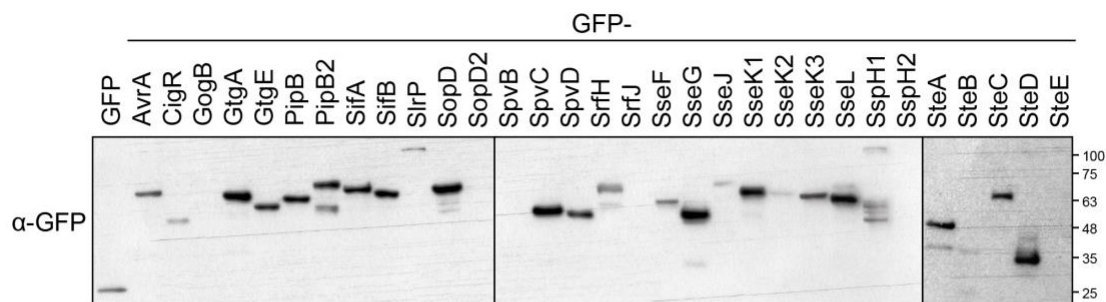
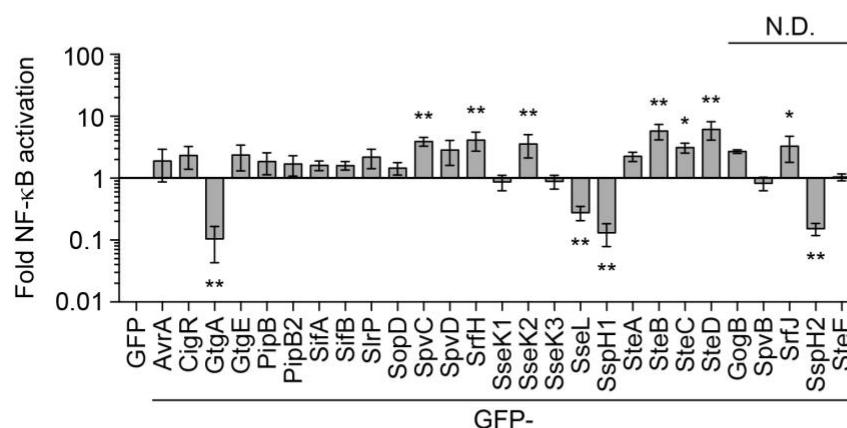
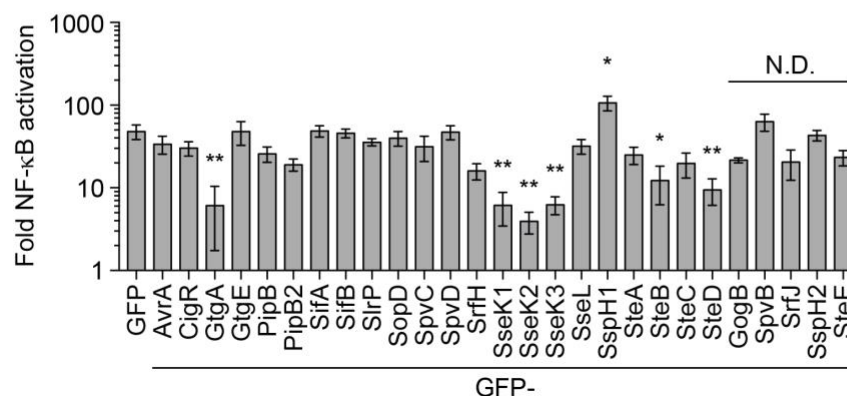
A**B****C**

Figure 3.3: GtgA, SseK1, SseK2, SseK3, SteB, and SteD are sufficient to inhibit TNF- α -induced NF- κ B activation.

293ET cells were co-transfected with plasmids encoding an NF- κ B-dependent *Firefly* luciferase, a constitutively expressed *Renilla* luciferase and the indicated GFP-tagged effector protein. (A) SDS-PAGE and immunoblotting of transfected 293ET cells to determine the expression of GFP-tagged effectors proteins during NF- κ B luciferase assays. (B) NF- κ B luciferase reporter assay from unstimulated 293ET cells expressing the indicated GFP-tagged effector protein. Data are presented as the fold difference in NF- κ B activity relative to GFP-

expressing cells and represent the mean $\pm$ SEM of 3 independent experiments. (C) NF- κ B-dependent luciferase activity was measured in the cell lysates of 293ET cells expressing the indicated GFP-tagged effector protein stimulated for 18 h with 20 ng/ml TNF- α . Data are presented as the fold change in NF- κ B activity between unstimulated (shown in panel B) and TNF- α -stimulated 293ET cells and represent the mean $\pm$ SEM of 3 independent experiments. Statistical significances were computed between 293ET cells expressing GFP and each GFP-tagged effector protein (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$). Abbreviations: *N.D.*, not detected.

characterised SPI-2 T3SS effectors. As GtgA inhibited NF- κ B reporter activation in both unstimulated and TNF- α -stimulated cells, I decided to further characterise the function of GtgA.

3.1.3 GtgA, GogA, and PipA inhibit NF- κ B signalling dependent on a functional zinc metalloprotease motif

The *Salmonella* Typhimurium strain ATCC 14028s genome contains two genes: *gogA* and *pipA*, the protein products of which share high amino acid sequence identity with GtgA - 97% and 67% respectively. Although GogA and PipA have not been shown to be T3SS effector proteins previously, I hypothesised that these two proteins might also be sufficient to inhibit TNF- α -induced NF- κ B reporter activation in 293ET cells. Functional redundancy between this family of related proteins might explain why there was not a significant increase in NF- κ B reporter activity in Δ *gtgA* mutant-infected RAW264.7 reporter cells. To determine whether GogA and PipA function similarly to GtgA, the open-reading frames (ORFs) of *gogA* and *pipA* were amplified from *Salmonella* Typhimurium strain ATCC 14028s genomic DNA, and then ligated into a mammalian expression vector so that when expressed they are fused via their N-terminus to GFP. The ability of the unrelated SPI-2 T3SS effector PipB to influence TNF- α -induced NF- κ B reporter activity was also analysed as a negative control. These plasmids were then transiently transfected into 293ET cells along with the NF- κ B luciferase reporter plasmids described above. Cells were stimulated for 8 h with TNF- α prior to cell lysis

and subsequent analysis of luciferase activity. As expected NF- κ B reporter activation was inhibited in cells expressing GFP-GtgA but not GFP-PipB. Interestingly NF- κ B reporter activation was also significantly inhibited in cells expressing GFP-GogA and GFP-PipA (**Figure 3.4A**).

The C-terminal regions of GtgA, GogA, and PipA each contain a short metal-binding motif commonly found in zinc metalloproteases - 'HEXXH' (where 'X' is any amino acid) (Gomis-Ruth, 2009). In addition, bioinformatic analysis of GtgA using the Phyre2 structural prediction software (Kelley et al., 2015), predicted that the residues spanning this motif (GtgA residues 179 to 224) are structurally similar to NleC, a zinc metalloprotease T3SS effector from EPEC/EHEC (see Chapter 1.4.2). NleC cleaves NF- κ B transcription factor subunits during EPEC/EHEC infection thereby dampening activation of the NF- κ B signalling pathway (Yen et al., 2010, Baruch et al., 2011, Pearson et al., 2011, Muhlen et al., 2011). We confirmed these data: GFP-NleC inhibited NF- κ B reporter activation in both unstimulated and TNF- α -stimulated 293ET cells and mutation of the catalytically important glutamate residue in the HEXXH motif to an alanine (NleC^{E184A}) partially abrogated reporter inhibition (**Figure 3.4A**) despite equivalent levels of expression of the wild-type and mutant protein (**Figure 3.4B**). However, relative to cells expressing GFP, basal levels of NF- κ B reporter activation were inhibited in cells expressing GFP-NleC^{E184A} suggesting that NleC also inhibits NF- κ B signalling independent of its catalytic activity. Mutation of the catalytically important glutamate in GtgA, GogA, and PipA (GtgA^{E183A}, GogA^{E183A}, and PipA^{E181A}) completely abrogated the ability of these proteins to inhibit NF- κ B reporter activation demonstrating that inhibition of NF- κ B signalling by GtgA, GogA, and PipA is dependent on putative zinc metalloprotease activity (**Figure 3.4A**). SDS-PAGE and immunoblot analysis confirmed that the abundance of these mutants relative to wild-type proteins was indistinguishable (**Figure 3.4B**).

I next tested whether GtgA, GogA, PipA, and NleC modulate the activation of other innate immune signalling pathways including MAPK and type I interferon signalling using

activator protein-1- (AP-1-) and interferon-stimulated response element- (ISRE-) dependent

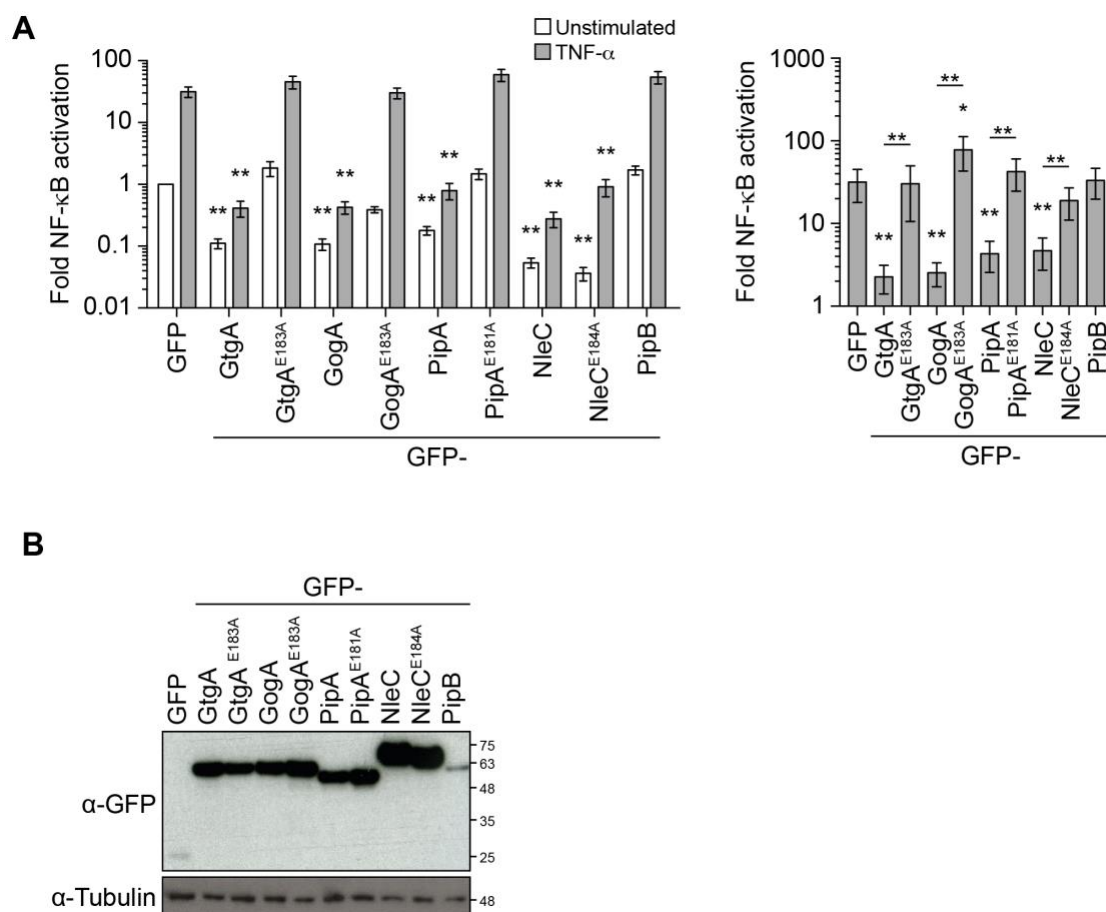


Figure 3.4: GtgA, GogA, and PipA inhibit the NF-κB signalling pathway.

(A) 293ET cells were co-transfected with plasmids encoding an NF-κB-dependent *Firefly* luciferase, a constitutively expressed *Renilla* luciferase, and the indicated GFP-tagged effector protein. Cells were then stimulated for 8 h with 20 ng/ml TNF-α prior to analysis of luciferase activity. Data are presented relative to the luciferase activity in unstimulated GFP-expressing cells (*left*), and to the luciferase activity in unstimulated cells expressing each indicated GFP-tagged effector protein (*right*). Data represents the mean ± SEM of 3 to 5 independent experiments. *Left* panel: Statistical significances were computed between unstimulated or stimulated GFP-expressing cells and each indicated GFP-tagged effector protein. *Right* panel: Statistical significances for the fold change in reporter activation after stimulation, were computed between 293ET cells expressing GFP and each indicated GFP-tagged effector protein, as well as GFP-tagged effector proteins and their putative catalytic mutant (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$). (B) Whole cell lysates for the experiment shown in panel A were separated by SDS-PAGE and immunoblotted with an α-GFP antibody to confirm expression of each GFP-tagged effector. Immunoblots are representative of 3 independent experiments.

luciferase reporter plasmids. 293ET cells were transfected with an AP-1 reporter plasmid and plasmids encoding GFP-tagged effector proteins and then stimulated for 8 h with PMA. As a positive control, reporter activation in cells expressing the phosphothreonine lyase effector protein SpvC, which irreversibly dephosphorylates the MAPKs JNK and p38 (Mazurkiewicz et al., 2008, Li et al., 2007), was also analysed. As expected, PMA treatment induced activation of the AP-1 reporter and this was inhibited in cells expressing GFP-SpvC (**Figure 3.5A**). However, AP-1 reporter activation was indistinguishable between cells expressing GFP and GFP-tagged GtgA, GogA, PipA, or PipB suggesting that these proteins do not inhibit MAPK signalling. Interestingly in cells expressing GFP-NleC, basal AP-1 reporter activity was reduced and there was an increase in the fold activation of AP-1 signalling between unstimulated and PMA-treated cells (**Figure 3.5B**). I also analysed whether GtgA, GogA, and PipA inhibit activation of an ISRE-dependent reporter stimulated for 8 h with IFN- β . There was no significant difference in IFN- β induced ISRE reporter activation between cells expressing GFP and GFP-tagged GtgA, GogA, PipA, or PipB but the ISRE reporter was inhibited in cells expressing GFP-NleC (**Figure 3.5B**). This was not dependent on the catalytic activity of NleC as the catalytic mutant GFP-NleC^{E184A} inhibited IFN- β -induced ISRE reporter activation to a similar level as wild-type NleC.

Therefore, these data demonstrate that GtgA, GogA, and PipA inhibit NF- κ B signalling, but not MAPK or type I interferon signalling. Inhibition of NF- κ B signalling is dependent on their putative zinc metalloprotease activity. This is in contrast to NleC, which when ectopically expressed in 293ET cells inhibited both NF- κ B and type I interferon signalling. Although NleC-mediated inhibition of TNF- α -induced NF- κ B signalling was dependent on NleC's catalytic activity, NleC also inhibited NF- κ B and type I interferon signalling in a catalytically-independent manner.

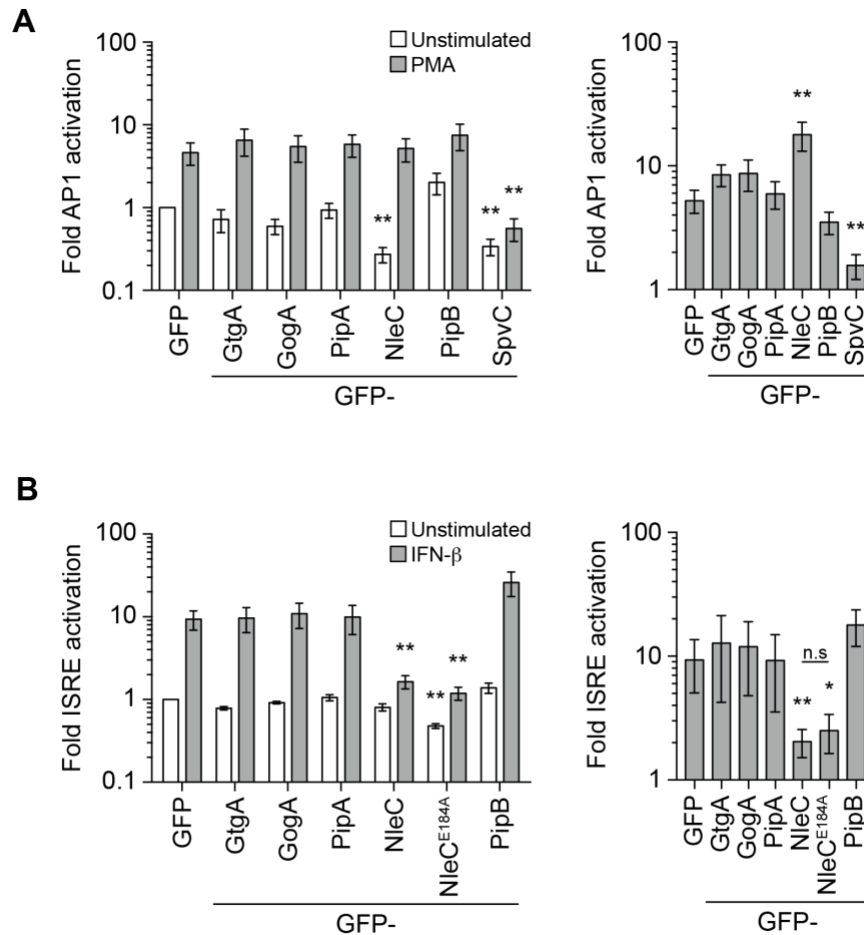


Figure 3.5: NleC inhibits IFN- β -induced ISRE signalling independently of its zinc metalloprotease activity.

293ET cells were co-transfected with plasmids encoding an AP1-dependent (A) or ISRE-dependent (B) *Firefly* luciferase reporter, a constitutively expressed *Renilla* luciferase and the indicated GFP-tagged effector protein. Cells were then stimulated for 8 h with 5 μ g/ml PMA (A) or 1360 U/ml IFN- β (B). Data are presented relative to the luciferase activity in unstimulated GFP-expressing cells (*left*), and to the luciferase activity in unstimulated cells expressing each indicated GFP-tagged effector protein (*right*). Data represents the mean $\pm$ SEM of 3 independent experiments. *Left* panel: Statistical significances were computed between unstimulated or stimulated GFP-expressing cells and each indicated GFP-tagged effector protein. *Right* panel: Statistical significances for the fold change in reporter activation after stimulation, were computed between 293ET cells expressing GFP and each indicated GFP-tagged effector protein, as well as GFP-tagged effector proteins and their putative catalytic mutant (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$). Abbreviations: *n.s.*, non-significant.

3.1.4 NleC residues 281-330 are required for inhibition of IFN- β -induced ISRE signalling

Truncation analysis of NleC has shown previously that the last 64 residues (residues 267-330) are dispensable for inhibition of NF- κ B signalling by NleC (Muhlen et al., 2011). As NleC inhibits ISRE signalling independently of its catalytic activity, and because this region is not present in GtgA, GogA, or PipA, I hypothesised that this region might be required for ISRE inhibition. To test this hypothesis, I ligated two truncated forms of *nleC* into a mammalian expression vector, to express GFP-tagged NleC¹⁻²³¹ and NleC¹⁻²⁸⁰. These truncations were based on the published crystal structures of NleC (Li et al., 2014, Turco and Sousa, 2014).

The ability of the truncated forms of NleC to inhibit NF- κ B and type I interferon signalling, was then tested in 293ET cells using the NF- κ B and ISRE reporter assays described above. As expected NF- κ B reporter activation was significantly inhibited in cells expressing NleC and NleC¹⁻²⁸⁰. Catalytically inactive NleC (NleC^{E184A}) only partially inhibited NF- κ B reporter activation, whereas GFP-NleC¹⁻²³¹ had no effect (**Figure 3.6A**). In contrast, ISRE reporter activation was significantly inhibited in cells expressing wild-type and catalytically inactive NleC (NleC^{E184A}) but not in cells expressing NleC¹⁻²³¹ or NleC¹⁻²⁸⁰ (**Figure 3.6B**). Therefore, NleC residues 281 to 330 are required for inhibition of type I interferon signalling by NleC.

3.1.5 GtgA, GogA, and PipA cleave the N-terminal domain of p65

NleC cleaves NF- κ B subunits within the NTD of the RHR thereby preventing NF- κ B transcription factors from interacting with DNA and inducing proinflammatory gene expression (Yen et al., 2010, Baruch et al., 2011, Pearson et al., 2011, Muhlen et al., 2011). As the C-terminal region of GtgA was predicted to be structurally similar to NleC, and because GtgA, GogA, and PipA inhibit NF- κ B signalling dependent on a putative zinc metalloprotease motif, I hypothesised that GtgA, GogA, and PipA might also function by cleaving NF- κ B transcription factor subunits.

To test this, I purified His₆-GST-tagged GtgA, GogA, and PipA as well as their putative

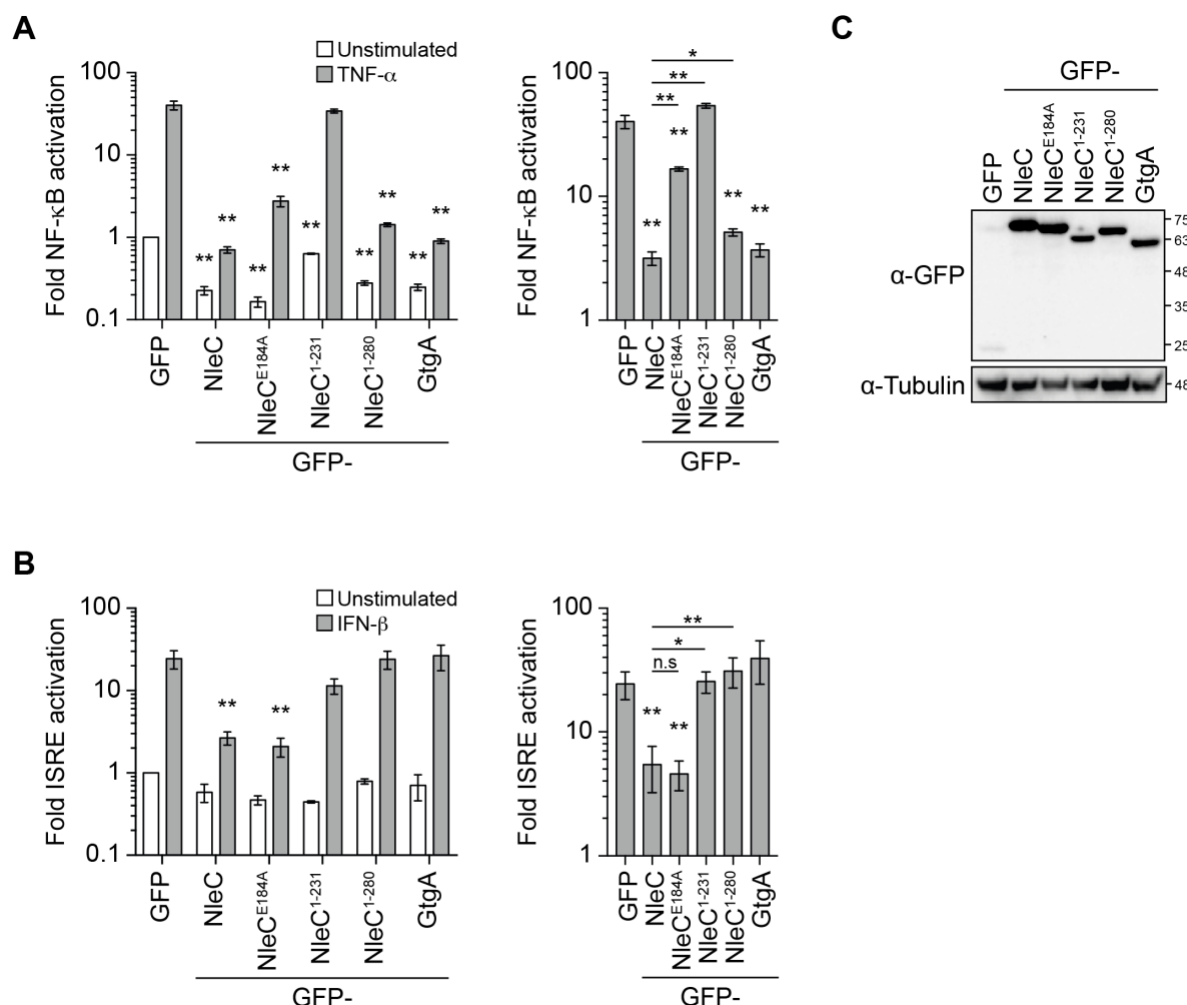


Figure 3.6: NleC residues 281 to 330 are required for ISRE reporter inhibition but not NF- κ B reporter inhibition.

293ET cells were co-transfected with plasmids encoding an NF- κ B-dependent (A) or ISRE-dependent (B) *Firefly* luciferase reporter, a constitutively expressed *Renilla* luciferase and the indicated GFP-tagged effector protein. Cells were then stimulated for 8 h with 20 ng/ml TNF- α (A), or 1360 U/ml IFN- β (B). Data are presented relative to the luciferase activity in unstimulated GFP-expressing cells (*left*), and to the luciferase activity in unstimulated cells expressing each indicated GFP-tagged effector protein (*right*). Data represents the mean $\pm$ SEM of 3 independent experiments. *Left* panel: Statistical significances were computed between unstimulated or stimulated GFP-expressing cells and each indicated GFP-tagged effector protein. *Right* panel: Statistical significances for the fold change in reporter activation after stimulation, were computed between 293ET cells expressing GFP and each indicated GFP-tagged effector protein, as well as between GFP-NleC and each GFP-NleC variant (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$). (C) Lysates from the experiments shown in panels A and B were separated using SDS-PAGE and

analysed by immunoblotting to confirm expression of GFP-tagged effectors proteins. Immunoblots are representative of 3 independent experiments.

catalytically inactive mutants from *E. coli* using affinity chromatography. As controls, I also purified His₆-GST-NleC and His₆-GST-NleC^{E184A}. Purified recombinant effectors were then incubated at a 1:50 molar ratio for 5 h at 37°C with the RHR of p65 (His₆-SUMO-p65²⁰⁻²⁹¹). The reaction was then analysed by SDS-PAGE and p65 cleavage revealed by Coomassie blue staining. Immunoblotting using an α -GST antibody was also done to confirm equal abundance of each His₆-GST-tagged protein in the reaction.

As expected, His₆-SUMO-p65²⁰⁻²⁹¹ was cleaved by NleC. Cleavage yielded three cleavage fragments, one of which was approximately 30 kDa in size and two that were approximately 19 kDa in size (**Figure 3.7A**). The p65 RHR was also cleaved by GtgA, GogA, and PipA; the fragments were similar in size to those generated by NleC suggesting that GtgA, GogA, and PipA cleave p65 at a similar position as NleC. p65 cleavage was dependent on the zinc metalloprotease motif of each protein as mutation of the putative catalytically important glutamate residue to an alanine abrogated cleavage of the p65 RHR. Additionally, when recombinant GtgA, GogA, PipA, and NleC were incubated with 100 mM of the metal-chelating agent EDTA for 30 min prior to the addition of His₆-SUMO-p65, p65 RHR cleavage was significantly reduced (**Figure 3.7B**). These data show that GtgA, GogA, and PipA are zinc metalloproteases that cleave the p65 RHR, directly.

Truncation analysis of p65 has shown that the NTD of p65 (residues 19-187) is the smallest region that can be cleaved by NleC (Giogha et al., 2015). To determine whether GtgA, GogA, and PipA also cleave the p65 NTD I incubated recombinant GtgA, GogA, and PipA with His₆-SUMO-p65²⁰⁻¹⁸⁸. Similar to NleC, His₆-SUMO-p65²⁰⁻¹⁸⁸ was cleaved by GtgA, GogA, and PipA (**Figure 3.7C**). Densitometry analysis of the Coomassie blue stained polyacrylamide gels, showed that the greatest amount of cleavage was observed following p65 cleavage by GtgA (60% cleavage) and GogA (63%). In comparison, PipA and NleC

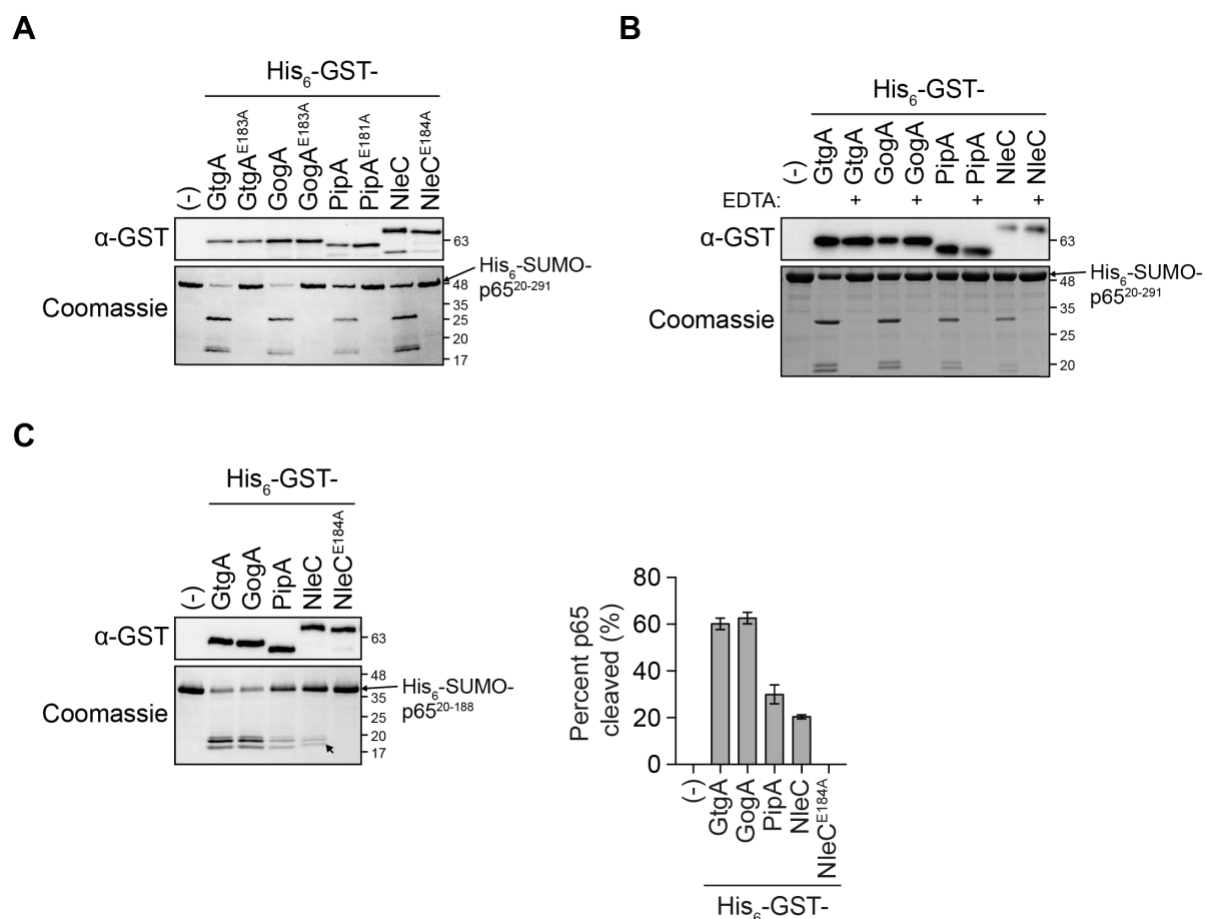


Figure 3.7: GtgA, GogA, and PipA directly cleave the p65 N-terminal domain.

In vitro cleavage assays using 0.1 μ M of the indicated His₆-GST-tagged effector protein and 5 μ M His₆-SUMO-p65²⁰⁻²⁹¹ (A and B) or His₆-SUMO-p65²⁰⁻¹⁸⁸ (C). Putative proteases and substrates, purified by affinity chromatography, were co-incubated in 40 μ l reaction buffer for 5 h at 37°C. The reaction was quenched by the addition of Laemmli buffer and proteins separated and visualised by SDS-PAGE followed by Coomassie blue staining. In panel C, the arrow highlights the change in the size of the cleavage product generated when His₆-SUMO-p65²⁰⁻¹⁸⁸ is cleaved by NleC. p65 cleavage was quantified using densitometry analysis (*right*). The Coomassie blue stained polyacrylamide gel and α -GST immunoblot are representative of 3 independent experiments.

cleaved 32% and 21%, respectively. Interestingly the smallest fragment generated following p65 cleavage by GtgA, GogA, and PipA was smaller in size (indicated by the arrow in **Figure 3.7C**), when compared to the smallest fragment generated by NleC, suggesting a different cleavage site. Together these data demonstrate that like NleC, GtgA, GogA, and PipA are

zinc metalloproteases that cleave the NTD of p65 directly. In addition, GtgA, GogA, and PipA cleave p65 at a similar, but not identical cleavage site as NleC.

3.1.6 GtgA, GogA, and PipA cleave p65, cRel, and RelB, but not NF- κ B1 (p50/p105), NF- κ B2 (p52/p100) or NFAT transcription factor subunits

In addition to cleaving p65, NleC has been reported to cleave other NF- κ B subunits including cRel, RelB, and NF- κ B1 (p105/p50). It has not yet been determined whether NleC cleaves NF- κ B2 (p100/p52). Therefore, I hypothesised that GtgA, GogA, and PipA might also cleave other NF- κ B transcription factors. To test this, 293ET cells were transiently transfected with the indicated GFP-tagged effector or catalytically inactive mutant-encoding vectors and then lysates were separated by SDS-PAGE and immunoblotted for endogenous or ectopically expressed NF- κ B subunits as described below.

Using an antibody raised against a C-terminal region of p65, I observed a reduction in the abundance of full length p65, and the presence of a cleaved form of p65 in cells expressing GFP-tagged GtgA, GogA, PipA, and NleC. p65 was not cleaved in cells expressing GFP, GFP-PipB nor in cells expressing catalytically inactive variants of GtgA, GogA, PipA, or NleC. This shows that cleavage is dependent on the zinc metalloprotease activity of each protein. Additionally, immunoblotting with an α -p50 antibody revealed that p50 is cleaved in cells expressing GFP-NleC but not GFP-tagged GtgA, GogA, or PipA (**Figure 3.8A**).

To analyse cleavage of RelB, cRel, and NF- κ B2 (p100/p52), I co-transfected cells with plasmids encoding the indicated GFP-tagged effector and RelB-FLAG, FLAG-cRel or FLAG-p100. Immunoblotting using α -GFP and α -FLAG antibodies was then done. FLAG-tagged RelB, and cRel were not detected in cells expressing GFP-tagged GtgA, GogA, PipA, or NleC despite being detected in cells expressing GFP, GFP-PipB, or catalytically inactive protease variants (**Figure 3.8A**). In contrast, FLAG-p100 was detected in all cells except those expressing GFP-NleC. Therefore, NleC is a more promiscuous protease that cleaves all five

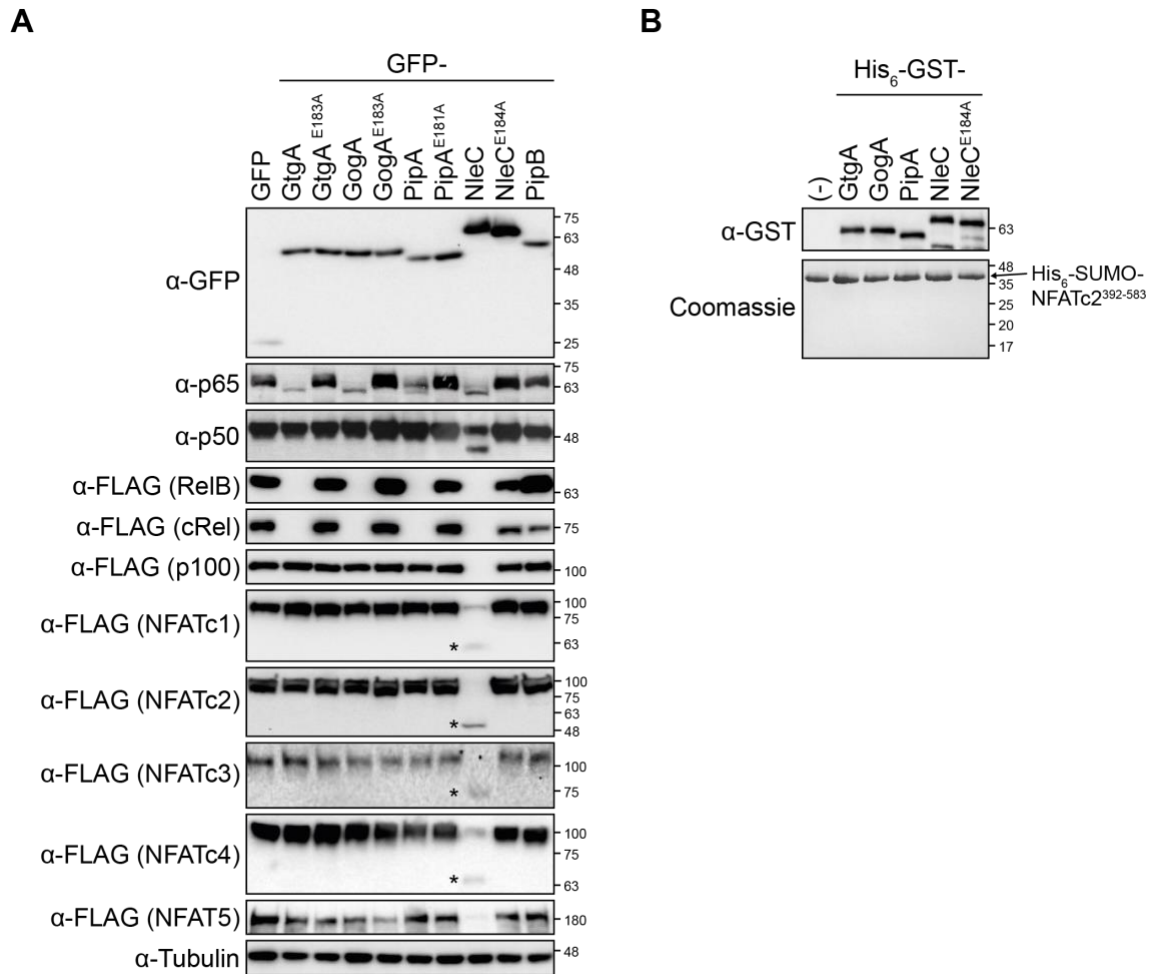


Figure 3.8: Substrate specificity of GtgA, GogA, PipA, and NleC.

(A) SDS-PAGE and immunoblot analysis of 293ET cells co-expressing the indicated GFP-tagged effector protein and FLAG-tagged NF-κB or NFAT transcription factor subunit. Endogenous p65 and p50 were detected with α-p65 and α-p50 antibodies. Immunoblotting with an α-Tubulin antibody was used as a loading control. Immunoblots are representative of 3 to 5 independent experiments. The * denotes NFAT subunit cleavage products. (B) *In vitro* cleavage assays using 0.1 μM GST-tagged effector proteins and 5 μM His₆-SUMO-NFATc2³⁹²⁻⁵⁸³. Putative proteases and substrates purified by affinity chromatography were co-incubated in 40 μl reaction for 5 hours at 37°C. The reaction was quenched by the addition of Laemmli buffer and proteins separated and visualised by SDS-PAGE followed by Coomassie blue staining or immunoblotting with an α-GST antibody. Coomassie blue stained polyacrylamide gels are representative of 3 independent experiments.

NF-κB subunits but GtgA, GogA, and PipA show clear substrate specificity for a subset of NF-κB subunits: p65, cRel, and RelB.

3.1.7 GtgA, GogA, and PipA do not cleave NFAT transcription factor subunits

The nuclear factor of activated T-cells (NFAT) family of transcription factors comprises five proteins: NFATc1, NFATc2, NFATc3, NFATc4, and NFAT5. These proteins were initially identified as regulators of T cell development and function; however, they have more recently been discovered to be important in other immune cells including DCs (Müller and Rao, 2010), a cell type within which *Salmonella* resides *in vivo* (Yrlid et al., 2000), and T3SS effector proteins are translocated into (Geddes et al., 2007). NFAT subunits contain a DNA-binding domain that is structurally similar to the RHR found in NF- κ B subunits (Chen et al., 1998a). I therefore hypothesised that GtgA, GogA, and PipA also cleave members of the NFAT family of transcription factors.

To test this hypothesis, 293ET cells were transfected with plasmids encoding each NFAT subunit tagged at their N-terminus with a FLAG-epitope tag, together with a GFP-tagged protease. Whole cell lysates were then separated using SDS-PAGE and immunoblotted using α -GFP and α -FLAG antibodies. **Figure 3.8A** shows that there was no change in the abundance of full-length FLAG-tagged NFATc1, NFATc2, NFATc3, NFATc4, or NFAT5 in cells expressing GFP-tagged GtgA, GogA, or PipA relative to cells expressing GFP or GFP-PipB. Therefore, the GtgA family of zinc metalloproteases do not cleave NFAT subunits. However, there was a reduction in the abundance of all five FLAG-tagged NFAT subunits in cells expressing GFP-NleC and this was dependent on the catalytic activity of NleC (**Figure 3.8A**). Additionally, in cells co-transfected with GFP-NleC and FLAG-tagged NFATc1, NFATc2, NFATc3, or NFATc4, a cleavage product of the size that would be expected if each protein was cleaved in the RHR was visible (indicated by an * on each blot). Next, I purified the N-terminal domain of NFATc2 (residues 392-583) fused via its N-terminus to a His₆-SUMO expression tag by affinity chromatography and carried out an *in vitro* cleavage assay using His₆-GST-tagged effectors. The NTD of NFATc2 was not directly cleaved by NleC when recombinant protease and substrate were incubated at a 1:50 molar ratio. Importantly, lack of

cleavage was not due a lack of protease activity, as these assays were done at the same time as the experiments shown in **Figure 3.7C**.

In this chapter, I identified a family of three effector proteins comprising GtgA, GogA, and PipA, that share over 65% amino acid sequence identity and inhibit activation of an NF- κ B-dependent luciferase reporter in 293ET cells. GtgA, GogA, and PipA zinc metalloproteases are functionally similar to the T3SS effector NleC from EPEC/EHEC that cleaves the N-terminal domain of NF- κ B transcription factor subunits. However, the substrate specificity of the three *Salmonella* proteases differs to the specificity of NleC. Whereas NleC cleaves all five NF- κ B subunits, GtgA, GogA, and PipA cleave p65, cRel, and RelB, but not NF- κ B1 (p105/p50) or NF- κ B2 (p100/p52).

4 NF- κ B inhibition by GtgA, GogA, and PipA in *Salmonella*-infected cells

In Chapter 3, I demonstrated that GFP-tagged GtgA, GogA, and PipA inhibit NF- κ B signalling by directly cleaving NF- κ B transcription subunits directly. However, infection of RAW264.7 macrophages with a Δ *gtgA* mutant strain did not elicit increased NF- κ B reporter activation relative to wild-type infected cells. As GtgA, GogA, and PipA are apparently functionally very similar, if not identical, it is probable that they function redundantly in *Salmonella*-infected cells; however, at the time these experiments were conducted only GtgA had been shown to a T3SS effector protein.

GtgA was discovered to be a SPI-2 T3SS effector protein using an *in vitro* high-throughput proteomic approach, in which mass spectrometry was used to identify proteins secreted *in vitro* by *Salmonella* cultured in SPI-2 T3SS-inducing conditions (Niemann et al., 2011). In the same experiments, translocation of PipA into host cells was not detected. GtgA was also demonstrated to be translocated into J774 cells infected with SPI-1-expressing, logarithmic phase *Salmonella*. In these infection conditions GtgA was translocated into host cells dependent on the SPI-2 T3SS and not the SPI-1 T3SS. Additionally, peptide fragments of GtgA, GogA, and PipA, were identified in the culture supernatant by mass-spectrometry when *Salmonella* was grown in SPI-1-inducing conditions (Spano et al., 2011). However, the analysis was unable to distinguish which of the three proteins was detected. Therefore, GtgA, GogA, and PipA might also be translocated into host cells by the SPI-1 T3SS.

In this chapter I describe the generation of *gtgA*, *gogA*, and *pipA*, single, double and triple mutant strains. The requirement of *gtgA*, *gogA*, and *pipA* for intracellular replication, cleavage of p65 and inhibition of NF- κ B signalling in *Salmonella*-infected cells was then analysed. The last section of the chapter demonstrates that GtgA and GogA are translocated in host cells dependent on the SPI-2 T3SS and following translocation, they localise to the nucleus.

4.1 Results

4.1.1 Construction of *Salmonella* Typhimurium *gtgA*, *gogA*, and *pipA* deletion mutant strains

Salmonella Typhimurium strain ATCC 14028s single deletion mutants were constructed using the λ -red recombinase method (outlined in **Figure 4.1A**) (Datsenko and Wanner, 2000). Primers with 40-nucleotide 5'-overhangs homologous to regions flanking the gene of interest were designed and used to amplify the kanamycin resistance cassette from the plasmid pKD4 by PCR. PCR products were then transformed into wild-type *Salmonella* expressing the λ -red recombinase from the plasmid pKD46 and positive transformants selected on agar plates supplemented with kanamycin. Colonies were then screened using PCR with primers complementary to sequences flanking the gene of interest (**Figure 4.1B** and **Table 4.1**).

To generate a $\Delta gtgA \Delta gogA$ double mutant strain, $\Delta gtgA::kan$ pKD46 was transformed with a chloramphenicol resistance cassette amplified using PCR. Positive transformants were selected on agar plates supplemented with both kanamycin and chloramphenicol and then screened by colony PCR (**Figure 4.1B**). After curing the strain of the temperature sensitive plasmid pKD46, $\Delta gtgA::kan \Delta gogA::cm$ was then transformed with the FLP recombinase encoding plasmid pCP20 to remove the resistance cassettes by inducing recombination of the FLP recombinase target (FRT) sequences. p22 phage transduction was then used to transfer the *pipA::kan* gene locus from a $\Delta pipA::kan$ single deletion mutant into a clean $\Delta gtgA \Delta gogA$ mutant strain, generating the $\Delta gtgA \Delta gogA \Delta pipA::kan$ triple mutant strain (**Figure 4.1B**). Mutant strains are herein referred to as $\Delta gtgA$, $\Delta gogA$, $\Delta pipA$, $\Delta gtgA \Delta gogA$, and $\Delta gtgA \Delta gogA \Delta pipA$.

4.1.2 Characterisation of *gtgA*, *gogA*, and *pipA* mutant strains: intracellular replication

Before analysing if *gtgA*, *gogA*, and *pipA* are important for NF- κ B transcription factor subunit cleavage and the inhibition of NF- κ B signalling in *Salmonella*-infected cells I determined if *gtgA*, *gogA*, and *pipA* are important for intracellular replication of *Salmonella*.

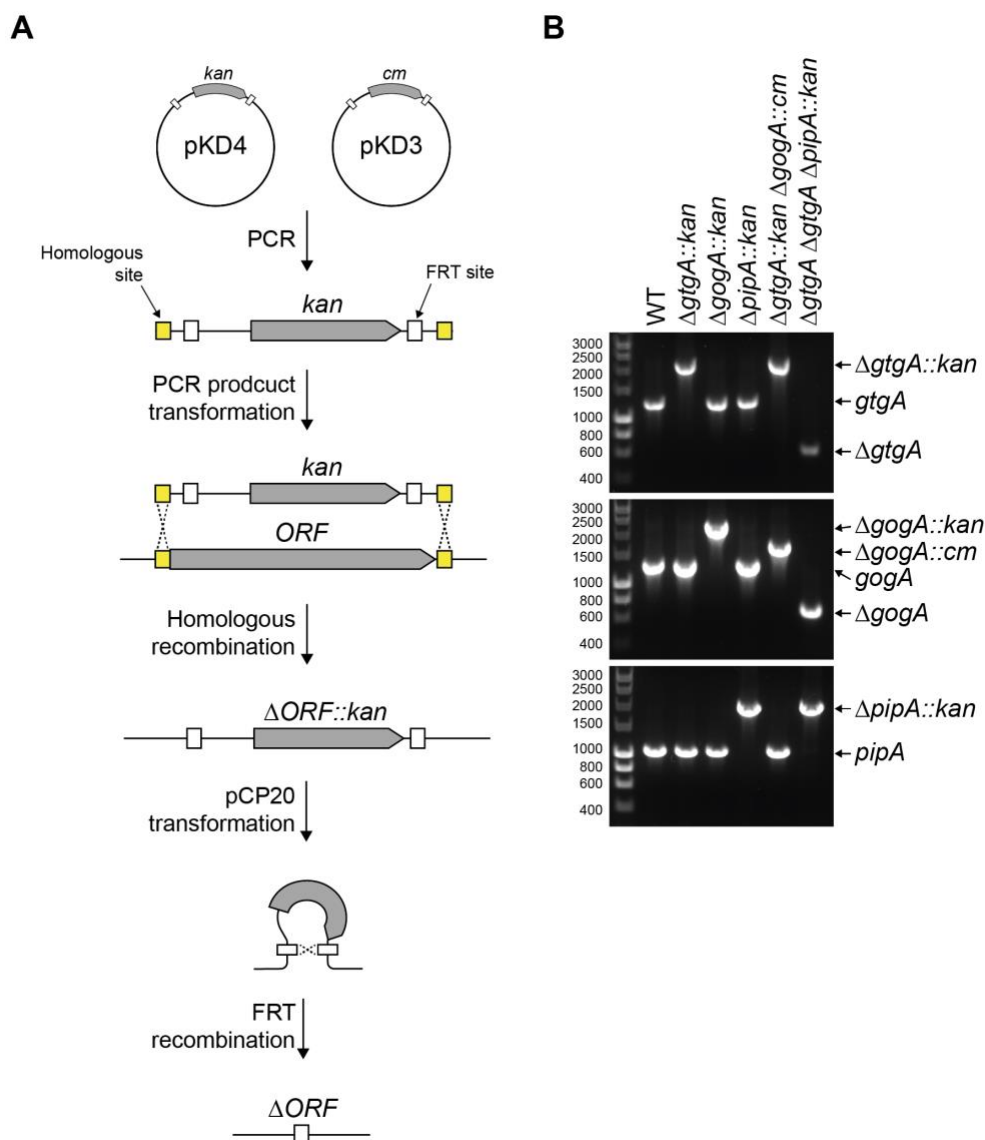


Figure 4.1: Construction of *gtgA*, *gogA*, and *pipA* mutant strains.

(A) Diagrammatic representation of the λ -red recombination method for generating deletion mutant strains. Kanamycin or chloramphenicol resistance cassettes (*kan* and *cm*) are amplified by PCR, using primers with 40-nucleotide 5'-overhangs homologous to regions flanking the ORF to be deleted. The PCR product is then transformed into *Salmonella* containing the plasmid pKD46, which encodes the λ -red recombinase from an arabinose-inducible promoter. Bacteria are then selected on agar plates supplemented with kanamycin or chloramphenicol. Transformation of colonies with the plasmid pCP20, which encodes the FLP recombinase, then removes the resistance cassette by recombining the FRT sequences flanking the resistance cassette. (B) Colony PCRs were performed using primers complementary to nucleotides approximately 100 to 300 nucleotides adjacent to the indicated gene. PCR products were then resolved by agarose gel electrophoresis. **Table 4.1** details the expected size of each PCR product. Abbreviations: *FRT*, FLP recombinase target.

Table 4.1: Expected sizes of PCR products (base pairs)

	<i>gtgA</i>	<i>gogA</i>	<i>pipA</i>
Wild-type	1228	1284	1055
$\Delta ORF::kan$	2021	2077	1854
$\Delta ORF::cm$	n/a	1632	n/a
$\Delta ORF\ clean$	~620	~700	n/a

HeLa cells were infected with wild-type *Salmonella* or the various *gtgA*, *gogA*, and *pipA* mutant strains carrying the GFP-expressing plasmid pFPV25.1. Cells were harvested at both 1 and 10 h post invasion (h p.i.) and the percentage of infected cells and the relative bacterial load per cell analysed by flow cytometry. 1 h p.i., the percentage of infected cells (**Figure 4.2A**) and the relative bacterial load per cell (GFP-geometric mean of infected cells) (**Figure 4.2B**) was similar between wild-type *Salmonella*-infected and mutant-infected cells demonstrating that the deletion mutant strains are not invasion-deficient. Next, fold bacterial replication was calculated by dividing the GFP-geometric mean of infected cells at 10 h p.i. by the GFP-geometric mean of infected cells at 1 h p.i. **Figure 4.2C** demonstrates that there was a 1.9-fold (± 0.1) increase in the GFP-geometric mean in wild-type-infected HeLa cells, whereas there was only a 1.3-fold (± 0.1) increase in the GFP geometric mean in cells infected with the SPI-2 T3SS null mutant strain ($\Delta ssaV$). However, fold replication of *gtgA*, *gogA*, and *pipA* single, double, and triple mutant strains was not significantly different from that of wild-type *Salmonella*.

I also determined if *gtgA*, *gogA*, and *pipA* are required for *Salmonella* intra-macrophage replication. RAW264.7 cells were inoculated with the same GFP-expressing mutant strains and at 2 and 16 h p.u. cells were analysed by flow cytometry. Bacterial uptake was similar for all strains analysed (**Figure 4.3A** and **B**), but the fold replication of a $\Delta ssaV$ mutant strain from 2 to 16 h p.u. was reduced relative to wild-type *Salmonella* (**Figure 4.3C**). The difference in fold replication between wild-type and a $\Delta ssaV$ mutant strain was not statistically significant. Replication of *gtgA*, *gogA*, and *pipA* single, double and triple mutant strains was indistinguishable from wild-type *Salmonella*. Together these data suggest that *gtgA*, *gogA*,

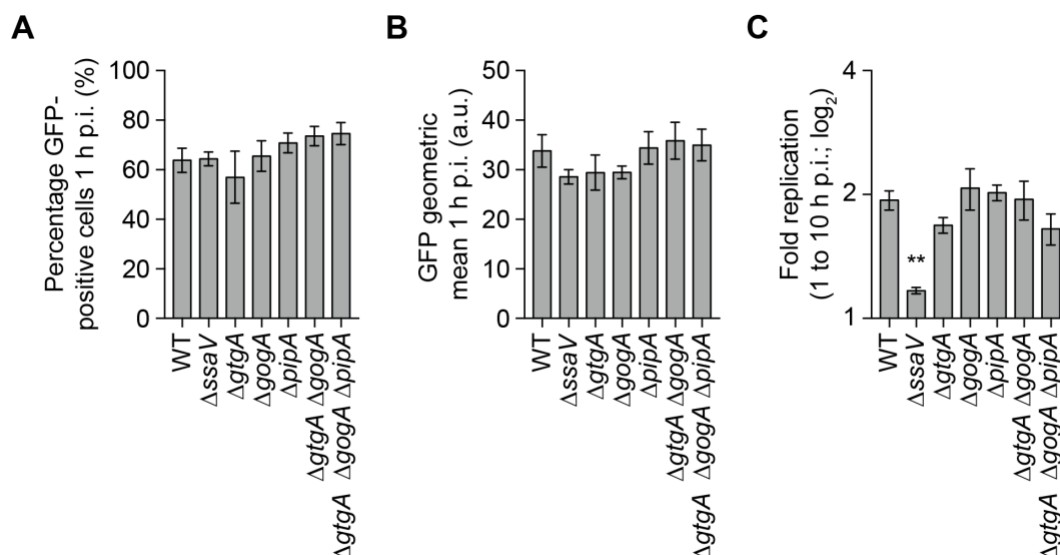


Figure 4.2: *gtgA*, *gogA*, and *pipA* are not required for *Salmonella* replication in HeLa cells.

HeLa cells were infected with GFP-expressing wild-type (WT) *Salmonella* or the indicated isogenic mutant. At 1 and 10 h p.i., flow cytometry was done to identify the percentage of infected cells (A), the relative bacterial load of infected cells (B) and fold replication (C). Gating on GFP-positive events identified infected cells and fold replication was calculated as the fold change in the GFP geometric mean of infected cells from 1 to 10 h p.i. Data are represented as the mean $\pm$ SEM of 3 independent experiments. Statistical significances were computed between WT and each isogenic mutant strain (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$). Abbreviations: a.u., arbitrary units.

and *pipA* are dispensable for *Salmonella* replication in both HeLa cells and RAW264.7 macrophages and also demonstrate that any difference observed in NF- κ B reporter activation between wild-type and *gtgA*, *gogA*, and *pipA* mutant strains is not due to differences in bacterial load.

4.1.3 p65 cleavage in *Salmonella*-infected cells is dependent on *gtgA*, *gogA*, and *pipA*

Next, I analysed if p65 is cleaved in *Salmonella*-infected cells and if this is dependent on the SPI-2 T3SS and *gtgA*, *gogA*, and *pipA*. As the SPI-1 T3SS is required for *Salmonella*-induced invasion of HeLa cells, it is not possible to test the role of effector proteins translocated

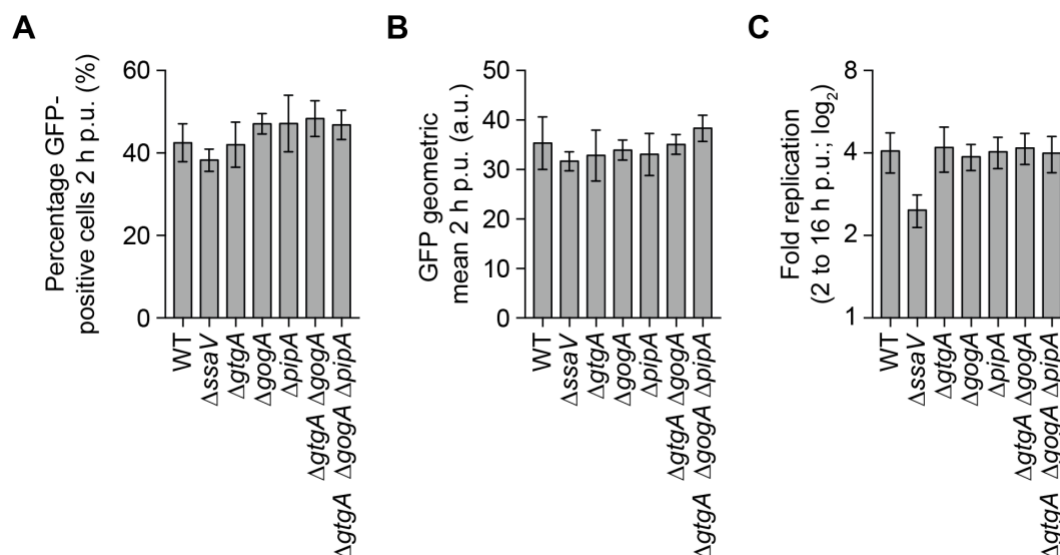


Figure 4.3: *gtgA*, *gogA*, and *pipA* are not required for *Salmonella* replication in RAW264.7 macrophages.

RAW264.7 macrophages were infected with GFP-expressing wild-type (WT) *Salmonella* or the indicated isogenic mutant. At 2 and 16 h p.u., flow cytometry was done to identify the percentage of infected cells (A), the relative bacterial load of infected cells (B) and fold replication (C). Gating on GFP-positive events identified infected cells and fold replication was calculated as the fold change in the GFP-geometric mean of infected cells from 2 to 16 h p.u. Data are represented as the mean $\pm$ SEM of 3 independent experiments. Statistical significances were computed between WT and each isogenic mutant strain (ordinary one-way ANOVA with *post-hoc* Dunnett's test; no significant differences were found). Abbreviations: *a.u.*, arbitrary units.

by this secretion system in this experimental set-up. HeLa cells were infected with wild-type *Salmonella*, a SPI-2 T3SS null mutant strain ($\Delta ssaV$) and a $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant strain. 3 h p.i., cells were harvested and whole cell lysates separated by SDS PAGE and analysed by immunoblotting with an antibody raised against the C-terminal region of p65. **Figure 4.4A** shows that there was no difference in the abundance of full-length p65 in *Salmonella*-infected cells.

In EPEC/EHEC-infected cells, p65 is cleaved by NleC and the resulting cleavage products are degraded by the proteasome (Yen et al., 2010). Therefore, I hypothesised that

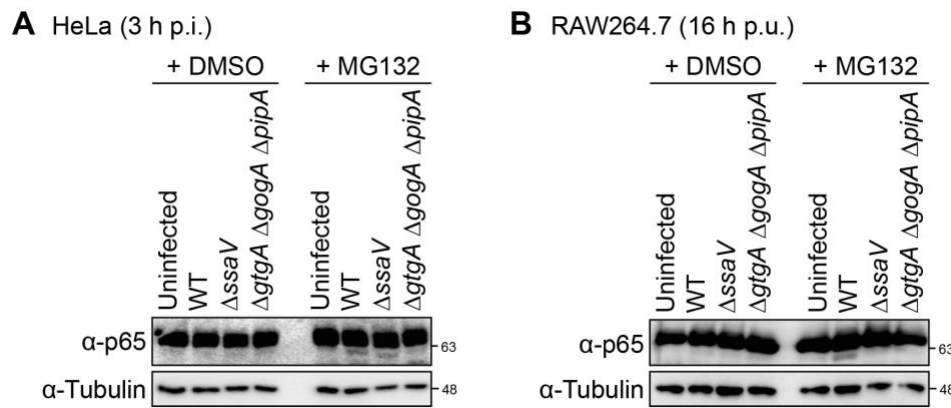


Figure 4.4: p65 is cleaved and degraded by the proteasome, dependent on *gtgA*, *gogA*, and *pipA* in HeLa cells and RAW264.7 macrophages.

(A) HeLa cells were infected with wild-type (WT) *Salmonella* or the indicated isogenic mutant strain. 1 h p.i., cells were treated with DMSO or 10 μ M MG132 for 2 h. Cells were then lysed in Laemmli buffer and whole cell lysates analysed by SDS-PAGE and immunoblot analysis using α -p65 and α -Tubulin antibodies. Immunoblots are representative of 4 independent experiments. (B) RAW264.7 macrophages were inoculated with WT or the indicated isogenic mutant strain. 14 h p.u., cells were treated for 2 h with DMSO or 10 μ M MG132. Cells were then lysed in Laemmli buffer and whole cell lysates analysed by SDS-PAGE and immunoblot analysis. Immunoblots are representative of 5 independent experiments.

the p65 cleavage products generated by GtgA, GogA, or PipA might also be degraded by the proteasome. To test this, uninfected and infected HeLa cells were treated with the proteasomal inhibitor MG132 from 1 to 3 h p.i. and immunoblotting of whole cell lysates with a p65 antibody was then done. In MG132-treated, wild-type infected cells, there was an additional band that migrated further through the polyacrylamide gel than full-length p65 (**Figure 4.4A**). This band is of a similar size to the cleavage product observed in cells expressing GFP-tagged GtgA, GogA, or PipA (approximately 60 kDa) (**Figure 3.8**). Interestingly, only a small fraction of the total pool of p65 was cleaved; however, it is important to note that the sample includes both infected and uninfected cells and that the subcellular localisation of p65, which is only transcriptionally active in the nucleus, has not been considered. The p65 cleavage product was also present in cells infected with a $\Delta ssaV$ mutant strain but was absent in cells infected with a $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant strain (**Figure**

4.4A). These data demonstrate that a small percentage of p65 is cleaved and then degraded by the proteasome in *Salmonella*-infected cells. Cleavage is dependent on members of the GtgA family and, in HeLa cells at the time point analysed (3 h p.i.), is independent of the SPI-2 T3SS (**Figure 4.4A**). This suggests that at least one member of the family is translocated into host cells by the SPI-1 T3SS.

Cleavage of p65 in *Salmonella*-infected RAW264.7 macrophages was also analysed. RAW264.7 macrophages were infected for 16 h with either wild-type *Salmonella*, Δ ssaV, or Δ gtgA Δ gogA Δ pipA mutant strains, with cells undergoing treatment with DMSO or MG132 from 14 to 16 h p.u. Immunoblot analysis of whole cell lysates revealed that there was no change in the abundance of full-length p65 in *Salmonella*-infected RAW264.7 macrophages. However, cleaved p65 was present in wild-type infected cells treated with MG132 and this was dependent on *gtgA*, *gogA*, and *pipA* (**Figure 4.4B**). In contrast to HeLa cells, p65 was not cleaved in Δ ssaV mutant-infected cells. The SPI-2 T3SS therefore might be required for p65 cleavage in RAW264.7 macrophages, however as the Δ ssaV mutant showed a reduction in replication at 16 h p.u. (**Figure 4.3**), it remains possible that the difference in bacterial load between wild-type and Δ ssaV-infected cells could explain why I did not detect p65 cleavage in these cells.

4.1.4 Characterisation of *gtgA*, *gogA*, and *pipA* mutant strains: NF- κ B reporter activation

To determine the effect that p65 cleavage has on gene transcription in *Salmonella*-infected cells I next infected HeLa cells transfected with NF- κ B reporter plasmids with wild-type *Salmonella* and *gtgA*, *gogA*, and *pipA* single, double and triple deletion mutant strains. Cells were harvested and luciferase activity analysed at two time points: 3 h p.i. and 10 h p.i.

Relative to uninfected cells, HeLa cells infected with wild-type *Salmonella* induced a 13.2-fold (± 2.7) and 12.8-fold (± 2.1) increase in NF- κ B reporter activation at 3 and 10 h p.i., respectively. There was no significant difference in NF- κ B reporter activation between wild-type infected and Δ ssaV or Δ gtgA, Δ gogA, and Δ pipA single mutant infected cells at both time points, however there was a modest yet non-significant increase in NF- κ B reporter activation

in cells infected with a $\Delta gtaA \Delta gogA$ double mutant and $\Delta gtaA \Delta gogA \Delta pipA$ triple mutant strain (**Figure 4.5A and B**). Relative to wild-type *Salmonella*, the $\Delta gtaA \Delta gogA$ double mutant strain induced a 1.74- and 1.71-fold increase in NF- κ B reporter activation and the $\Delta gtaA \Delta gogA \Delta pipA$ triple mutant strain induced a 1.85- and 1.92-fold increase in NF- κ B reporter activation at 3 and 10 h p.i., respectively (**Figure 4.5A and B**).

To further explore if GtgA, GogA, and PipA inhibit NF- κ B signalling in *Salmonella*-infected cells, I infected RAW264.7 NF- κ B reporter macrophages (described in Chapter 3) with the same *gtgA*, *gogA*, and *pipA* single, double, and triple mutant strains. As observed previously, infection of RAW264.7 macrophages with a $\Delta gtaA$ single deletion mutant strain had no effect on NF- κ B reporter activation in comparison to wild-type *Salmonella*. In addition, there was no statistically significant difference in NF- κ B reporter activation between wild-type *Salmonella*-infected cells or cells infected with either $\Delta ssaV$, $\Delta gogA$, $\Delta pipA$, $\Delta gtaA \Delta gogA$, or $\Delta gtaA \Delta gogA \Delta pipA$ mutant strains.

Despite a lack of statistical significance in NF- κ B reporter activation between wild-type and $\Delta gtaA \Delta gogA \Delta pipA$ mutant-infected HeLa or RAW264.7 macrophages, there was a small yet reproducible increase in reporter activation in cells infected with a $\Delta gtaA \Delta gogA \Delta pipA$ triple mutant strain (**Figure 4.5**). This suggests that the NF- κ B reporter cells lack the required level of sensitivity to accurately characterise these mutant strains. Therefore, I tested if NF- κ B reporter activation was inhibited in cells infected with a $\Delta gtaA \Delta gogA \Delta pipA$ triple mutant strain expressing either HA-tagged GtgA, GogA, or PipA from a low-copy number plasmid, pWSK29 (Wang and Kushner, 1991). *gtgA* and *gogA*, along with 1000 nucleotides upstream of each gene, were amplified using PCR and ligated into the low-copy number plasmid pWSK29 ($p_{gtgA}gtgA$ -2HA and $p_{gogA}gogA$ -2HA). *pipA* is the second gene in the *pipBA* operon therefore to preserve physiologically relevant expression conditions, 500 nucleotides upstream of the operon, *pipB* and *pipA* were amplified and ligated into pWSK29 ($p_{pipB}pipBA$ -2HA). To control for the additional presence of *pipB* in this construct, a PipB-encoding plasmid was also

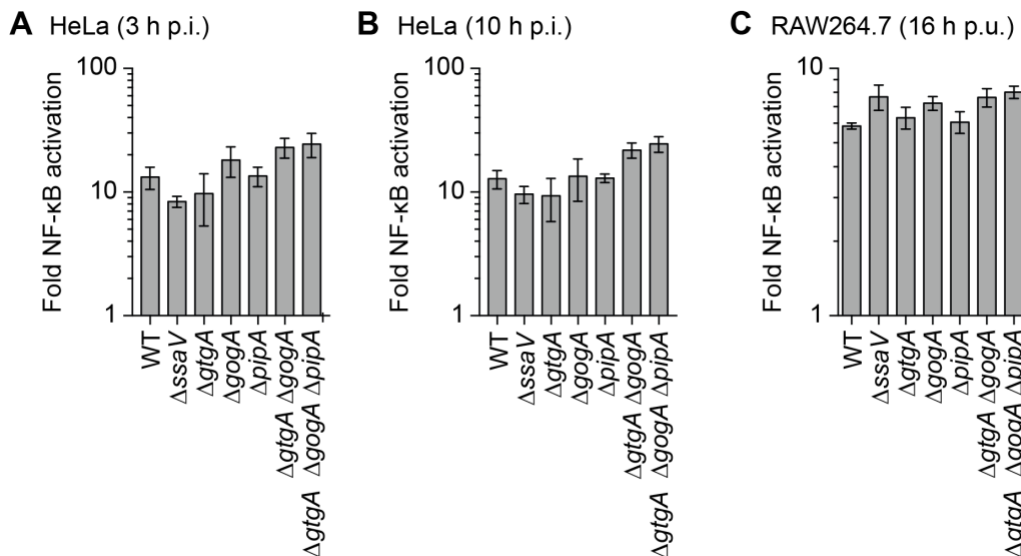


Figure 4.5: Analysis of NF-κB reporter activation by *gtgA*, *gogA*, and *pipA* mutant strains in *Salmonella*-infected cells

(A and B) HeLa cells co-transfected with NF-κB luciferase reporter plasmids were infected with wild-type (WT) *Salmonella* or the indicated isogenic mutant strain. (A) 3 and (B) 10 h p.i. cells were lysed and luciferase activity analysed. (C) RAW264.7 NF-κB reporter macrophages were inoculated with WT or the indicated isogenic mutant strain. 16 h p.u. cells were lysed and luciferase activity analysed. Data are presented relative to NF-κB activity in uninfected cells and represent the mean $\pm$ SEM of 3 independent experiments. Statistical significances were computed between WT and each isogenic mutant strain (ordinary one-way ANOVA with *post-hoc* Dunnett's test; no significant differences were found).

constructed (*p_{pipB}pipB-2HA*). To enable protein expression and translocation into host cells to be analysed, each protein (with the exception of *pipB* in the plasmid *p_{pipB}pipBA-2HA*) was tagged at its C-terminus with 2 tandem copies of the HA-epitope. The ORFs of *gtgA*, *gogA*, and *pipA* as well as the Shine-Dalgarno sequence of each gene were also amplified and cloned into pWSK29 with their expression controlled under the *ssaG* promoter, the activity of which is strongly induced in low pH and low nutrient conditions, and in intracellular vacuolated *Salmonella* (Kroger et al., 2013). Each protein was tagged at its C-terminus with 2 tandem copies of the HA-epitope as above.

HeLa cells transfected with NF- κ B luciferase reporter plasmids were then infected for 10 h with a Δ gtgA Δ gogA Δ pipA mutant strain transformed with the indicated plasmid. Plasmid maintenance negatively affects the fitness of *Salmonella* in HeLa cells and RAW264.7 macrophages (Knodler et al., 2005). Therefore, as a control for the plasmid burden conferred by pWSK29, wild-type *Salmonella* and the Δ gtgA Δ gogA Δ pipA mutant strains were also transformed with pWSK29-empty (pEmpty). To confirm expression of the tagged proteins, whole cell lysates were separated by SDS-PAGE and analysed by immunoblotting with an α -HA antibody. An antibody that recognises the bacterial chaperone DnaK, was used as a control to assess bacterial load in each sample (**Figure 4.6A**). Despite equal bacterial loads in each infected sample, neither GtgA-2HA or PipA-2HA were detected by Western blot at 10 h p.i. when expressed from their endogenous promoters. GogA-2HA and PipB-2HA were detected however relative to PipB-2HA, the abundance of GogA-2HA was noticeably reduced (**Figure 4.6A**). Furthermore, GtgA-2HA and GogA-2HA were detected when expressed from the *ssaG* promoter, whereas PipA-2HA was not.

Next, immunofluorescence microscopy was done to check if the expressed proteins were translocated into host cells. Cells seeded on coverslips that had been infected simultaneously with the previous experiment were fixed with paraformaldehyde and immunolabelled with an α -HA antibody and an antibody raised against the structural components of *Salmonella* LPS (α -CSA-1). In accordance with previously published data, PipB-2HA localised to the *Salmonella*-containing vacuole and *Salmonella*-induced tubules (Knodler et al., 2004), however when expressed from their endogenous promoters I did not detect GtgA-2HA, GogA-2HA or PipA-2HA (data not shown). GogA-2HA was detected by immunoblot analysis (**Figure 4.6A**), therefore it is likely that the abundance of GogA-2HA is below the limit of detection for immunofluorescence microscopy. When under the control of the *ssaG* promoter, GtgA-2HA and GogA-2HA were detected by Western blotting (**Figure 4.6A**) and were translocated into HeLa cells, but PipA-2HA was not (**Figure 4.6C**). In infected

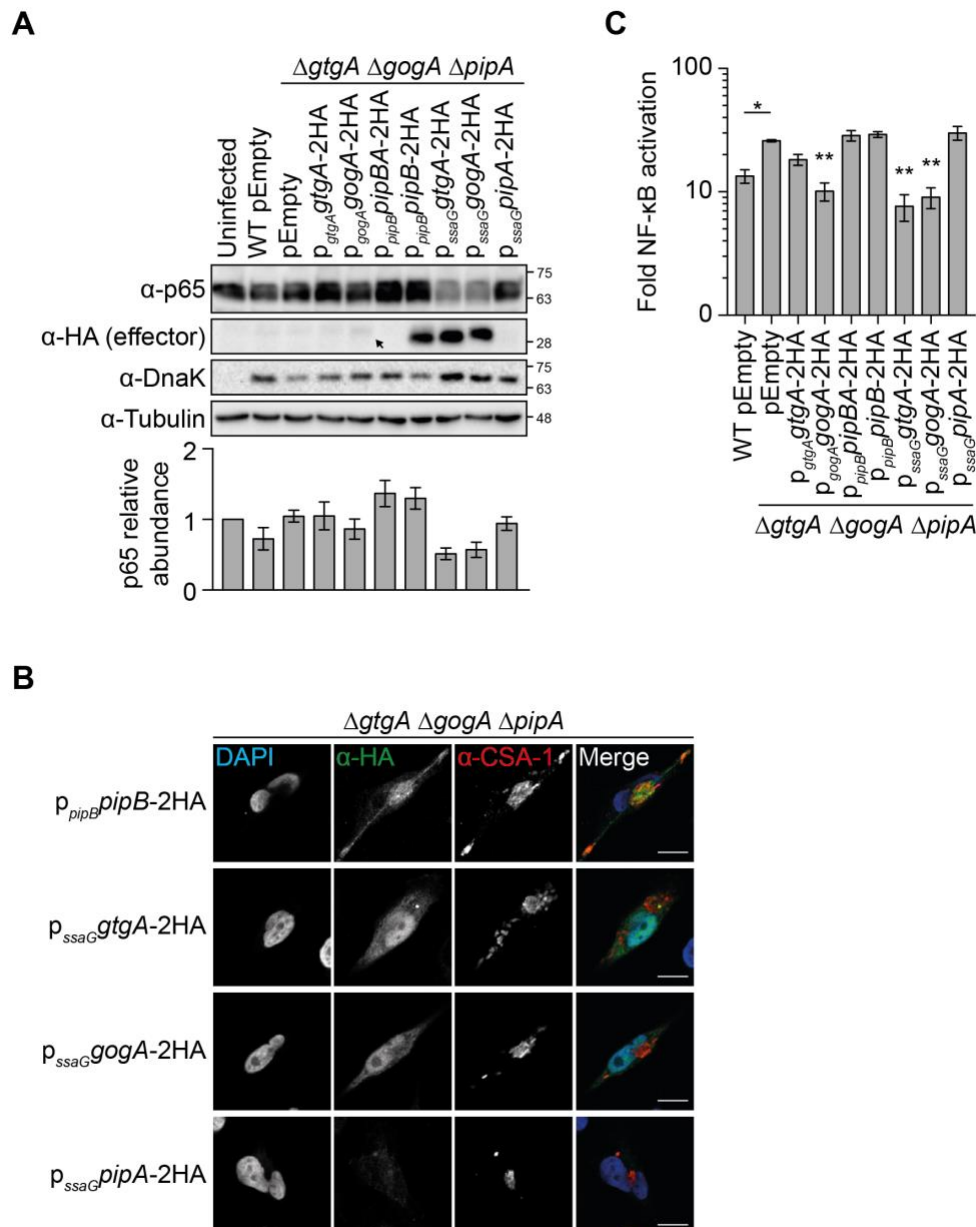


Figure 4.6: GtgA and GogA expressed from a low copy-number plasmid inhibit NF-κB reporter activation in *Salmonella*-infected HeLa cells.

(A) HeLa cells co-transfected with NF-κB reporter plasmids were infected with wild-type (WT) *Salmonella* or the indicated isogenic mutant strain. 10 h p.i. cells were lysed and whole cell lysates were analysed by SDS-PAGE and immunoblot analysis; the abundance of p65 relative to uninfected cells, was also quantified by densitometry. Immunoblots are representative of 3 independent experiments and immunoblot quantification represents the mean $\pm$ SEM of these 3 experiments. Statistical significances were computed between WT pEmpty and Δ gtgA Δ gogA Δ pipA pEmpty as well as between Δ gtgA Δ gogA Δ pipA pEmpty and the indicated Δ gtgA Δ gogA Δ pipA strain (ordinary one-way ANOVA with *post-hoc* Dunnett's test; no significant differences were found). The arrow indicates GogA-2HA expressed from its endogenous

promoter. (B) Representative confocal immunofluorescence microscopy images of HeLa cells infected for 10 h with the indicated *Salmonella* strain. HA-tagged effector proteins were expressed from the *ssaG* promoter and were encoded on the low-copy number plasmid pWSK29. DNA (DAPI, blue); HA-tagged effectors (α -HA, green); *Salmonella* (α -CSA-1, red). Scale bar: 10 μ m. (C) Luciferase activity in cell lysates from the experiment shown in panel A were analysed. Data are presented relative to NF- κ B activity in uninfected cells and represent the mean $\pm$ SEM of 3 independent experiments. Statistical significances were computed between WT pEmpty and Δ *gtgA* Δ *gogA* Δ *pipA* pEmpty as well as between Δ *gtgA* Δ *gogA* Δ *pipA* pEmpty and the indicated Δ *gtgA* Δ *gogA* Δ *pipA* strain (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$).

cells, GtgA-2HA and GogA-2HA localised to the cell cytosol and nucleus, which was labelled using DAPI (shown in blue) (**Figure 4.6B**).

Immunoblotting was also done using an α -p65 antibody (**Figure 4.6A**) and luciferase activity measured in cell lysates (**Figure 4.6C**). The relative abundance of p65 in whole cell lysates was indistinguishable between uninfected cells and cells infected with the majority of the mutant strains, however there was a reproducible reduction in the abundance of full-length p65 in cells infected with the triple mutant expressing GtgA-2HA or GogA-2HA under the control of the *ssaG* promoter (**Figure 4.6A**). Densitometry analysis of three biological repeats demonstrated that this reduction was not significant.

In contrast to earlier experiments (**Figure 4.5**), infection of HeLa cells with a Δ *gtgA* Δ *gogA* Δ *pipA* triple mutant strain induced a statistically significant increase in NF- κ B reporter activation relative to wild-type infected cells (**Figure 4.6C**). When expressed from their endogenous promoter, increased NF- κ B reporter activation elicited by the Δ *gtgA* Δ *gogA* Δ *pipA* triple mutant strain was complemented with GogA-2HA but not GtgA-2HA, PipA-2HA, or PipB-2HA. Additionally, both GtgA-2HA and GogA-2HA complemented the triple deletion mutant strains when expressed from the *ssaG* promoter.

Collectively, these data demonstrate that the GtgA family of effectors dampen activation of NF- κ B-dependent gene transcription in *Salmonella*-infected cells. However, as GogA-2HA

expressed from its endogenous promoter, which was barely detected by immunoblot analysis, could fully complement the $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant strain, it is likely that the abundance of these proteins in infected cells is low.

4.1.5 Translocation of GtgA and GogA into RAW264.7 macrophages is dependent on the SPI-2 T3SS

In the previous experiments, I did not detect PipA-2HA in *Salmonella*-infected HeLa cells when expressed under the control of its endogenous promoter or the *ssaG* promoter. The expression of *pipA* is highly induced following the internalisation of *Salmonella* by macrophages (Kroger et al., 2013), therefore I next analysed if I could detect PipA-2HA expression and translocation in RAW264.7 macrophages. RAW264.7 macrophages, seeded on glass coverslips, were infected with wild-type *Salmonella* or a $\Delta ssaV$ mutant strain expressing either GtgA-2HA, GogA-2HA, and PipA-2HA from their endogenous promoter or the *ssaG* promoter. To account for the attenuated replication of the SPI-2 T3SS null strain, RAW264.7 macrophages were infected with wild-type *Salmonella* at a MOI of 10 and a $\Delta ssaV$ mutant strain at a MOI of 100. Translocation of the SPI-2 T3SS effector protein PipB-2HA by wild-type and a $\Delta ssaV$ mutant strain was also analysed. Cells were fixed 16 h p.u., and immunolabelled with α -HA and α -CSA-1 antibodies to detect HA-tagged effectors and *Salmonella*-infected cells, respectively.

Similar to HeLa cells, I did not detect GtgA-2HA, GogA-2HA, and PipA-2HA by immunofluorescence microscopy when they were expressed under the control of their endogenous promoters (data not shown). However, PipB-2HA was detected in approximately 40% of infected cells and localised in close vicinity to *Salmonella* (**Figure 4.7**). As the cells were permeabilised with 0.1% Triton X-100, which permeabilises both host and bacterial membranes, I was unable to distinguish between non-translocated and translocated PipB. However, the majority of PipB-2HA detected in WT-infected cells is likely to have been translocated into the host cell as in $\Delta ssaV$ -mutant infected cells, PipB-2HA co-localised to only

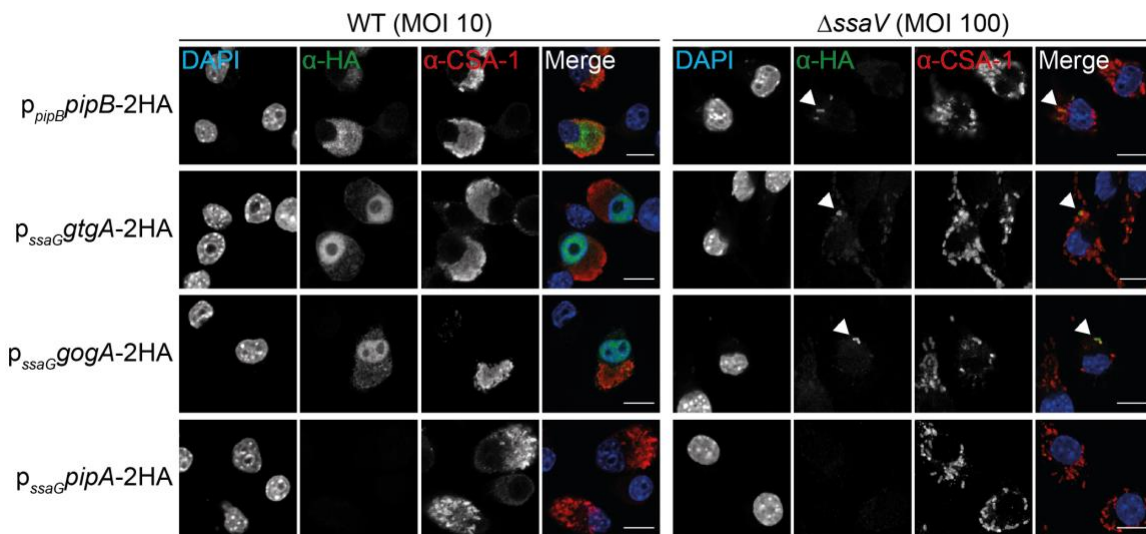
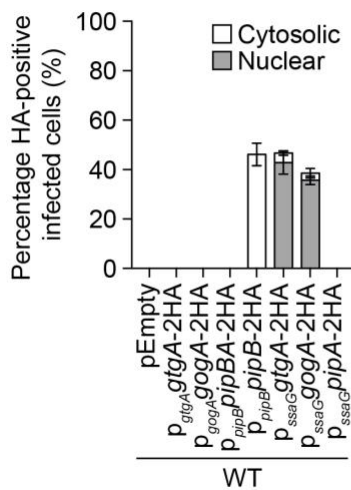
A**B**

Figure 4.7: GtgA and GogA are translocated into RAW264.7 macrophages dependent on the SPI-2 T3SS and localise to the nucleus.

(A) Representative confocal immunofluorescence microscopy images of RAW264.7 macrophages infected for 16 hours with wild-type (WT) *Salmonella* at a MOI of 10 or a SPI-2 T3SS null (Δ ssaV) mutant strain at a MOI of 100. HA-tagged effector proteins were expressed from the *ssaG* promoter and were encoded on the low-copy number plasmid pWSK29. DNA (DAPI, blue); HA-tagged effectors (α -HA, green); *Salmonella* (α -CSA-1, red). Co-localisation of HA-tagged effectors with *Salmonella* in Δ ssaV-infected macrophages are highlighted with arrowheads. Scale bar: 10 μ m. (B) Percentage of infected cells from the experiment shown in panel A with translocated HA-tagged effectors scored as predominantly nuclear or cytosolic in localisation. A total of 450 infected cells were counted across 3 independent experiments. Data are presented as mean $\pm$ SEM of 3 independent experiments.

a small percentage of bacteria (indicated by the white arrows in **Figure 4.7A**). GtgA-2HA and GogA-2HA, but not PipA-2HA, were detected when expressed under the control of the *ssaG* promoter. Similar to PipB, GtgA and GogA were translocated into approximately 40% of infected RAW264.7 macrophages where they localised to both the cytosol and the nucleus (**Figure 4.7A and B**). 2HA-tagged GtgA or GogA was not detected in the nucleus or cytosol of cells infected with a Δ *ssaV* mutant strain, but instead colocalised with a small percentage of *Salmonella*. These data suggest that translocation of GtgA and GogA into RAW264.7 cells is dependent on the SPI-2 T3SS and demonstrate that when translocated into host cells from bacteria, GtgA and GogA localised to the host cell cytosol but also accumulate strongly in the nucleus.

As I was unable to detect translocation of PipA-2HA into *Salmonella*-infected cells and was therefore unable to analyse the sub-cellular localisation of PipA, I next determined whether GFP-PipA localises to the nucleus. 293ET cells were stably transduced with GFP-tagged GtgA, GogA, and PipA and imaged using confocal microscopy. Similar to translocated GtgA and GogA, GFP-tagged GtgA, GogA, and PipA all localised to the nucleus (**Figure 4.8**). Furthermore, catalytically inactive variants also localised to the nucleus demonstrating that catalytic activity is not required for subcellular localisation (**Figure 4.8**).

In this chapter I have shown that during *Salmonella* infection of epithelial cells and macrophages, the GtgA family comprising GtgA, GogA, and PipA, are required for cleavage of p65 and are important for inhibition of NF- κ B signalling in *Salmonella*-infected cells. Furthermore, I provide evidence that following SPI-2 T3SS-dependent translocation of GtgA and GogA, these effectors localise to the host cell cytosol and nucleus. I was unable to analyse if PipA is an effector protein as I could not detect HA-tagged PipA in infected cells, however the subcellular localisation of PipA expressed ectopically was similar to that of GtgA and GogA.

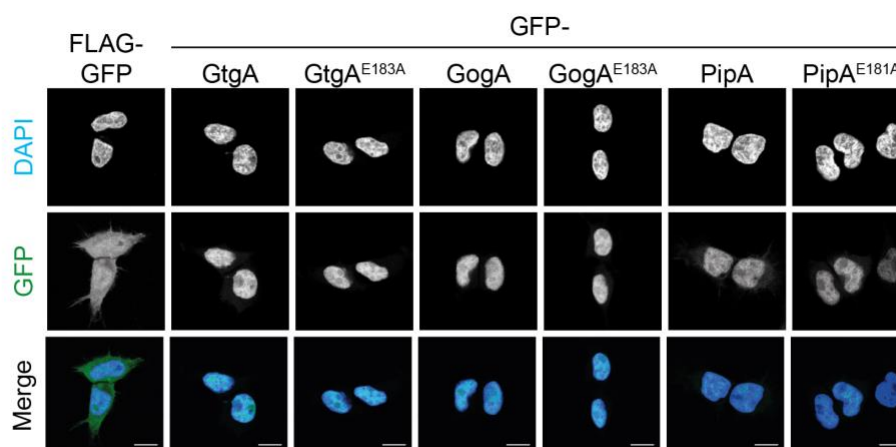


Figure 4.8: GFP-tagged GtgA, GogA, and PipA localise to the nucleus independent of their catalytic activity.

Representative confocal microscopy images of 293ET cells stably expressing FLAG-tagged GFP or the indicated GFP-tagged effector. DNA (DAPI, blue); GFP (green). Scale bar: 10 μ m.

5 Structural insights into the substrate specificity of GtgA

During the course of conducting the experiments described in chapter 4, another laboratory published data that fully corroborated my results. Sun et al., (2016) demonstrated that GtgA, GogA, and PipA are zinc metalloproteases that cleave p65 and RelB but not NF- κ B1 (p105/p50) or NF- κ B2 (p100/p52). Cleavage of cRel by the GtgA family of effectors was not analysed. Amino terminal amino acid sequencing of p65 following cleavage by GtgA, GogA, and PipA *in vitro*, identified that GtgA, GogA, and PipA cleave p65 between residues G40 and R41, two amino acids away from the C38/E39 cleavage site targeted by NleC. Additionally, the authors provided evidence that relative to wild-type *Salmonella*, a Δ gtgA Δ gogA Δ pipA triple mutant strain induces increased activation of the NF- κ B signalling pathway and increased expression of proinflammatory genes. The triple mutant strain also displayed increased virulence in *Nramp1*^{+/+} C57BL/6 mice that had been inoculated orally. The authors hypothesised that this was due to the induction of a cytokine storm in mice infected with the triple mutant strain. Therefore, rather than exploring the physiological relevance of GtgA-, GogA-, and PipA-dependent NF- κ B inhibition further, I next investigated the molecular and structural basis of substrate recognition for GtgA, GogA, and PipA.

Although the crystal structure of NleC has been published previously, the mechanism of substrate recognition is not fully understood. Despite only sharing weak amino acid similarity to other zinc metalloproteases, NleC retains structural features of zincin superfamily members. In addition, two separate groups remarked that the active site cleft of NleC is similar in shape to the major groove of DNA and that the surface is highly negatively charged. These observations led to the hypothesis that NleC recognises NF- κ B subunits by mimicking the shape of the DNA major groove and the charge of the DNA phosphate backbone.

In this chapter, I describe the molecular and structural characterisation of GtgA. Mutational analysis of the p65 cleavage site was done and identified a residue in p65 that is critical for substrate recognition, explaining the differences in substrate specificity between the GtgA family of effectors and NleC. Furthermore, I present the crystal structure of GtgA alone

and in complex with the N-terminal domain of p65. These crystal structures show that despite the low sequence identity between GtgA and NleC, the active site cleft of GtgA also mimics the shape of the DNA major groove and the negative charge of the DNA phosphate backbone. Accordingly, residues in the p65 NTD which interact with DNA in p65-DNA complexes published previously, also interact with GtgA. Mutational analysis of GtgA residues that interact with p65 in the complex structure was then done, and led to the identification of residues that are important for GtgA-p65 complex formation and GtgA's catalytic activity.

5.1 Results

5.1.1 The P1' residue R41 in p65 is critical for substrate specificity of GtgA, GogA, and PipA

Protease nomenclature dictates that residues surrounding the cleavage site are numbered according to their position relative to scissile bond (Schechter and Berger, 1967). Substrate residues extending away from the scissile bond towards the proteins N-terminus are numbered P1, P2, P3, and P4, whereas substrate residues extending towards the proteins C-terminus are numbered P1', P2', P3', and P4'. Subsites or pockets in the surface of the protease, into which substrate residues are inserted are numbered similarly – S4, S3, S2, S1, (scissile bond) S1', S2' S3', and S4'.

An amino acid alignment of residues surrounding the G40/R41 cleavage site in p65 with the corresponding residues in the four other NF- κ B subunits revealed that there are three non-conserved residues in p105/p50 and p100/p52 in close proximity to the scissile bond (**Figure 5.1A**). These include residues corresponding to the P4, P1', and P3' sites in p65 (residues K37, R41, and A43). GtgA, GogA, and PipA do not cleave p105/p50 and p100/p52, therefore I hypothesised that these non-conserved residues might explain the substrate specificity of these proteases. To test this, I mutated each non-conserved residue in p65 to the corresponding residue in p105/p50. 293ET cells were then transfected with plasmids encoding either FLAG-epitope-tagged wild-type p65, p65^{K37V}, p65^{R41P}, or p65^{A43H}, as well as either GFP

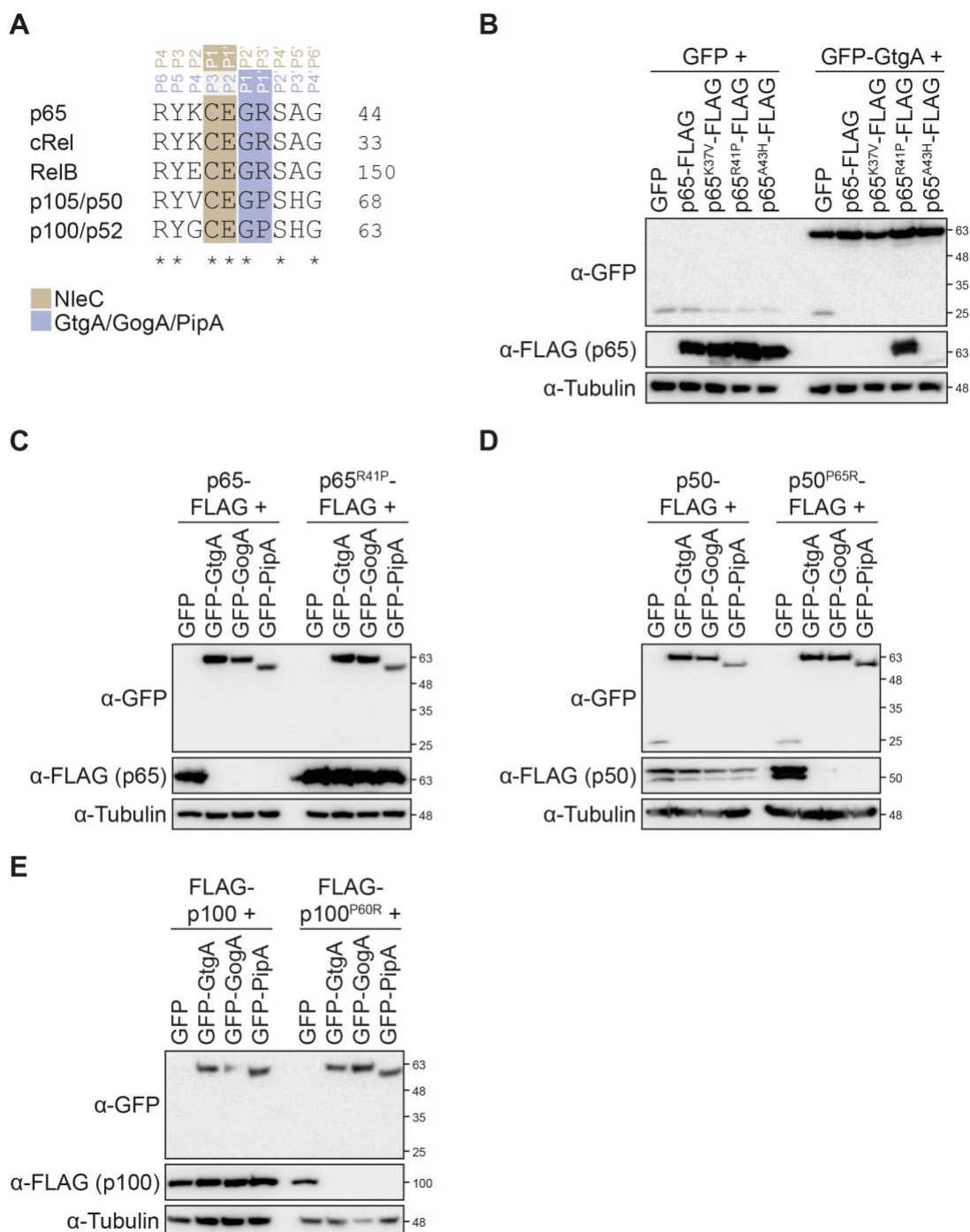


Figure 5.1: Substrate specificity of the GtgA family of effector proteins is governed by the P1' site residue.

(A) Amino acid alignment of the p65 cleavage site and the corresponding residues in cRel, RelB, p105/p50, and p100/p52. Uniprot: Q04206, Q04864, Q01201, P19838, and Q00653. (B to E) 293ET cells were transiently transfected with plasmids encoding the indicated FLAG-tagged NF- κ B subunit variant, together with a plasmid encoding GFP or a GFP-tagged effector protein. Whole cell lysates were separated by SDS-PAGE and immunoblotted using α -GFP

and α -FLAG antibodies. Immunoblotting with an α -Tubulin antibody was used as a loading control. Immunoblots are representative of at least 3 independent experiments.

or GFP-GtgA. Whole cell lysates were separated by SDS-PAGE and the abundance of each FLAG-tagged p65 variant was analysed by immunoblotting with an α -FLAG antibody. In cells expressing GFP, there was an equal abundance of each FLAG-tagged p65 variant, however in cells expressing GFP-GtgA, only FLAG-p65^{R41P} was detected (**Figure 5.1B**). Similar observations were made in cells expressing GFP-GogA and GFP-PipA: wild-type FLAG-p65 was not detected, whereas the abundance of FLAG-p65^{R41P} was similar in GFP, GFP-GtgA, GFP-GogA, and GFP-PipA expressing cells (**Figure 5.1C**). Mutation of the p50 residue P65, and the p100 residue P60 to an arginine had the inverse effect. The abundance of wild-type FLAG-tagged p50 and p100 was indistinguishable in cells expressing GFP or GFP-tagged GtgA, GogA, or PipA, whereas p50^{P65R} and p100^{P60R} were not detected in GFP-GtgA, GFP-GogA, and GFP-PipA expressing cells, despite being detected in cells expressing GFP (**Figure 5.1D and E**).

Together these data demonstrate that GtgA, GogA, and PipA cleave a subset of NF- κ B subunits comprising p65, cRel, and RelB, because the P1' site residue is not conserved in p105/p50 and p100/p52. In comparison, NleC, which cleaves p65 between residues C38 and E39, cleaves all 5 NF- κ B subunits because the P1' residue is conserved (**Figure 5.1A**) (Li et al., 2014, Turco and Sousa, 2014).

5.1.2 Crystal structure of GtgA

To gain insight into the mechanism of substrate recognition by GtgA, GogA, and PipA, I solved the crystal structure of GtgA alone and in complex with the N-terminal domain of p65 (residues 20-188). In order to prevent cleavage of p65 by GtgA, I purified a GtgA mutant in which the catalytically required glutamate residue in the HEXXH motif was mutated to a glutamine (E183Q). The N-terminal region of T3SS effector proteins are frequently disordered and can prevent protein crystallisation (Esposito et al., 2018, Yao et al., 2014), therefore the

19 N-terminal amino acids of GtgA^{E183Q} were removed (Esposito et al., 2018, Yao et al., 2014). To determine if this affected the ability of GtgA to cleave p65 *in vitro* I purified His₆-GST-GtgA²⁰⁻²²⁸ and performed an *in vitro* cleavage assay. Cleavage of His₆-SUMO-p65²⁰⁻²⁹¹ by His₆-GST-tagged GtgA and GtgA²⁰⁻²²⁸ was indistinguishable therefore the 19 N-terminal residues of GtgA are not important for p65 cleavage (**Figure 5.2**).

For crystallisation trials, GtgA^{20-228 E183Q}, tagged at its N-terminus with a TEV-cleavable His₆-GST-tag was expressed in *E. coli* strain PC2 (Cherepanov, 2007) and then purified by affinity chromatography. The soluble expression tag was then removed following overnight incubation with purified recombinant TEV protease before GtgA^{20-228 E183Q} was purified further using size-exclusion chromatography. **Figure 5.3A** shows that GtgA^{20-228 E183Q} eluted at a volume that is indicative of a monomeric protein for a protein with an expected molecular weight of 24.1 kDa. Similarly, the p65 N-terminal domain (residues 20-188) was expressed fused via its N-terminus to a His₆-SUMO expression tag and purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resin. The His₆-SUMO expression tag was removed using purified recombinant ULP1 and p65²⁰⁻¹⁸⁸ then purified further by size-exclusion chromatography (**Figure 5.3C and D**). p65²⁰⁻¹⁸⁸, which has an expected molecular weight of 19.3 kDa, eluted at a volume that is indicative of a monomeric protein (**Figure 5.3D**).

To crystallise GtgA in complex with p65, crystallisation trials were conducted in which GtgA^{20-228 E183Q} was mixed at an equimolar ratio with p65²⁰⁻¹⁸⁸ immediately prior to crystallisation. Crystallisation trays were then incubated at 20°C and monitored for crystal growth. In the first crystallisation trial comprising approximately 1000 different commercially available buffer compositions, GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸ crystals grew within 48 h in a buffer comprising 0.1 M Tris pH 8.5, 28% (w/v) PEG 6000, and 0.5 M LiCl. Attempts to optimise GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸ crystals using hit optimisation strategies including microseed matrix screening, where crystals are crushed and then mixed with conditions unrelated to the original crystal hit, were done (D'Arcy et al., 2014). However, plate-like crystals grown without seeding in a condition similar to the initial hit (0.1 M Tris pH 8.3, 32.5% [w/v] PEG 6000 and 0.5 M LiCl)

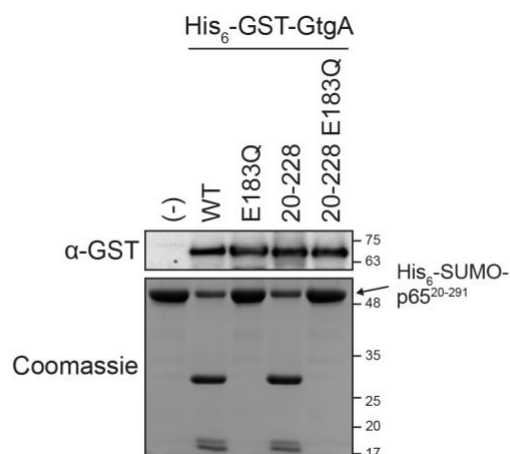


Figure 5.2: The first 19 amino acids of GtgA are dispensable for GtgA's catalytic activity.

In vitro cleavage assay in which the indicated His₆-GST-GtgA variant was incubated with His₆-SUMO-p65²⁰⁻²⁹¹ at a molar ratio of 1:50 at 37°C for 5 h. The reaction was quenched by the addition of 2 x Laemmli buffer, then separated by SDS-PAGE and proteins visualised both by immunoblotting with an α -GST antibody and by Coomassie blue staining. The Coomassie blue stained polyacrylamide gel and immunoblot are representative of 3 independent experiments.

diffracted to 2.1 Å resolution. The crystal structure of Zn²⁺-free GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸ was then solved by molecular replacement using the structure of the p65 NTD (PDB 2RAM) as search template (**Figure 5.4** and **Table 5.1**). GtgA^{20-228 E183Q}-p65²⁰⁻¹⁸⁸ crystals belong to space group *P2₁2₁2₁* with a single copy of the complex in the asymmetric unit. GtgA^{20-228 E183Q} residues 20-27, 137-145, and N228, as well as p65²⁰⁻¹⁸⁸ residues R187 and A188 are not visible in the electron density.

Crystallisation of GtgA^{20-228 E183Q} alone was performed in a similar manner. In initial crystallisation screens, thin needle crystals grew in 12 h in buffers with acidic to neutral pH containing small PEGs (PEG 300 and PEG 400) and calcium salts. Hit optimisation was initially concentrated around these conditions but I was unable to significantly improve crystal morphology. However, 34 days after the initial crystallisation screens were setup, thicker needle-like crystals grew in 0.1 M Tris pH 8.5, 30% (v/v) isopropanol and 30% (w/v) PEG 3350. Following hit optimisation, GtgA^{20-228 E183Q} crystals grew in 48 h in 0.1 M Tris pH 8.5, 20% (v/v) 2-Propanol, and 20% (w/v) PEG 3350 and these diffracted to 2.6 Å resolution. The

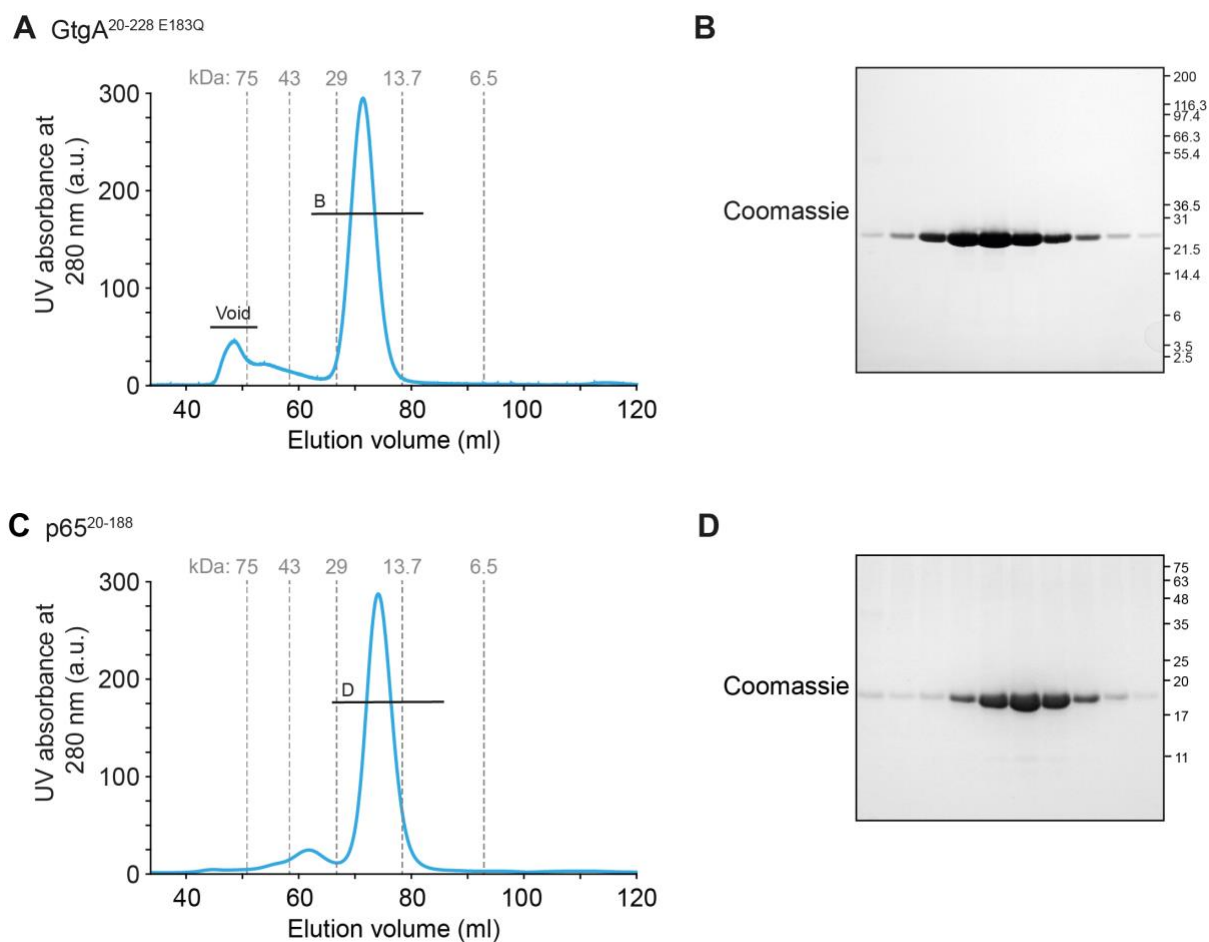


Figure 5.3: Size exclusion chromatography of GtgA^{20-228 E183Q} and p65²⁰⁻¹⁸⁸.

Elution profile of (A) GtgA^{20-228 E183Q} and (C) p65²⁰⁻¹⁸⁸ separated by size exclusion chromatography. The grey dashed lines depict the calibration curve for standard proteins. (B and D) Peak elution fractions were separated by SDS-PAGE and visualised by Coomassie blue staining. Abbreviations: *a.u.*, Arbitrary units.

crystal structure of Zn²⁺-bound GtgA^{20-228 E183Q} was then solved by molecular replacement using GtgA from the complex structure (**Figure 5.5**). GtgA^{20-228 E183Q} crystals belong to space group *I*121 with 2 copies in the asymmetric unit linked covalently by a disulphide bond between residue C44 of each chain. This is likely a crystallisation artefact as in solution GtgA^{20-228 E183Q} was monomeric (**Figure 5.3A**) and the disulphide bond is not present in the GtgA-p65 complex structure (**Figure 5.4**). No electron density was visible for residues 20-24, 157, 158, and 228, and the two molecules in the asymmetric unit are nearly identical with a root mean square deviation (RMSD; C α of residues 27-154 and 160-227) of 0.2 Å.

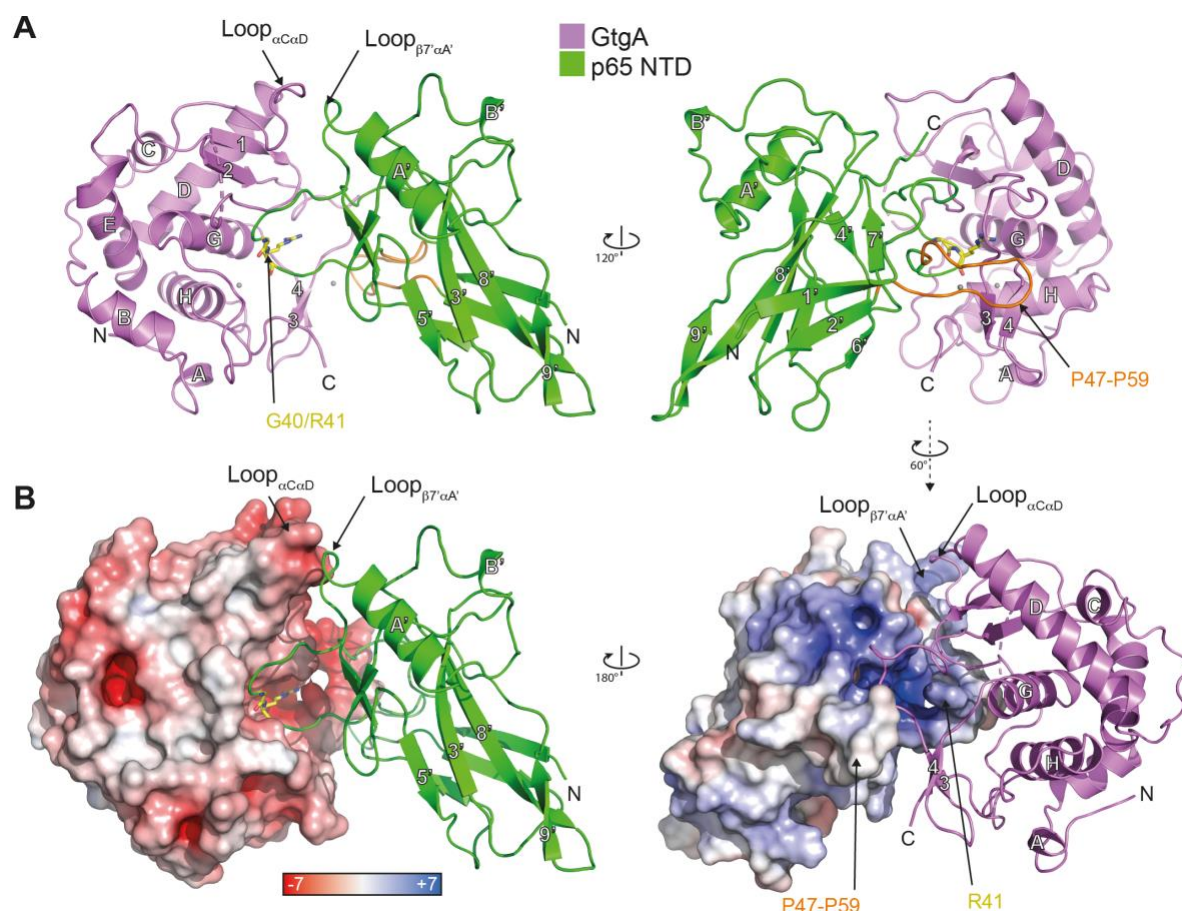


Figure 5.4: Crystal structure of Zn^{2+} -free GtgA^{20-228 E183Q} in complex with p65²⁰⁻¹⁸⁸.

(A) GtgA and p65 are shown in a cartoon representation, with the exception of the p65 cleavage site residues G40 and R41 which are shown in a stick representation and coloured in yellow. α -helices and β -strands in GtgA are labelled A to H and 1 to 4, respectively. α -helices and β -strands in p65 are labelled A' and B', and 1' to 9', respectively. The grey spheres represent chloride ions. (B) Surface and cartoon representation of the GtgA-p65 complex structure. The electrostatic surface potential of GtgA and p65 were calculated using the Adaptive Poisson-Boltzmann Solver (APBS) in PyMOL (Baker et al., 2001).

Members of the Zincin superfamily of proteases contain a common structural catalytic core in which the active site helix sits at the bottom of a cleft formed between an upper N-terminal subdomain (NSD) and a lower C-terminal subdomain (CSD) (**Figure 1.7**). The NSD contains backing helices that supports a 3 to 5 stranded β -sheet and the active site helix (Cerdà-Costa and Xavier Gomis-Rüth, 2014). These features are present in both the apo and

Table 5.1: Crystallographic data collection and refinement statistics

	GtgA ^{20-228 E183Q}	GtgA ^{20-228 E183Q} -p65 ²⁰⁻¹⁸⁸
PDB ID	6GGO	6GGR
Data Collection statistics		
Wavelength (Å)	0.9686	0.9159
Resolution range (Å)	70.28 - 2.6	55.93 - 2.097
Highest resolution range (Å)	2.72 - 2.6	2.172 - 2.097
Space group	<i>I</i> 121	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Cell dimensions:		
a, b, c (Å)	95.78 40.68 112.161	39.39 85.83 111.87
α , β , γ (°)	90 94.0027 90	90 90 90
Total reflections	43450 (4336)	714811 (61672)
Unique reflections	13578 (1345)	22948 (2217)
Multiplicity	3.2 (3.2)	31.1 (27.8)
Completeness (%)	99.8 (99.8)	99.0 (97.7)
Mean I/sigma (I)	5.3 (1.3)	13.9 (3.4)
Wilson B-factor	59.91	32.36
R-merge	0.102 (0.617)	0.1894 (1.419)
R-meas	0.122 (0.743)	0.1927 (1.446)
R-pim	0.067 (0.408)	0.03482 (0.2735)
CC1/2	0.992 (0.919)	0.995 (0.946)
Refinement statistics		
Reflections used in refinement	13394 (1304)	22770 (2208)
Reflections used for R-free	594 (63)	1129 (107)
R-work	0.2175 (0.4119)	0.2061 (0.2901)
R-free	0.2564 (0.4379)	0.2520 (0.3280)
Number of non-hydrogen atoms	3243	3028
Macromolecules	3182	2877
Ligands	4	2
Solvent	57	149
Protein residues	397	359
RMS (bonds) (Å)	0.003	0.006
RMS (angles) (°)	0.51	0.65
Ramachandran favoured (%)	98.71	98.87
Ramachandran allowed (%)	1.29	1.13
Ramachandran outliers (%)	0	0
Rotamer outliers (%)	0.56	0.31
Clashscore	2.44	2.49
Average B-factor	93.53	49.83
Macromolecules	93.95	50.2
Ligands	72.71	30.71
Solvent	71.91	43.02

Highest-resolution shell values are given in parentheses

complex form of GtgA: the GtgA NSD comprises two backing helices (α D and α E) that fold into a V-shape, the active site helix (α G) and unusually, a 2-stranded β -sheet formed by strands β 1 and β 2 (see below; **Figure 5.5A**). Additionally, residues N152 to D175 frame the

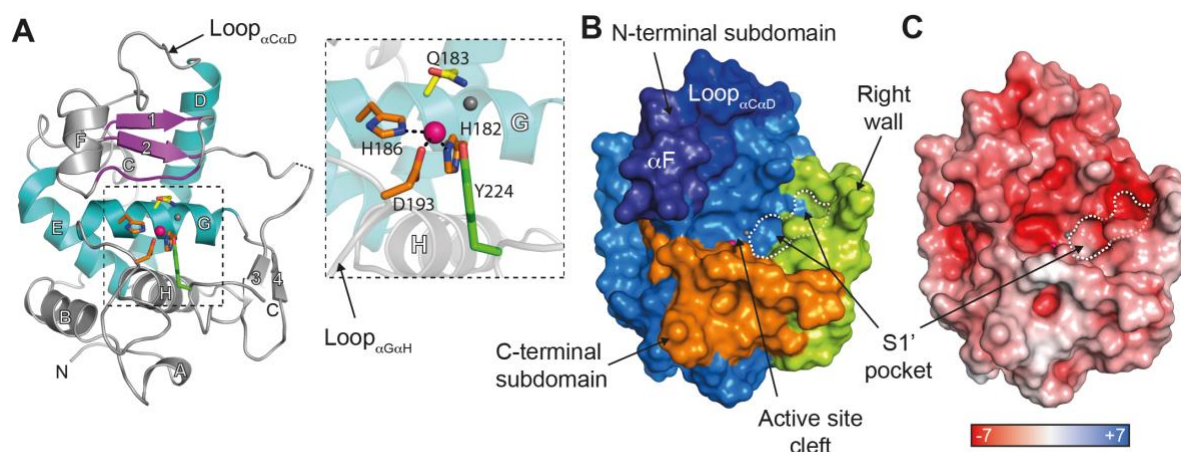


Figure 5.5: Crystal structure of Zn^{2+} -bound GtgA^{20-228 E183Q}.

(A) GtgA is shown in cartoon representation with the Zincin-like catalytic core coloured as in Cerdà-Costa and Xavier Gomis-Rüth (2014); α -helices are coloured teal, whereas the $\beta 1\beta 2$ β -sheet and the active site upper rim residues are coloured purple. *Inset*: close-up view of the active site; the zinc and chloride ion are represented as pink and grey spheres, respectively. Zinc-coordinating residues are coloured orange. Q183 and Y224 are coloured yellow and green, respectively. (B) Surface representation of GtgA coloured according to its active site subdomains. Features in the N-terminal subdomain are coloured in different shades of blue. (C) Surface representation of GtgA coloured according to the electrostatic surface potential.

right side of the active cleft and will be referred to herein as the ‘right wall’ (**Figure 5.5B**). The CSD includes all residues after helix αG including helix αH .

In the GtgA apo structure, the zinc ion is tetrahedrally coordinated by residues H183, H186 and D193 and a chloride ion (**Figure 5.5A**). In other Zincins (e.g. Asticin [PDB 1AST] (Bode et al., 1992)), the zinc ion is coordinated by a catalytically required water molecule, hydrogen bonded with the carboxyl group of the glutamate residue in the HEXXH motif. The presence of a chloride ion at this position in the GtgA apo structure is likely a crystallisation artefact as the catalytically important residue E183 in the HEXXH motif was mutated to a glutamine. The GtgA residue Y224 is also in close proximity to the zinc ion however the distance is too great for Y224 to be a coordinating residue (3.9 Å). The corresponding residue in NleC (Y227), was important for p65 cleavage *in vitro*, whereas NleC variants where the

zinc-coordinating residues were individually mutated to an alanine residue were catalytically inactive (Li et al., 2014).

In the structures of other Zincins, the NSD β -sheet usually contains 3 to 5 β -strands with the 3 N-terminal strands forming a ψ -loop motif. The lowermost strand of the ψ -loop motif, forms part of the active site cleft known as the 'upper-rim' and residues in this strand hydrogen bond to the substrate backbone (Cerdà-Costa and Xavier Gomis-Rüth, 2014). GtgA is unusual: the ψ -loop motif is incomplete and the NSD β -sheet comprises only 2 β -strands (**Figure 5.5A**). However, hydrogen bonds are formed between the upper-rim of GtgA and the backbone of p65 (**Figure 5.6A**) demonstrating that the interaction of the substrate with the active site cleft is similar to that which has been observed for other Zincins previously.

Like NleC (Li et al., 2014, Turco and Sousa, 2014), the active site cleft of GtgA is highly negatively charged (**Figure 5.5C**) and shaped similar to the DNA major groove. Therefore, GtgA might recognise NF- κ B subunits by mimicking the negative charge of the DNA phosphate backbone.

5.1.3 Crystal structure of GtgA in complex with the N-terminal domain of p65

In the complex structure, GtgA and the N-terminal domain of p65 interact via a buried surface area of 1081 Å². The majority of inter-chain hydrogen bonds are between the active site cleft of GtgA and the loop in p65 connecting the β -strands $\beta 1'$ and $\beta 2'$. Additional interactions occur between the surface of the loop connecting helices αC and αD and the surface of the loop connecting sheet $\beta 7'$ and $\alpha A'$ in p65 (**Figure 5.4**). GtgA in complex with p65 is highly similar to the apo structure with an RMSD ($C\alpha$ of residues 28-136, 146-154 and 159-227) of 0.36 Å, however in the complex structure helix αF is invisible in the electron density. An overlay of the apo structure with GtgA in complex with p65 demonstrates that residues in helix αF clash sterically with the p65 chain (**Figure 5.7**).

Residues surrounding the p65 cleavage site extend horizontally across the active site

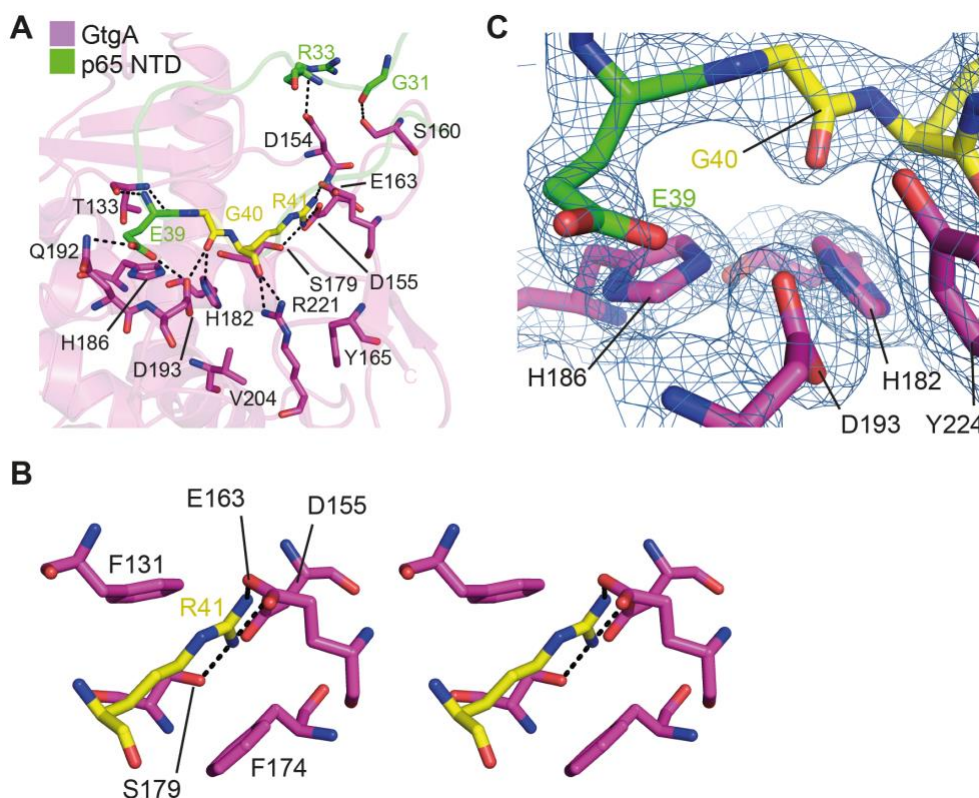


Figure 5.6: The GtgA-p65 interaction interface.

(A) Close-up view of the GtgA-p65 interacting residues. GtgA is coloured purple and p65 is coloured green with the exception of the p65 cleavage site residues (G40/R41) which are coloured in yellow. Black dashed lines represent inter-chain hydrogen bonds. (B) Wall-eyed stereo view of the P1' residue R41 inserted into the S1 pocket of GtgA. (C) 2FoFc electron density map of the GtgA active site. The density map is contoured at 1 σ .

cleft of GtgA, anti-parallel to the upper rim residues of GtgA. Backbone-backbone hydrogen bonds similar to those observed in a β -sheet are formed between the p65 P2 residue E39 and the GtgA upper rim residue T133. In addition, the carboxyl side chain of the P2 residue E39 is hydrogen bonded to two residues in the loop connecting GtgA helices α G and α H - Q192 and D193. The p65 cleavage site (G40/R41) sits in the middle of the GtgA active site cleft, such that the carbonyl oxygen of the P1 residue G40 is situated directly above the zinc coordinating residues of GtgA and the guanidino group of the P1' residue R41 is inserted into the negatively charged S1' pocket formed by the upper rim residue F131, residues D155, E163

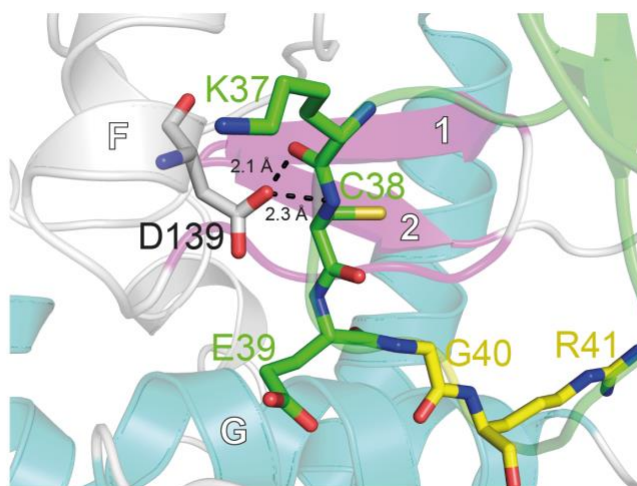


Figure 5.7: Residues in helix α F clash in the GtgA apo structure sterically with the p65 N-terminal domain.

The GtgA apo structure was superimposed with GtgA in complex with p65. The GtgA apo structure is coloured as in Cerdà-Costa and Xavier Gomis-Rüth (2014): α -helices are coloured teal, whereas the β 1 β 2 β -sheet and the active site upper rim residues are coloured purple. GtgA from the complex structure is not shown. p65 is coloured in green with the exception of the G40/R41 cleavage site which is coloured in yellow.

and F174 in the right wall of the GtgA active site cleft, as well as S179 in helix α G. The benzyl rings of F131 and F174 form hydrophobic contacts with the 3-carbon aliphatic straight chain of R41, whereas GtgA residues D155, E163, and S179 are hydrogen bonded to the guanidino group of R41 (**Figure 5.6B**). Also, the carbonyl oxygen of the P1' residue R41 is hydrogen bonded by GtgA residue R221. As well as forming part of the S1' pocket, residues in the right wall of the GtgA active site cleft - D154 and S160, hydrogen bond with p65 residues R33 and G31, respectively (**Figure 5.6A**). In addition, p65 residues P47 to P59 loop around the back of the GtgA right wall, forming long-range inter-chain van der Waals contacts (**Figure 5.4**).

Although the zinc ion is absent from the complex structure (**Figure 5.6C**), the position of the carbonyl oxygen of the p65 residue G40, relative to the zinc coordinating residues in GtgA is similar to that observed in other zinc metalloprotease-substrate complexes such as insulin degrading enzyme (IDE) in complex with a peptide substrate (PDB 2G54) and Astacin in complex with a transition state analogue (PDB 1QJI). In the GtgA-p65 complex structure

presented here, the carbonyl oxygen of p65 residue G40 is hydrogen bonded to GtgA residues H182 and D193 (**Figure 5.6A**).

Superimposition of the p65 NTD from the GtgA-p65 complex structure with structures of the p65 RHR in complex with DNA, revealed that the structure of p65 NTD reported here is highly similar to those published previously with C α RMSD values between 0.91 Å (PDB 5U01.B) to 1.43 Å (PDB 1RAM.A). One of the only differences between these structures is that when in complex with GtgA, the side-chains of the P2 and P1' residues (E38 and R41) are rotated approximately 180° so that the guanidino group of R41 is inserted into the GtgA S1' pocket.

5.1.4 Mutagenic analysis of GtgA-p65 interacting residues

To explore the importance of residues in GtgA that interact with the p65 N-terminal domain in the complex structure, I generated a panel of plasmids expressing variants of GtgA^{E183A}, in which an additional residue was mutated to an alanine. The mutant panel included catalytically inactive GtgA D154A, D155A, D159A, S160A, E163A, S179A, Q192A, and R221A. A luminescence-based mammalian interactome mapping (LUMIER) assay was then done to analyse the requirement of individual residues for the formation of a stable complex between GtgA and p65. 293ET cells were transfected with a plasmid encoding p65 fused via its C-terminus to the Renilla luciferase. Next, these cells were lysed, and the post-nuclear supernatants (PNS) incubated for 2 h with purified GST, wild-type His₆-GST-GtgA^{E183A} or a His₆-GST-GtgA^{E183A} variant. Glutathione sepharose was then used to perform a GST-pulldown and the binding of each GtgA variant to p65-Renilla analysed using a Renilla luciferase assay (**Figure 5.8A and B**). As expected p65-Renilla was pulled-down with His₆-GST-GtgA^{E183A} but not with GST alone, demonstrating that catalytically inactive GtgA forms a stable complex with p65 in this experimental set-up. p65-Renilla was pulled down with a similar level of efficiency to GtgA^{E183A} by the GtgA^{E183A} variants D159A, S160A, Q192A, and R221A, whereas the interaction of GtgA^{E183A} variants D154A, D155A, E163A, and S179A with

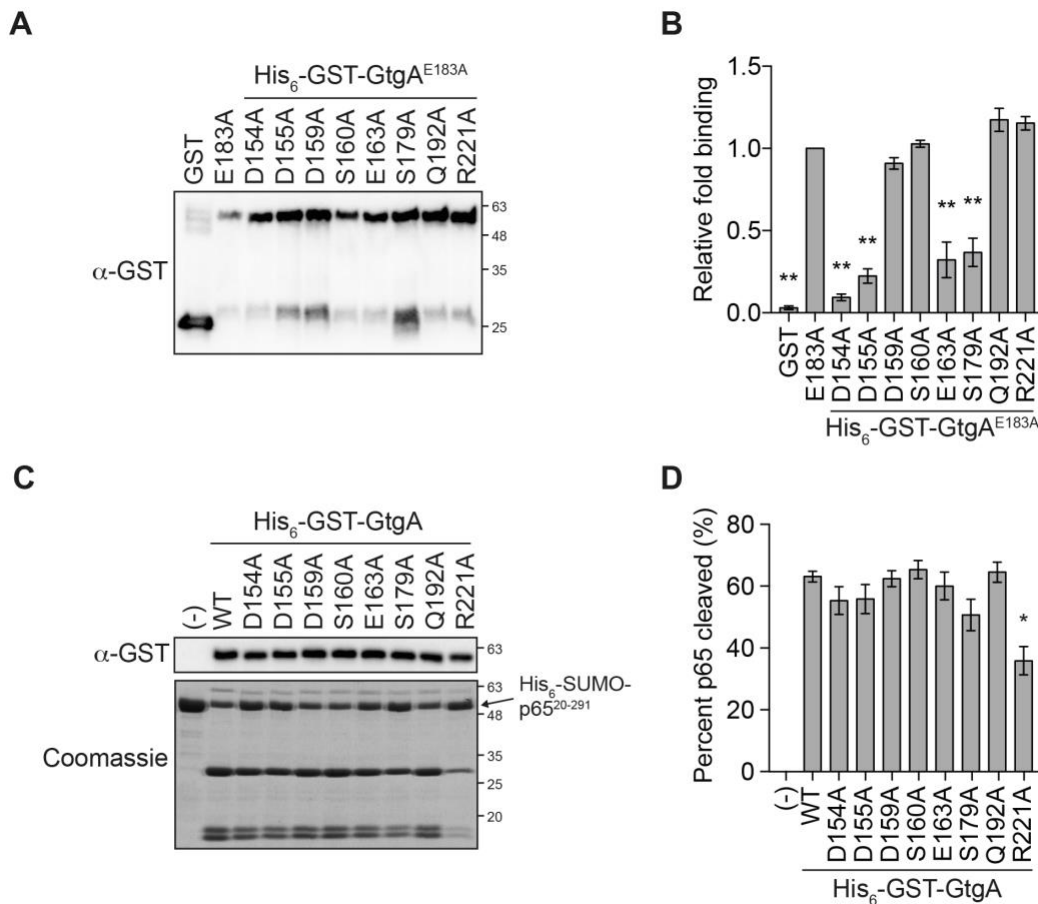


Figure 5.8: Identification of GtgA residues important for GtgA-p65 stable complex formation and *in vitro* catalytic activity.

(A and B) LUMIER binding assay. 293ET cells were transfected with a plasmid encoding p65-Renilla. Cells were then lysed and post-nuclear supernatant incubated with the indicated His₆-GST-tagged effector protein. Following a GST-pulldown, *Renilla* luciferase activity was measured and immunoblot analysis using an α-GST antibody performed. Data are presented as the fold change in luciferase activity relative to His₆-GST-GtgA^{E183A} and represented as the mean ± SEM of 3 independent experiments. Statistical significances were computed between His₆-GST-GtgA^{E183A} and each GtgA^{E183A} variant (ordinary one-way ANOVA with *post-hoc* Dunnett's multiple comparisons test; *, $P < 0.05$; **, $P < 0.01$). (C) *In vitro* cleavage assay in which the indicated His₆-GST-GtgA variant was incubated with His₆-SUMO-p65²⁰⁻²⁹¹ at a molar ratio of 1:50 at 37 °C for 5 h. The reaction was quenched by the addition of 2 x Laemmli buffer, then separated by SDS-PAGE and proteins visualised both by immunoblot analysis using an α-GST antibody and by Coomassie blue staining. The Coomassie blue stained polyacrylamide gel and immunoblot are representative of 3 independent experiments. (D) Densitometry analysis of the experiment shown in panel C was done to determine the abundance of the cleavage products, relative to the abundance of full length His₆-SUMO-p65²⁰⁻²⁹¹. The percentage of p65 cleaved represents the intensity of cleaved p65 relative to full length p65.

Statistical significances were computed between His₆-GST-GtgA and each GtgA variant (ordinary one-way ANOVA with *post-hoc* Dunnett's multiple comparisons test; *, $P < 0.05$; **, $P < 0.01$).

p65-Renilla was significantly reduced. GtgA residues D155, E163, and S179 form the S1' pocket and hydrogen bond to the guanidino group of the P1' residue R41, further demonstrating the importance of the P1' residue in substrate recognition.

Next, I analysed if residues important for formation of a stable complex between GtgA and p65 were required for GtgA's catalytic activity. I purified the same panel of GtgA point mutants with an intact HEXXH motif and an *in vitro* cleavage assay was done. Wild-type or mutant His₆-GST-GtgA was incubated for 5 h with His₆-SUMO-p65²⁰⁻²⁹¹ and then p65 cleavage analysed using SDS-PAGE and Coomassie blue staining (**Figure 5.8C**). The percentage of His₆-SUMO-p65²⁰⁻²⁹¹ cleaved by wild-type or mutant GtgA was then quantified using densitometry analysis (**Figure 5.8D**). Approximately 60% of p65 was cleaved by wild-type GtgA, GtgA^{D159A}, GtgA^{S160A}, GtgA^{E163A} or GtgA^{Q192A}, whereas there was a small non-significant decrease in p65 cleavage when cleaved by GtgA^{D154A}, GtgA^{D155A} or GtgA^{S179A}. Notably, mutation of R221 to an alanine had the greatest effect on the catalytic activity of GtgA: GtgA^{R221A} cleaved approximately 35% of His₆-SUMO-p65²⁰⁻²⁹¹ despite GtgA^{E183A} R221A interacting with p65 with a similar efficiency as GtgA^{E183A}.

Next, an NF- κ B luciferase assay in 293ET cells was done to determine the effect of the above mutations on GtgA's ability to inhibit activation of the NF- κ B signalling pathway. 293ET cells were transfected with NF- κ B reporter plasmids and a plasmid encoding GFP, GFP-GtgA or the indicated GFP-GtgA variant. Cells were then stimulated with TNF- α for 8 h and cell lysates harvested. Then, SDS-PAGE and immunoblot analysis of whole cell lysates confirmed that each GFP-GtgA variant was expressed to a similar level as wild-type GFP-GtgA (**Figure 5.9A**). I also determined that the nuclear localisation of each GFP-GtgA variant was unaffected by the alanine substitutions (**Figure 5.9B**). **Figure 5.9C** shows that TNF- α -induced NF- κ B

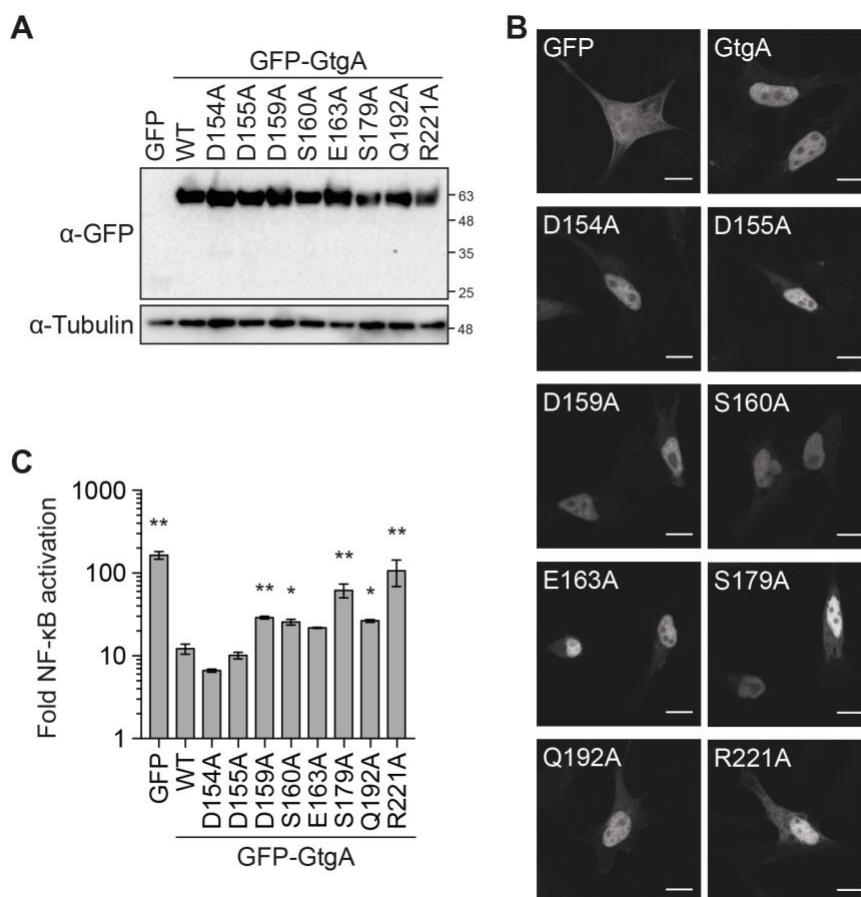


Figure 5.9: Identification of GtgA residues required for NF-κB reporter inhibition.

(A) 293ET cells were transiently transfected with NF-κB reporter plasmids and the indicated GFP-GtgA variant. Cells were then stimulated for 8 h with 20 ng/ml TNF-α prior to cell lysis and analysis by SDS-PAGE and immunoblotting. (B) Representative confocal microscopy images of HeLa cells transiently expressing GFP or the indicated GFP-GtgA variant. Scale bar: 10 μm. (C) Luciferase reporter activity measured in lysates from the experiment shown in panel A. Data are presented as the fold change in NF-κB activity between unstimulated and TNF-α-stimulated 293ET cells and represent the mean ± SEM of 3 independent experiments. Statistical significances were computed between 293ET cells expressing wild-type GFP-GtgA and each GtgA variant (ordinary one-way ANOVA with *post-hoc* Dunnett's test; *, $P < 0.05$; **, $P < 0.01$).

reporter activation was strongly inhibited in cells expressing wild-type GFP-GtgA but not GFP alone. In line with the results of the *in vitro* cleavage assay (Figure 5.8C and D) there was a significant reduction in the ability of GFP-GtgA^{R221A} and GFP-GtgA^{S179A} to inhibit TNF-α-induced NF-κB reporter activation. There was also a small increase in NF-κB reporter

activation in cells expressing GFP-tagged GtgA variants D159A, S160A and Q192A in comparison to cells expressing wild-type GFP-GtgA.

In this chapter I demonstrated that the P1' site is critical for determining substrate specificity of GtgA, GogA, and PipA. In cleavable NF- κ B subunits (p65, cRel, and RelB), an arginine residue is present at this site, whereas in non-cleavable NF- κ B subunits (NF- κ B1 and NF- κ B2) a proline residue is present. The crystal structure of GtgA alone and in complex with the NTD of p65 explains the importance of the P1 residue and shows that like NleC, the active site cleft of GtgA is highly negatively charged. Mutational analysis further revealed GtgA residues that are important for p65 cleavage and NF- κ B reporter inhibition in 293ET cells, as well as residues important for formation of the GtgA-p65 complex.

6 Discussion

Work presented in this thesis revealed that two families of *Salmonella* effector proteins – the SseK family comprising SseK1, SseK2, and SseK3, and the GtgA family comprising GtgA, GogA, and PipA, are sufficient to inhibit activation of the NF- κ B signalling pathway. Furthermore, GtgA, GogA, and PipA were identified to be zinc metalloproteases that cleave the NF- κ B transcription factor subunits p65, RelB, and cRel but not NF- κ B1 (p105/p50) or NF- κ B2 (p100/p52). Accordingly, cleavage of p65 in *Salmonella*-infected cells required *gtgA*, *gogA*, and *pipA*. Mutational analysis of residues surrounding the p65 cleavage site (G40/R41) revealed that the P1' residue R41 in p65 is critical for substrate specificity; a proline residue at the corresponding position in NF- κ B1 and NF- κ B2 prevents cleavage by GtgA, GogA, and PipA. In addition, I solved the crystal structure of GtgA alone and in complex with the N-terminal domain of p65. The complex structure explained the importance of the P1' residue in substrate recognition, and demonstrated that the active site cleft of GtgA is highly negatively charged and similar in shape to the DNA major groove.

6.1 NF- κ B inhibition by SPI-2 T3SS effectors

Previously, the SPI-2 T3SS effector proteins GogB, SseK1, SseK2, SseK3, SpvD, SspH1, and SseL have been described to inhibit activation of the NF- κ B signalling pathway. Specifically, GogB, SseK1, SseK3, and SpvD inhibit the expression of NF- κ B regulated genes in *Salmonella*-infected cells (Pilar et al., 2012, Gunster et al., 2017, Rolhion et al., 2016), and GogB, SseK1, SseK2, SseK3, SpvD, and SspH1 inhibit activation of an NF- κ B-responsive reporter when expressed ectopically (Pilar et al., 2012, Yang et al., 2015, Gunster et al., 2017, Rolhion et al., 2016, Grabe et al., 2016, Haraga and Miller, 2003). However, some of these data are controversial (e.g. SseL), and an unbiased systematic analysis of the importance of individual effector proteins for NF- κ B inhibition in *Salmonella*-infected cells has not been done. Therefore, I infected RAW264.7 NF- κ B reporter macrophages with a panel of *Salmonella* deletion mutant strains and analysed reporter activity as a proxy for NF- κ B activation. NF- κ B

reporter activation was specifically analysed in macrophages because during systemic infection, *Salmonella* replicates intracellularly in macrophages dependent on the SPI-2 T3SS (Cirillo et al., 1998). Intestinal epithelial cells represent another cell type in which *Salmonella* resides *in vivo*, but whereas stationary phase *Salmonella*, which do not express the SPI-1 T3SS, are readily phagocytosed by macrophages *in vitro*, *Salmonella* invasion of epithelial cells is dependent on the SPI-1 T3SS (Galan and Curtiss, 1989). Therefore, a screen in epithelial cells would not have been able to distinguish between SPI-1 and SPI-2 T3SS-dependent phenotypes.

Initial experiments that analysed NF- κ B reporter activation at 16 h p.u. only identified members of the SseK family as important for NF- κ B inhibition in *Salmonella*-infected RAW264.7 macrophages. This timepoint was analysed because a Δ ssaV mutant strain unable to assemble a functional SPI-2 T3SS and translocate any SPI-2 T3SS effector proteins has previously been demonstrated to induce increased NF- κ B reporter activation at this timepoint relative to wild-type *Salmonella* (Gunster et al., 2017). However, at 16 h p.u. mutant strains deleted of effectors required for intravacuolar replication (e.g. Δ sseF and Δ sseG) are attenuated for replication and it is possible that the differences in bacterial load between wild-type *Salmonella* and these mutant strains could affect NF- κ B reporter activation.

In contrast to previous reports, *gogB* and *spvD* were not important for NF- κ B inhibition in the experimental conditions used in this study. Previously, a Δ *gogB* mutant strain was shown to elicit increased NF- κ B reporter activation relative to wild-type *Salmonella* in RAW264.7 macrophages 20 h p.u. (Pilar et al., 2012), whereas I analysed NF- κ B activity at 16 h p.u. Therefore, GogB might inhibit NF- κ B activation at late time points after phagocytosis. Additionally, an Δ *spvD* mutant strain induced increased expression of NF- κ B-responsive genes in *Tlr4*^{-/-} BMDMs but not in wild-type BMDMs, suggesting that SpvD inhibits the expression of genes induced by a stimulus that is not LPS (Rolhion et al., 2016). The same study suggested that NF- κ B activity induced by LPS prior to the translocation of SPI-2 T3SS effector proteins masks the activities of SpvD and this is why the phenotype was observed

only in *Tlr4*^{-/-} macrophages. Conceivably, this could explain why infection of LPS-responsive RAW264.7 macrophages with an Δ *spvD* mutant strain did not lead to increased NF- κ B reporter activation.

SseL has also been reported to inhibit ubiquitination of I κ B α leading to NF- κ B inhibition in *Salmonella*-infected cells (Le Negrate et al., 2008). However, these data are controversial, as a subsequent report failed to observe any effect of SseL on NF- κ B signalling (Mesquita et al., 2013). In my experiments, I did not detect increased NF- κ B reporter activation in cells infected with an Δ *sseL* mutant strain suggesting that SseL does not modulate NF- κ B signalling during *Salmonella* infection.

As the infection screen did not identify any new effectors that are important for NF- κ B inhibition in *Salmonella*-infected macrophages, I next tested if any SPI-2 T3SS effectors are sufficient to inhibit NF- κ B reporter activation. The advantage of this experiment is that any effectors that function redundantly in *Salmonella*-infected cells would be revealed. I confirmed previous results that SseK1, SseK2, and SseK3 inhibit TNF- α -induced NF- κ B reporter activation (Yang et al., 2015, Gunster et al., 2017), but did not observe a significant reduction in reporter activation in cells expressing SpvD or SspH1. Myc-tagged SpvD and untagged SspH1 have previously been reported to inhibit PMA- and LPS-induced NF- κ B reporter activation, respectively (Grabe et al., 2016, Haraga and Miller, 2003). As these effectors did not inhibit TNF- α -induced NF- κ B reporter activation, they might specifically inhibit NF- κ B signalling downstream of these different stimuli. Alternatively, the activities of these effectors might have been affected by the N-terminal GFP tag that was used in this study.

Despite this, the sufficiency screen was successful in identifying GtgA, a previously uncharacterised effector protein, as a potent inhibitor of both basal and TNF- α -induced NF- κ B reporter activation. Two proteins which share high amino acid sequence identity with GtgA but had not been shown to be T3SS effector proteins - GogA and PipA, also inhibited NF- κ B signalling induced by TNF- α when expressed ectopically. In my assays, NF- κ B reporter inhibition by GtgA, GogA, and PipA required the metal-binding motif HEXXH found in the C-

terminal region of each protein, suggesting that these proteins are catalytically active zinc metalloproteases. As I only screened SPI-2 T3SS effectors for sufficiency to inhibit TNF- α -induced NF- κ B reporter activation I cannot rule out the possibility that other SPI-2 T3SS effectors inhibit NF- κ B signalling activated by other stimuli. SseL, SspH1, and SspH2 were each sufficient to inhibit basal NF- κ B reporter activity, therefore these effectors might be worthy of further investigation in this respect. This could involve screening SPI-2 T3SS effectors for sufficiency to inhibit NF- κ B signalling downstream of the DNA and RNA cytosolic PRRs RIG-I and MDA-5, or NF- κ B signalling induced by ADP-heptose, which is detected by alpha-kinase 1 (Zhou et al., 2018).

6.2 Catalytically independent functions of NleC

GtgA, GogA, and PipA did not inhibit PMA-induced AP-1 reporter activation, or IFN- β -induced ISRE reporter activation suggesting that they specifically inhibit the NF- κ B signalling pathway. In contrast, the EPEC effector NleC, which was used as a control in these assays, inhibited IFN- β -induced activation of an ISRE luciferase reporter independent of its catalytic activity, and TNF- α -induced NF- κ B reporter activation in both a catalytically-dependent and -independent manner. Although inhibition of NF- κ B signalling by NleC dependent on its catalytic activity has been described previously, NleC has not been demonstrated to inhibit innate immune signalling independently of its zinc metalloprotease activity. Truncation analysis revealed that catalytically-independent phenotypes required the C-terminal region of NleC (residues 281-330). This region is not present in GtgA, GogA, and PipA explaining the phenotypic differences between the GtgA family and NleC.

The ISRE reporter is used to measure the transcriptional activity of the ISGF3 complex comprising STAT1, STAT2, and IRF9. ISGF3-dependent gene transcription is initiated following cellular stimulation by type I interferons and requires the activity of the histone acetyltransferase p300 (Bhattacharya et al., 1996). p300 is also recruited to κ B-sites bound by p65-containing NF- κ B transcription factors (Gerritsen et al., 1997), and is important for p65-

dependent gene transcription (Mukherjee et al., 2013). Intriguingly, NleC has been described previously to interact with p300 (Shames et al., 2011), however the physiological relevance of this interaction is unclear. NF- κ B and ISRE reporter inhibition by catalytically inactive NleC might be a consequence of the NleC-p300 interaction. p65, STAT2, and NleC all interact with the TAZ1 domain of p300, therefore NleC might competitively inhibit p65 and STAT2 from interacting with p300. This might not be limited to p65- and ISGF3-induced gene transcription, as other transcription factors including p53 interact with the p300 TAZ1 domain (Krois et al., 2016b). However, further work is required to ascertain if this phenotype is physiologically relevant because the abundance of NleC in cells transfected with a plasmid encoding GFP-NleC under the control of the strong CMV promoter is likely to be far greater than the abundance of NleC translocated into cells infected with EPEC/EHEC. Specifically, ISRE-reporter activation could be analysed in cells infected with wild-type EPEC or a $\Delta nleC$ mutant strain, followed by expression of NleC deletion mutants in the $\Delta nleC$ mutant background. Furthermore, immunoprecipitation experiments could be performed to determine if the C-terminal region of NleC is required for NleC to interact with p300.

6.3 GtgA, GogA, and PipA are zinc metalloproteases that cleave NF- κ B subunits

Due to the predicted structural similarity between members of the GtgA family and NleC, I hypothesised that GtgA, GogA, and PipA are zinc metalloproteases that inhibit NF- κ B signalling by cleaving NF- κ B transcription factor subunits. Indeed, recombinant, purified GST-tagged GtgA, GogA, and PipA cleaved the NTD of p65 *in vitro*. Mutation of the catalytically important glutamate residue in the HEXXH motif to an alanine, or the addition of the metal-chelating agent EDTA to the reaction buffer, abrogated p65 cleavage confirming that GtgA, GogA, and PipA are zinc metalloproteases. Furthermore, immunoblot analysis of individual NF- κ B subunits in cells expressing members of the GtgA family ectopically, revealed that GtgA, GogA, and PipA cleave a subset of NF- κ B subunits comprising p65, RelB, and cRel, but not NF- κ B1 (p105/p50) or NF- κ B2 (p100/p52). In comparison, all five subunits were cleaved in cells expressing NleC. NFAT subunits, which contain a DNA binding domain that

is structurally similar to the RHR found in NF- κ B subunits, were also cleaved in NleC-expressing cells suggesting that these proteins might be an additional substrate of NleC. However, *in vitro* NleC did not cleave the NTD of NFATc2. This might have been because additional post-translational modifications or co-factors are required for NFAT cleavage or cleavage in NleC-expressing cells is indirect. It is also possible that NFAT proteins do not represent a physiologically relevant substrate of NleC. Future work should be done to determine if NFAT subunits are cleaved in EPEC/EHEC-infected cells dependent on NleC.

The physiological relevance of the differing substrate specificities of the GtgA family and NleC was not explored in this thesis, however it is conceivable that the difference in substrate specificity might affect the transcription of different subsets of NF- κ B-regulated genes in cells infected by *Salmonella* and EPEC/EHEC, respectively. This is because different NF- κ B dimers have affinity for different κ B-sites and thus regulate different genes. For example, p65 homodimers preferentially activate transcription at A/T-centric κ B-sites, whereas the p52 homodimer in complex with the Bcl3, induces transcription at G/C-centric κ B-sites (Wang et al., 2012). Furthermore, it would be interesting to determine if cleavage of a subset of NF- κ B subunits by GtgA, GogA, and PipA is advantageous for *Salmonella* replication *in vivo*.

During the course of this thesis, another group published data showing that GtgA, GogA, and PipA cleave p65 and RelB but not NF- κ B1 or NF- κ B2 (Sun et al., 2016). Additionally, they demonstrated that GtgA, GogA, and PipA cleave p65 between residues G40 and R41, two amino acids away from the primary site in p65 cleaved by NleC (C38/E39) (Baruch et al., 2011). My results are consistent with these data as the sizes of the p65 cleavage products generated by GtgA, GogA, and PipA, although similar, are different to those generated by NleC. However, work in this thesis is the first to demonstrate that GtgA, GogA, and PipA cleave cRel and that NleC cleaves NF- κ B2 and is the first to describe the molecular basis for the differing substrate specificities of these proteases.

GtgA, GogA, and PipA only share weak amino acid sequence identity with NleC, suggesting that the GtgA family and NleC are not homologs. Instead cleavage of NF- κ B

subunits by a T3SS effector protein has evolved twice. This demonstrates the importance of the NF- κ B signalling pathway in innate immunity and shows that genes regulated by the NF- κ B signalling pathway confer a strong selective pressure on Gram-negative bacteria. Several other bacterial virulence factors have evolved to cleave NF- κ B transcription factor subunits thereby dampening NF- κ B-induced gene transcription. The Gram-negative pathogenic bacterium *Photobacterium damsela piscicida*, encodes an AB toxin called AIP56, that is secreted by a type II secretion system (do Vale et al., 2017). AIP56 comprises an N-terminal catalytic domain with homology to NleC, and a C-terminal domain that is important for binding of the toxin to host cells and for toxin internalisation. Once inside host cells, AIP56 cleaves the N-terminal domain of sea bass p65 between residues C39 and E40 (C38 and E39 in human p65) and induces macrophage and neutrophil apoptosis (Silva et al., 2013). Additionally, strains belonging to the *Neisseria meningitidis* ST-11 clonal complex encode a secreted IgA protease that cleaves the TAD of p65 (Besbes et al., 2015), and *Chlamydia trachomatis* encodes two proteases – CT441 and CPAF, which cleave p65 between the RHR and TAD (Lad et al., 2007, Christian et al., 2010). p65 cleavage as a mechanism for immune evasion is not only restricted to bacterial pathogens. p65 is cleaved towards its C-terminus in poliovirus- and rhinovirus-infected cells by the 3C protein (Neznanov et al., 2005).

6.4 Physiological relevance of NF- κ B cleavage

Characterisation of GtgA, GogA, and PipA *in vitro* or when expressed ectopically in 293ET cells suggested that these three proteins are functionally identical. Functional redundancy in *Salmonella*-infected cells might explain why infection of RAW264.7 NF- κ B reporter macrophages with a Δ gtgA mutant strain did not induce increased NF- κ B reporter activation relative to wild-type *Salmonella*. To address this hypothesis, I analysed NF- κ B reporter activation in cells infected with *Salmonella* mutant strains deleted of one or more members of the GtgA family. NF- κ B reporter activation in cells infected with *gtgA*, *gogA*, and *pipA* single deletion mutant strains was indistinguishable from NF- κ B reporter activation in

wild-type infected cells, however there was a reproducible increase in NF- κ B reporter activity in cells infected with a $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant strain. Although this increase was not statistically significant, this suggests that GtgA, GogA, and PipA might function redundantly in *Salmonella*-infected cells. A caveat of this experimental setup is that the reporter assay measures NF- κ B activity in a heterogeneous population of infected and uninfected bystander cells. To overcome this limitation, single cell analysis could have been done. For example, flow cytometry could have been used to analyse activation of a fluorescence-based NF- κ B reporter in infected cells.

SseK1 and SseK3 function to inhibit NF- κ B signalling in *Salmonella*-infected macrophages, therefore another reason why I did not detect a significant increase in reporter activation in $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant infected cells might be because of the activities of these two effectors. Future work should be done to analyse if the GtgA family are important for NF- κ B inhibition in mutant strains additionally deleted for *sseK1* and *sseK3*.

Despite this the increase in NF- κ B reporter activation in cells infected with a $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant strain was complemented by plasmid-encoded GogA but not GtgA or PipA when these were expressed under the control of their endogenous promoter. GtgA and PipA were not detected by immunoblot analysis of whole cell lysates, and the abundance of GogA was significantly less than that of the unrelated SPI-2 T3SS effector protein PipB, suggesting that these effectors might be weakly expressed under physiologically relevant conditions. An inability to detect PipA was surprising, especially as the abundance of *pipA* mRNA is strongly upregulated in *Salmonella*-infected macrophages (Srikumar et al., 2015). However, it is possible that *pipA* mRNA is not efficiently translated into protein during macrophage infection.

A separate report also demonstrated that GtgA, GogA, and PipA inhibit NF- κ B signalling *in vivo* (Sun et al., 2016). A $\Delta gtgA \Delta gogA \Delta pipA$ triple mutant strain was more virulent than wild-type *Salmonella* in *Nramp1*^{+/+} C57BL6 mice, inducing increased expression of proinflammatory cytokines in the small intestine and causing mice to rapidly succumb to

infection. These phenotypes only occurred after infection by oral gavage and not intraperitoneal injection, suggesting that GtgA, GogA, and PipA predominantly function to inhibit NF- κ B signalling in the small intestine. However, cytokine expression was not measured at secondary sites of infection such as the spleen, in mice infected by intraperitoneal infection, so a function for GtgA, GogA, and PipA in systemic disease cannot be ruled out and should be explored further.

The abundance of full length p65 was not reduced in cells infected with wild-type *Salmonella* relative to uninfected cells. However, a p65 cleavage product was present in *Salmonella*-infected cells treated with the proteasomal inhibitor MG132, suggesting that only a small fraction of the total pool of p65 is cleaved and that the larger cleavage product is degraded by the proteasome. p65 cleavage was not detected in cells infected with a Δ gtgA Δ gogA Δ pipA triple mutant strain demonstrating that members of the GtgA family of effectors function to cleave NF- κ B transcription factor subunits in *Salmonella*-infected cells. In EPEC/EHEC-infected cells cleavage of p65 by NleC generates two cleavage products; the larger fragment is degraded by the proteasome (Yen et al., 2010), whereas the smaller fragment interacts with the transcriptional co-activator ribosomal protein S3 (Rps3) (Hodgson et al., 2015). This interaction prevents the nuclear translocation of Rps3, thereby inhibiting expression of Rps3-dependent NF- κ B regulated genes. As the sizes of the fragments generated by members of the GtgA family and NleC are almost identical I hypothesise that Rps3-dependent gene transcription is inhibited in a similar manner in *Salmonella*-infected cells.

Analysis of a SPI-2 T3SS-null (Δ ssaV) mutant strain revealed cleavage of p65 in infected HeLa cells was independent of the SPI-2 T3SS, but in infected RAW264.7 macrophages was dependent on the SPI-2 T3SS. HeLa cells were actively invaded by SPI-1 T3SS-expressing *Salmonella* grown to logarithmic phase, whereas RAW264.7 macrophages were inoculated with stationary phase bacteria and uptake was by phagocytosis. These data therefore provide indirect evidence in support of both SPI-1 and SPI-2 T3SS-dependent translocation of the

GtgA family of effector proteins. However, one caveat of these data is that the Δ ssaV mutant is attenuated for replication in RAW264.7 macrophages at 16 h p.u., meaning that the lack of p65 cleavage in Δ ssaV-infected cells might be due to a reduction in bacterial load. For this reason, it was essential to carry out direct attempts to detect the translocation of GtgA, GogA, and PipA in a SPI-2 T3SS dependent manner (see below).

Overall, the data presented in this thesis suggest that two families of effector proteins – the SseK family and the GtgA family, inhibit activation of an NF- κ B reporter in *Salmonella*-infected cells. The SseK family specifically inhibits TNF- α -induced NF- κ B activity probably due to GlcNAcylation of death domain proteins, and also inhibits necroptotic cell death (Gunster et al., 2017). Although the substrates of the SseK family have not been fully characterised, it is clear that each SseK family member has different substrates (Gunster et al., 2017) and therefore the exact functions of SseK1, SseK2, and SseK3 might be slightly different. This is in contrast to GtgA, GogA, and PipA which appear to be functionally identical and inhibit NF- κ B signalling regardless of the stimuli. It is not clear why the *Salmonella* genome has retained three versions of what appears to be virtually the same protein. The genes encoding GtgA and GogA are found on bacteriophages, whereas *pipA* is present in an operon with the gene for the SPI-2 T3SS effector PipB. Therefore, these might have been retained due to their proximity to other virulence genes. Alternatively, they may function during different stages of infection, perhaps delivered into host cells by different T3SSs, and functioning in different cell types. In support of this hypothesis expression of *gtgA* and *gogA* is upregulated in late exponential phase and early stationary phase, whereas *pipA* is expressed in SPI-2-inducing conditions and in macrophages (Kroger et al., 2013). In addition, the expression of *gtgA* and *gogA* is regulated by the regulatory protein HilD which controls the expression of SPI-1 genes, and the expression of *pipA* is regulated by the *phoP/phoQ* two component regulatory system (Colgan et al., 2016).

6.5 Nuclear localisation of GtgA, GogA, and PipA

Following translocation into host cells, effector proteins localise to different subcellular compartments thereby targeting their activities towards a localised environment. Several SPI-2 T3SS effectors localise to the SCV and SIT membrane, whereas others localise to the host cell cytosol and nucleus. To analyse the subcellular localisation of GtgA, GogA, and PipA, I infected HeLa and RAW264.7 macrophages with *Salmonella* strains expressing GtgA, GogA, and PipA tagged at their C-terminus with 2 tandem copies of the HA-epitope. However, when they were expressed under the control of their endogenous promoters I was unable to detect GtgA, GogA, and PipA translocated into host cells using immunofluorescence microscopy. Therefore, to analyse their subcellular localisation, GtgA, GogA, and PipA were expressed under the control of the *ssaG* promoter, which is strongly activated in SPI-2-inducing conditions (Kroger et al., 2013). Immunofluorescence microscopy revealed that GtgA and GogA were translocated into RAW264.7 macrophages where they localised to the host cell nucleus, whereas PipA was not detected in this experimental setup. Translocation of GtgA and GogA was dependent on the SPI-2 T3SS suggesting that these proteins might be SPI-2 T3SS effector proteins. However, it remains to be determined if this is physiologically relevant as I could not detect GtgA or GogA by immunofluorescence microscopy when they were expressed from their endogenous promoter.

Interestingly, GtgA and GogA were only detected in less than half of all infected cells, suggesting heterogeneity of *ssaG* promoter activity, leading to variation in the amount of translocated protein. As *ssaG* encodes a structural component of the SPI-2 T3SS apparatus, the variation in GtgA detected could indicate that not all intracellular bacteria express the SPI-2 T3SS. Heterogeneous expression of virulence factors has been demonstrated previously, and increases the fitness of the bacterial population. For example, not all bacteria in the intestinal lumen express the SPI-1 T3SS. SPI-1 T3SS-expressing bacteria invade the intestinal epithelium and induce intestinal inflammation, which aids the replication of SPI-1 T3SS-negative bacteria in the intestinal lumen (Diard et al., 2013).

GFP-tagged GtgA, GogA, and PipA localised to the nucleus when expressed ectopically, demonstrating that the context of infection and other bacterial proteins are not required for their subcellular localisation. NleC has been demonstrated to localise to the nucleus when expressed ectopically (Pearson et al., 2011), however HA-epitope-tagged NleC, translocated into cells during EPEC infection localised to the site of translocon insertion (Yen et al., 2010). This discrepancy has yet to be resolved, however it is logical that these effectors localise to the nucleus so that they only cleave NF- κ B subunits in activated transcription factors. This localised activity of GtgA, GogA, and PipA might explain why the total pool of p65 is not decreased upon infection whilst remaining sufficient for these proteins to alter immune responses.

GtgA, GogA, PipA, and NleC are not predicted to contain a NLS, so the mechanism by which these effector proteins localise to the nucleus is unclear. Each effector is small enough to diffuse through the nuclear pore, therefore nuclear localisation might be a passive process. Alternatively, nuclear localisation could be an active process that requires a non-canonical NLS, or a post-translational modification. The *Shigella* T3SS effector protein OspF is SUMOylated following its translocation into host cells, and SUMOylation is thought to be required for OspF nuclear localisation (Jo et al., 2017). Intriguingly, immunoblot analysis of cells expressing GFP-GtgA, occasionally revealed a second, higher molecular weight (HMW) band (data not shown), which might indicate that GFP-GtgA is post-translationally modified. The change in size between GFP-GtgA and the HMW band was consistent with the addition of a single ubiquitin or ubiquitin-like molecule such as SUMO (data not shown). Furthermore, the online SUMOylation prediction tool JASSA, predicted with high confidence that residue K26 in GtgA and GogA, and residue K123 in NleC, might be SUMOylated. Perhaps like OspF, nuclear localisation of GtgA, GogA, and NleC is facilitated by SUMOylation. To determine if these effectors are SUMOylated, GtgA, GogA, and NleC could be immunoprecipitated from infected cells and then samples immunoblotted using antibodies that recognise SUMO-1, and SUMO-2/3. Mass spectrometry analysis could then be used to pinpoint the identity of the modified residue and point mutants constructed to determine if the PTM is important for

nuclear localisation. If a point mutant of GtgA is identified that no longer translocates to the nucleus, it would then be interesting to determine the importance of nuclear localisation in the function of GtgA.

6.6 Structural characterisation of GtgA

Previously, NleC has been hypothesised to recognise NF- κ B subunits by mimicking the negative charge of the DNA phosphate backbone and the shape of the DNA major groove. This hypothesis was supported by the observation that NleC point mutants, where negatively charged residues across the surface of the active site cleft (E115, E150, and E153) were individually mutated to an alanine, are less efficient at cleaving p65 *in vitro* (Li et al., 2014, Turco and Sousa, 2014). To determine if GtgA, GogA, and PipA recognise NF- κ B subunits using a similar mechanism, and to investigate the molecular determinants of substrate specificity, I solved the crystal structure of GtgA alone and in complex with the p65 NTD in collaboration with the Rittinger laboratory at the Francis Crick Institute.

Although present in the GtgA apo structure, no electron density was recorded for the catalytic zinc ion in the complex structure. It is not clear why the zinc ion was not present; bacteria were grown in the presence of additional zinc and metal-chelating agents such as EDTA were not used in the protein purification process. Once I had solved the crystal structure of GtgA in complex with p65 and it became apparent that the zinc ion was absent, I attempted to crystallise GtgA in complex with the NTD using new protein preparations. Unfortunately, I was unable to recrystallise the protein, possibly because the initial protein preparation contained a heterogeneous population of Zn²⁺-bound and Zn²⁺-free GtgA molecules. Presumably the Zn²⁺-free population bound to the p65 NTD and crystallised. Despite the absence of zinc in complex structure, the carbonyl oxygen of the p65 P1 residue G40 is placed above the zinc coordinating residues of GtgA and superimpositions of other Zn²⁺-bound zincin superfamily members in complex with a peptide substrate (PDB 2G54) or a transition state analogue (PDB 1QJI), demonstrate that the distance of the P1 carbonyl oxygen from the zinc-coordinating residues are similar.

A DALI server search for the closest structural homologues of GtgA, revealed unsurprisingly that the closest structural homolog of GtgA is NleC (PDB 4Q3J; Z score = 10.7, RMSD = 3.0). In addition, the catalytic domains of tetanus toxin (PDB 5N0C; Z score = 7.1, RMSD = 3.4) and botulinum neurotoxin type E (PDB 3FFZ; Z score = 7.0, RMSD = 3.3) were identified to share weak structural similarity with GtgA. **Figure 6.1** shows that although the catalytic cores of NleC and GtgA are highly similar, variable regions in each protein assemble to form the active site cleft. In GtgA, the first variable region (VR1) forms the uppermost section of the negatively charged active site cleft. The loop connecting helices α C and α D folds down over the NSD β -sheet, and contacts positively charged residues in p65. In NleC, this loop is shorter, and instead a long helix - helix α B, stacks against the reverse of the catalytic core without forming part of the active site cleft. The second variable region (VR2) in both enzymes includes the right side of the active site cleft. In GtgA, this includes strands β 3 and β 4 and two negatively charged residues that form the S1' pocket – D155 and E163. VR2 in NleC is much larger, and includes an extension of the ψ -loop comprising sheets β 2 and β 3, and helix α F. The ψ -loop extension is supported by helix α I, for which there are no corresponding residues in GtgA, GogA, and PipA. The guanidino head group of a single residue in helix α I (R239), occupies the same structural position as residues that form the S1' pocket in GtgA. The positive charge of residue R239 in the S1' pocket of NleC, might partially explain NleC's preference for the P1' residue E39, whereas negatively charged residues in the S1' pocket of GtgA might explain GtgA's preference for R41. In support of this hypothesis, NleC residue R239 is conserved in the NleC homolog AIP56 (**Figure 6.2**).

Electrostatic surface potential calculations revealed that like the active site cleft of NleC, the active site cleft of GtgA is negatively charged. However, analysis of the distribution of glutamate and aspartate residues across the active site cleft of GtgA and NleC, reveals that these negatively charged residues are differentially located. Conceivably, this could contribute to cleavage site specificity as the p65 NTD might be tilted slightly differently when bound to the active site cleft.

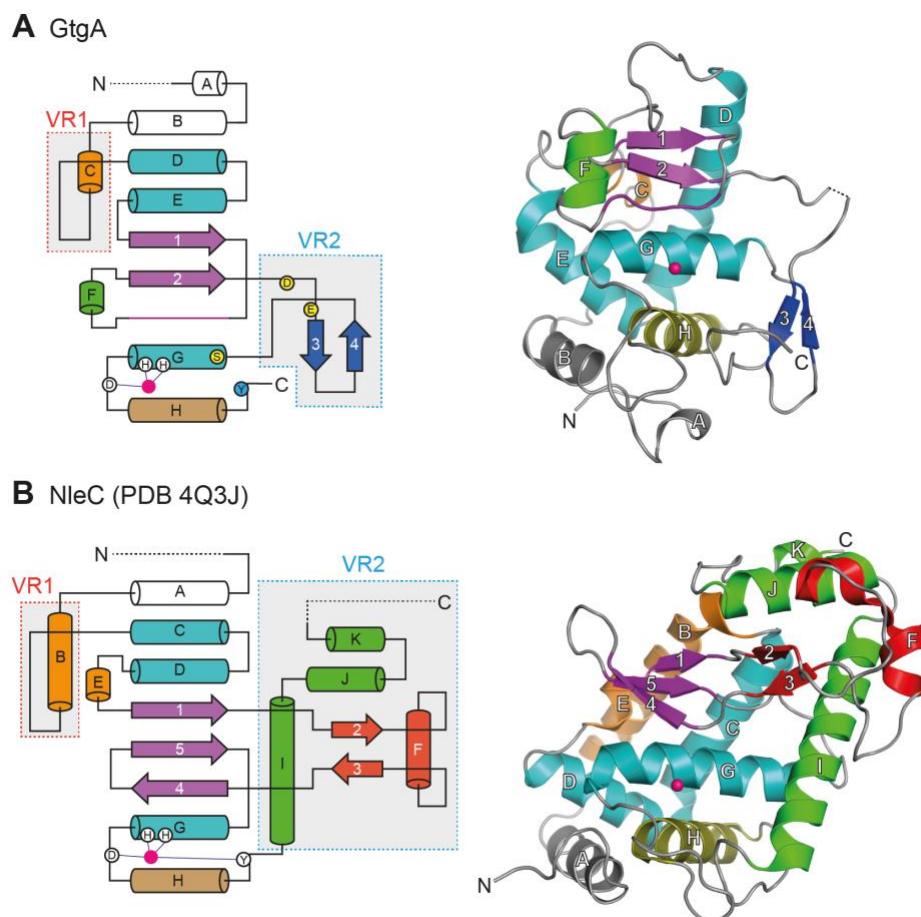


Figure 6.1: Structural comparison of GtgA and NleC.

Topological and cartoon representation of (A) GtgA and (B) NleC (PDB 4Q3J). The Zincin-like catalytic cores is coloured as in Cerdà-Costa and Xavier Gomis-Rüth (2014); α -helices are coloured teal, β -strands are coloured purple. Variable regions are coloured orange, blue, green and red. In the topological diagrams, residues that coordinate the catalytic zinc ion are shown in white circles, residues that form the GtgA S1' pocket are coloured yellow, and the GtgA residue T224 is coloured blue. The catalytic zinc ion is represented as a pink circle or sphere. Abbreviations: VR, variable region.

In the GtgA-p65 complex structure the negatively charged surface of the loop connecting helices α C and α D in GtgA, interacts with the positively charged surface of the loop connecting strand β 7' and helix α A' in p65 (**Figure 6.3A**). p65 residues which form part of this loop (K122, K123, and R124), as well as residues in the flexible linker and dimerisation domain interact with the negatively charged phosphate backbone of DNA in structures of the p65 RHR in

NleC	-MKIPSLQSNFNFSAAGYSAPIAPNRAENAYADYVLDIGKRIPLSAADLSNVYESVIRA	59
AIP56	MTAIFSLAINSNFV---LANNDKPDASDDKYADYVVRGSEHPLNHTQIIELSSAVSRA	56
	* * * * * : * : : : * : : * : : * : : * : *	
NleC	VHDSRSRLIDQHTVDMIGNTVLDALSRSTFRDAVSYGIHNEKVHIGCIKYRNEYELNEE	119
AIP56	VLLSYPNIIDRYTAAATEYTVIDALFHSPTRHIVSFGLHNQQENLGHIRYTNIEYINN	116
	* * . : * : : * : * : * : * : * : * : * : * : *	
NleC	SSVKIDDIQSLTCNELYEYDVGQEPFIPICEAGEN-DNEEPYVSFSVAPDTSYEMPSWQ	178
AIP56	REDEFSLVSEVSYYDDIKSSNAQQVPLVAFYEAREDRATGTPIVNMGVAPSLFSGRYSWWQ	176
	. : . : . : : : : . : . * * : . : * * : . * * : . * * : . * * : . *	
NleC	EGLIHEIIHHVTGSSDPSGDSNIELGPTEILARRVAQELGWSVPDFKGYAEPEREHLRL	238
AIP56	EALIHEIVHHVTGSSDTHHEE--NKQGPTTEILAQMVAAELHWAIPTFKGYSDPARVEAIQE	234
	* . * * * * : * * * * * : : * * * * * : * * * * * : * * * * * : *	
NleC	RNLNALRQAAMRHEENERAFFERLGTISDRYEASPDFTEYSAVSNIGYGFIQQHDFPGLA	298
AIP56	RDFHSLNMFQRHGSELGFLFTRLATIAKGGKASPDFGTLTSFCSEGISSF----PKYP	289
	* : : : * : * * : : * * * * : : * * * * : : * * : : *	
NleC	INDNLQDANQIQLYHGAPYIFTFGDVK-----	326
AIP56	DHDDD-----FNGGGAFFLPSASADSSVECTFDVLNRIEPVDDSIKFEGGNLLIKNDF	342
	: * : : * : : * : : * : *	
NleC	--HNQR-----	330
AIP56	KNLNLRLVAQLSFLNAKKGSGFYRKNDWSKSWYQASSWKNGLSGLYGYGHDESEGNLIY	402
	* *	
NleC	-----	330
AIP56	SPYGITFNDGSFSIGFSSRKHINDNTKDDNFVKLNANWSSFYAGQMFFDKNKRPVALV	462
NleC	-----	330
AIP56	ITEPLNAAFAGWSYIYKDGKWHYEAQDDWDQRLFKDSTLSLDPHAPQFIN	513

Figure 6.2: Amino acid alignment of NleC and AIP56.

(A) Sequence alignment of NleC from *E. coli* strain 0127:H6 E2348/69 and AIP56 from *Photobacterium damsela piscicida* (Uniprot: B7UNX4 and Q2VL32). NleC residue R239, and the equivalent residue in AIP56 are highlighted in red, and the grey rectangle highlights NleC helix α I and the equivalent residues in AIP56.

complex with DNA published previously (**Figure 6.3B**). Although the p65 construct crystallised in complex with GtgA did not include the dimerisation domain, when previous structures of the p65 RHR was superimposed with the GtgA-p65 complex structure, the dimerisation domain was positioned in close proximity to a second negatively charged groove on the surface of GtgA that is adjacent to the active site cleft. Conceivably, GtgA might recognise NF- κ B

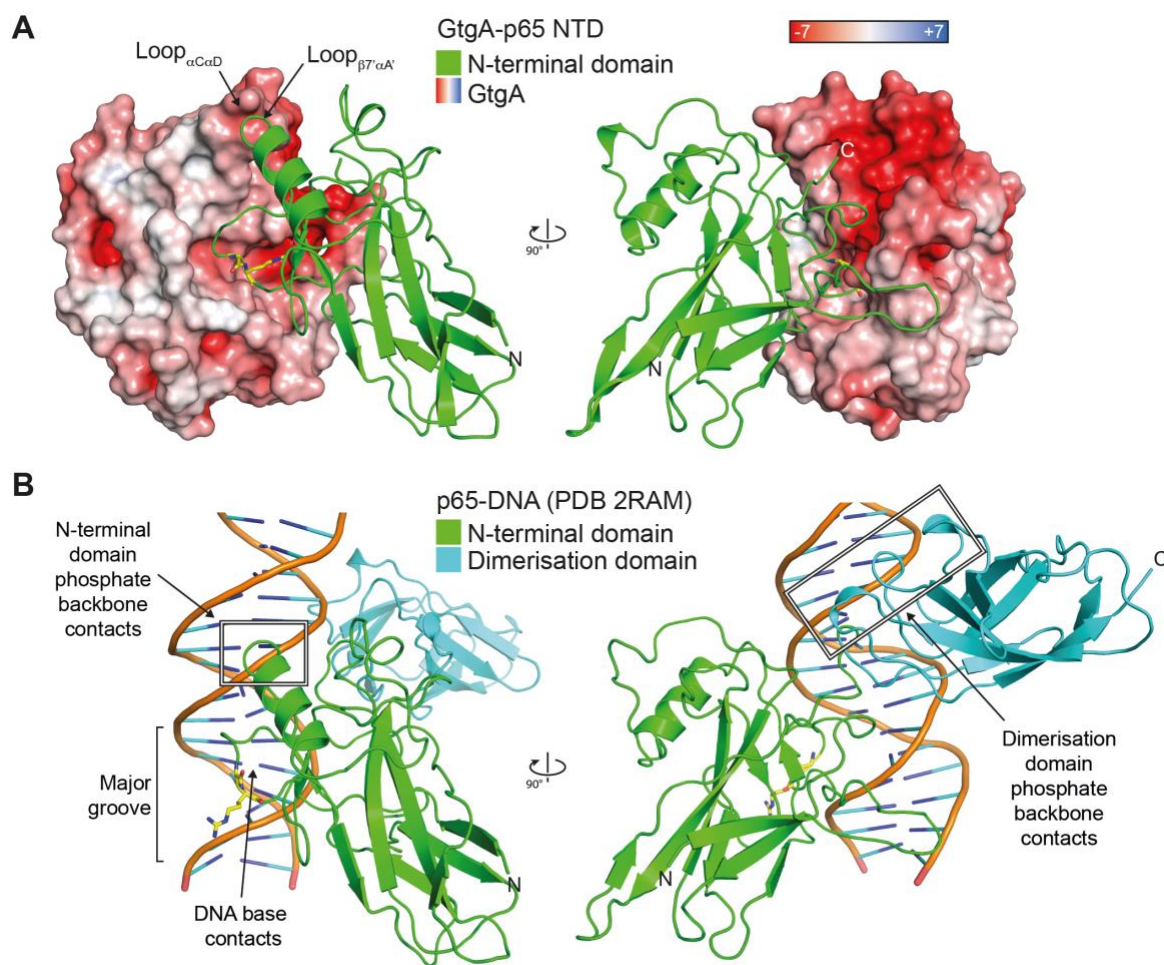


Figure 6.3: GtgA recognises NF-κB transcription factor subunits by mimicking DNA.

(A) GtgA in complex with the p65 N-terminal domain. GtgA is shown in surface representation coloured according to its electrostatic surface potential, and the p65 NTD is shown in cartoon representation. (B) p65 Rel homology region in complex with DNA (PDB 2RAM). In both panels, GtgA residues G40/R41 are represented as yellow sticks.

subunits using a bi-modal mechanism of recognition, whereby the two negatively charged grooves on the surface of GtgA interact with positively charged residues in the p65 NTD and DD (**Figure 6.3**). To test this, mutational analysis of negatively charged residues in GtgA, and positively charged residues on the surface of p65 could be done. Together these data demonstrate that GtgA mimics DNA to facilitate substrate recognition.

The surface of GogA and PipA homology models, generated using the structure of GtgA are similarly negatively charged. However, residues in PipA that form helix αF are not

conserved and the surface of helix α F is not negatively charged (**Figure 6.4**). This difference might explain why PipA is less efficient at cleaving p65 *in vitro* than GtgA and GogA, especially as the majority of residues identified by structure-based mutagenic analysis to be important for p65 cleavage *in vitro*, and for NF- κ B reporter inhibition in 293ET cells are conserved across GtgA, GogA, and PipA. Unfortunately, helix α F was not visible in the electron density of GtgA in complex with the p65 NTD, so its contribution to substrate binding could not be assessed.

The complex structure additionally led to the identification of GtgA residues important for p65 cleavage and NF- κ B inhibition. Mutation of GtgA residue R221 to an alanine substantially impaired p65 cleavage *in vitro*, and partially abrogated GtgA's ability to inhibit NF- κ B reporter activation in 293ETs cells when expressed ectopically. The side chain of R221 is sandwiched between hydrophobic residues in the helix α H and strand β 3 – V204 and Y165 respectively, and the guanidino group extends into the active site cleft adjacent to the catalytic zinc ion. R221 probably stabilises the right wall of the active site cleft and the C-terminal tail of the enzyme which includes the catalytically important residue Y224. GtgA residues D159, S170, S179 and Q192 were also important for inhibiting NF- κ B reporter activity in 293ET cells. Accordingly, three of these four residues (S160, S179, and Q192) are conserved in GogA and PipA (**Figure 6.4A**).

In summary, this thesis characterises the function of a previously uncharacterised family of type III secretion system effectors comprising GtgA, GogA, and PipA. This family of effectors inhibit NF- κ B signalling redundantly in *Salmonella*-infected cells by cleaving a subset of NF- κ B transcription factor subunits comprising p65, RelB, and cRel. Proteolytic activity required a conserved HEXXH motif and was inhibited by the addition of EDTA to *in vitro* cleavage assays demonstrating that GtgA, GogA, and PipA are zinc metalloproteases. The structure of GtgA alone and in complex with the p65 shed further light on the mechanism of substrate recognition by NF- κ B-degrading zinc metalloprotease effector proteins, confirming previous hypotheses that the structurally similar T3SS effector protein NleC from EPEC/EHEC recognises NF- κ B subunits by mimicking the shape and charge of DNA.

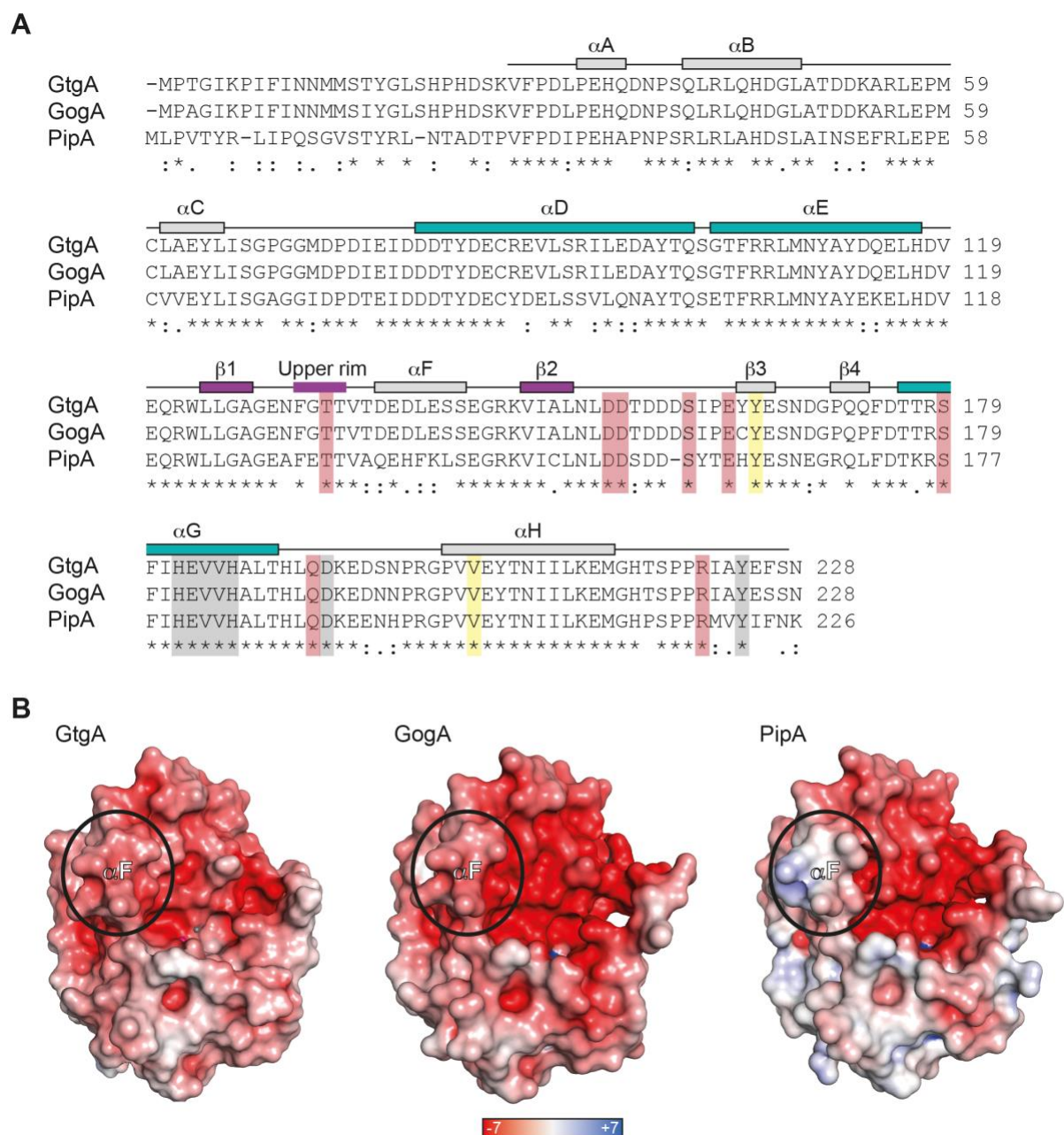


Figure 6.4: Differential surface charge of the active site cleft of PipA.

(A) Sequence alignment of GtgA, GogA, and PipA from *Salmonella* Typhimurium strain ATCC 14028s (Uniprot: A0A0F6AZI6, A0A0F6B537, and A0A0F6AZQ0). The 'HEXXH' zinc metalloprotease motif, GtgA residues D193, and Y224 as well as the corresponding residues in GogA and PipA are highlighted in grey, residues that are hydrogen bonded to the p65 NTD in the GtgA-p65 complex structure are highlighted in red, and residues Y165 and V204, which sandwich GtgA residue R221, are highlighted in yellow. The secondary structure of GtgA is shown above the alignment. (B) Surface representation of GtgA and a model of GogA and PipA coloured according to the electrostatic surface potential as calculated using APBS (Baker et al., 2001). Differential surface charge of helix α F is highlighted by the black circle.

7 References

- ADAMS, P. D., AFONINE, P. V., BUNKOCZI, G., CHEN, V. B., DAVIS, I. W., ECHOLS, N., HEADD, J. J., HUNG, L. W., KAPRAL, G. J., GROSSE-KUNSTLEVE, R. W., MCCOY, A. J., MORIARTY, N. W., OEFFNER, R., READ, R. J., RICHARDSON, D. C., RICHARDSON, J. S., TERWILLIGER, T. C. & ZWART, P. H. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr*, 66, 213-21.
- AJENE, A. N., WALKER, C. L. F. & BLACK, R. E. 2013. Enteric Pathogens and Reactive Arthritis: A Systematic Review of *Campylobacter*, *Salmonella* and *Shigella*-associated Reactive Arthritis. *Journal of Health, Population, and Nutrition*, 31, 299-307.
- AKIRA, S., UEMATSU, S. & TAKEUCHI, O. 2006. Pathogen Recognition and Innate Immunity. *Cell*, 124, 783-801.
- ÁLVAREZ-ORDÓÑEZ, A., PRIETO, M., BERNARDO, A., HILL, C. & LÓPEZ, M. 2012. The Acid Tolerance Response of *Salmonella* spp.: An adaptive strategy to survive in stressful environments prevailing in foods and the host. *Food Research International*, 45, 482-492.
- ARENA, E. T., AUWETER, S. D., ANTUNES, L. C., VOGL, A. W., HAN, J., GUTTMAN, J. A., CROXEN, M. A., MENENDEZ, A., COVEY, S. D., BORCHERS, C. H. & FINLAY, B. B. 2011. The deubiquitinase activity of the *Salmonella* pathogenicity island 2 effector, SseL, prevents accumulation of cellular lipid droplets. *Infect Immun*, 79, 4392-400.
- AUWETER, S. D., BHAVSAR, A. P., DE HOOG, C. L., LI, Y., CHAN, Y. A., VAN DER HEIJDEN, J., LOWDEN, M. J., COOMBES, B. K., ROGERS, L. D., STOYNOV, N., FOSTER, L. J. & FINLAY, B. B. 2011. Quantitative mass spectrometry catalogues *Salmonella* pathogenicity island-2 effectors and identifies their cognate host binding partners. *J Biol Chem*, 286, 24023-35.
- AUWETER, S. D., YU, H. B., ARENA, E. T., GUTTMAN, J. A. & FINLAY, B. B. 2012. Oxysterol-binding protein (OSBP) enhances replication of intracellular *Salmonella* and binds the *Salmonella* SPI-2 effector SseL via its N-terminus. *Microbes Infect*, 14, 148-54.
- BAKER, N. A., SEPT, D., JOSEPH, S., HOLST, M. J. & MCCAMMON, J. A. 2001. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc Natl Acad Sci U S A*, 98, 10037-41.
- BARTHEL, M., HAPFELMEIER, S., QUINTANILLA-MARTINEZ, L., KREMER, M., ROHDE, M., HOGARDT, M., PFEFFER, K., RUSSMANN, H. & 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, 2839-58.
- BARUCH, K., GUR-ARIE, L., NADLER, C., KOBY, S., YERUSHALMI, G., BEN-NERIAH, Y., YOGEV, O., SHAULIAN, E., GUTTMAN, C., ZARIVACH, R. & ROSENSHINE, I. 2011. Metalloprotease type III effectors that specifically cleave JNK and NF- κ B. *EMBO J*, 30, 221-31.
- BAYER-SANTOS, E., DURKIN, C. H., RIGANO, L. A., KUPZ, A., ALIX, E., CERNY, O., JENNINGS, E., LIU, M., RYAN, A. S., LAPAQUE, N., KAUFMANN, STEFAN H. & HOLDEN, D. W. 2016. The *Salmonella* Effector SteD Mediates MARCH8-Dependent Ubiquitination of MHC II Molecules and Inhibits T Cell Activation. *Cell Host & Microbe*, 20, 584-595.
- BERG, H. C. & ANDERSON, R. A. 1973. Bacteria Swim by Rotating their Flagellar Filaments. *Nature*, 245, 380.
- BESBES, A., LE GOFF, S., ANTUNES, A., TERRADE, A., HONG, E., GIORGINI, D., TAHA, M. K. & DEGHMANE, A. E. 2015. Hyperinvasive Meningococci Induce Intra-nuclear Cleavage of the NF- κ B Protein p65/RelA by Meningococcal IgA Protease. *PLoS Pathog*, 11, e1005078.

- BEUZON, C. R., BANKS, G., DEIWICK, J., HENSEL, M. & HOLDEN, D. W. 1999. pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of *Salmonella* Typhimurium. *Mol Microbiol*, 33, 806-16.
- BEUZON, C. R., MERESSE, S., UNSWORTH, K. E., RUIZ-ALBERT, J., GARVIS, S., WATERMAN, S. R., RYDER, T. A., BOUCROT, E. & HOLDEN, D. W. 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J*, 19, 3235-49.
- BEUZON, C. R., SALCEDO, S. P. & HOLDEN, D. W. 2002. Growth and killing of a *Salmonella enterica* serovar Typhimurium sifA mutant strain in the cytosol of different host cell lines. *Microbiology*, 148, 2705-15.
- BHATTACHARYA, S., ECKNER, R., GROSSMAN, S., OLDREAD, E., ARANY, Z., D'ANDREA, A. & LIVINGSTON, D. M. 1996. Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. *Nature*, 383, 344-7.
- BHAVSAR, A. P., BROWN, N. F., STOEPEL, J., WIERMER, M., MARTIN, D. D., HSU, K. J., IMAMI, K., ROSS, C. J., HAYDEN, M. R., FOSTER, L. J., LI, X., HIETER, P. & FINLAY, B. B. 2013. The *Salmonella* type III effector SspH2 specifically exploits the NLR co-chaperone activity of SGT1 to subvert immunity. *PLoS Pathog*, 9, e1003518.
- BIASINI, M., BIENERT, S., WATERHOUSE, A., ARNOLD, K., STUDER, G., SCHMIDT, T., KIEFER, F., GALLO CASSARINO, T., BERTONI, M., BORDOLI, L. & SCHWEDE, T. 2014. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res*, 42, W252-8.
- BIRMINGHAM, C. L., SMITH, A. C., BAKOWSKI, M. A., YOSHIMORI, T. & BRUMELL, J. H. 2006. Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *J Biol Chem*, 281, 11374-83.
- BISPHAM, J., TRIPATHI, B. N., WATSON, P. R. & WALLIS, T. S. 2001. *Salmonella* Pathogenicity Island 2 Influences Both Systemic Salmonellosis and *Salmonella*-Induced Enteritis in Calves. *Infection and Immunity*, 69, 367-377.
- BODE, W., GOMIS-RUTH, F. X., HUBER, R., ZWILLING, R. & STOCKER, W. 1992. Structure of astacin and implications for activation of astacins and zinc-ligation of collagenases. *Nature*, 358, 164-7.
- BOUCROT, E., HENRY, T., BORG, J. P., GORVEL, J. P. & MERESSE, S. 2005. The intracellular fate of *Salmonella* depends on the recruitment of kinesin. *Science*, 308, 1174-8.
- BRINK, T., LEISS, V., SIEGERT, P., JEHLE, D., EBNER, J. K., SCHWAN, C., SHYMANETS, A., WIESE, S., NURNBERG, B., HENSEL, M., AKTORIES, K. & ORTH, J. H. C. 2018. *Salmonella* Typhimurium effector Ssel inhibits chemotaxis and increases host cell survival by deamidation of heterotrimeric Gi proteins. *PLoS Pathog*, 14, e1007248.
- BROWNE, S. H., HASEGAWA, P., OKAMOTO, S., FIERER, J. & GUINEY, D. G. 2008. Identification of *Salmonella* SPI-2 secretion system components required for SpvB-mediated cytotoxicity in macrophages and virulence in mice. *FEMS Immunol Med Microbiol*, 52, 194-201.
- BRUNO, V. M., HANNEMANN, S., LARA-TEJERO, M., FLAVELL, R. A., KLEINSTEIN, S. H. & GALÁN, J. E. 2009. *Salmonella* Typhimurium Type III Secretion Effectors Stimulate Innate Immune Responses in Cultured Epithelial Cells. *PLOS Pathogens*, 5, e1000538.
- BUCKLE, G. C., WALKER, C. L. F. & BLACK, R. E. 2012. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *Journal of Global Health*, 2, 010401.
- CARNELL, S. C., BOWEN, A., MORGAN, E., MASKELL, D. J., WALLIS, T. S. & STEVENS, M. P. 2007. Role in virulence and protective efficacy in pigs of *Salmonella enterica* serovar Typhimurium secreted components identified by signature-tagged mutagenesis. *Microbiology*, 153, 1940-1952.
- CERDÀ-COSTA, N. & XAVIER GOMIS-RÜTH, F. 2014. Architecture and function of metallopeptidase catalytic domains. *Protein Science*, 23, 123-144.

- CHEN, H. M., WANG, Y., SU, L. H. & CHIU, C. H. 2013. Nontyphoid *Salmonella* infection: microbiology, clinical features, and antimicrobial therapy. *Pediatr Neonatol*, 54, 147-52.
- CHEN, L., GLOVER, J. N. M., HOGAN, P. G., RAO, A. & HARRISON, S. C. 1998a. Structure of the DNA-binding domains from NFAT, Fos and Jun bound specifically to DNA. *Nature*, 392, 42.
- CHEN, Y. Q., GHOSH, S. & GHOSH, G. 1998b. A novel DNA recognition mode by the NF- κ B p65 homodimer. *Nat Struct Biol*, 5, 67-73.
- CHEREPANOV, P. 2007. LEDGF/p75 interacts with divergent lentiviral integrases and modulates their enzymatic activity in vitro. *Nucleic Acids Res*, 35, 113-24.
- CHRISTIAN, J., VIER, J., PASCHEN, S. A. & HACKER, G. 2010. Cleavage of the NF- κ B family protein p65/RelA by the chlamydial protease-like activity factor (CPAF) impairs proinflammatory signaling in cells infected with Chlamydiae. *J Biol Chem*, 285, 41320-7.
- CIRILLO, D. M., VALDIVIA, R. H., MONACK, D. M. & FALKOW, S. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol Microbiol*, 30, 175-88.
- CLARK, M. A., JEPSON, M. A., SIMMONS, N. L. & HIRST, B. H. 1994. Preferential interaction of *Salmonella* Typhimurium with mouse Peyer's patch M cells. *Res Microbiol*, 145, 543-52.
- COLGAN, A. M., KROGER, C., DIARD, M., HARDT, W. D., PUENTE, J. L., SIVASANKARAN, S. K., HOKAMP, K. & HINTON, J. C. 2016. The Impact of 18 Ancestral and Horizontally-Acquired Regulatory Proteins upon the Transcriptome and sRNA Landscape of *Salmonella enterica* serovar Typhimurium. *PLoS Genet*, 12, e1006258.
- CRUMP, J. A. & HEYDERMAN, R. S. 2015. A Perspective on Invasive *Salmonella* Disease in Africa. *Clin Infect Dis*, 61, S235-40.
- CRUMP, J. A., LUBY, S. P. & MINTZ, E. D. 2004. The global burden of typhoid fever. *Bull World Health Organ*, 82, 346-53.
- D'ARCY, A., BERGFORS, T., COWAN-JACOB, S. W. & MARSH, M. 2014. Microseed matrix screening for optimization in protein crystallization: what have we learned? *Acta Crystallogr F Struct Biol Commun*, 70, 1117-26.
- D'COSTA, V. M., BRAUN, V., LANDEKIC, M., SHI, R., PROTEAU, A., MCDONALD, L., CYGLER, M., GRINSTEIN, S. & BRUMELL, J. H. 2015. *Salmonella* Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7. *Cell Rep*.
- DATSENKO, K. A. & WANNER, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*, 97, 6640-5.
- DESAI, P. T., PORWOLLIK, S., LONG, F., CHENG, P., WOLLAM, A., BHONAGIRI-PALSIKAR, V., HALLSWORTH-PEPIN, K., CLIFTON, S. W., WEINSTOCK, G. M. & MCCLELLAND, M. 2013. Evolutionary Genomics of *Salmonella enterica* Subspecies. *MBio*, 4.
- DIARD, M., GARCIA, V., MAIER, L., REMUS-EMSERMANN, M. N. P., REGOES, R. R., ACKERMANN, M. & HARDT, W.-D. 2013. Stabilization of cooperative virulence by the expression of an avirulent phenotype. *Nature*, 494, 353.
- DO VALE, A., PEREIRA, C., OSORIO, C. R. & DOS SANTOS, N. M. S. 2017. The Apoptogenic Toxin AIP56 Is Secreted by the Type II Secretion System of *Photobacterium damsela* subsp. piscicida. *Toxins (Basel)*, 9.
- DOMINGUES, L., HOLDEN, D. W. & MOTA, L. J. 2014. The *Salmonella* effector SteA contributes to the control of membrane dynamics of *Salmonella*-containing vacuoles. *Infect Immun*, 82, 2923-34.
- DUMMLER, A., LAWRENCE, A. M. & DE MARCO, A. 2005. Simplified screening for the detection of soluble fusion constructs expressed in *E. coli* using a modular set of vectors. *Microb Cell Fact*, 4, 34.
- EMSLEY, P., LOHKAMP, B., SCOTT, W. G. & COWTAN, K. 2010. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr*, 66, 486-501.

- ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol*, 35, 495-516.
- ESPOSITO, D., GÜNSTER, R. A., MARTINO, L., EL OMARI, K., WAGNER, A., THURSTON, T. L. M. & RITTINGER, K. 2018. Structural basis for the glycosyltransferase activity of the *Salmonella* effector SseK3. *Journal of Biological Chemistry*, 293, 5064-5078.
- EULALIO, A., FROHLICH, K. S., MANO, M., GIACCA, M. & VOGEL, J. 2011. A candidate approach implicates the secreted *Salmonella* effector protein SpvB in P-body disassembly. *PLoS One*, 6, e17296.
- EVANS, P. R. & MURSHUDOV, G. N. 2013. How good are my data and what is the resolution? *Acta Crystallographica Section D: Biological Crystallography*, 69, 1204-1214.
- FARACHE, J., KOREN, I., MILO, I., GUREVICH, I., KIM, K. W., ZIGMOND, E., FURTADO, G. C., LIRA, S. A. & SHAKHAR, G. 2013. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity*, 38, 581-95.
- FEASEY, N. A., DOUGAN, G., KINGSLEY, R. A., HEYDERMAN, R. S. & GORDON, M. A. 2012. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. *Lancet*, 379, 2489-99.
- FEASEY, N. A., HADFIELD, J., KEDDY, K. H., DALLMAN, T. J., JACOBS, J., DENG, X., WIGLEY, P., BARQUIST, L., LANGRIDGE, G. C., FELTWELL, T., HARRIS, S. R., MATHER, A. E., FOOKES, M., ASLETT, M., MSEFULA, C., KARIUKI, S., MACLENNAN, C. A., ONSARE, R. S., WEILL, F. X., LE HELLO, S., SMITH, A. M., MCCLELLAND, M., DESAI, P., PARRY, C. M., CHEESBROUGH, J., FRENCH, N., CAMPOS, J., CHABALGOITY, J. A., BETANCOR, L., HOPKINS, K. L., NAIR, S., HUMPHREY, T. J., LUNGUYA, O., COGAN, T. A., TAPIA, M. D., SOW, S. O., TENNANT, S. M., BORNSTEIN, K., LEVINE, M. M., LACHARME-LORA, L., EVERETT, D. B., KINGSLEY, R. A., PARKHILL, J., HEYDERMAN, R. S., DOUGAN, G., GORDON, M. A. & THOMSON, N. R. 2016. Distinct *Salmonella* Enteritidis lineages associated with enterocolitis in high-income settings and invasive disease in low-income settings. *Nat Genet*, 48, 1211-1217.
- FIGUEIRA, R., WATSON, K. G., HOLDEN, D. W. & HELAINE, S. 2013. Identification of *Salmonella* pathogenicity island-2 type III secretion system effectors involved in intramacrophage replication of *S. enterica* serovar typhimurium: implications for rational vaccine design. *MBio*, 4, e00065.
- FIROZ MIAN, M., PEK, E. A., CHENOWETH, M. J. & ASHKAR, A. A. 2011. Humanized mice are susceptible to *Salmonella* Typhi infection. *Cellular and Molecular Immunology*, 8, 83-87.
- FU, Y. & GALAN, J. E. 1999. A *Salmonella* protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature*, 401, 293-7.
- FUJIMOTO, T. & PARTON, R. G. 2011. Not Just Fat: The Structure and Function of the Lipid Droplet. *Cold Spring Harbor Perspectives in Biology*, 3, a004838.
- GALAN, J. E. 2009. Common themes in the design and function of bacterial effectors. *Cell Host Microbe*, 5, 571-9.
- GALAN, J. E. & CURTISS, R., 3RD 1989. Cloning and molecular characterization of genes whose products allow *Salmonella* Typhimurium to penetrate tissue culture cells. *Proc Natl Acad Sci U S A*, 86, 6383-7.
- GALKIN, V. E., ORLOVA, A., VANLOOCK, M. S., ZHOU, D., GALAN, J. E. & EGELMAN, E. H. 2002. The bacterial protein SipA polymerizes G-actin and mimics muscle nebulin. *Nat Struct Biol*, 9, 518-21.
- GALLUZZI, L., VITALE, I., AARONSON, S. A., ABRAMS, J. M., ADAM, A., AGOSTINIS, P., ALNEMRI, E. S., ALTUCCI, L., AMERLIO, I., ANDREWS, D. W., ANNICCHIRICO-PETRIZZELLI, M., ANTONOV, A. V., ARAMA, E., BAEHRECKE, E. H., BAREV, N. A., BAZAN, N. G., BERNASSOLA, F., BERTRAND, M. J. M., BIANCHI, K., BLAGOSKLONNY, M. V., BLOMGREN, K., BORNER, C., BOYA, P., BRENNER, C., CAMPANELLA, M., CANDI, E., CARMONA-GUTIERREZ, D., CECCONI, F., CHAN, F. K., CHANDEL, N. S., CHENG, E. H., CHIPUK, J. E., CIDLOWSKI, J. A.,

- CIECHANOVER, A., COHEN, G. M., CONRAD, M., CUBILLOS-RUIZ, J. R., CZABOTAR, P. E., D'ANGIOLELLA, V., DAWSON, T. M., DAWSON, V. L., DE LAURENZI, V., DE MARIA, R., DEBATIN, K. M., DEBERARDINI, R. J., DESHMUKH, M., DI DANIELE, N., DI VIRGILIO, F., DIXIT, V. M., DIXON, S. J., DUCKETT, C. S., DYNLACHT, D. B., EL-DEIRY, W. S., ELROD, J. W., FIMIA, G. M., FULDA, S., GARCIA-SAWEZ, A. J., GARG, A. D., GARRIDO, C., GAVATHIOTIS, E., GOLSTEIN, P., GOTTLIEB, E., GREEN, D. R., GREENE, L. A., GRONEMEYER, H., GROSS, A., HAJNOCZKY, G., HARDWICK, J. M., HARRIS, I. S., HENGARTNER, M. O., HETZ, C., ICHIO, H., JAATTELA, M., JOSEPH, B., JOST, P. J., JUIN, P. P., KAISERW, W. J., KARIN, M., KAUFMANN, T., KEPP, O., KIMCHI, A., KITSIS, R. N., KLIONSKY, D. J., KNIGHT, R. A., KUMAR, S., LEE, S. W., LEMASTERS, J. J., LEVINE, B., LINKERMANN, A., LIPTON, S. A., LOCKSIN, R. A., LOPEZ-OTIN, C., LOWE, S. W., LUEDDE, T., LUGLI, E., MACFARLANE, M., MADEO, F., MALEXWICZ, M., MALORNI, W., MANIC G., MARINE, J. C., MARIN, S. J., MARTINOU, J. C., MEDEMA, J. P., MEHLEN, P., MEIR, P., MELINO, S., MIAO, E. A., MOLKENTIN, J. D., MOLL, U. M., MUOZ-PINEDO, C., NAGATA, S., NUNEZ, G., OBERST, A., OREN, M., OVERHOLTZER, M., PAGANO, M., PANARETAKIS, T., PASPARAKIS, M., PENNINGER, J. M., PERERA, D. M., PERVAIZ, S., PETER, M. E., PIACENTINI, M., PINTON, P., PREHN, J. H. M., PUTHALAKATH, H., RABINOVICH, G. A., REHM, M., RIZZUTO, R., RODRIGUES, C. M. P., RBINSZTEIN, D. C., RUDEL, T., RYAN, K. M., SAYAN, E., SCORRANO, L., SHAO, F., SHI, Y., SILKE, J., SIMON, H. U., SISTIGU, A., STOCKWELL, B. R., STRASSER, A., SZABADKAI, G., TAIT, S. W. G., TANG, D., TAVERNARAKIS, N., THORBURN, A., TSUJIMOTO, Y., TURK, B., VANDEN BERGE, T., VANDENABEELE, P., VANDER HEIDEN, M. G., VILLUNGER, A., VIRGIN, H. W., VOUSDEN, K. H., VUCIC, D., WAGNER, E. F., WALCZAK, H., WALLACH, D., WANG, Y., WELLS, J. A., WOOD, W., YUAN, J., ZAKERI, Z., ZHIVOTOVSKY, B., ZITVOGEL, L., MELINO, G. & KROEMER, G. 2018. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ*, 25, 486-541.
- GARCIA-DEL PORTILLO, F., ZWICK, M. B., LEUNG, K. Y. & FINLAY, B. B. 1993. *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 10544-10548.
- GASTEIGER, E., HOOGLAND, C., GATTIKER, A., DUVAUD, S. E., WILKINS, M. R., APPEL, R. D. & BAIROCH, A. 2005. Protein Identification and Analysis Tools on the ExPASy Server. In: WALKER, J. M. (ed.) *The Proteomics Protocols Handbook*. Totowa, NJ: Humana Press.
- GEDDES, K., CRUZ, F. & HEFFRON, F. 2007. Analysis of cells targeted by *Salmonella* type III secretion in vivo. *PLoS Pathog*, 3, e196.
- GERRITSEN, M. E., WILLIAMS, A. J., NEISH, A. S., MOORE, S., SHI, Y. & COLLINS, T. 1997. CREB-binding protein/p300 are transcriptional coactivators of p65. *Proc Natl Acad Sci U S A*, 94, 2927-32.
- GHOSH, G., VAN DUYN, G., GHOSH, S. & SIGLER, P. B. 1995. Structure of NF- κ B p50 homodimer bound to a κ B site. *Nature*, 373, 303-10.
- GIOGHA, C., WONG FOK LUNG, T., MUHLEN, S., PEARSON, J. S. & HARTLAND, E. L. 2015. Substrate recognition by the zinc metalloprotease effector NleC from enteropathogenic *Escherichia coli*. *Cell Microbiol*.
- GOMIS-RUTH, F. X. 2009. Catalytic domain architecture of metzincin metalloproteases. *J Biol Chem*, 284, 15353-7.
- GRABE, G. J., ZHANG, Y., PRZYDACZ, M., ROLHION, N., YANG, Y., PRUNEDA, J. N., KOMANDER, D., HOLDEN, D. W. & HARE, S. A. 2016. The *Salmonella* Effector SpvD Is a Cysteine Hydrolase with a Serovar-specific Polymorphism Influencing Catalytic Activity, Suppression of Immune Responses, and Bacterial Virulence. *J Biol Chem*, 291, 25853-25863.

- GRIMONT, P. & WEILL, F.-X. 2007. *Antigenic Formulae of the Salmonella serovars*, (9th ed.) Paris: WHO Collaborating Centre for Reference and Research on Salmonella.
- GUNSTER, R. A., MATTHEWS, S. A., HOLDEN, D. W. & THURSTON, T. L. 2017. SseK1 and SseK3 T3SS effectors inhibit NF- κ B signalling and necroptotic cell death in *Salmonella*-infected macrophages. *Infect Immun*.
- HANEDA, T., ISHII, Y., SHIMIZU, H., OHSHIMA, K., IIDA, N., DANBARA, H. & OKADA, N. 2012. *Salmonella* type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection. *Cell Microbiol*, 14, 485-99.
- HARAGA, A. & MILLER, S. I. 2003. A *Salmonella enterica* Serovar Typhimurium Translocated Leucine-Rich Repeat Effector Protein Inhibits NF- κ B-Dependent Gene Expression. *Infection and Immunity*, 71, 4052-4058.
- HARAGA, A. & MILLER, S. I. 2006. A *Salmonella* type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1. *Cell Microbiol*, 8, 837-46.
- HARDT, W. D., CHEN, L. M., SCHUEBEL, K. E., BUSTELO, X. R. & GALAN, J. E. 1998. S. Typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell*, 93, 815-26.
- HARRIS, J. C., DUPONT, H. L. & HORNICK, R. B. 1972. Fecal leukocytes in diarrheal illness. *Ann Intern Med*, 76, 697-703.
- HAYDEN, M. S. & GHOSH, S. 2012. NF- κ B, the first quarter-century: remarkable progress and outstanding questions. *Genes Dev*, 26, 203-34.
- HAYWARD, R. D. & KORONAKIS, V. 1999. Direct nucleation and bundling of actin by the SipC protein of invasive *Salmonella*. *Embo j*, 18, 4926-34.
- HECKMAN, K. L. & PEASE, L. R. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc*, 2, 924-32.
- HENRY, T., COUILLAUT, C., ROCKENFELLER, P., BOUCROT, E., DUMONT, A., SCHROEDER, N., HERMANT, A., KNODLER, L. A., LECINE, P., STEELE-MORTIMER, O., BORG, J. P., GORVEL, J. P. & MERESSE, S. 2006. The *Salmonella* effector protein PipB2 is a linker for kinesin-1. *Proc Natl Acad Sci U S A*, 103, 13497-502.
- HENSEL, M., NIKOLAUS, T. & EGELSEER, C. 1999. Molecular and functional analysis indicates a mosaic structure of *Salmonella* pathogenicity island 2. *Mol Microbiol*, 31, 489-98.
- HENSEL, M., SHEA, J. E., GLEESON, C., JONES, M. D., DALTON, E. & HOLDEN, D. W. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science*, 269, 400-3.
- HENSEL, M., SHEA, J. E., WATERMAN, S. R., MUNDY, R., NIKOLAUS, T., BANKS, G., VAZQUEZ-TORRES, A., GLEESON, C., FANG, F. C. & HOLDEN, D. W. 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol Microbiol*, 30, 163-74.
- HESS, J., LADEL, C., MIKO, D. & KAUFMANN, S. H. 1996. *Salmonella* Typhimurium aroA-infection in gene-targeted immunodeficient mice: major role of CD4⁺ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. *J Immunol*, 156, 3321-6.
- HIGGINSON, E. E., SIMON, R. & TENNANT, S. M. 2016. Animal Models for Salmonellosis: Applications in Vaccine Research. *Clinical and Vaccine Immunology*, 23, 746.
- HOCHMANN, H., PUST, S., VON FIGURA, G., AKTORIES, K. & BARTH, H. 2006. *Salmonella enterica* SpvB ADP-ribosylates actin at position arginine-177-characterization of the catalytic domain within the SpvB protein and a comparison to binary clostridial actin-ADP-ribosylating toxins. *Biochemistry*, 45, 1271-7.
- HODGSON, A., WIER, E. M., FU, K., SUN, X., YU, H., ZHENG, W., SHAM, H. P., JOHNSON, K., BAILEY, S., VALLANCE, B. A. & WAN, F. 2015. Metalloprotease NleC suppresses

- host NF- κ B/inflammatory responses by cleaving p65 and interfering with the p65/RPS3 interaction. *PLoS Pathog*, 11, e1004705.
- IMAMI, K., BHAVSAR, A. P., YU, H., BROWN, N. F., ROGERS, L. D., FINLAY, B. B. & FOSTER, L. J. 2013. Global impact of *Salmonella* pathogenicity island 2-secreted effectors on the host phosphoproteome. *Mol Cell Proteomics*, 12, 1632-43.
- JASLOW, S. L., GIBBS, K. D., FRICKE, W. F., WANG, L., PITTMAN, K. J., MAMMEL, M. K., THADEN, J. T., FOWLER, V. G., JR., HAMMER, G. E., ELFENBEIN, J. R. & KO, D. C. 2018. *Salmonella* Activation of STAT3 Signaling by SarA Effector Promotes Intracellular Replication and Production of IL-10. *Cell Rep*, 23, 3525-3536.
- JENNINGS, E., THURSTON, T. L. M. & HOLDEN, D. W. 2017. *Salmonella* SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences. *Cell Host Microbe*, 22, 217-231.
- JO, K., KIM, E. J., YU, H. J., YUN, C. H. & KIM, D. W. 2017. Host Cell Nuclear Localization of *Shigella flexneri* Effector OspF Is Facilitated by SUMOylation. *J Microbiol Biotechnol*, 27, 610-615.
- JOHNSON, R., MYLONA, E. & FRANKEL, G. 2018. Typhoidal *Salmonella*: Distinctive virulence factors and pathogenesis. *Cell Microbiol*, e12939.
- JOLLY, C., WINFREE, S., HANSEN, B. & STEELE-MORTIMER, O. 2014. The Annexin A2/p11 complex is required for efficient invasion of *Salmonella* Typhimurium in epithelial cells. *Cell Microbiol*, 16, 64-77.
- JONES, R. M., WU, H., WENTWORTH, C., LUO, L., COLLIER-HYAMS, L. & NEISH, A. S. 2008. *Salmonella* AvrA Coordinates Suppression of Host Immune and Apoptotic Defenses via JNK Pathway Blockade. *Cell Host Microbe*, 3, 233-44.
- JORGENSEN, I. & MIAO, E. 2015. Pyroptotic cell death defends against intracellular pathogens. *Immunol Rev*, 265, 130-142.
- KAMANOVA, J., SUN, H., LARA-TEJERO, M. & GALÁN, J. E. 2016. The *Salmonella* Effector Protein SopA Modulates Innate Immune Responses by Targeting TRIM E3 Ligase Family Members. *PLOS Pathogens*, 12, e1005552.
- KAYAGAKI, N., STOWE, I. B., LEE, B. L., O'ROURKE, K., ANDERSON, K., WARMING, S., CUELLA, T., HALEY, B., ROOSE-GIRMA, M., PHUNG, Q. T., LIU, P. S., LILL, J. R., LI, H., WU, J., KUMMERFELD, S. ZHANG, J., LEE, W. P., SNIPAS, S. J., SALVESEN, G. S., MORRIS, L. X., FITZGERALD, L., ZHANG, Y., BERTRAM, E. M., GOODNOW, C. C. & DIXIT, V. M. 2015. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*, 426, 666-671.
- KEESTRA, A. M., WINTER, M. G., KLEIN-DOUWEL, D., XAVIER, M. N., WINTER, S. E., KIM, A., TSOLIS, R. M. & BAUMLER, A. J. 2011. A *Salmonella* virulence factor activates the NOD1/NOD2 signaling pathway. *MBio*, 2.
- KELLEY, L. A., MEZULIS, S., YATES, C. M., WASS, M. N. & STERNBERG, M. J. E. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protocols*, 10, 845-858.
- KESHET, Y. & SEGER, R. 2010. The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. *Methods Mol Biol*, 661, 3-38.
- KESZEI, A. F., TANG, X., MCCORMICK, C., ZEQRARAJ, E., ROHDE, J. R., TYERS, M. & SICHERI, F. 2014. Structure of an SspH1-PKN1 complex reveals the basis for host substrate recognition and mechanism of activation for a bacterial E3 ubiquitin ligase. *Mol Cell Biol*, 34, 362-73.
- KIDWAI, A. S., MUSHAMIRI, I., NIEMANN, G. S., BROWN, R. N., ADKINS, J. N. & HEFFRON, F. 2013. Diverse secreted effectors are required for *Salmonella* persistence in a mouse infection model. *PLoS One*, 8, e70753.
- KIM, Y. G., KIM, J. H. & KIM, K. J. 2009. Crystal structure of the *Salmonella enterica* serovar Typhimurium virulence factor SrfJ, a glycoside hydrolase family enzyme. *J Bacteriol*, 191, 6550-4.
- KINGSLEY, R. A., MSEFULA, C. L., THOMSON, N. R., KARIUKI, S., HOLT, K. E., GORDON, M. A., HARRIS, D., CLARKE, L., WHITEHEAD, S., SANGAL, V., MARSH, K.,

- ACHTMAN, M., MOLYNEUX, M. E., CORMICAN, M., PARKHILL, J., MACLENNAN, C. A., HEYDERMAN, R. S. & DOUGAN, G. 2009. Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res*, 19, 2279-87.
- KLEMM, E. J., SHAKOOR, S., PAGE, A. J., QAMAR, F. N., JUDGE, K., SAEED, D. K., WONG, V. K., DALLMAN, T. J., NAIR, S., BAKER, S., SHAHEEN, G., QURESHI, S., YOUSAFZAI, M. T., SALEEM, M. K., HASAN, Z., DOUGAN, G. & HASAN, R. 2018. Emergence of an Extensively Drug-Resistant *Salmonella enterica* Serovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation Cephalosporins. *MBio*, 9.
- KNODLER, L. A., BESTOR, A., MA, C., HANSEN-WESTER, I., HENSEL, M., VALLANCE, B. A. & STEELE-MORTIMER, O. 2005. Cloning vectors and fluorescent proteins can significantly inhibit *Salmonella enterica* virulence in both epithelial cells and macrophages: implications for bacterial pathogenesis studies. *Infect Immun*, 73, 7027-31.
- KNODLER, L. A., CROWLEY, S. M., SHAM, H. P., YANG, H., WRANDE, M., MA, C., ERNST, R. K., STEELE-MORTIMER, O., CELLI, J. & VALLANCE, B. A. 2014. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. *Cell Host Microbe*, 16, 249-56.
- KNODLER, L. A., VALLANCE, B. A., CELLI, J., WINFREE, S., HANSEN, B., MONTERO, M. & STEELE-MORTIMER, O. 2010. Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proceedings of the National Academy of Sciences*, 107, 17733-17738.
- KNODLER, L. A., VALLANCE, B. A., HENSEL, M., JÄCKEL, D., FINLAY, B. B. & STEELE-MORTIMER, O. 2004. *Salmonella* type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. *Molecular Microbiology*, 49, 685-704.
- KOŁODZIEJEK, A. M. & MILLER, S. I. 2015. *Salmonella* modulation of the phagosome membrane, role of SseJ. *Cell Microbiol*, 17, 333-41.
- KROGER, C., COLGAN, A., SRIKUMAR, S., HANDLER, K., SIVASANKARAN, S. K., HAMMARLOF, D. L., CANALS, R., GRISSOM, J. E., CONWAY, T., HOKAMP, K. & HINTON, J. C. 2013. An infection-relevant transcriptomic compendium for *Salmonella enterica* Serovar Typhimurium. *Cell Host Microbe*, 14, 683-95.
- KROIS, A. S., FERREON, J. C., MARTINEZ-YAMOUT, M. A., DYSON, H. J. & WRIGHT, P. E. 2016a. Recognition of the disordered p53 transactivation domain by the transcriptional adapter zinc finger domains of CREB-binding protein. *Proceedings of the National Academy of Sciences of the United States of America*, 113, E1853-E1862.
- KROIS, A. S., FERREON, J. C., MARTINEZ-YAMOUT, M. A., DYSON, H. J. & WRIGHT, P. E. 2016b. Recognition of the disordered p53 transactivation domain by the transcriptional adapter zinc finger domains of CREB-binding protein. *Proc Natl Acad Sci U S A*, 113, E1853-62.
- LAD, S. P., YANG, G., SCOTT, D. A., WANG, G., NAIR, P., MATHISON, J., REDDY, V. S. & LI, E. 2007. Chlamydial CT441 is a PDZ domain-containing tail-specific protease that interferes with the NF- κ B pathway of immune response. *J Bacteriol*, 189, 6619-25.
- LAI, J. J., LAI, K. P., CHUANG, K. H., CHANG, P., YU, I. C., LIN, W. J. & CHANG, C. 2009. Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF- α expression. *J Clin Invest*, 119, 3739-51.
- LARA-TEJERO, M., SUTTERWALA, F. S., OGURA, Y., GRANT, E. P., BERTIN, J., COYLE, A. J., FLAVELL, R. A. & GALAN, J. E. 2006. Role of caspase-1 inflammasome in *Salmonella* Typhimurium pathogenesis. *J Exp Med*, 203, 1407-1412.
- LAROCK, D. L., CHAUDHARY, A. & MILLER, S. I. 2015. Salmonellae interactions with host processes. *Nat Rev Microbiol*, 13, 191-205.
- LASKOWSKI, R. A. 2001. PDBsum: summaries and analyses of PDB structures. *Nucleic Acids Research*, 29, 221-222.

- LAWLEY, T. D., CHAN, K., THOMPSON, L. J., KIM, C. C., GOVONI, G. R. & MONACK, D. M. 2006. Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathog*, 2, e11.
- LE NEGRATE, G., FAUSTIN, B., WELSH, K., LOEFFLER, M., KRAJEWSKA, M., HASEGAWA, P., MUKHERJEE, S., ORTH, K., KRAJEWSKI, S., GODZIK, A., GUINEY, D. G. & REED, J. C. 2008. *Salmonella* Secreted Factor L Deubiquitinase of *Salmonella* Typhimurium Inhibits NF- κ B, Suppresses I κ B α Ubiquitination and Modulates Innate Immune Responses. *The Journal of Immunology*, 180, 5045-5056.
- LESNICK, M. L., REINER, N. E., FIERER, J. & GUINEY, D. G. 2001. The *Salmonella* spvB virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Molecular Microbiology*, 39, 1464-1470.
- LI, H., XU, H., ZHOU, Y., ZHANG, J., LONG, C., LI, S., CHEN, S., ZHOU, J. M. & SHAO, F. 2007. The phosphothreonine lyase activity of a bacterial type III effector family. *Science*, 315, 1000-3.
- LI, S., ZHANG, L., YAO, Q., LI, L., DONG, N., RONG, J., GAO, W., DING, X., SUN, L., CHEN, X., CHEN, S. & SHAO, F. 2013. Pathogen blocks host death receptor signalling by arginine GlcNAcylation of death domains. *Nature*, 501, 242-6.
- LI, W., LIU, Y., SHENG, X., YIN, P., HU, F., LIU, Y., CHEN, C., LI, Q., YAN, C. & WANG, J. 2014. Structure and mechanism of a type III secretion protease, NleC. *Acta Crystallogr D Biol Crystallogr*, 70, 40-7.
- LIEBL, D., QI, X., ZHE, Y., BARNETT, T. C. & TEASDALE, R. D. 2017. SopB-Mediated Recruitment of SNX18 Facilitates *Salmonella* Typhimurium Internalization by the Host Cell. *Front Cell Infect Microbiol*, 7, 257.
- LILIC, M., GALKIN, V. E., ORLOVA, A., VANLOOCK, M. S., EGELMAN, E. H. & STEBBINS, C. E. 2003. *Salmonella* SipA polymerizes actin by stapling filaments with nonglobular protein arms. *Science*, 301, 1918-21.
- LIN, S. L., LE, T. X. & COWEN, D. S. 2003. SptP, a *Salmonella* Typhimurium type III-secreted protein, inhibits the mitogen-activated protein kinase pathway by inhibiting Raf activation. *Cell Microbiol*, 5, 267-75.
- LISS, V., SWART, A. L., KEHL, A., HERMANN, N., ZHANG, Y., CHIKKABALLI, D., BOHLES, N., DEIWICK, J. & HENSEL, M. 2017. *Salmonella enterica* Remodels the Host Cell Endosomal System for Efficient Intravacuolar Nutrition. *Cell Host Microbe*.
- LIU, X., ZHANG, Z., RUAN, J., PAN, Y., MAGUPALLI, V. G., WU, H. & LIEBERMAN, J. 2016. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature*, 535, 153-158.
- LOSSI, N. S., ROLHION, N., MAGEE, A. I., BOYLE, C. & HOLDEN, D. W. 2008. The *Salmonella* SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid : cholesterol acyltransferase activity. *Microbiology*, 154, 2680-8.
- MAJOWICZ, S. E., MUSTO, J., SCALLAN, E., ANGULO, F. J., KIRK, M., O'BRIEN, S. J., JONES, T. F., FAZIL, A., HOEKSTRA, R. M. & INTERNATIONAL COLLABORATION ON ENTERIC DISEASE 'BURDEN OF ILLNESS, S. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin Infect Dis*, 50, 882-9.
- MAZURKIEWICZ, P., THOMAS, J., THOMPSON, J. A., LIU, M., ARBIBE, L., SANSONETTI, P. & HOLDEN, D. W. 2008. SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Mol Microbiol*, 67, 1371-83.
- MCEWAN, D. G., RICHTER, B., CLAUDI, B., WIGGE, C., WILD, P., FARHAN, H., MCGOURTY, K., COXON, F. P., FRANZ-WACHTEL, M., PERDU, B., AKUTSU, M., HABERMANN, A., KIRCHOF, A., HELFRICH, M. H., ODGREN, P. R., VAN HUL, W., FRANGAKIS, A. S., RAJALINGAM, K., MACEK, B., HOLDEN, D. W., BUMANN, D. & DIKIC, I. 2015. PLEKHM1 regulates *Salmonella*-containing vacuole biogenesis and infection. *Cell Host Microbe*, 17, 58-71.
- MCGHIE, E. J., HAYWARD, R. D. & KORONAKIS, V. 2001. Cooperation between actin-binding proteins of invasive *Salmonella*: SipA potentiates SipC nucleation and bundling of actin. *Embo j*, 20, 2131-9.

- MCGOURTY, K., THURSTON, T. L., MATTHEWS, S. A., PINAUD, L., MOTA, L. J. & HOLDEN, D. W. 2012. *Salmonella* inhibits retrograde trafficking of mannose-6-phosphate receptors and lysosome function. *Science*, 338, 963-7.
- MCLAUGHLIN, L. M., GOVONI, G. R., GERKE, C., GOPINATH, S., PENG, K., LAIDLAW, G., CHIEN, Y. H., JEONG, H. W., LI, Z., BROWN, M. D., SACKS, D. B. & MONACK, D. 2009. The *Salmonella* SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration. *PLoS Pathog*, 5, e1000671.
- MÉRESSE, S., UNSWORTH, K. E., HABERMANN, A., GRIFFITHS, G., FANG, F., MARTÍNEZ-LORENZO, M. J., WATERMAN, S. R., GORVEL, J.-P. & HOLDEN, D. W. 2001. Remodelling of the actin cytoskeleton is essential for replication of intravacuolar *Salmonella*. *Cellular Microbiology*, 3, 567-577.
- MESMIN, B., BIGAY, J., MOSER VON FILSECK, J., LACAS-GERVAIS, S., DRIN, G. & ANTONNY, B. 2013. A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. *Cell*, 155, 830-43.
- MESQUITA, F. S., HOLDEN, D. W. & ROLHION, N. 2013. Lack of effect of the *Salmonella* deubiquitinase SseL on the NF- κ B pathway. *PLoS One*, 8, e53064.
- MESQUITA, F. S., THOMAS, M., SACHSE, M., SANTOS, A. J., FIGUEIRA, R. & HOLDEN, D. W. 2012. The *Salmonella* deubiquitinase SseL inhibits selective autophagy of cytosolic aggregates. *PLoS Pathog*, 8, e1002743.
- MIAO, E. A., BRITTNACHER, M., HARAGA, A., JENG, R. L., WELCH, M. D. & MILLER, S. I. 2003. *Salmonella* effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. *Mol Microbiol*, 48, 401-15.
- MIAO, E. A., LEAF, I. A., TREUTING, P. M., MAO, D. P., DORS, M., SARKAR, A., WARREN, S. E., WEWERS, M. D. & ADEREM, A. 2010a. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol*, 11, 1136-42.
- MIAO, E. A., MAO, D. P., YUDKOVSKY, N., BONNEAU, R., LORANG, C. G., WARREN, S. E., LEAF, I. A. & ADEREM, A. 2010b. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc Natl Acad Sci U S A*, 107, 3076-80.
- MONACK, D. M., BOULEY, D. M. & FALKOW, S. 2004. *Salmonella* Typhimurium persists within macrophages in the mesenteric lymph nodes of chronically infected *Nramp1*^{+/+} mice and can be reactivated by IFN- γ neutralization. *J Exp Med*, 199, 231-41.
- MOTA, L. J., RAMSDEN, A. E., LIU, M., CASTLE, J. D. & HOLDEN, D. W. 2009. SCAMP3 is a component of the *Salmonella*-induced tubular network and reveals an interaction between bacterial effectors and post-Golgi trafficking. *Cell Microbiol*, 11, 1236-53.
- MUHLEN, S., RUCHAUD-SPARAGANO, M. H. & KENNY, B. 2011. Proteasome-independent degradation of canonical NF- κ B complex components by the NleC protein of pathogenic *Escherichia coli*. *J Biol Chem*, 286, 5100-7.
- MUKHERJEE, S. P., BEHAR, M., BIRNBAUM, H. A., HOFFMANN, A., WRIGHT, P. E. & GHOSH, G. 2013. Analysis of the RelA:CBP/p300 Interaction Reveals Its Involvement in NF- κ B-Driven Transcription. *PLOS Biology*, 11, e1001647.
- MULLER, A. J., HOFFMANN, C., GALLE, M., VAN DEN BROEKE, A., HEIKENWALDER, M., FALTER, L., MISSELWITZ, B., KREMER, M., BEYAERT, R. & HARDT, W. D. 2009. The *S. Typhimurium* effector SopE induces caspase-1 activation in stromal cells to initiate gut inflammation. *Cell Host Microbe*, 6, 125-36.
- MULLER, C. W., REY, F. A., SODEOKA, M., VERDINE, G. L. & HARRISON, S. C. 1995. Structure of the NF- κ B p50 homodimer bound to DNA. *Nature*, 373, 311-7.
- MÜLLER, M. R. & RAO, A. 2010. NFAT, immunity and cancer: a transcription factor comes of age. *Nature Reviews Immunology*, 10, 645.
- MURSHUDOV, G. N., VAGIN, A. A. & DODSON, E. J. 1997. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr*, 53, 240-55.

- NAWABI, P., CATRON, D. M. & HALDAR, K. 2008. Esterification of cholesterol by a type III secretion effector during intracellular *Salmonella* infection. *Mol Microbiol*, 68, 173-85.
- NEZNANOV, N., CHUMAKOV, K. M., NEZNANOVA, L., ALMASAN, A., BANERJEE, A. K. & GUDKOV, A. V. 2005. Proteolytic cleavage of the p65-RelA subunit of NF- κ B during poliovirus infection. *J Biol Chem*, 280, 24153-8.
- NIEMANN, G. S., BROWN, R. N., GUSTIN, J. K., STUFKENS, A., SHAIKH-KIDWAI, A. S., LI, J., MCDERMOTT, J. E., BREWER, H. M., SCHEPMOES, A., SMITH, R. D., ADKINS, J. N. & HEFFRON, F. 2011. Discovery of novel secreted virulence factors from *Salmonella enterica* serovar Typhimurium by proteomic analysis of culture supernatants. *Infect Immun*, 79, 33-43.
- NORRIS, F. A., WILSON, M. P., WALLIS, T. S., GALYOV, E. E. & MAJERUS, P. W. 1998. SopB, a protein required for virulence of *Salmonella* Dublin, is an inositol phosphate phosphatase. *Proc Natl Acad Sci U S A*, 95, 14057-9.
- NUCCIO, S. P. & BAUMLER, A. J. 2014. Comparative analysis of *Salmonella* genomes identifies a metabolic network for escalating growth in the inflamed gut. *MBio*, 5, e00929-14.
- ODENDALL, C., ROLHION, N., FORSTER, A., POH, J., LAMONT, D. J., LIU, M., FREEMONT, P. S., CATLING, A. D. & HOLDEN, D. W. 2012. The *Salmonella* kinase SteC targets the MAP kinase MEK to regulate the host actin cytoskeleton. *Cell Host Microbe*, 12, 657-68.
- OHLSON, M. B., HUANG, Z., ALTO, N. M., BLANC, M. P., DIXON, J. E., CHAI, J. & MILLER, S. I. 2008. Structure and function of *Salmonella* SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation. *Cell Host Microbe*, 4, 434-46.
- PATEL, J. C. & GALAN, J. E. 2005. Manipulation of the host actin cytoskeleton by *Salmonella*-all in the name of entry. *Curr Opin Microbiol*, 8, 10-5.
- PEARSON, J. S., GIOGHA, C., ONG, S. Y., KENNEDY, C. L., KELLY, M., ROBINSON, K. S., LUNG, T. W., MANSELL, A., RIEDMAIER, P., OATES, C. V., ZAID, A., MUHLEN, S., CREPIN, V. F., MARCHES, O., ANG, C. S., WILLIAMSON, N. A., O'REILLY, L. A., BANKOVACKI, A., NACHBUR, U., INFUSINI, G., WEBB, A. I., SILKE, J., STRASSER, A., FRANKEL, G. & HARTLAND, E. L. 2013. A type III effector antagonizes death receptor signalling during bacterial gut infection. *Nature*, 501, 247-51.
- PEARSON, J. S., RIEDMAIER, P., MARCHES, O., FRANKEL, G. & HARTLAND, E. L. 2011. A type III effector protease NleC from enteropathogenic *Escherichia coli* targets NF- κ B for degradation. *Mol Microbiol*, 80, 219-30.
- PELMENSCHIKOV, V., BLOMBERG, M. R. & SIEGBAHN, P. E. 2002. A theoretical study of the mechanism for peptide hydrolysis by thermolysin. *J Biol Inorg Chem*, 7, 284-98.
- PILAR, A. V., REID-YU, S. A., COOPER, C. A., MULDER, D. T. & COOMBES, B. K. 2012. GogB is an anti-inflammatory effector that limits tissue damage during *Salmonella* infection through interaction with human FBXO22 and Skp1. *PLoS Pathog*, 8, e1002773.
- POH, J., ODENDALL, C., SPANOS, A., BOYLE, C., LIU, M., FREEMONT, P. & HOLDEN, D. W. 2008. SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodelling. *Cell Microbiol*, 10, 20-30.
- PORTALIOU, A. G., TSOLIS, K. C., LOOS, M. S., ZORZINI, V. & ECONOMOU, A. 2016. Type III Secretion: Building and Operating a Remarkable Nanomachine. *Trends Biochem Sci*, 41, 175-89.
- PRUNEDA, J. N., DURKIN, C. H., GEURINK, P. P., OVAA, H., SANTHANAM, B., HOLDEN, D. W. & KOMANDER, D. 2016. The Molecular Basis for Ubiquitin and Ubiquitin-like Specificities in Bacterial Effector Proteases. *Mol Cell*, 63, 261-76.
- RAFFATELLU, M., WILSON, R. P., WINTER, S. E. & BAUMLER, A. J. 2008. Clinical pathogenesis of typhoid fever. *J Infect Dev Ctries*, 2, 260-6.
- RANDOW, F. & SALE, J. E. 2006. Retroviral transduction of DT40. In: BUERSTEDDE, J.-M. & TAKEDA, S. (eds.) *Reviews and Protocols in DT40 Research: Subcellular Biochemistry*. Dordrecht: Springer Netherlands.

- RAO, S., SCHIEBER, A. M., O'CONNOR, C. P., LEBLANC, M., MICHEL, D. & AYRES, J. S. 2017. Pathogen-Mediated Inhibition of Anorexia Promotes Host Survival and Transmission. *Cell*, 168, 503-516 e12.
- RANDOW, F., MACMICKING, J. D. & JAMES, L. C. 2013. Cellular self-defense: how cell autonomous immunity protects against pathogens. *Science*, 340, 701-706.
- RATHMAN, M., BARKER, L. P. & FALKOW, S. 1997. The unique trafficking pattern of *Salmonella* Typhimurium-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry. *Infect Immun*, 65, 1475-85.
- ROCHE, P. A. & FURUTA, K. 2015. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol*, 15, 203-16.
- ROLHION, N., FURNISS, R. C., GRABE, G., RYAN, A., LIU, M., MATTHEWS, S. A. & HOLDEN, D. W. 2016. Inhibition of Nuclear Transport of NF- κ B p65 by the *Salmonella* Type III Secretion System Effector SpvD. *PLoS Pathog*, 12, e1005653.
- RYTKONEN, A., POH, J., GARMENDIA, J., BOYLE, C., THOMPSON, A., LIU, M., FREEMONT, P., HINTON, J. C. & HOLDEN, D. W. 2007. SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. *Proc Natl Acad Sci U S A*, 104, 3502-7.
- SCHECHTER, I. & BERGER, A. 1967. On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun*, 27, 157-62.
- SCHEICH, C., KÜMMEL, D., SOUMAILAKAKIS, D., HEINEMANN, U. & BÜSSOW, K. 2007. Vectors for co-expression of an unrestricted number of proteins. *Nucleic Acids Research*, 35, e43-e43.
- SCHROEDER, N., HENRY, T., DE CHASTELLIER, C., ZHAO, W., GUILHON, A. A., GORVEL, J. P. & MERESSE, S. 2010. The virulence protein SopD2 regulates membrane dynamics of *Salmonella*-containing vacuoles. *PLoS Pathog*, 6, e1001002.
- SHAM, H. P., SHAMES, S. R., CROXEN, M. A., MA, C., CHAN, J. M., KHAN, M. A., WICKHAM, M. E., DENG, W., FINLAY, B. B. & VALLANCE, B. A. 2011. Attaching and effacing bacterial effector NleC suppresses epithelial inflammatory responses by inhibiting NF- κ B and p38 mitogen-activated protein kinase activation. *Infect Immun*, 79, 3552-62.
- SHAMES, S. R., BHAVSAR, A. P., CROXEN, M. A., LAW, R. J., MAK, S. H., DENG, W., LI, Y., BIDSHARI, R., DE HOOG, C. L., FOSTER, L. J. & FINLAY, B. B. 2011. The pathogenic *Escherichia coli* type III secreted protease NleC degrades the host acetyltransferase p300. *Cell Microbiol*, 13, 1542-57.
- SHEA, J. E., BEUZON, C. R., GLEESON, C., MUNDY, R. & HOLDEN, D. W. 1999. Influence of the *Salmonella* Typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse. *Infect Immun*, 67, 213-9.
- SHI, J., ZHAO, Y., WANG, K., SHI, X., WANG, Y., HUANG, H., ZHUANG, Y., CAI, T., WANG, F. & SHAO, F. 2015. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature*, 526 660-665.
- SILVA, D. S., PEREIRA, L. M., MOREIRA, A. R., FERREIRA-DA-SILVA, F., BRITO, R. M., FARIA, T. Q., ZORNETTA, I., MONTECUCCO, C., OLIVEIRA, P., AZEVEDO, J. E., PEREIRA, P. J., MACEDO-RIBEIRO, S., DO VALE, A. & DOS SANTOS, N. M. 2013. The apoptogenic toxin AIP56 is a metalloprotease A-B toxin that cleaves NF- κ B p65. *PLoS Pathog*, 9, e1003128.
- SPANNO, S. & GALAN, J. E. 2012. A Rab32-dependent pathway contributes to *Salmonella* Typhi host restriction. *Science*, 338, 960-3.
- SPANNO, S., GAO, X., HANNEMANN, S., LARA-TEJERO, M. & GALAN, J. E. 2016. A Bacterial Pathogen Targets a Host Rab-Family GTPase Defense Pathway with a GAP. *Cell Host Microbe*, 19, 216-26.
- SPANNO, S., LIU, X. & GALAN, J. E. 2011. Proteolytic targeting of Rab29 by an effector protein distinguishes the intracellular compartments of human-adapted and broad-host *Salmonella*. *Proc Natl Acad Sci U S A*, 108, 18418-23.

- SRIKUMAR, S., KRÖGER, C., HÉBRARD, M., COLGAN, A., OWEN, S. V., SIVASANKARAN, S. K., CAMERON, A. D. S., HOKAMP, K. & HINTON, J. C. D. 2015. RNA-seq Brings New Insights to the Intra-Macrophage Transcriptome of *Salmonella* Typhimurium. *PLOS Pathogens*, 11, e1005262.
- STECHER, B., HAPFELMEIER, S., MÜLLER, C., KREMER, M., STALLMACH, T. & HARDT, W.-D. 2004. Flagella and Chemotaxis Are Required for Efficient Induction of *Salmonella enterica* Serovar Typhimurium Colitis in Streptomycin-Pre-treated Mice. *Infection and Immunity*, 72, 4138-4150.
- SUN, L., WANG, H., WANG, Z., HE, S., CHEN, S., LIAO, D., WANG, L., YAN, J., LIU, W., LEI, X. & WANG, X. 2012. Mixed lineage kinase domain-like protein mediates necrosis signalling downstream of RIP3 kinase. *Cell*, 148, 213-227.
- SUN, H., KAMANOVA, J., LARA-TEJERO, M. & GALAN, J. E. 2016. A Family of *Salmonella* Type III Secretion Effector Proteins Selectively Targets the NF- κ B Signaling Pathway to Preserve Host Homeostasis. *PLoS Pathog*, 12, e1005484.
- TEREBIZNIK, M. R., VIEIRA, O. V., MARCUS, S. L., SLADE, A., YIP, C. M., TRIMBLE, W. S., MEYER, T., FINLAY, B. B. & GRINSTEIN, S. 2002. Elimination of host cell PtdIns(4,5)P₂ by bacterial SigD promotes membrane fission during invasion by *Salmonella*. *Nature Cell Biology*, 4, 766.
- TERWILLIGER, T. C., GROSSE-KUNSTLEVE, R. W., AFONINE, P. V., MORIARTY, N. W., ZWART, P. H., HUNG, L.-W., READ, R. J. & ADAMS, P. D. 2008. Iterative model building, structure refinement and density modification with the PHENIX AutoBuild wizard. *Acta Crystallographica Section D: Biological Crystallography*, 64, 61-69.
- THELEN, M. & STEIN, J. V. 2008. How chemokines invite leukocytes to dance. *Nat Immunol*, 9, 953-9.
- THIENNIMITR, P., WINTER, S. E., WINTER, M. G., XAVIER, M. N., TOLSTIKOV, V., HUSEBY, D. L., STERZENBACH, T., TSOLIS, R. M., ROTH, J. R. & BAUMLER, A. J. 2011. Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. *Proc Natl Acad Sci U S A*, 108, 17480-5.
- THURSTON, T. L. M., MATTHEWS, S. A., JENNINGS, E., ALIX, E., SHAO, F., SHENOY, A. R., BIRRELL, M. A. & HOLDEN, D. W. 2016. Growth inhibition of cytosolic *Salmonella* by caspase-1 and caspase-11 precedes host cell death. *Nature Communications*, 7, 13292.
- THURSTON, T. L. M., WANDEL, M. P., VON MUHLINEN, N., FOEGLEIN, A. & RANDOW, F. 2012. Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature*, 482, 414-418.
- TURCO, M. M. & SOUSA, M. C. 2014. The Structure and Specificity of the Type III Secretion System Effector NleC Suggest a DNA Mimicry Mechanism of Substrate Recognition. *Biochemistry*, 53, 5131-5139.
- UCHE, I. V., MACLENNAN, C. A. & SAUL, A. 2017. A Systematic Review of the Incidence, Risk Factors and Case Fatality Rates of Invasive Nontyphoidal *Salmonella* (iNTS) Disease in Africa (1966 to 2014). *PLoS Negl Trop Dis*, 11, e0005118.
- VALDIVIA, R. H. & FALKOW, S. 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella* typhimurium acid-inducible promoters by differential fluorescence induction. *Mol Microbiol*, 22, 367-78.
- VAN DER VELDEN, A. W., BAUMLER, A. J., TSOLIS, R. M. & HEFFRON, F. 1998. Multiple fimbrial adhesins are required for full virulence of *Salmonella* Typhimurium in mice. *Infect Immun*, 66, 2803-8.
- VAZQUEZ-TORRES, A., JONES-CARSON, J., BAUMLER, A. J., FALKOW, S., VALDIVIA, R., BROWN, W., LE, M., BERGGREN, R., PARKS, W. T. & FANG, F. C. 1999. Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature*, 401, 804-8.
- VIDAL, S. M., PINNER, E., LEPAGE, P., GAUTHIER, S. & GROS, P. 1996. Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains. *The Journal of Immunology*, 157, 3559.

- WANG, R. F. & KUSHNER, S. R. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene*, 100, 195-9.
- WANG, V. Y., HUANG, W., ASAGIRI, M., SPANN, N., HOFFMANN, A., GLASS, C. & GHOSH, G. 2012. The transcriptional specificity of NF- κ B dimers is coded within the κ B DNA response elements. *Cell Rep*, 2, 824-39.
- WATSON, K. G. & HOLDEN, D. W. 2010. Dynamics of growth and dissemination of *Salmonella* in vivo. *Cell Microbiol*, 12, 1389-97.
- WEENING, E. H., BARKER, J. D., LAARAKKER, M. C., HUMPHRIES, A. D., TSOLIS, R. M. & BAUMLER, A. J. 2005. The *Salmonella enterica* serotype Typhimurium lpf, bcf, stb, stc, std, and sth fimbrial operons are required for intestinal persistence in mice. *Infect Immun*, 73, 3358-66.
- WESSLING-RESNICK, M. 2015. Nramp1 and Other Transporters Involved in Metal Withholding during Infection. *The Journal of Biological Chemistry*, 290, 18984-18990.
- WINTER, S. E., THIENNIMITR, P., WINTER, M. G., BUTLER, B. P., HUSEBY, D. L., CRAWFORD, R. W., RUSSELL, J. M., BEVINS, C. L., ADAMS, L. G., TSOLIS, R. M., ROTH, J. R. & BAUMLER, A. J. 2010. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature*, 467, 426-9.
- WOOD, M. W., JONES, M. A., WATSON, P. R., HEDGES, S., WALLIS, T. S. & GALYOV, E. E. 1998. Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. *Mol Microbiol*, 29, 883-91.
- YANG, Z., SODERHOLM, A., LUNG, T. W., GIOGHA, C., HILL, M. M., BROWN, N. F., HARTLAND, E. & TEASDALE, R. D. 2015. SseK3 Is a *Salmonella* Effector That Binds TRIM32 and Modulates the Host's NF- κ B Signalling Activity. *PLoS One*, 10, e0138529.
- YAO, Q., ZHANG, L., WAN, X., CHEN, J., HU, L., DING, X., LI, L., KARAR, J., PENG, H., CHEN, S., HUANG, N., RAUSCHER, F. J., 3RD & SHAO, F. 2014. Structure and specificity of the bacterial cysteine methyltransferase effector NleE suggests a novel substrate in human DNA repair pathway. *PLoS Pathog*, 10, e1004522.
- YEN, H., OOKA, T., IGUCHI, A., HAYASHI, T., SUGIMOTO, N. & TOBE, T. 2010. NleC, a type III secretion protease, compromises NF- κ B activation by targeting p65/RelA. *PLoS Pathog*, 6, e1001231.
- YIP, Y. Y., PERNIGO, S., SANGER, A., XU, M., PARSONS, M., STEINER, R. A. & DODDING, M. P. 2016. The light chains of kinesin-1 are autoinhibited. *Proc Natl Acad Sci U S A*, 113, 2418-23.
- YRLID, U., SVENSSON, M., JOHANSSON, C. & WICK, M. J. 2000. *Salmonella* infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS Immunol Med Microbiol*, 27, 313-20.
- YU, X. J., LIU, M. & HOLDEN, D. W. 2016. *Salmonella* Effectors SseF and SseG Interact with Mammalian Protein ACBD3 (GCP60) To Anchor Salmonella-Containing Vacuoles at the Golgi Network. *MBio*, 7.
- ZHANG, Q., LENARDO, M. J. & BALTIMORE, D. 2017. 30 Years of NF- κ B: A Blossoming of Relevance to Human Pathobiology. *Cell*, 168, 37-57.
- ZHOU, D., CHEN, L. M., HERNANDEZ, L., SHEARS, S. B. & GALAN, 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, 248-59.
- ZHOU, D., MOOSEKER, M. S. & GALÁN, J. E. 1999. Role of the *S. Typhimurium* Actin-Binding Protein SipA in Bacterial Internalization. *Science*, 283, 2092-2095.
- ZHOU, P., SHE, Y., DONG, N., LI, P., HE, H., BORIO, A., WU, Q., LU, S., DING, X., CAO, Y., XU, Y., GAO, W., DONG, M., DING, J., WANG, D. C., ZAMYATINA, A. & SHAO, F. 2018. Alpha-kinase 1 is a cytosolic innate immune receptor for bacterial ADP-heptose. *Nature*.