Host DNA damage responses to the typhoid toxin of *Salmonella enterica*

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Declaration

I, Daniel Stark, confirm that this thesis is my own work. I am aware of the University’s Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university.

Daniel Stark, July 2022
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Abstract

The typhoid toxin is a virulence factor of the bacterial pathogen *Salmonella enterica*, which causes typhoid fever. The toxin has been shown to cause a DNA damage response in intoxicated human cells and to promote infection (Ibler *et al.*, 2019). DNA damage responses have been shown to activate innate immune pathways via leakage of self-DNA into the cytosol and activation of the cGAS-STING pathway (Wolf *et al.*, 2016). This thesis shows that purified typhoid toxin upregulates a type-I interferon-like response, including the antiviral ubiquitin-like interferon-stimulated gene 15 (ISG15), in a STING-dependent manner. ISG15 was upregulated in response to toxigenic *Salmonella* infection and overexpression of ISG15 reduced *Salmonella* burden, suggesting a role in host defence. Chronic *Salmonella* infection has been linked to gallbladder cancer (Di Domenico *et al.*, 2017), and ISG15 has been implicated as a regulator of P53 and thus tumour suppression in response to DNA damage (Park *et al.*, 2016). The toxin induces cell death in wild-type MEFs, whereas ISG15 KO MEFs survive and proliferate despite hallmarks of genomic instability such as micronuclei. This suggests that ISG15 may protect the host from pathogen-induced genomic instability. Taken together, this thesis provides new insights into host responses to the typhoid toxin, and the findings may be applicable to other bacterial genotoxins.
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List of Abbreviations

53BP1  p53-binding protein 1 
AMR  antimicrobial resistant 
APH  aphisidicolin 
APS  ammonium persulfate 
ATM  Ataxia Telangiectasia Mutated 
ATR  Ataxia telangiectasia and Rad3 related 
ATRIP  ATR interacting protein 
BSA  bovine serum albumin 
CDK  cyclin-dependent kinase 
CDT  cytolethal distending toxin 
CFU  colony forming unit 
cGAS  cyclic GMP-AMP synthase 
CldU  5-chloro-2'-deoxyuridine 
DAMP  damage-associated molecular pattern 
DDR  DNA damage response 
DMEM  Dulbecco’s modified Eagle medium 
DNA-PKc  DNA-dependent protein kinase, catalytic subunit 
DSB  double-strand break 
dsDNA  double-stranded DNA 
DTT  Dithiothreitol 
EdU  5-Ethynyl-2-deoxyuridine 
ER  endoplasmic reticulum 
ERAD  ER-associated degradation 
ETP  etoposide 
FBS  Foetal Bovine Serum 
FISH  fluorescence in situ hybridisation 
FT  flow through 
GST  glutathione S-transferase
HA human influenza hemagglutinin
HDR homology directed repair
His histidine
HMGB High mobility group box protein
HQ toxin catalytically inactive toxin with CdtB-H160Q mutation
IFIH1 interferon induced with helicase C domain 1 (also known as MDA5)
IFIT interferon induced protein with tetratricopeptide repeat
IFN interferon
IKK IκB kinase
IL interleukin
INT3 integrator complex subunit 3
iNTS invasive non-typhoidal Salmonella
IPTG Isopropyl β- d-1-thiogalactopyranoside
IR infrared radiation
IRAK interleukin 1 receptor associated kinase
IRF interferon regulatory factor
ISG interferon stimulated gene
ISG15 interferon stimulated gene 15
ISGF3 interferon stimulated gene factor 3
ISRE interferon-sensitive response element
JAK Janus kinase
LB Lysogeny broth
LPS Lipopolysaccharide
MDR multidrug-resistant
MEF mouse embryonic fibroblast
MES 2-(N-morpholino) ethanesulfonic acid
MHC major histocompatibility complex
MLN mesenteric lymph nodes
MOI multiplicity of infection
MQ Milli-Q water
MRN Mre11-Rad50-Nbs1 complex
NABP Nucleic Acid Binding Protein
Neu5Ac N-Acetylneuraminic acid
Neu5Gc N-Glycolylneuraminic acid
NFκβ Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHEJ non-homologous end joining
NiNTA nickel-nitrilotriacetic acid
NK natural killer
ns non-significant
NT non-targeting
NTS non-typhoidal *Salmonella*
OAS 2'-5'-oligoadenylate synthetase
OD optical density
PAMP pathogen-associated molecular pattern
PBS phospho-buffered saline
pcDNA plasmid cloning DNA
PCR polymerase chain reaction
PFA paraformaldehyde
PLT pertussis-like toxin
PVDF polyvinylidene difluoride
qRT-PCR Real-Time Quantitative Reverse Transcription PCR
RING response induced by a genotoxin
ROS reactive oxygen species
RPA replication protein A
rpm revolutions per minute
SASP senescence associated secretory phenotype
SBP ssDNA-binding protein
SCV Salmonella containing vacuole
SDS sodium dodecyl sulphate
SDS-PAGE SDS–polyacrylamide gel electrophoresis
Sif Salmonella induced filaments
siRNA small interfering RNA
SOC super optimal broth
SOSS sensor of single-stranded DNA
SPI Salmonella pathogenicity island
SSB single-strand break
ssDNA single-stranded DNA
ST Salmonella Typhimurium
STAT signal transducer and activator of transcription
STING Stimulator of interferon genes
T3SS type 3 secretion system
TBK tank-binding kinase
TBS tris-buffered saline
TEMED tetramethylethylenediamine
Tris trisaminomethane
TLR toll-like receptor
TLS translesion DNA synthesis
TYK tyrosine kinase
UBE1L Ubiquitin-like modifier-activating enzyme 7
USP18 Ubiquitin Specific Peptidase 18
UV ultraviolet
WHO World Health Organisation
WT wild type
XDR extensively drug resistant
γH2AX phosphorylated H2A histone family member X
Part 1: Literature Review

1 Salmonella infection

1.1 Introduction

The typhoidal serovars of Salmonella enterica, including Typhi and Paratyphi (henceforth S. Typhi and S. Paratyphi) are intracellular Gram-negative bacteria that cause typhoid fever and paratyphoid fever respectively, collectively known as enteric fever. Enteric fever is a life-threatening infectious disease transmitted to humans by contaminated food and water and threatens human populations in regions lacking access to clean water and good sanitation (Parry et al., 2002; Crump and Mintz, 2010; Galán, 2016). Typhoid fever is the best characterised and will be discussed henceforth.

Typhoid fever is a human-specific disease that has threatened humanity since the earliest recorded plagues, with evidence that it was cause of the plague of Athens in 430 BC (Papagrigorakis et al., 2006). In modern history, typhoid fever was a major cause of illness in the USA and Europe during the 19th Century, but has been largely eradicated in high-income countries over the preceding century following improvements in sanitation (Parry et al., 2002). Today, typhoid fever affects low- to middle-income countries, predominantly in sub-Saharan Africa, South Asia, South East Asia and Oceania (World Health Organization, 2018). Although the overall global burden of typhoid fever appears to have reduced since the 1990s, global morbidity and mortality remains high (Als et al., 2018). As of 2018 there were between 11 to 21 million cases worldwide, and of these cases, 128,000 to 161,000 people die every year (World Health Organization, 2018). There are gaps in the data available and inconsistencies in data acquisition between regions, making the true extent of the global burden of typhoid fever uncertain (Als et al., 2018).

Besides typhoid fever itself, S. Typhi infection is associated with other global health issues (Gunn et al., 2014). Chronic carriage of S. Typhi can be asymptomatic, which increases infection spread and complicates diagnosis and treatment. Furthermore,
chronic infection has been linked to increased incidence of gallbladder cancer. In this chapter I will describe how typhoidal *Salmonella* causes infection and how this can lead to typhoid fever, chronic carriage, and cancer.

### 1.2 Disease outcomes of *Salmonella* infection

There are more than 2600 known serovars of *Salmonella enterica* which differ in host specificity and disease outcome (Brenner *et al.*, 2000; Gal-Mor, Boyle and Grassl, 2014). In terms of those that affect humans, these serovars can be divided into typhoidal and non-typhoidal *Salmonella*, referring to whether infection can lead to typhoid fever. They can also be categorised as invasive or non-invasive, based on whether they establish systemic or localised infection (Fig. 1.1).
Non-invasive, non-typhoidal *Salmonella* (NTS) typically causes rapid onset of gastroenteritis 6 – 72 h after infection. Infection is generally self-limiting and lasts an average of 10 days in immunocompetent individuals. Symptoms include vomiting and diarrhoea. Infection can still be lethal in immunocompromised individuals, and NTS infection still causes approximately 150,000 deaths per year. The most common NTS serovar affecting humans is *S. Typhimurium* sequence type 19 (ST19) (Gal-Mor, Boyle and Grassl, 2014).
Invasive non-typhoidal *Salmonella* (iNTS) is associated with a reduced host inflammatory response and the ability to replicate within macrophages (Ramachandran *et al.*, 2015). iNTS can cause systemic infection, symptoms similar to typhoid fever, and higher fatality rates than other NTS serovars, particularly in immunocompromised individuals (Crump *et al.*, 2015; Stanaway *et al.*, 2019). iNTS serovars include *Salmonella enterica* Javiana (R. A. Miller and Wiedmann, 2016), the multidrug resistant *S. Typhi* sequence type 313 (ST313) and *Salmonella Enteritidis* ST11 (Ramachandran *et al.*, 2015; Kanteh *et al.*, 2021).

**Typhoidal Salmonella** includes serovars Typhi, Paratyphi A, B and C, and Sendai (Gal-Mor, Boyle and Grassl, 2014). Unlike NTS infection, *S. Typhi* incubation ranges from 3 to 60 days and causes minimal gastrointestinal inflammation. Typhoidal *Salmonella* establishes a systemic infection, colonising the intestine, mesenteric lymph nodes, liver, spleen, and bone marrow (Parry *et al.*, 2002; Raffatellu *et al.*, 2008). Symptoms of infection include a fever of >38°C, lethargy and other influenza-like symptoms, which last for an average of 3 weeks. Up to 10% of patients infected with *S. Typhi* shed the bacteria in their faeces for up to three months following infection. 1-4% become chronic carriers, shedding the bacteria for more than a year (Parry *et al.*, 2002; Gal-Mor, Boyle and Grassl, 2014; Gal-Mor, 2019). Of these long-term carriers, most carry the infection asymptotically, and 25% have no history of acute typhoid symptoms, suggesting that the initial infection was asymptomatic or misdiagnosed (Parry *et al.*, 2002). These chronic carriers retain the pathogen in the population and can transmit the infection to others via their faeces, which can then present as typhoid fever. A famous example of an asymptomatic chronic carrier is Mary Mallon, also known as ‘Typhoid Mary’, an Irish cook in New York in the early 1900s who inadvertently killed over 50 people by serving them contaminated food, whilst never suffering symptoms of typhoid fever herself (Gal-Mor, Boyle and Grassl, 2014; Galán, 2016).
1.3 Diagnostics and Treatment

1.3.1 Diagnosis of *Salmonella* infection

Accurate diagnosis of typhoid fever is complicated by the lack of distinct symptoms, which leads to cases being confused with malaria, dengue and other febrile disease (Parry *et al.*, 2002). Asymptomatic chronic carriage also complicates clinical diagnosis of *Salmonella* infection, to the extent that the WHO has made development of new tools to identify and treat chronic carriers a research priority (World Health Organization, 2018).

*S*. *Typhi* can be cultured from patient samples including faeces, blood, and bone marrow (Gilman *et al.*, 1975). In each case, test accuracy is dependent on the amount of sample cultured but can still correctly diagnose typhoid even after antibiotic courses have started. Bone marrow samples are the most sensitive, positive in 80 – 95% of typhoid patients, followed by blood samples, positive in 60 – 80% of typhoid patients, and stool samples, which are positive in 30% of typhoid patients (Hussein Gasem *et al.*, 1995; Wain *et al.*, 2001; Parry *et al.*, 2002).

In addition to culture tests, there are several antibody tests available. The most common is Widal's Test, which measures levels of antibody against O and H antigens of *S*. *Typhi* (Andrews and Ryan, 2015). However, levels of these antibodies do not directly correlate to the extent of infection and vary between populations and individuals. Furthermore, the O and H antigens of *S*. *Typhi* are common to other serovars of *Salmonella*. A test that measures antibody levels against the Typhi-specific Vi polysaccharide antigen has been shown to be 70 – 80% sensitive and 80 – 95% specific. Overall, antibody tests are useful diagnostic tools when corrected for differences in population and when used in conjunction with other tests (Parry *et al.*, 2002, 2011).

A recent study was able to identify distinct metabolite signatures between acute and chronic typhoid cases, and were able to narrow their findings to five metabolic markers (Näsström *et al.*, 2018). Machine learning techniques on patient gene expression profiles have also discovered a diagnostic signature that can differentiate typhoid fever from other febrile illnesses, even in cases where
Salmonella could not be cultured from samples. PCR-based diagnostics are a promising diagnostic tool (Blohmke et al., 2019).

1.3.2 Antibiotic Resistance

Salmonella infection is ordinarily treated with antibiotics (Gal-Mor, Boyle and Grassl, 2014). However, multidrug resistant (MDR) strains of S. Typhi have increased over the past decades, resistant to first line antibiotics such as ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol (World Health Organization, 2018; Yang, Chong and Song, 2018). For example, MDR S. Typhi caused treatment failures in Nepal (Thanh et al., 2016) and the incidence of MDR S. Typhi in Malawi increased from 7% to 97% between 2010 and 2014 (Feasey et al., 2015). The resultant switch to the use of second- and third-line antibiotics for treatment has seen the emergence of extensively drug resistant (XDR) strains in Pakistan, with resistance to fluoroquinolones and third generation cephalosporins as well as first line antibiotics (Klemm et al., 2018). There are few treatment options for XDR S. Typhi infection, prompting the World Health Organisation to make research into new treatment measures a research priority (World Health Organization, 2018).

1.3.3 Vaccination

In addition to antibiotic treatment, there are three vaccines for typhoid fever currently licensed for use by the WHO. These include a typhoid conjugate vaccine (TCV), unconjugated Vi polysaccharide (ViPS) and a live vaccine (Ty21A) (World Health Organization, 2018).

TCV consists of, and stimulates an immune response against, the Vi polysaccharide antigen of S. Typhi and tetanus toxin protein. TCV was shown to be 54.6% effective at preventing all possible typhoid fever symptoms in adults in randomised controlled trials, with an efficacy of 87.1% in preventing the field definition of typhoid fever: >38°C fever and bacteraemia (Jin et al., 2017). There is currently insufficient data to validate the efficacy of TCV in the field following natural exposure to typhoid fever (Milligan et al., 2018). However, its introduction is still
recommended by the WHO in countries with a severe typhoid burden due to its superior immunological properties compared to the other vaccine options (World Health Organization, 2018).

ViPS, consisting of the Vi polysaccharide antigen alone, displays differences in vaccine efficacy dependent on the population tested. For instance, 52% of healthy typhoid-naïve adults taking part in a randomised control trial were protected, compared to 64%-72% of patients in Nepal, China and South Africa where typhoid is endemic and interaction with S. Typhi more likely (Jin et al., 2017; World Health Organization, 2018). Furthermore, age affects the efficacy of the vaccine: field studies in India revealed 56% protection in children aged 5-14 years and 80% protection in children aged 2-4 years (World Health Organization, 2018). Overall, a meta-analysis of typhoid vaccine studies concluded that ViPS has an efficacy of 45% to 69% two years after vaccination, with a three year cumulative efficacy suggested to be 55% (Milligan et al., 2018).

Ty21A is an attenuated strain of S. Typhi lacking virulence genes including the Vi capsule and stimulates an immune response against O, H, and other surface antigens of S. Typhi. Field trials in Chile and Egypt indicate it prevented typhoid fever in 62% to 96% of cases up to 7 years after vaccination (World Health Organization, 2018).

Mathematical modelling has predicted that vaccination alone is not sufficient to eradicate typhoid (Pitzer et al., 2014). Therefore, improvements in diagnostics and alternative treatments are an international research focus. To achieve this, a better understanding of the pathogenesis of S. Typhi is needed.

### 1.4 Pathogenesis of S. Typhi

#### 1.4.1 Salmonella entry into host cells

Salmonella infections are caused by ingestion of contaminated food or water. Salmonella enters the gastrointestinal (GI) system and survives due to a high tolerance for the acidic conditions in the stomach (Ohl and Miller, 2001). Bacteria progress through the GI tract into the small intestine, where they adhere to intestinal epithelial cells, favouring specialised cells known as M cells (Fig. 1.1) (Fàbrega and
Vila, 2013; Dougan and Baker, 2014). These cells are found within Peyer’s patches, which are the key interface between the lymph system and the intestine. M cells transport antigens from the intestinal lumen to immune cells in the lymph nodes, providing S. Typhi with a route to disseminate further into the body via phagosomes in the lymph system (Dougan and Baker, 2014).

NTS invasion of host cells stimulates an inflammatory response, such as secretion of pro-inflammatory cytokines, pyroptosis of infected epithelial and macrophage cells, and recruitment of neutrophils (Knodler et al., 2014). However, S. Typhi invasion elicits a weaker and subclinical inflammatory response, allowing it to disseminate to other organ systems and establish systemic infection (Dougan and Baker, 2014; Winter et al., 2015).

As S. Typhi is host restricted to humans there have been efforts to find effective animal models to model S. Typhi infection. Much of the work on Salmonella pathogenesis has been done using S. Typhimurium in mouse or in vitro models (Dougan and Baker, 2014). However, it is important to note differences between S. Typhimurium and S. Typhi invasion, virulence and immune evasion mechanisms.

Roughly 90% of the S. Typhi genome consists of genes homologous to S. Typhimurium serovars, showing that there is a conserved Salmonella core genome (Chan et al., 2003). Salmonella pathogenicity is controlled by genomic segments known as Salmonella pathogenicity islands (SPIs), which encode genes with roles in cell attachment and invasion, immune evasion, and bacterial effector secretion (Dougan and Baker, 2014). Salmonella invasion of host cells is mediated by SPI-1 and SPI-2, which together facilitate cell entry by bacterial-mediated macropinocytosis, and formation of an intracellular niche within a Salmonella-containing vacuole (SCV). Work on SPI-1 and SPI-2 has predominantly been done in S. Typhimurium, and whereas Typhi does encode a functional SPI-1 and SPI-2, there are differences in effectors and the virulence strategies that S. Typhimurium and S. Typhi employ.
1.4.2 SPI-1

SPI-1 encodes a type-III secretion system (T3SS), a needle-like appendage found in certain species of Gram-negative bacteria that acts to sense eukaryotic host cells and translocate effector proteins into them. In S. Typhimurium, SPI-1 effector proteins include SipA (McGhie, Hayward and Koronakis, 2001, 2004) and SopE (Hardt et al., 1998; Humphreys et al., 2012), which subvert host cell signalling pathways responsible for rearrangement of the actin cytoskeleton. This results in the formation of membrane ruffles, forming large vesicles that engulf the adherent Salmonellae and draw them into the cell (McGhie et al., 2009; Lorkowski et al., 2014). The SPI-1 effector SopB is a phosphoinositide phosphatase that interrupts progression of the SCV through the normal endosomal maturation pathway, causing it to become enlarged and thus creating a spacious environment for Salmonella growth and replication (Hernandez et al., 2004).

SPI-1 effectors also modulate immune responses to Salmonella infection, which differ between S. Typhimurium and S. Typhi. Inflammatory responses are triggered by SPI-1 effectors such as SopA (Wood et al., 2000; Zhang et al., 2006) and are favoured by S. Typhimurium, as neutrophil recruitment reduces the intestinal microbiota to give Salmonella a competitive advantage (Sekirov et al., 2010). However, SopA is a pseudogene in S. Typhi (Dougan and Baker, 2014) which correlates with the fact that inflammation is not a characteristic of S. Typhi infection. Furthermore, S. Typhi has mutations in genes such as ttrS that would otherwise enable NTS to capitalise on the high levels of reactive oxygen species generated by neutrophils (Dougan and Baker, 2014).

S. Typhimurium has also evolved SPI-1 dependent mechanisms to evade recognition and host defence responses. SPI-1 deletion S. Typhimurium mutants invaded primary porcine alveolar macrophages less efficiently than wild type, and SPI-1 effectors suppressed pro-inflammatory cytokine production and promoted macrophage death (Pavlova et al., 2011). Salmonella effector SopB can prevent infection-induced apoptosis by maintaining levels of the pro-survival kinase Akt (Knodler, Finlay and Steele-Mortimer, 2005). Other S. Typhimurium SPI-1 effectors with anti-inflammatory effects including avrA, which inhibits NF-KB signalling and
contributes to intracellular bacterial survival *in vivo* (Collier-Hyams *et al*., 2002; Wu, Jones and Neish, 2012), and *sptP*, which reduces membrane ruffling following bacterial entry and downregulates proinflammatory cytokine release (Lin, Le and Cowen, 2003; Johnson *et al*., 2017). Although *sptP* is also expressed in *S. Typhi* with a 94% sequence identity, it is not translocated into the host, indicating that *S. Typhi* and NTS employ different virulence strategies (Johnson *et al*., 2017).

### 1.4.3 SPI-2

Once *Salmonella* is internalised within the SCV, SPI-2 encodes effectors that are released across the phagosomal membrane into the host cell cytosol. These act to interrupt the normal progression of vesicle trafficking and maturation, allowing the SCV to escape from lysosomal degradation and instead act as an intracellular niche for *Salmonella* to replicate in (McGhie *et al*., 2009; Fàbrega and Vila, 2013; Liss *et al*., 2017). For example, a key SPI-2 effector is SopD2, which targets both Rab7 and Rab32 and inhibits normal endosomal trafficking (D’Costa *et al*., 2015; Spanò *et al*., 2016). SopD2 deletion in *S. Typhimurium* attenuates virulence in mice, although interestingly SopD2 is a pseudogene in *S. Typhi*, suggesting that *S. Typhi* employs a different evasion mechanism (Parkhill *et al*., 2001; Spanò *et al*., 2016). Indeed, a study in human macrophages suggested that SPI-2 was not essential for *S. Typhi* survival, whereas SPI-2 deficient *S. Typhimurium* is highly attenuated in mice (Hensel *et al*., 1998; Forest *et al*., 2010).

Other SPI-2 effectors in *S. Typhimurium* include SpiC, which inactivates the host protein Hook3, a key component of endosomes responsible for endosomal fusion with organelles (Uchiya *et al*., 1999; Shotland,Krämer and Groisman, 2003). *S. Typhimurium* can also recruit host proteins to the autophagosome, such as focal adhesion kinase (FAK) which inhibits autophagy (Owen, Anderson and Casanova, 2016).

Studies in *S. Typhimurium* have shown that SCVs traffic to the perinuclear region of the host cell, adjacent to the Golgi apparatus, where the replicative niche is reinforced by the formation of tubulovesicular *Salmonella*-induced filaments (SIFs) through the action of the SPI-2 effector SifA (Stein *et al*., 1996). SIFs form a network
that extends outwards into the host cell, using the host microtubular network, which serves to provide nutrients to the SCV (Rajashekar et al., 2008; Knuff and Finlay, 2017; Liss et al., 2017).

As well as modulating endosomal trafficking, multiple SPI-2 effectors also inhibit innate immune signalling in S. Typhimurium (Jennings, Thurston and Holden, 2017). For example, SpyC dephosphorylates ERK, p38 and MAPKs, thus inhibiting proinflammatory cytokine transcription (Mazurkiewicz et al., 2008). GtgA suppresses inflammation by cleavage of the DNA binding loop in p65 and RelB (Sun et al., 2016). SseK2 and SseK3 inhibit TNFa-stimulated NF-kB signalling (Yang et al., 2015; Günster et al., 2017). GogB mutants exhibit severe caecal inflammation in mice (Pilar et al., 2012). Finally, SpvD binds Exportin-2 and thus interrupts the import and export of KPNA1, thus inhibiting nuclear import of p65 (Rolhion et al., 2016).

1.4.4 S. Typhi specialisation

A comparative genomic analysis divided S. enterica into two subpopulations, referred to as clade A and clade B (den Bakker et al., 2011). S. Typhi and S. Paratyphi A were found in a subclade of clade A and shared SPIs including SPI-18, which has roles in invasion, and a cytolethal distending toxin (CDT) islet, which will be covered in greater detail in the following section. These islets were unique to the Typhi subclade and were not found in the other clade A serovars studied, which included NTS such as S. Typhimurium. Of the 98 species of S. enterica studied approximately 20% were in clade B, which were found to also contain SPI-18 and the CDT islet, as well as β-glucoronidase and S-fimbrial operons (den Bakker et al., 2011; Rodriguez-Rivera et al., 2015). These clade B serovars included S. Javiana and S. Montevideo, which are examples of disease-causing iNTS (den Bakker et al., 2011; R. A. Miller and Wiedmann, 2016).

Although S. Typhi and S. Typhimurium have a common core genome, S. Typhi shows specialised methods of immune evasion and dissemination within the host. S. Typhi has reduced approximately 10% of the NTS genome into pseudogenes, including genes that contribute to virulence and host interactions in S. Typhimurium
Furthermore, the S. Typhi genome contains 300-400 Typhi-specific genes in unique SPIs, including SPI-7, -15, -17 and -18 (Chan et al., 2003; Dougan and Baker, 2014).

SPI-7 encodes genes implicated in attachment to human cells, including a specialised type IVB pilus that binds to the host epithelial cystic fibrosis transmembrane conductance regulator (CFTR) and enables host adhesion (Tsui et al., 2003). This differs from NTS such as S. Typhimurium, which adhere to intestinal epithelial cells via specialised fimbriae (Bäumler, Tsolis and Heffron, 1996). The genetic sequences for these fimbriae were found to be compromised in S. Typhi and other serovars capable of causing systemic infection (Townsend et al., 2001; Bishop et al., 2008; Kisiela et al., 2012; Dougan and Baker, 2014).

SPI-7 also encodes the Vi capsule, an α(1→4)-D-GalpANAc homopolymer that encapsulates the S. Typhi bacterium and shields surface antigens from host innate immune recognition and response (Pickard et al., 2003; Dougan and Baker, 2014). The Vi capsule was shown to prevent TLR4-dependent recognition of S. Typhi and thus prevent macrophage production of pro-inflammatory IL-6 (Wilson et al., 2008). Furthermore, neutrophils did not extend chemotactic pseudopodia towards Vi-positive S. Typhi, but did towards S. Typhimurium and E.coli (Wangdi et al., 2014). This allows S. Typhi to evade an acute host inflammatory response and establish persistent and systemic infection. Indeed, S. Typhimurium engineered to express the Vi capsule was shown to persist in mice when compared to infection with the wild-type (Jansen et al., 2011).

S. Typhi is able to downregulate production of pattern-associated molecular patterns (PAMPs) to reduce host inflammatory responses. For example, S. Typhi encodes the transcriptional regulator TviA, which allows it to repress expression of flagellin which otherwise stimulates host production of proinflammatory IL-8 and pyroptosis (Winter et al., 2008, 2015).

S. Typhi infects dendritic cells or CD18+ phagocytes in the gut-associated lymph tissue (Watson and Holden, 2010). Rather than be degraded within the lysosome, S. Typhi can survive and replicate, and can use infected phagocytes to be disseminated throughout the body. Invasion of the lymph system allows S. Typhi to
access the liver, spleen and, via bile, the gallbladder, where it has been shown to establish replicative niches (Watson and Holden, 2010).

### 1.4.5 Gallbladder cancer

S. Typhi and S. Typhimurium encode environmental sensor kinases PhoQ and PhoP, which induce activation of genes providing resistance to the hostile environment of the gallbladder (van Velkinburgh and Gunn, 1999). This resistance enables S. Typhi to form biofilms on gallstones and to establish a chronic and asymptomatic infection within the gallbladder (Prouty, Schwesinger and Gunn, 2002; Crawford et al., 2010).

Chronic S. Typhi carriage within the gallbladder has been linked with gallbladder cancer, a cancer with poor prognosis (Di Domenico et al., 2017). Gallbladder cancer is rare in the western world but relatively common in India and Pakistan where 85% of worldwide typhoid cases occur (Scanu et al., 2015). There is a positive correlation between the presence of gallstones and gallbladder carcinoma with presence of both S. Typhi Vi and flagellin (Dutta et al., 2000; Nath et al., 2008). For example, 44% of gallbladder cancer patients were positive for S. Typhi in Chile, where typhoid is endemic (Koshiol et al., 2016). Between 1922 and 1975 in New York, chronic typhoid carriers were 6 times more likely to die of cancer than controls (Welton, Marr and Friedman, 1979).

S. Typhi infection was shown to transform susceptible mice, gallbladder organoids and mouse embryonic fibroblasts (MEFs) via activation of AKT and MAPK pathways (Scanu et al., 2015). Susceptibility to transformation was dependent on mutations in P53 and amplification of c-Myc, suggesting that patients with mutations in these oncogenes may be predisposed to developing gallbladder cancer following S. Typhi infection (Scanu et al., 2015).

The study did not identify which S. Typhi effectors were responsible for AKT and MAPK-dependent transformation. However, it is well established that a common trigger of oncogenesis and malignant transformation is DNA damage, and S. Typhi encodes a genotoxic virulence factor known as the typhoid toxin. Thus, the typhoid
toxin is an interesting candidate for linking S. Typhi infection and gallbladder cancer.

1.5 The typhoid toxin

1.5.1 Cytolethal distending toxins

The typhoid toxin was first identified in S. Typhi as a cytolethal distending toxin (CDT), with its name coined because of its suggested link to typhoid fever symptoms (Haghjoo and Galán, 2004). CDTs induce a DNA damage response (DDR), cellular and nuclear distension, and subsequently cell death, in eukaryotic cells. They have been identified in multiple bacterial species including Campylobacter spp., enteropathogenic Escherichia coli (Pérès et al., 1997), Shigella dysenteriae (Okuda, Kurazono and Takeda, 1995), Haemophilus ducreyi (Cope et al., 1997), Actinobacillus actinomycetemcomitans (Sugai et al., 1998; Yamano et al., 2003), and Helicobacter hepaticus (Young, Knox and Schauer, 2000). Interestingly, since the discovery of the typhoid toxin more than 40 NTS serovars have been found to encode CDT-toxins distinct from the typhoid toxin, which also cause DDRs and have been shown to influence infection (den Bakker et al., 2011; Rodriguez-Rivera et al., 2015; R. Miller and Wiedmann, 2016; Miller et al., 2018).

CDTs in most species are tripartite oligomeric proteins that are secreted by bacteria during infection. They consist of the CdtA, CdtB and CdtC subunits. CdtA and CdtC are responsible for delivery of the DNase-I like CdtB into the host nucleus, where CdtB acts as the catalytic subunit and induces a DDR and cell cycle arrest (Lara-Tejero and Galán, 2000; Hassane et al., 2001; Lara-Tejero and Galán, 2001, 2002; Li et al., 2002; Hassane, Lee and Pickett, 2003; Guerra et al., 2011). This damage was shown to be via induction of single strand breaks in DNA that then developed into double strand breaks during DNA replication (Fedor et al., 2013). CDTs have been shown to induce apoptosis in intoxicated cells (Gelfanova, Hansen and Spinola, 1999; Cortes-Bratti et al., 2001; Alaoui-El-Azher et al., 2010). Chronic exposure to CDTs has been linked to increases in chromosomal instability and mutation frequency (Guidi, Guerra, et al., 2013).
Intriguingly, CdtB has attenuated nuclease activity relative to DNase I, displaying only 0.01% efficiency compared to bovine DNase I (Elwell et al., 2001). However, mutagenesis of conserved residues between DNase I and CdtB abrogates the ability of CdtB to cause cell cycle arrest (Elwell and Dreyfus, 2000). For example, two histidines (H160 and H274) were found to be critical for CdtB toxicity, and a H160Q mutation was sufficient to abolish toxicity (Nešić, Hsu and Stebbins, 2004). Furthermore, comet assays and pulsed field gel electrophoresis have shown that DNA is fragmented by CdtB (Elwell and Dreyfus, 2000; Frisan et al., 2003; Fedor et al., 2013; Fahrer et al., 2014).

The S. Typhi genome was found to contain a sequence with 50% sequence similarity to the CdtB subunit of other CDTs. Interestingly, however, S. Typhi contains no homologues for CdtA or CdtC (Haghjoo and Galán, 2004). Instead, S. Typhi was found to encode two sequences homologous to the pertussis toxin of Bordetella pertussis: pertussis-like toxin A and B (PltA and PltB) (Fig. 1.2). The typhoid toxin is a hybrid toxin formed of one subunit of each of CdtB and PltA, with a PltB pentamer, together formed into a pyramid-like structure. CdtB performs the same DNase-like function as seen in other CDTs, and the ADP-ribosylating subunit PltA acts as a linker region to PltB, which binds glycoproteins on the host cell-surface membrane to mediate toxin uptake (Spanò, Ugalde and Galán, 2008; Song, Gao and Galan, 2013).
Typhoid toxin orthologues are also found in iNTS serovars. Predicted peptide products of typhoid toxin orthologues across Typhi and the iNTS serovars Javiana, Montevideo, Oranienburg and Mississippi was found to have 98.1%, 96.1%, and 99.4% conserved amino acids for pltA, pltB, and cdtB (R. A. Miller and Wiedmann, 2016). The toxin of iNTS S. Javiana was found to induce DNA damage and cell cycle arrest in a similar manner to typhoid toxin, but to elicit different clinical presentations dependent on differences in the receptor binding subunit pltB (R. A. Miller and Wiedmann, 2016; Lee et al., 2020).

1.5.2 Secretion and delivery of the typhoid toxin

The toxin is synthesised by S. Typhi and secreted into the SCV lumen via a holin/endolysin system (Galán, 2016). The timing of toxin secretion is dependent on the *Salmonella* PhoP-PhoQ sensing system, which induces toxin expression upon
detection of the environment conditions within the SCV (Fowler and Galán, 2018). The transcriptional regulator IgeR also prevents CdtB production when *Salmonella* is in an extracellular environment (Haghjoo and Galán, 2007).

Toxin subunits are individually exported from the bacterial cytoplasm into the periplasm, where the full toxin is assembled. The endolysin TtsA (typhoid toxin secretion A), an N-acetyl-b-D-muramidase, is encoded in the same genomic islet as the subunits of the typhoid toxin. TtsA is released into the S. Typhi periplasmic space via pores in the inner membrane formed by holins (Geiger *et al.*, 2018). Once delivered, TtsA catalyses localised and controlled disruption of the peptidoglycan layer of the bacterial cell wall, allowing for selected proteins, such as the typhoid toxin, to be secreted through it (Hodak and Galán, 2013). TtsA was found to not be necessary for CDT-toxin secretion in non-typhoidal *Salmonella* serovars (Miller *et al.*, 2018).

Once in the SCV lumen, the toxin is exocytosed from the host cell via outer membrane vesicle intermediates in a process dependent on SPI-2 effectors including SifA (Spanò, Ugalde and Galán, 2008; Guidi, Levi, *et al.*, 2013). The toxin can then be endocytosed in an autocrine or paracrine manner.

Plasma membrane binding on the target cell is mediated by the PltB pentamer, which recognises and binds specific N-linked surface glycans. The toxin was found to bind tri-antennary sialylated glycoproteins with the greatest binding affinity, specifically with the consensus sequence Neu5Ac2-3Galβ1-3/β1-4Glc/GlcNAc (Song, Gao and Galan, 2013; Deng *et al.*, 2014; Galán, 2016). However, it could bind to a wide range of N-linked glycoproteins and even glycolipids, though with reduced affinity, allowing the toxin to bind a wide variety of cell types (Song, Gao and Galan, 2013). The presence of Neu5Ac in the consensus sequence is particularly interesting. Human sialoglycans primarily terminate with Neu5Ac, as humans do not express CMP-N-acetylneuraminic acid hydroxylase (CMAH), which converts Neu5Ac to Neu5Gc (Varki *et al.*, 2011). CMAH is expressed in most mammals, meaning that most non-human mammalian sialoglycans are terminated in Neu5Gc. Whilst the typhoid toxin binds Neu5Ac terminated glycans with high affinity, it does not recognise Neu5Gc, meaning that the toxin can recognise and
bind human cells but not cells of many other mammals. This is another example of the human specificity of S. Typhi, and could be reason for it only causing disease in humans (Deng et al., 2014). For example, S. Typhi only causes mild symptoms consistent with NTS infection in chimpanzees (Edsall et al., 1960; Gaines, Tully and Tigertt, 1968) which express CMAH and thus Neu5Gc. Mice, however, do still express Neu5Ac and therefore can be used as models for investigating the effects of the toxin.

In a process similar to other CDTs, the typhoid toxin has been shown to be transported in a retrograde manner from endosomes to the Golgi and endoplasmic reticulum, exploiting the endoplasmic reticulum associated degradation (ERAD) pathway to be translocated into the cytosol and then into the nucleus via nuclear pores (Guerra et al., 2005, 2009; Frisan, 2016; Chang et al., 2019). An N-terminal segment of Actinobacillus actinomycetemcomitans CdtB was found to be necessary for active nuclear localisation, although it is currently unknown exactly how the typhoid toxin enters the nucleus (Nishikubo et al., 2003). Once in the nucleus, CdtB of the typhoid toxin has been shown to cause DNA damage and cell cycle arrest, which is consistent with CdtB of other CDTs (Song, Gao and Galan, 2013; Ibler et al., 2019).

1.5.3 *In vivo* effects of the typhoid toxin

The typhoid toxin is currently seen as one of the principal virulence factors of S. Typhi. Many pathogens use genotoxic virulence effectors to exploit host DDRs as a strategy to promote infection and pathogen survival (Weitzman and Weitzman, 2014; Grasso and Frisan, 2015; Chumduri et al., 2016). When the typhoid toxin was first discovered it was suggested that it had a role in causing typhoid fever symptoms (Song, Gao and Galan, 2013), but more recently studies have suggested a role in promoting chronic and systemic infection, as well as having a significant effect on bacteraemia (Del Bel Belluz et al., 2016; Miller et al., 2018; Gibani et al., 2019). Furthermore, the toxin was shown to induce an anti-inflammatory response in healthy mice, suggesting a role in immunomodulation and possibly immune evasion (Martin et al., 2021).
Peritoneal injection of the toxin into mice caused symptoms of typhoid fever, including lethargy, weight loss, and a reduction of leukocytes and neutrophils, with death in all mice occurring after 5 days (Song, Gao and Galan, 2013). The phenotype was dependent on the catalytic site of CdtB, as an H160 mutant did not have the same effect. Constitutive expression of Neu5Gc-glycosylated receptors in the mice was sufficient to provide resistance to the effects of the toxin, showing that receptor-binding of the toxin was necessary for the phenotype (Deng et al., 2014).

However, in an infection model using recombinant S. Typhimurium engineered with the toxin islet, the presence of the toxin promoted mouse survival and significantly reduced gut inflammation in the early stages of infection (Del Bel Belluz et al., 2016). Using S. Typhimurium strains developed to induce typhoid-fever symptoms in mice, the study showed that infection with toxin-negative S. Typhimurium resulted in death for 40% of the infected population after 15 days with excessive inflammation identified in the gut. However, all mice infected with toxin-expressing S. Typhimurium survived the full course of the experiment. Interestingly, whereas the toxin reduced the gut inflammatory response it enhanced the inflammatory response in the liver. The toxin also promoted chronic carriage, with toxigenic S. Typhimurium isolated from livers, caeca, and MLNs of infected mice 180 days post-infection. No Salmonella was recovered at the 180-day timepoint from mice infected with control non-toxigenic strains indicating toxin-dependent chronic carriage (Del Bel Belluz et al., 2016). Expression of the toxin was found to promote infection of the liver in mice by the toxigenic NTS serovar S. Javiana, suggesting a role in immune evasion and systemic dissemination (Miller et al., 2018). Furthermore, infection with toxigenic Salmonella was shown to cause both a DDR and senescence in vivo, but interestingly also a toxin-dependent anti-inflammatory environment which is characteristic of S. Typhi infection (Martin et al., 2021).

In 2019 a human challenge study suggested that the toxin in fact had no role in the initiation of typhoid fever symptoms (Gibani et al., 2019). 40 human volunteers were infected with wild-type or toxin-negative S. Typhi and monitored for 14 days until treatment with antibiotics. Interestingly there was no significant difference in the rate of typhoid infection or clinical manifestations between infection strains, aside
from a significantly prolonged bacteraemia in volunteers treated with the toxin-negative strain (i.e., wild-type 48h, toxin-negative 96h). Understandably, the study could not investigate differences in severe typhoid disease or bacterial carriage (Gibani et al., 2019).

Based on current \textit{in vivo} data it was hypothesised that the typhoid toxin is secreted by \textit{S. Typhi} to facilitate infection strategies such as immune evasion, dissemination and chronic carriage. However, it is unknown how this hypothesis correlates with \textit{in vitro} studies of the toxin, which show that it has a severe genotoxic effect on host cells. It is possible that the toxin is the transformative effector causing gallbladder cancer in cases of chronic \textit{S. Typhi} infection. My PhD thesis aims to explore how the host responds to intoxication and counteracts dangerous phenotypes linked to DNA damage, such as cancer. To address this, I will first describe the host DDR.
2 DNA Damage Response

DNA is a fragile molecule that is prone to damage. This damage can be caused exogenously, such as by genotoxic molecules or radiation, or endogenously, such as in the process of DNA replication. Tens of thousands of DNA damage events occur in a single human cell every day, meaning that DNA metabolism and maintenance is a constantly active process (Jackson and Bartek, 2009).

Damage can be characterised by the appearance of single or double strand breaks (DSBs and SSBs respectively) in the DNA backbone, mismatches, base modifications such as alkylation or deamination or the appearance of crosslinks between stacked bases (Jackson and Bartek, 2009; Deans and West, 2011). All of these are potentially mutagenic, and all can cause the replication machinery to stall, putting the cell in a state of replication stress.

A variety of DDR pathways stabilise vulnerable sites, recognise damage, recruit repair proteins, and pause the cell cycle, allowing for the cell to initiate repair before the damage can escalate (Jackson and Bartek, 2009).

2.1 Causes of DNA Damage

2.1.1 Exogenous causes

Exogenous damage to DNA can occur through high energy radiation such as ultraviolet (UV) or ionising radiation (IR), or via numerous chemical agents, including bacterial genotoxins and chemotherapeutic drugs.

UV radiation, particularly at >280 nm wavelength, induces reactions and covalent structural rearrangements in pyrimidines leading to the formation of cytotoxic derivatives (Rastogi et al., 2010). IR can ionise either DNA itself or lead to the formation of high-energy radical oxygen species that subsequently interact with DNA. This can result in both SSBs and DSBs as well as base modifications and DNA-protein crosslinks, which can proliferate by generation of further radicals (Mavragani et al., 2019).
DNA damaging agents are often targeted against the components of the replication fork or associated machinery. For example, camptothecin and doxorubicin stabilise the interaction between topoisomerase and DNA or block it from binding, preventing it from relieving torsional stress in unreplicated DNA upstream of replication forks that leads to DNA breaks (Hsiang *et al.*, 1985; Wassermann *et al.*, 1990; Sørensen *et al.*, 1994). Hydroxyurea (HU) inhibits synthesis of deoxyribonucleotides, thus depleting the pool necessary for DNA replication and resulting in stalling of the replication fork (Collins and Oates, 1987). Aphidicolin is an inhibitor of DNA polymerase α (Pol-α), forming a bond with DNA close to the nucleotide binding site of polymerase that results in replication stress and DNA breaks (Baranovskiy *et al.*, 2014).

### 2.1.2 Endogenous causes

DNA damage can occur through errors in DNA replication, damage by reactive oxygen species and spontaneous hydrolysis at 37°C (Lindahl and Barnes, 2000). For example, DNA polymerases have an error rate of 1 in every 1000-30000 bp, leading to a high rate of incorporated mismatched bases. However proper function of the DNA repair response reduces these mismatches to 1 in $10^{'10}$-10$^{11}$ (Kunkel and Loeb, 1981).

Helicases unwind the DNA double helix for replication or transcription, and in doing so inflict significant torsional stress on adjacent double stranded DNA, which if untreated would lead to DNA breakage. Topoisomerases reduce this tension upstream of helicase by introducing SSBs in double strand DNA (Wang, 1985; Pommier *et al.*, 1998). These cleavages are resealed, but in the case that they are not, for example if the replication machinery stalls and breaks down, then this also leaves untreated single strand nicks in the DNA backbone.

### 2.2 Replication stress

Sites of DNA damage can block the progression of DNA polymerase. During DNA replication, helicases generate two single strand templates that are then processed by DNA polymerase. If DNA polymerase stalls while helicase continues to race
ahead, long tracts of single-strand DNA (ssDNA) form, resulting in a state of replication stress. Unless stabilised, stalled forks are vulnerable to collapse. ssDNA is more fragile than double-strand DNA (dsDNA), and indeed ssDNA at the replication fork has been shown to be a precursor to chromosomal breakage (Feng et al., 2011). Widespread fork breakdown is known as replication catastrophe, characterised by massive DNA breakage and disruption of the entire genome (Toledo et al., 2014; Toledo, Neelsen and Lukas, 2017). DNA replication is therefore a carefully monitored process.

2.3 Apical Kinases

Cells use a variety of sensing pathways to identify mismatched bases, cross-linking, SSBs or DSBs. Mismatched bases, base modifications, and cross-linked bases can be excised and replaced by the correct base (Jalal, Earley and Turchi, 2011). However longer tracts of ssDNA or DSBs cannot be repaired in this manner and require more complex process. In these cases, three kinases are recruited to sites of damage using evolutionarily conserved motifs, where they regulate cell cycle checkpoints, prevent origin of replication firing, and trigger accumulation of specific repair factors (Shechter, Costanzo and Gautier, 2004; Falck, Coates and Jackson, 2005; Bekker-Jensen et al., 2006). DNA dependent protein kinase (DNA-PK) and Ataxia Telangiectasia mutated (ATM) are recruited at DSBs (Caron et al., 2015), whereas ATM and RAD3-related (ATR) is recruited to SSBs (Blackford and Jackson, 2017) (Fig. 2.1).
**Fig. 2.1 DNA damage responses to single- and double-strand DNA breaks**

ATM is recruited to DSBs by the MRN complex, where it phosphorylates multiple effectors including CHK2 and BRCA1, and 53BP1 via a phosphorylation and ubiquitination cascade. BRCA1 phosphorylation triggers homology directed repair, whereas 53BP1 can trigger non-homologous end joining. (B) Ku 70/80 cap either end of a DSB and recruit DNA-PKcs, which recruits repair factors including LIG4, Artemis and XRCC4. (C) Stalled DNA polymerase during replication results in lengthening tracts of fragile ssDNA. RPA stabilises ssDNA and recruits ATR via the adaptor protein ATRIP. RPA also stimulates the binding of the RAD9-RAD1-HUS1 (9-1-1) checkpoint clamp, which binds TopBP1, which subsequently activates ATR. ATR phosphorylates effector proteins such as CHK1, which reduces replication fork progression, origin firing and the G2/M transition. This reduces the burden of replication stress on the cell, prevents further damage, and allows the cell to activate DNA repair pathways. (D) Phosphorylated CHK1 and CHK2 inhibit CDC25A and thus cell cycle progression. CHK1 and 53BP1 also activate P53, which is a crucial transcription factor regulating cell fate decisions including entry into apoptosis or senescence via target genes including P21.

2.3.1 Double-strand break response

**ATM** is the master regulator of the cell response to DNA DSBs. A kinase, it phosphorylates a variety of substrates involved in DNA repair, the cell cycle and cell fate decisions. ATM is recruited to DSBs by the MRE11-RAD50-NBS1 (MRN) complex and undergoes autophosphorylation at numerous sites, resulting in ATM monomerization and activation (Bakkenist and Kastan, 2003; Kozlov et al., 2011).
ATM can also be activated in an MRN-independent manner, in response to oxidative stress or certain conformational changes in chromatin (Kim et al., 2009; Guo et al., 2010).

Upon activation, ATM initiates a phosphorylation and ubiquitination signalling cascade. A key phosphorylation target is H2AX, one of four histone H2A variants that makes up 11-25% of H2A within the cell (West and Bonner, 1980; Kinner et al., 2008). H2A phosphorylation at Ser139 is a marker of DNA damage and leads to formation of phospho-H2AX (γH2AX). Phosphorylation occurs quickly following damage and has been seen as soon as 1 minute after exposure to IR whilst peaking 15-20 minutes after exposure (Rogakou et al., 1999; Redon et al., 2009). γH2AX does not act as the initial sensor of DNA damage, but rather a platform for recruitment of further DNA repair factors, and acts to transmit signal up to 15 Mbp away from the site of damage via further H2AX phosphorylation (Rogakou et al., 1999; Celeste et al., 2003; Bewersdorf, Bennett and Knight, 2006; Dellaire, Kepkay and Bazett-Jones, 2009). This amplification signal both recruits DNA repair factors from further away and arrests transcription or replication in proximity to the break to prevent further damage.

As depicted in Fig. 2.1A, γH2AX recruits mediator of DNA damage checkpoint 1 (MDC1), bringing it into proximity with ATM where it is phosphorylated (Stucki et al., 2005). This initiates ubiquitination of H1 and H2A by ubiquitin ligases RNF8 and RNF168 (Mattiroli et al., 2012). The scaffold protein P53 binding protein 1 (53BP1) is then recruited to the DSB, which recruits further DNA repair proteins (Blackford and Jackson, 2017).

DNA-PKcs is recruited to DSBs by Ku70/80 (Fig. 2.1B), a basket-shaped heterodimer that caps each dsDNA end of the break (Walker, Corpina and Goldberg, 2001). The resulting holoenzyme, DNA-PK, binds both Ku ‘caps’ and thus brings the DNA ends together for ligation.

Both ATM and DNA-PK initiate repair of DSBs by non-homologous end joining (NHEJ), a cell cycle independent repair process (Riballo et al., 2004; Jette and Lees-Miller, 2015). NHEJ does not use a template for repair, making it potentially error prone and mutagenic, although a low rate of errors is observed (Bétermier,
Bertrand and Lopez, 2014). DNA-PK autophosphorylation allows recruitment of NHEJ factors including Artemis, which processes both ends using 5’ to 3’ endonuclease activity (Ma et al., 2002). Ligation of DNA ends is carried out by DNA-ligase IV and stabilising factor XRCC4 (Sibanda et al., 2001).

ATM and Artemis can also promote homology directed repair (HDR) in G2 phase by phosphorylation of the tumour suppressor BRCA1 (Beucher et al., 2009). HDR uses a replicated intact sister chromatid as a template for repair, and directly competes with 53BP1-dependent NHEJ activation. DNA ends are resected into ssDNA overhangs by nuclease to allow invasion of a template strand. Elongation and ligation complete the repair (Pardo, Gómez-González and Aguilera, 2009).

### 2.3.2 Single-strand break response

**ATR** is recognised as being the main responder to replication stress (Fig. 2.1C). Inhibition of DNA-PK (but not ATM) has been shown to cause replication stress, suggesting a role as well (Liu et al., 2012). ATR is key to preventing collapse of fragile ssDNA sites into more severe DSBs. ATR deficiencies have been shown to lead to an increase in fragile sites on chromosomes, which increases the chances of replication stress or mutagenesis (Casper et al., 2002). Repetitive DNA sequences are prone to forming secondary structures and are thus vulnerable to polymerase stalling and replication stress during DNA replication. ATR was found to be essential for prevention of fork collapse at these sites (Shastri et al., 2018).

The ATR pathway is activated by binding of replication protein A (RPA) bound to ssDNA. RPA is a highly conserved heterotrimer formed of 70 kDa, 32 kDa and 14 kDa subunits (Wold, 1997). RPA protects ssDNA from collapse into DSB, prevents formation of fork stalling hairpins, and prevents untimely reannealing of ssDNA during homology directed DNA repair. RPA is phosphorylated upon binding to ssDNA and acts as a dynamic scaffold which regulates association and dissociation of repair factors (Fanning, Klimovich and Nager, 2006). Exhaustion of nuclear pools of RPA lead to escalation of replication stress and resultant replication catastrophe, signified by nuclear-wide chromosomal breakage which drives the cell into
senescence (Toledo et al., 2014; Toledo, Neelsen and Lukas, 2017; Ibler et al., 2019).

Independently, two complexes translocate to the RPA-ssDNA complex. ATR, via ATR-interacting protein (ATRIP), binds to ssDNA via RPA (Cortez et al., 2001; Zou and Elledge, 2003). Meanwhile, RPA stimulates the binding of the RAD17-Rfc2-5 (RSR) complex to ssDNA, and facilitates the subsequent binding of the RAD9-RAD1-HUS1 (9-1-1) checkpoint clamp in an ATP-dependent reaction (Bermudez et al., 2003; Zou, Liu and Elledge, 2003). Rad9 binds DNA topoisomerase 2-binding protein 1 (TopBP1), which subsequently activates ATR (Kumagai et al., 2006; Lee, Kumagai and Dunphy, 2007). Another protein, ETAA1, also activates ATR and is brought into proximity to ATR by direct binding with RPA (Bass et al., 2016; Feng et al., 2016; Haahr et al., 2016; Lee et al., 2016). Whether TOPBP1 and ETAA1 act as redundancy measures towards each other, or whether they are necessary for ATR activation in different scenarios is unknown.

ATR activation also leads to γH2AX foci formation, suggesting that γH2AX has a role in surveillance of replication. γH2AX was shown to colocalise with PCNA, BRCA1 and 53BP1 at arrested replication forks in S phase cells (Ward and Chen, 2001).

Another protein known as Schlafen-11 (SLFN11) also binds RPA in an ATR-independent manner and acts to inhibit replication fork progression, suggesting that there are redundant pathways for sensing replication stress (Murai et al., 2018).

2.4 Cell cycle regulation

The cell cycle is a carefully regulated series of phases and checkpoints that control DNA replication and cell division. Diploid cells begin in G1 phase with two sets of 23 chromosomes. They undergo DNA replication in S phase, upon which they enter G2 phase. Cells then undergo mitosis (M phase) and split into two G1 daughter cells. Cells can also enter G0 phase, where they adopt quiescence and the cell cycle is temporarily paused (Vermeulen, Van Bockstaele and Berneman, 2003).

Progression through the cell cycle is controlled by cyclins, which control cyclin-dependent kinases (CDKs) and a resultant phosphorylation cascade promoting
progression of the cell cycle. A series of checkpoints monitor entry of the cell into each phase of the cell cycle and will arrest the cell cycle in certain conditions such as unrepaired DNA damage. Cell cycle arrest is controlled by CDK inhibitors such as P21, a P53 target, which prevents formation of the CDK-cyclin complex (Deshpande, Sicinski and Hinds, 2005) (Fig. 2.1D). P21, alongside another CDK inhibitor P16, prevents CDK-driven phosphorylation of retinoblastoma tumour suppressor protein (Rb). In its unphosphorylated state, Rb sequesters the E2F transcription factor and thus prevents transcription of genes essential for G1 to S phase transition (Ohtani et al., 2004).

ATR serves as a key S/G2 phase checkpoint, preventing early S phase exit and premature entry into mitosis (Saldivar et al., 2018). CHK1 is recruited by the mediator protein Claspin and phosphorylated by ATR (Kumagai and Dunphy, 2000; Liu et al., 2000). Phosphorylated CHK1 (pCHK1) is a kinase that inhibits origin of replication firing, preventing accumulation of further DNA damage (Maya-Mendoza et al., 2007) (Fig. 2.1D).

ATM recruits a similar kinase, CHK2, which also regulates a key cell cycle checkpoint. CHK1 and CHK2 phosphorylate CDC25 (cell division control protein 25), a phosphatase that activates cyclin dependent kinases CDK1 and CDK2. Phosphorylation promotes CDC25 degradation and blocks CDK-1 and -2 dependent mitotic entry (Furnari, Rhind and Russell, 1997; Peng et al., 1997; Sanchez et al., 1997; Matsuoka et al., 2000) (Fig. 2.1D).

When a cell undergoes DNA damage, it can temporarily exit from the cell cycle and activate DNA repair pathways to fix the damage. If the damage is too excessive to be repaired, the cell must prevent further propagation of mutations which could lead to tumorigenesis. It can do this via controlled self-destruction (apoptosis) or permanent cell cycle arrest (senescence). A key regulator of these cell fates is P53.

2.4.1 P53

P53 is a key transcription factor that determines whether the cell activates pro-survival or apoptotic pathways (Tyner et al., 2002; Dumble et al., 2007; Shu, Li and Wu, 2007). It is phosphorylated and activated by CHK1 via ATR and 53BP1 via ATM
(Tibbetts et al., 1999; Cuella-Martin et al., 2016). ATR activation was found to induce P53-dependent cell cycle arrest and senescence, even in the absence of any DNA damage (Toledo et al., 2008).

P53 is continually produced and degraded within the cell by Mdm2-dependent ubiquitination (Haupt et al., 1997). P53 phosphorylation at Ser-15 and -37 inhibits ubiquitination and degradation and results in accumulation of activated P53 (Shieh et al., 1997). When activated, P53 acts as a transcription factor for target genes involved in apoptosis, as well as cell cycle arrest, including CDK inhibitors. This includes P21, which inhibits cyclin B and CDC2, leading to cell cycle arrest during any phase of the cell cycle (El-Deiry et al., 1993; Agarwal et al., 1995, 1998; Innocente et al., 1999).

2.4.2 Apoptosis

If DNA damage is extensive enough that it cannot be repaired, the cell can self-destruct in a programmed way that prevents release of immunogenic debris (Taylor, Cullen and Martin, 2008). DNA-PK phosphorylation of γH2AX is required for apoptosis (Mukherjee et al., 2006). γH2AX foci migrate to the nuclear periphery during early apoptosis, forming an ‘apoptotic ring’ and localising with ATM, DNA-PK and Chk2 (Solier et al., 2009; Solier and Pommier, 2014). P53 mediates inactivation of the pro-survival gene B-cell lymphoma 2 (Bcl-2) via a downstream GTPase Cdc42 (cell division control protein 42) (Thomas, Giesler and White, 2000). P53 also upregulates the homologous Bcl-2-associated X (BAX), which penetrates the outer mitochondrial membrane to release pro-apoptotic markers that trigger a caspase cascade (Toshiyuki and Reed, 1995; Gross et al., 1998; Tait and Green, 2010). Caspases are proteases that cleave substrates including PARP and lamins (Lazebnik et al., 1994, 1995). Morphological features of apoptotic cells include nuclear fragmentation, shrinkage, and condensation of chromatin, as the cell is carefully packaged into small blebs that can be endocytosed and degraded by immune cells (Kerr, Wyllie and Currie, 1972).
2.4.3 Senescence

Senescence is permanent cell cycle arrest, characterised by cell distension, flattening, and secretion of a senescence-associated secretory phenotype (SASP) (Hernandez-Segura, Nehme and Demaria, 2018). Common markers of senescent cells include expression of senescence-associated $\beta$-galactosidase, tumour suppressor P16 and a persistent DNA damage phenotype (Hernandez-Segura, Nehme and Demaria, 2018).

What triggers the cell to enter senescence is unclear and is likely based on context and different factors. However, the P53 target gene P21 is known to be an important regulator of entry into senescence. Persistence of DNA repair intermediates leads to accumulation of P21 and nuclear entrapment of cyclin B1, leading to permanent G2 arrest (Feringa et al., 2018). The decision to enter senescence appears to be based in the relative levels of P21 and high mobility group box 1 (HMGB1). Doxorubicin-induced senescent cells showed elevated levels of P21 and HMGB1 as opposed to apoptotic cells, which displayed reduced levels of both (Lee et al., 2019).

Although senescence is typically viewed as a mechanism of preventing cancer, senescence can be pro-tumorigenic. Senescence can induce a senescence-associated inflammatory response which can be pro- or anti-tumorigenic depending on P53 activity (Pribluda et al., 2013). SASP can include pro-inflammatory factors including IL-6, IL-1, and IL-8, which can promote inflammation and immune cell migration. This can trigger aberrant effects in surrounding cells, including secondary senescence (Secher et al., 2013; Hernandez-Segura, Nehme and Demaria, 2018; Ibler et al., 2019). Chronic inflammation can cause tissue damage and lead to tumorigenesis (Coppé et al., 2010).

2.5 The DDR to the typhoid toxin

Previous studies in the Humphreys lab revealed that the typhoid toxin activated both ATR and ATM, suggesting the formation of both SSBs and DSBs. The toxin induced hyperphosphorylation of RPA and replication stress in S/G2 phase, leading
to ATR activation and downstream phosphorylation of P53 and CHK1. The toxin also induced DSBs labelled by 53BP1 in G0/G1 phase (Ibler et al., 2019).

Furthermore, toxin-induced replication stress was shown to induce a senescence-like phenotype. This was characterised by cell distension, permanent cell cycle arrest and a secretory phenotype that induced secondary senescence in bystander cells. Cells treated with secretomes from intoxicated cells underwent senescence and became more susceptible to Salmonella infection, suggesting that the toxin was creating replicative niches and facilitating infection spread (Ibler et al., 2019). This correlated with in vivo data implicating the toxin in systemic bacterial dissemination, chronic carriage, bacteraemia and suppression of host inflammatory responses (Del Bel Belluz et al., 2016; Miller et al., 2018; Gibani et al., 2019).

Toxin nuclease activity saturated the RPA pathway through excessive SSB production, which provided mechanistic details of how the toxin-induced replication stress caused this senescence response facilitating infection (Ibler et al., 2019). Senescence caused by replication stress has been implicated in many disease states, such as progeria, which is characterised by premature ageing. Expression of a truncated form of lamin, known as progerin, results in loss of lamin function and leads to replication stress (Kreienkamp et al., 2018). In this study and in others, replication stress has been shown to cause leakage of fragments of damaged DNA into the cytosol, activating an immune response that resulted in cellular senescence (Wolf et al., 2016; Kreienkamp et al., 2018). The DDR has been shown to directly activate immune responses in different contexts, characterised with induction of antimicrobial peptides and recruitment of immune cells (Nakad and Schumacher, 2016). Furthermore, recent studies have shown that other bacterial genotoxins such as E. coli CDT induce immune responses in a DNA damage-dependent manner (Pons et al., 2021).

The immune response is key to host recognition of threats, including DNA damage. Innate immune pathways recognise markers of damage and regulate cell fate decisions to suppress tumorigenic effects. This includes triggering apoptosis or senescence to prevent replication of potentially cancerous cells. It was hypothesised that the toxin was triggering a replication stress-induced immune
response to promote senescence and thus a *Salmonella*-permissive microenvironment. To explore this hypothesis, I will first describe the innate immune response with a focus on the downstream responses to DNA damage.
3 Innate Immune Responses to DNA damage

3.1 Introduction

Pathogen invasion is typically accompanied by release of numerous molecular motifs such as bacterial lipopolysaccharides, flagellin, and bacterial and viral nucleic acids RNAs (Akira, Uematsu and Takeuchi, 2006; Li and Wu, 2021). These motifs are known as pathogen associated molecular patterns (PAMPs). Cellular stresses, such as such as genomic instability within cancer cells, cause release of damage-associated molecular patterns (DAMPs). For example, a major DAMP is cytoplasmic DNA released from the nucleus or mitochondria (Dempsey and Bowie, 2015; Grazioli and Pugin, 2018). Replication stress leads to formation of micronuclei containing aggregates of dsDNA breaks (Xu et al., 2011), which can rupture and spill free DNA into the cytoplasm (MacKenzie et al., 2017; Bakhoum et al., 2018).

These PAMPs and DAMPs act as ligands for an array of pattern recognition receptors (PRRs), which stimulate antimicrobial or proinflammatory responses including interferon (IFN) induction in order to contain the threat (Haller, Kochs and Weber, 2007) (Fig. 3.1).
A  PRR activation of the type 1 IFN pathway

B  The Type 1 IFN pathway
DNA damage or pathogen invasion introduce immunogenic nucleic acids into the cytosol. Cytosolic DNA and RNA are detected by different pattern recognition receptors such as cGAS, Ku70/DNA-PK, RIG-I and MDA5, among others. These PRRs, once activated, initiate signalling responses via STING, which phosphorylates the kinase TBK1. TBK1 in turn phosphorylates IRF3, causing it to dimerise and translocate to the nucleus and promote expression of IFNs. (B) Type-I IFNs bind their cognate receptor, activating a JAK-STAT system and stimulating phosphorylation of STAT1. Phosphorylated STAT1 forms the heterotrimeric transcription factor ISGF3 with IRF9. ISGF3 translocates to the nucleus and promotes expression of a wide variety of IFN stimulated genes.

3.2 Interferons

When host cells are challenged by pathogen invasion, they mount a rapid innate immune response to counteract the threat. This may take the form of direct inactivation of pathogenic virulence factors, or activation of different cell fate pathways to prevent infection spread. Conversely, some pathogens have evolved mechanisms by which they are able to manipulate these immune responses to their advantage.

DNA damage also causes induction of IFNs. For example, the DNA damage inducer etoposide was shown to initiate type I and II IFNs, IFN stimulated genes (ISGs) and IFN regulatory factors-1 and -7 in an NF-κB dependent manner (Brzostek-Racine et al., 2011). IFN induction has also been linked to replication stress (Wolf et al., 2016; Kreienkamp et al., 2018).

The IFN system is an important component of the innate immune response. IFNs were first discovered in 1957 as secreted factors that exerted an antiviral effect, so named because they were able to ‘interfere’ with viral replication (Isaacs and Lindenmann, 1988). They are a group of cytokines secreted by damaged or infected cells that signal to bystander cells to increase host defences. This may be via upregulation of immune pathways, activation of immune cells such as T cells, or upregulation of antigen presentation (Crouse, Kalinke and Oxenius, 2015; Boxx and Cheng, 2016). There are more than twenty IFN genes in humans, which are divided into three classes, type-I, -II and -III (Haller, Kochs and Weber, 2007; Boxx and Cheng, 2016). Mammalian type-I IFNs include 14 subtypes of IFNα, as well as IFNβ, IFNω, IFNκ, IFNζ, IFNδ, IFNτ and IFNε. In humans, IFNα and IFNβ are the most abundant, IFNω is expressed in certain contexts and the remaining isoforms are
encoded as a single IFNκ isoform. Type-II IFNs solely include IFNγ, whereas type-III IFNs comprise of 4 subtypes of IFNλ (Boxx and Cheng, 2016).

Type-I and -II IFNs are used in the defence against either viruses or bacteria in different contexts (Haller, Kochs and Weber, 2007; Boxx and Cheng, 2016). Type-III IFNs are structurally distinct from type-I IFNs but were initially thought to have redundant functions. However, more recent studies have shown that type-III IFNs have distinct roles, such as reinforcing epithelial barriers during bacterial infection (Odendall, Voak and Kagan, 2017). Furthermore, whereas type-I IFNs induced a strong acute response, type III IFNs induced a weaker but longer term response, resulting in the creation of unique antiviral environments in different cell types (Pervolaraki et al., 2018).

### 3.3 IFN induction by pattern recognition receptors

Type-I IFN induction is driven by IFN regulatory factors (IRFs), which are phosphorylated by IRF kinases including TBK1 (TANK binding kinase 1) and the IKK (IκB kinase) family. IFN regulatory factor (IRF)-3 is constitutively expressed in the cytosol and following phosphorylation by TBK1 undergoes dimerization and nuclear translocation (Fig. 3.1A). Once in the nucleus, it binds to coactivators CBP and p300 and binds to the IFN promoter region to induce IFN transcription. Another regulatory factor, IRF-7, initially exists at low levels, but upon IFN-induction is upregulated and forms a heterodimer with IRF-3, acting as a amplifying transcription factor that boosts IFN production (Honda, Takaoka and Taniguchi, 2006).

#### 3.3.1 TLRs, RLRs and NLRs

The IRF kinases are activated by a wide array of PRRs, which recognise different ligands and trigger different signalling cascades to initiate a response. One such class are the toll-like receptors (TLRs), a family of 14 transmembrane receptors that monitor the cell surface and endosomal compartments for viral and bacterial PAMPs including nucleic acids (Medzhitov, 2007; Ishii et al., 2008). Some TLRs, including TLR7 and TLR9, have been shown to recognise self-nucleic acids as well
which has been linked to autoimmune disorders such as systemic lupus erythematosus (Barrat et al., 2005; Celhar, Magalhães and Fairhurst, 2012). A subset of TLRs induce type-I IFN expression including endosomal TLR7, TLR8, TLR9 and TLR13, which recognise pathogenic nucleic acids within endosomal compartments (Mancuso et al., 2009; Eigenbrod et al., 2015; Castiglia et al., 2016; Martínez-Campos, Burguete-García and Madrid-Marina, 2017). For TLRs, IFN production is induced by MyD88-dependent phosphorylation of the IFN regulatory factor (IRF) kinases IRAK (interleukin-1 receptor associated kinase) and IKKα (Boxx and Cheng, 2016). TLR4 also induces type-I IFN expression but in a slightly different manner, it is located at the plasma membrane where it surveys the extracellular space for bacterial cell surface components. Upon ligation, it is endocytosed within autophagosomes and triggers type-I IFN induction in a mechanism dependent on the TRAM-TRIF adaptor and the IRF kinases TBK1 and IKKε (Kagan et al., 2008). TBK1 and the IKK family are key signal transducers between PRRs and IFN regulation and are conserved in several other mechanisms (Fitzgerald et al., 2003; Sharma et al., 2003).

Cytoplasmic PAMPs are detected by RIG-I-like receptors (RLRs) and nucleotide-binding and oligomerising domain (NOD)-like receptors (NLRs), both of which also act via phosphorylation of TBK1 and IKKε (Boxx and Cheng, 2016). RLRs include RIG-I (retinoic acid-inducible gene 1) itself and MDA5 (melanoma-associated differentiation protein 5), which recognise viral double stranded RNAs and bind to the protein adaptor MAVS (mitochondrial antiviral-signalling protein, also known as VISA) upon ligation, which triggers IRF phosphorylation and IFN induction (Ishii et al., 2008; Boxx and Cheng, 2016).) NLRs, including NOD1 and NOD2, bind bacterial cell wall peptides such as the muropeptide iE-DAP (Bi et al., 2017) and signal via the adaptor RIP2 (Pandey et al., 2009; Watanabe et al., 2010).

### 3.3.2 Cytoplasmic DNA sensors

Cytoplasmic DNA is a marker of either pathogen infection or genomic instability and is recognised by a variety of DNA sensors, leading to pro-inflammatory responses
and programmed cell death (Paludan and Bowie, 2013; Paludan, Reinert and Hornung, 2019).

Many DNA sensors signal through activation of the adaptor protein **STING** (stimulator for IFN genes, also known as MITA), a transmembrane protein in the endoplasmic reticulum (Ishikawa and Barber, 2008; Bhat and Fitzgerald, 2014). STING activation triggers IFN induction via recruitment of TBK1, which phosphorylates both it and IRF-3 (Zhong et al., 2008; C. Zhang et al., 2019). Interestingly, STING has also been shown to directly induce autophagy via translocation to ER-Golgi intermediate compartments in a process independent of TBK1 and IFNs (Gui et al., 2019). Abrogation of STING function via epigenetic silencing or missense mutations in many tumours has been shown to impede IFN and production of other pro-inflammatory cytokines following DNA damage, thus allowing damaged and potentially cancerous cells to evade immune cells (Konno et al., 2018).

DNA sensors acting via STING include cGAS (cyclic GMP-AMP synthase), DAI (DNA dependent activator of IRFs), Mre11, DDX41 (DexD/H box helicase) and IFI16 (gamma IFN-inducible protein 16).

**cGAS** binds to cytosolic DNA and catalyses the reaction of GMP and AMP into cyclic-GMP-AMP (cGAMP), which acts as a second messenger in activating STING (Sun et al., 2013; Wu et al., 2013; Shang et al., 2019). cGAS has been shown to detect both pathogenic and self-DNA in the cytoplasm (Sun et al., 2013; Wolf et al., 2016). A recent study has indicated that rather than being a cytoplasmic sensor, cGAS actually localised to specific phosphoinositides on the plasma membrane, thus helping it distinguish between self- and viral-DNA (Barnett et al., 2019). Detection of self-DNA has been linked to aberrant inflammatory responses with links to metabolic and autoimmune disorders such as diabetes (Ablasser and Chen, 2019; Bai and Liu, 2019). cGAS has been described as essential to senescence, with cGAS deletion abolishing SASP and causing spontaneous immortalisation of mouse embryonic fibroblasts upon treatment with DNA damaging agents (Yang et al., 2017). cGAS was found to be essential in detection of cytosolic DNA released by cells undergoing replication stress (Wolf et al., 2016).
DAI has been shown to upregulate type-I IFNs via NF-κB and IRF-3 in response to poly(dA:dT) (Takaoka et al., 2007). Mre11, better known as a component of the MRN complex in DNA repair, has been shown to activate STING in response to cytosolic DNA (Kondo et al., 2013). DDX41 binds dsDNA, as well as the bacteria-specific metabolites cyclic-di-AMP and cyclic-di-GMP (Z. Zhang et al., 2011; Parvatiyar et al., 2012). IFI16 is an AIM2-like receptor (ALR) which detects viral DNA (Unterholzn et al., 2010) and which can cooperate with cGAS in response to herpes simplex infection (Orzalli et al., 2015). It has been shown to activate STING in a non-canonical ATM-dependent manner following DNA damage (Dunphy et al., 2018).

Beyond STING-dependent processes, there are several DNA sensors that use unique mechanisms to trigger immune responses. For example, RNA-polymerase III transcribes AT rich dsDNA into an RNA intermediate that is then recognised by RIG-I leading to IFNβ induction (Ablasser et al., 2009; Chiu, MacMillan and Chen, 2009). LRRFIP1 (Leucine-Rich Repeat Flightless-Interacting Protein 1) was found to induce IFNβ following Listeria monocytogenes infection or dsDNA treatment via phosphorylation of β-catenin and subsequent recruiter of the IFN coactivator p300 (Yang et al., 2010). AIM2 has been shown to detect cytoplasmic viral dsDNA and facilitate ASC recruitment and caspase-1 activation (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

Other DNA repair factors also perform a DNA sensing role. Ku70 was shown to bind longer tracts of DNA (>500 bp) in the cytosol and trigger production of type-III IFNs via IRF1 and IRF-7 (X. Zhang et al., 2011). RAD50 has been shown to induce IL-1β in response to viral DNA (Roth et al., 2014).

### 3.4 IFN signalling

Type-I IFNs signal in an autocrine or paracrine manner through a heterodimeric IFNα/β receptor (IFNAR). Ligand binding causes crosslinking of the IFNAR1 and IFNAR2 subunits, causing the cytoplasmic tails of the heterodimer to activate Janus-kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (Fig. 3.1B). These kinases
phosphorylate members of the STAT family and stimulate formation of STAT dimers. In the case of type-I IFNs, this is predominantly a STAT1 and STAT2 heterodimer, which forms a heterotrimeric complex with IRF-9 known as the IFN-stimulated gene factor 3 (ISGF3) complex. ISGF3 binds to IFN-stimulated response elements (ISREs) located in the promoter regions of ISGs (Levy et al., 1989). Type-I and -II IFNs can also activate STAT1 homodimers, which bind to γ-activated sequences and lead to transcription of regulatory genes such as IRF1 (Boxx and Cheng, 2016).

The IFN response is self-sustaining, as STATs and IRFs have ISREs themselves and thus are stimulated by IFN. As well as phosphorylated ISGF3, the initial pulse of IFNβ also leads to formation of an unphosphorylated ISGF3 complex (uISGF3), which binds a distinct group of ISREs and results in a long-term constitutive antiviral response. Constant exposure to low levels of IFNs, seen in cancers and chronic infections, results in increased expression of uISGF3-induced proteins (Cheon et al., 2013; Wang et al., 2017; Michalska et al., 2018).

ISG induction can occur independently of IFNs. For example, STATs can be directly phosphorylated and activated by TLR2, TLR4 and TLR9 (Luu et al., 2014). IRF-3 can directly induce transcription of ISGs, including IFIT1 (Grandvaux et al., 2002).

Finally, some ISGs such as ISG15 have been shown to have P53-response elements (Park et al., 2016).

ISGs are a large pool of more than 300 genes with various roles in innate immunity, inflammation and cell fate decisions (Cho et al., 2008; Yu et al., 2015). The role of ISGs in response to pathogen invasion is well established (Haller, Kochs and Weber, 2007; Boxx and Cheng, 2016; Alphonse, Dickenson and Odendall, 2021), however many ISGs are activated in response to DNA damage as well. For example, the P53 gene has an ISRE and is induced by IFN. P53 acts as an enhancer of the IFN response by promoting transcription of regulatory factors including IRF-9 (Muñoz-Fontela et al., 2008). IFN-activated P53 has been shown to induce senescence in response to DNA damage (Yu et al., 2015). ISGs such as STAT1, the IFIT family (IFN induced proteins with tetratricopeptide repeats), the OAS family (oligoadenylate synthase), and ISG15 were upregulated following
progerin-induced replication stress, in which context ISG upregulation was linked to senescence (Kreienkamp et al., 2018).

3.5 IFN signalling and host-pathogen interactions

IFN signalling is activated in response to a wide variety of pathogens, often as a defensive mechanism. However, pathogens have evolved ways to subvert IFN signalling to promote virulence, and excessive or chronic IFN signalling in response to infection can have negative effects on the host.

3.5.1 IFN signalling and viruses

The type-I IFN response is well recognised as an antiviral response, and indeed ISGs have varying roles that interfere with viral replication and infection. For example, viperin was shown to inhibit replication, budding and egress of multiple viruses including Influenza A, HIV and Bunyamwera virus (Helbig and Beard, 2014). The OAS family synthesise 2′-5′ oligoadenylates that activate rNase L, which degrades viral RNA and thus inhibits the viral life cycle (Liang, Quirk and Zhou, 2006). IFI6 is localised to the ER and has roles in inhibition of hepatitis C virus and flavivirus, and regulation of apoptosis in response to dengue virus 2 (Meyer et al., 2015; Qi et al., 2015; Richardson et al., 2018). EGR1 is a transcription factor with a broad range of targets that has been shown to enhance signal transduction in response to viral replication, including foot and mouth disease virus (Zhu et al., 2018). ISG15 has antiviral roles against influenza, herpes and Sindbis virus, among others (Lenschow et al., 2005, 2007; Giannakopoulos et al., 2009).

3.5.2 IFN signalling and bacteria

Bacterial infection has also been shown to induce type-I IFN responses, which can be both protective and detrimental to the host depending on context (Alphonse, Dickenson and Odendall, 2021). For example, type-I IFNs prevent hyperinflammation caused by *Streptococcus pyogenes* (Castiglia et al., 2016),
prevent transmigration of *Streptococcus pneumoniae* across the lung (LeMessurier et al., 2013), and promote CXCL10-dependent cell recruitment in response to *Helicobacter pylori* (Watanabe et al., 2010). ISGs including OAS and ISG15 have been shown to restrict *Mycobacterium tuberculosis* (Bogunovic et al., 2012; Leisching et al., 2019), and viperin restricts *Shigella flexneri* replication (Helbig et al., 2019). Bacteria have therefore evolved mechanisms to block IFN signalling, such as *Shigella*, which secretes OspC to block calcium signalling and thus interrupt JAK/STAT signalling (Alphonse et al., 2022). Furthermore, some species of bacteria exploit IFN signalling, such as *Listeria monocytogenes* and *Francisella tularensis*. In these examples, infection leads to IFN-dependent suppression of IL-17, which is detrimental to the host and helps promote infection (Henry et al., 2010).

### 3.5.3 IFN signalling and *Salmonella*

*Salmonella* induces IFN signalling in different manners depending on host cell type. For example, *S. Typhimurium* mRNA is detected by RIG-I, which induces a type-I IFN response in non-phagocytic cells (Schmolke et al., 2014). In phagocytic cells, *Salmonella* LPS is recognised by TLR4, which also drives a type-I IFN response (Boxx and Cheng, 2016). However the Vi capsule prevents TLR-4 dependent recognition in *S. Typhi* (Wilson et al., 2008).

IFN signalling have both beneficial and detrimental impacts on *Salmonella* infection. Type-III IFNs can protect epithelial barriers from damage induced by *S. Typhimurium* (Odendall, Voak and Kagan, 2017). However, *S. Typhimurium* can subvert the type-I IFN response to its advantage, promoting macrophage necroptosis and repressing host defensive responses such as IL-1 cytokine and neutrophil chemokine release (Robinson et al., 2012; Perkins et al., 2015). IFNAR1-deficient mice survive better than wild-type mice, and display a reduced *Salmonella* burden in the liver and spleen (Robinson et al., 2012). The impact of IFN signalling on *S. Typhi* is less clear, although transcriptomic data has revealed the upregulation of ISGs such as the IFIT family and EGR1 in response to *S. Typhi* infection (Hannemann and Galán, 2017).
It is possible that S. Typhi may stimulate an IFN response via secretion of the typhoid toxin. There are examples of bacteria inducing IFN and inflammatory responses via activity of other genotoxins, such as CDTs. As discussed previously (section 2.5), genotoxic stress can lead to activation of DNA sensing pathways and IFN signalling. Indeed, the type-I IFN response was recently found to be activated by DNA damage caused by E. coli CDT (Pons et al., 2021), and CDT of H. ducreyi was shown to trigger a senescence phenotype including secretion of pro-inflammatory cytokines such as IL-6 (Péré-Védrenne et al., 2017). Beyond CDTs, E. coli also secretes a genotoxin known as colibactin, which causes interstrand DNA crosslinks. Colibactin has been shown to induce a senescence phenotype including secretion of inflammatory SASP (Secher et al., 2013). Further study will be needed to examine host responses to the typhoid toxin and its wider role in Salmonella virulence.

### 3.6 Host responses to the typhoid toxin

The typhoid toxin causes ssDNA nicks that induce replication stress and senescence in host cells (Ibler et al., 2019). Toxin-induced senescence responses lead to formation of microenvironments of cells that are susceptible to Salmonella infection, thus suggesting that S. Typhi secretes the toxin to promote its infection of the host (Ibler et al., 2019). This correlates to in vivo studies showing that the toxin promotes systemic dissemination and chronic carriage (Del Bel Belluz et al., 2016; Miller et al., 2018; Gibani et al., 2019). However, S. Typhi has also been implicated in gallbladder cancer, and the genotoxic effects of the toxin could induce mutagenesis and cancer (Di Domenico et al., 2017). It is possible that the host cell undergoes senescence to defend itself from tumorigenesis and in doing so presents an opportunity for Salmonella to hijack the process and facilitate its dissemination.

In other disease states such as progeria, replication stress leads to DNA leakage into the cytosol, activation of cGAS and a type-I IFN response (Wolf et al., 2016; Kreienkamp et al., 2018). IFNβ was shown to amplify DDRs to promote senescence while inactivation of the IFN pathway prevents progeria and extends lifespan (Yu et al., 2015). E. coli CDT has been shown to promote an IFN response in response to
DNA damage (Pons et al., 2021), although it remains to be seen whether the typhoid toxin does the same.

Some patients infected with S. Typhi will chronically carry the infection asymptptomatically and go on to develop gallbladder cancer. To understand the role of the toxin in different manifestations of S. Typhi infection a greater understanding of the wider host response to the toxin, particularly the innate defences counteracting bacterial genotoxins, is needed.

### 3.7 Aims and Hypothesis

I hypothesise that the host induces an immune response to prevent genotoxic, potentially cancerous, phenotypes induced by the toxin. Understanding this aspect of the host-pathogen interaction will illuminate interplay between the DDR, innate immunity and *Salmonella* infection.

This thesis aims to:

1. Characterise the host responses to the typhoid toxin by determining toxin-dependent differences in the host transcriptome.
2. Identify specific factors that contribute to the host response to the toxin.
3. Determine whether the toxin can induce cancerous phenotypes and how the host can protect itself against these.

This will help elucidate the functional role of the typhoid toxin, provide a mechanistic link between the genotoxic effects of the toxin and observable phenotypes of intoxication, and possibly discover relevant links between the typhoid toxin, chronic carriage, and cancer.
Part 2: Results

4 Host responses to the typhoid toxin

4.1 Introduction

The typhoid toxin of *Salmonella* Typhi causes replication stress and senescence *in vitro* (Ibler *et al.*, 2019). Both phenotypes have been linked to cancer and bacterial-associated oncogenesis (Cougnoux *et al.*, 2014). Indeed, *S.* Typhi is a significant risk factor for gallbladder cancer (Scanu *et al.*, 2015). Thus, it stands to reason that diverse host defence mechanisms operate to protect humans from the effects of genotoxic *Salmonella*, but no mechanism has been reported for typhoid toxin. With the aim of gaining a greater understanding of host responses to the toxin, GeneChip microarray analysis was exploited to investigate transcriptional changes in cultured cells treated with typhoid toxin.

4.2 Results

4.2.1 Purification of recombinant typhoid toxin

There are two possible methods to study the host responses to the typhoid toxin. One is to infect cells with toxigenic *Salmonella*, such as *S.* Typhi, the NTS serovar S. Javiana (Miller *et al.*, 2018), or engineered toxigenic *S.* Typhimurium (Del Bel Belluz *et al.*, 2016). However, *Salmonella* also encodes other virulence effectors that may also activate DDRs and innate host defences. To uncouple the toxin from other *Salmonella* virulence factors, recombinant toxin was purified from *E. coli* as described in Ibler *et al.* 2019. This approach has been used to study the effects of the toxin in other studies (Song, Gao and Galan, 2013; Ibler *et al.*, 2019).

The DE3 *E. coli* strain C41 Rosetta was transformed with pETDuet-1 expression vector encoding epitope-tagged toxin subunits pltA-Myc, pltB-His and cdtB-FLAG under the control of T7 RNA polymerase promoter. Addition of the lactose analogue IPTG (Isopropyl β-D-1-thiogalactopyranoside) activated expression of T7 RNA
polymerase in C41 that drove expression of toxin subunits, which, in the absence of any secretory mechanism, accumulate in the bacterial periplasm as a fully assembled holotoxin. NiNTA affinity chromatography was used to isolate the toxin via pltB-His from the C41 E. coli lysate (Fig. 4.1).

**Fig. 4.1 Purification of recombinant typhoid toxin**
Immunoblots of FLAG (CdtB-FLAG) and Myc (PltA-Myc), and Coomassie stains, of each fraction of the NiNTA purification of WT-toxin and HQ-toxin. The uninduced and induced fractions are E. coli lysate before and after IPTG treatment. The lysate is fractionated into soluble and insoluble fractions, and the soluble fraction is passed through the NiNTA column. The flow-through is the unbound fraction. The beads are then washed before the bound fraction is eluted from the beads. Purified GST-CdtB of known concentrations was used to create a standard curve to extrapolate the concentration of the eluted toxin fraction using intensities measured in Image Studio. Purification was performed together with Mohamed ElGhazaly, who took the gel images.

Simultaneous preparations were made of a wild-type typhoid toxin and a mutant cdtB-H160Q toxin with attenuated catalytic activity (henceforth WT-toxin and HQ-toxin) as previously described (Ibler et al., 2019). Coomassie stain analysis of the protein gel of the fractions showed that the eluted fractions were a crude preparation including other E. coli proteins. However, use of the HQ-toxin acted as a control for the potential effects of other E. coli contaminants in downstream assays in cultured cells. The presence of CdtB-FLAG, PltA-Myc and PltB-His was confirmed in the eluted fraction using Western-blotting for FLAG and Myc antibodies respectively. CdtB-FLAG and PltA-Myc were detected in the HQ-toxin
elution but at 10-fold lower levels than in the WT-toxin elution. This was possibly due to low expression of the His-tag on PltB.

CdtB-FLAG densitometry was compared to that of a GST-CDTB purification. CdtB-GST was shown by Coomassie to be ~80% homogeneity and concentration was calculated by Bradford assay using GST-CDTB as a standard. This enabled extrapolation of the concentration of toxin from a standard curve of GST-CDTB preparations.

To confirm that the purified toxin was functional, an in vitro intoxication assay was used as described in Ibler et al. 2019 in human fibrosarcoma HT1080 cells (henceforth standard intoxication assay). Briefly, 20 ng/ml of WT- and HQ-toxin was added to cells for 2 h to allow for toxin endocytosis, before removal and replacement with fresh media. Cells were fixed 24 h later and prepared for immunofluorescence analysis of the DNA damage marker γH2AX (Fig. 4.2A). WT-toxin treatment induced γH2AX in 82% of DAPI-stained nuclei (outlined in Fig. 4.2A), which was significantly higher than 33% of HQ-toxin treated cells (Fig. 4.2B). Positivity was defined as a greater γH2AX fluorescence intensity than the upper quartile of untreated γH2AX intensity, meaning the baseline γH2AX positivity was 25% in untreated cells. WT-toxin treatment induced a greater γH2AX response than that of 24 h continuous treatment with DNA-polymerase inhibitor aphidicolin (68% positivity), which acted as a positive control. Immunofluorescence also revealed that cell nuclei became distended upon WT-toxin treatment, which was consistent with observations in other studies (Haghjoo and Galán, 2004; Spanò, Ugalde and Galán, 2008; Guidi, Levi, et al., 2013; Ibler et al., 2019).
**Fig. 4.2 The typhoid toxin causes a DNA damage response and cell cycle arrest**

(A) The DNA damage response 24h post-intoxication by WT- and HQ-toxin preparations was assayed in HT1080 cells using immunofluorescence of γH2AX (green). Nuclei indicated by DAPI outline (grey). Scale bars are 20 μm. (B) Quantification of γH2AX-positive nuclei in (A). Nuclei were counted as positive if greater than the upper quartile of untreated γH2AX intensity. Each circle is an independent replicate each consisting of three technical replicates. One way ANOVA was used with Šidák’s multiple comparison test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD. (C) Heatmap shows percentages of HT1080 cells in G1, G2 and S phase at 2 hourly timepoints across 24 h following intoxication, determined by measurement of propidium iodide using flow cytometry. Experiment was performed together with Angela Ibler. One independent replicate consisting of three technical replicates.
To check whether the toxin caused cell cycle arrest, cells were synchronised by 24 h serum starvation before a standard intoxication assay was performed. Samples were prepared for flow cytometry analysis every 2h after intoxication over the 24h media chase. The overall effect of the toxin is best observed at 24h where flow cytometry analysis revealed that 40% of WT-toxin treated cells were in G2 phase compared to only 13% and 17% of cells in HQ-toxin treated and untreated cells respectively (Fig. 4.2C). This G2 accumulation coincided with a decrease in cells in G1 from 53% and 46% in HQ-toxin treated and untreated cells to 25% in WT-toxin treated cells. Thus, the toxin causes a G2 arrest in the cell cycle.

In addition, the time-course provided insight into the effects of the toxin. From 6h, the proportion of cells in S phase increases relative to HQ-toxin treated and untreated cells. At the 8 h timepoint through the 12 h timepoint, there were approximately 8% more WT-toxin treated cells in S phase than either HQ-toxin treated or untreated cells, suggesting that the toxin was stalling DNA replication forks in S phase, which is consistent with replication stress observed by Ibler et al. 2019. This delay and the accumulation of cells in G2 showed that the toxin was causing cell cycle arrest in S/G2 phase.

4.2.2 The toxin causes both ATR- and ATM-dependent DDRs

The toxin causes two distinct DDR phenotypes that lead to senescence: (i) RPA-labelled SSBs at replication forks in S/G2 phase resulting in DNA replication stress marked by phosphorylation of T21 in RPA, and (ii) 53BP1-labelled DSBs in G0/G1 (Ibler et al., 2019). In order to confirm that this was the case with purified toxin, RPA32 pT21 and 53BP1 were examined in intoxicated cells by immunofluorescence (Fig. 4.3A). In parallel, cells were incubated with the thymidine analogue EdU for 24 h before fixation to identify cells synthesising DNA and thus progressing through the cell-cycle. Both RPA32 pT21 and 53BP1 were increased in response to the WT-toxin compared to negative controls. RPA32 pT21 foci were induced in response to the WT-toxin in 33% of cells, compared to 2% of untreated and HQ-toxin treated cells (Fig. 4.3B). 6% of WT-toxin treated cells were positive for 53BP1 foci.
compared to 0% untreated and HQ-toxin treated cells. The toxin also caused a decrease in DNA replication. Whereas 87% and 73% of untreated and HQ-toxin treated cells were positive for EdU, showing that they were undergoing DNA replication, only 26% of WT-toxin treated cells were EdU positive.

There were no nuclei positive for both EdU and 53BP1, which was consistent with 53BP1 being recruited to DSBs in G0/G1. However, approximately 10% of nuclei were positive for both EdU and RPA32 pT21 in response to the WT-toxin, consistent with RPA phosphorylation occurring during DNA replication stress.
Fig. 4.3 The typhoid toxin causes distinct DNA damage responses in G0/G1 and G2/S phase

(A) The DNA damage response 24h post-intoxication by WT- and HQ-toxin preparations was assayed in HT1080 cells using immunofluorescence of phosphorylated RPA32 (RPA32 pT21, green), 53BP1 (red) and EdU (magenta). Distinct responses to the WT-toxin in G0/G1 and G2/S phase are indicated. Scale bars indicate 20μm. (B) Quantification of positive nuclei in (A). Nuclei were counted as positive if greater than the upper quartile of untreated intensity. Bars indicate total percentage across three technical replicates from one independent replicate.
4.2.3 The toxin activates a type-I IFN response

Having confirmed that purified typhoid toxin activates DDRs, host defence pathways were investigated by analysing the transcriptome of intoxicated HT1080 cells. The experiment was performed in collaboration with my colleague Angela Ibler who intoxicated HT1080 cells for 2h with either WT- or HQ-toxin before extraction of cellular RNA at 48h. The RNA was analysed using Clariom™S transcriptome profiling microarray at the Sheffield Microarray and Next Generation Sequencing Core Facility who provided data for analysis by myself (experimental pipeline indicated in Fig. 4.4A).

19460 genes were detected in both WT- and HQ-toxin treated samples, and of these 1885 genes were significantly differentially expressed (p < 0.05) with a log2 fold change > 1 or < -1 (Fig. 4.4B). Analysis of these 1885 genes performed with STRING v11.0 revealed the WT-toxin dependent enrichment of a variety of biological processes. This included 11 biological processes which were upregulated, of which 6 were related to immune responses (Fig. 4.4C, marked in bold). Of these six processes, the most upregulated was the type-I IFN signalling pathway, characterised by upregulation of 11 genes (Fig. 4.4D-E) which has been previously shown to be activated by replication stress and is implicated in premature senescence (Kreienkamp et al., 2018). The gene set included 2 ISG transcription factors (IRF-9 and STAT1) and 7 ISGs including IFN-induced proteins with tetratricopeptide repeats 1-3 (IFIT-1, -2 and -3), 2’-5’ oligoadenylate synthase 1 and 3 (OAS-1 and -3), early-growth response gene 1 (EGR1), viperin (also known as RSAD2), IFN-α inducible protein 6 (IFI6) and IFN-stimulated gene 15 (ISG15).
Fig. 4.4 The typhoid toxin induces a type-1 IFN signalling response

(A) Workflow for microarray analysing gene expression in HT1080 cells 48h post-intoxication by WT- and HQ-toxin. (B) Fold changes were calculated between treatments for 19460 genes. Of these, 1885 genes were significantly differentially regulated (p < 0.05) with a log2 fold change > 1 or < -1. Results from three independent replicates. (C) Heatmap shows significant enrichment of Gene Ontology (GO) terms detected by STRING in the set of 1885 genes indicated in (B). (D) Heatmap shows 11 genes related to the type-1 interferon signalling pathway, including ISGs upregulated in response to WT-toxin. (E) STRING network highlighting close functional and annotated relationships between the genes presented in (D), using same colour scale. Intoxication of HT1080 cells and RNA extraction were performed by Angela Ibler. I analysed the Affymetrix Gene Chip Microarray data, which was provided by the Sheffield Microarray and Next Generation Sequencing Core Facility.
4.2.4 The IFN response is not S/G2 dependent

To determine whether the type-I IFN response was triggered by toxin-induced replication stress, transcriptome changes were analysed following WT- and HQ-toxin treatment of HT1080s in the presence (10%) or absence (0%) of serum at 48h. Serum-starvation prevents entry into S phase, locking cells in G0/G1, which therefore inhibits toxin-induced replication stress (Ibler et al., 2019). Toxin-induced DSBs are permissive in the presence and absence of serum while SSBs in S/G2 are only permissive in the presence of serum (WT-tox, 10% serum), which enabled identification of 1195 genes associated with DNA replication stress that were significantly differentially expressed (p < 0.05) with a log2 fold change > 1 or < -1 (Fig. 4.5A). To identify genes associated with DSBs in G0/G1, the transcriptome of cells treated with WT-tox and HQ-tox in the absence of serum were analysed (Fig. 4.5B). Of these, 343 were significantly differentially expressed (p < 0.05) with a log2 fold change > 1 or < -1.

The type-I IFN response has been shown to be induced by replication stress, and to lead to senescence (Kreienkamp et al., 2018). Thus, it was hypothesised that the type-I IFN response was caused by toxin-induced replication stress, possibly due to host DNA leakage into the cytosol. The transcriptome experiment in Fig. 4.4 was also performed in the absence of serum to block entry into S phase and therefore replication stress (Fig. 4.5). Relative to HQ-toxin, WT-toxin induced a type-I IFN response in serum-starved cells showing that the IFN response occurred independently of S phase and was instead occurring in G0/G1 phase (Fig. 4.5C-D) where 53BP1-labelled DSBs were observed in Fig. 4.3). The major differences between the presence and absence of serum in intoxicated cells was in cell cycle and cell metabolic processes, most likely due to serum-starvation blocking replication and the supply of nutrients. When focussing on individual ISGs, the type-I IFN response in serum-starved cells was particularly evident for ISGs such as IFI6 and IFIT1 that were observed in G0/G1 but not for ISGs such as ISG15 and RSAD2, which were up-regulated in asynchronous replication-competent cells in the presence of serum (Fig. 4.5D). This suggests divergent mechanisms underlying ISG regulation.
Fig. 4.5 The toxin-dependent type-I IFN-like response occurs in G0/G1

(A) Fold changes were calculated 48h post-intoxication between WT-toxin treated cells in normal (10% serum) and serum-starved (0% serum) conditions for all detected genes, in order to determine toxin responses due to replication stress in S/G2M phase. 1195 genes were significantly differently regulated (p < 0.05) with a log2 fold change >1 or <1. Results from three independent replicates.

(B) Fold changes were calculated between WT- and HQ-toxin treated cells in serum-starved (0% serum) conditions for all detected genes, in order to determine toxin responses in G0/G1 phase. 343 genes were significantly differently regulated (p < 0.05) with a log2 fold change >1 or <1. Results from three independent replicates.

(C) GO Analysis of Gene Expression Changes

(D) Heatmap shows 11 genes related to the type-1 IFN signalling pathway upregulated in response to WT-toxin due to replication stress in S/G2M phase or damage in G0/G1 phase and compared to asynchronous responses from Fig. 4.4D.
4.2.5 Validation of the toxin-induced IFN response

As the type-I IFN response has been linked to both DDRs and senescence, the 11 type-I IFN related genes identified by the microarray were chosen for further analysis. To validate the microarray data, qRT-PCR was carried out in HT1080 cells on 9 of the IFN-related genes: IFI6, IFIT1, IFIT2, IFIT3, IRF9, ISG15, OAS1, OAS3 and STAT1. For 2 of the genes, RSAD2 and EGR1, efficient primers could not be designed. Untreated cells were used as a calibrator and cells were harvested 24 h following intoxication with WT- or HQ-toxin. 5 of the type-I IFN related genes were significantly upregulated in WT-toxin treated cells compared to untreated cells, compared to only 3 genes upregulated in HQ-toxin treated cells compared to untreated (Fig. 4.6A-I). Furthermore, 5 genes were significantly upregulated in WT-toxin compared to HQ-toxin (ISG transcription factor IRF-9 and ISGs IFIT1, IFIT2, IFIT3, and ISG15). Overall, this provided further evidence that some ISGs are upregulated in a toxin-dependent manner, although not all ISG expression changes found in the microarray could be validated.
Fig. 4.6 Validation of the toxin-dependent type-1 IFN-like response by qRT-PCR

(A-I) Quantification of mRNA levels in HT1080s of 9 type-1 IFN-related genes compared between untreated and 24h WT- and HQ-toxin treated samples. Bars represent mean and error bars indicate SD. Each circle is an individual technical replicate from minimum three independent replicates. One way ANOVA was used with Šidák’s multiple comparison test. Asterisks indicate significance.
To further validate the IFN-like response, levels of ISG15 and IFIT1 were assayed by immunoblotting, as these showed the greatest significance in mRNA levels between WT- and HQ-toxin treatment. Levels of phosphorylated STAT1 (pSTAT1) were also assayed, as STAT phosphorylation is a key component of ISG regulation (Fig. 4.7A). In contrast to mRNA (Fig. 4.6), immunoblot analysis revealed that there was no difference in IFIT1, pSTAT1, STAT1 or ISG15 protein levels 24 h after intoxication with WT-toxin (Fig. 4.7A). However, ISG15 protein levels were significantly increased at the 72 h timepoint compared to both untreated and HQ-toxin treated cells (Fig. 4.7A, B). IFIT1 was significantly upregulated at 72 h in WT-intoxicated cells compared to untreated cells and showed a non-significant increase compared to HQ intoxication (Fig. 4.7C). Both STAT1 and pSTAT1 showed an increase in response to WT toxin at 48 – 72 h, but this increase was not significant to either untreated or HQ-intoxicated cells. As a positive control, sustained 72 h treatment with purified IFNα triggered both phosphorylation and upregulation of STAT1, as well as upregulation of IFIT1 and ISG15 (Fig. 4.7A). IFNα also induced a smear of high molecular weight ISG15-conjugated (ISGylated) proteins which was not visible following intoxication where only free ISG15 was observed. No toxin induced ISGylation was observed in over exposed blots (data not shown). ISG15 is a ubiquitin-like protein and has been shown to act as a covalent adduct to other proteins throughout the cell (Morales and Lenschow, 2013; Perng and Lenschow, 2018).
Fig. 4.7 ISG15 and IFIT1 are upregulated in response to typhoid toxin
Immunoblots of protein levels in HT1080s of STAT1, phosphorylated STAT1 (pSTAT1), ISG15, and IFIT1 24 h, 48 h and 72 h after either treatment with WT-toxin, HQ-toxin or 100ng/ml purified IFN α. The pSTAT1 untreated band was not shown due to leakage from the lane containing the molecular weight marker. (B - E) Graph shows densitometry analysis (ImageStudio) of ISGs relative to tubulin and normalised to untreated (minimum 2 independent immunoblots). ISG15 densitometry is of the 15 kDa free ISG15 band. One way ANOVA was used with Šidák’s multiple comparison test. Asterisks indicate significance.

Immunofluorescence was also used to determine localisation of ISG15 in response to the toxin. Interestingly, ISG15 predominantly localised to the nucleus, suggesting it could be modulating function of nuclear proteins (Fig. 4.8A). ISG15 was significantly upregulated in the nucleus in WT-toxin treated cells and IFN-treated cells compared to untreated and HQ-toxin treated cells (Fig. 4.8B). Approximately 60% of WT intoxicated and IFNα-treated nuclei were positive for ISG15 compared to 20% of untreated and HQ-intoxicated nuclei.

In Fig. 4.5, the IFN response occurred in G0/G1 phase, but this was less clear for ISG15, which was best observed in asynchronous cells suggesting upregulation in both G0/G1 and S/G2. When ISG15 was assayed in WT- and HQ-intoxicated cells in serum-free conditions by immunofluorescence (Fig. 4.8C), there was significant variation in the data for HQ-intoxication and IFN-treatment, which made it difficult to interpret the results. However, there was a significant increase in ISG15 in WT-intoxicated cells compared to untreated cells (Fig. 4.8D). However, compared to around 60% positive cells in 10% serum, only 40% of cells were positive for ISG15 in response to the WT toxin in 0% serum.

ISG15 was assayed in WT- and HQ-intoxicated cells in serum-free conditions using immunoblotting. Consistent with the immunofluorescence data, WT-intoxication induced an increase in ISG15 in 0% serum, but not to the same extent as in 10% serum conditions (Fig. 4.8E). Taken together, this suggested that toxin induced ISG15 is not solely a product of the G0/G1 DDR and further suggests divergent regulation of ISG15 relative to other ISGs, e.g., IFI6 in Fig. 4.5D. It therefore seems possible that ISG15 is upregulated in response to the 53BP1-labelled DSBs in G1 and RPA-labelled SSBs in S/G2 observed in in Fig. 4.3.
Fig. 4.8 *Immunofluorescence analysis of ISG15*

(A) Immunofluorescence of ISG15 (red) 72h after treatment with WT-toxin, HQ-toxin or 100ng/ml purified IFNα in HT1080 cells. (B) Quantification of ISG15-positive nuclei in (A). Each circle is a technical replicate from two independent replicates. One way ANOVA was used with Šidák’s multiple comparison test. (C) Same as (A) in serum-starved (0% serum) conditions. (D) Quantification of ISG15-positive nuclei in (C). Each circle is a technical replicate from a single independent replicate. An unpaired t-test was used to compare Unt and WT-toxin conditions. (E) Immunoblot of protein levels in HT1080s of ISG15 72 h after treatment with WT-toxin, HQ-toxin or 100ng/ml purified IFNα in normal (10% serum) and serum-starved (0% serum) conditions.

Nuclei were counted as positive if greater than the upper quartile of untreated ISG15 intensity. Asterisks indicate significance. Bars indicate mean and error bars indicate SD. Nuclei indicated by DAPI outline (grey). Scale bars indicate 20µm.
4.2.6 Determining the role of IFNs in the toxin ISG response

ISGs are canonically regulated by IFNs binding to cognate receptors and activating a downstream JAK/STAT signalling pathway, triggering formation of heteromeric signalling complexes that bind to ISREs in ISG promoter sequences (Boxx and Cheng, 2016). Despite seeing upregulation of ISGs in the microarray (Fig. 4.4), no IFN genes were significantly differentially regulated between WT and HQ treatment, suggesting that ISG upregulation was IFN-independent (Fig. 4.9A). To examine this in further detail, IFNα and IFNβ mRNA levels were analysed by qRT-PCR. IFNα was significantly increased in both WT and HQ treatments compared to untreated but these were not significantly different to each other, suggesting that the increase was independent of toxin activity (Fig. 4.9B). IFNβ mRNA levels showed no significant difference between any treatments (Fig. 4.9C). It is important to note that the CT values for these IFNs were very high (approximately 39) compared to, for example, GAPDH (approximately 20), showing that mRNA levels of IFNs were low even in response to either WT- or HQ-toxin.

ISG15 expression was also examined following type-I IFN inhibition by B18R, which is encoded by vaccinia virus and competitively binds to IFN, thus inhibiting downstream signalling of IFN (Radoshevich et al., 2015). As expected, B18R abolished ISG15 upon addition of IFNα (Fig. 4.9D-E). However, whilst there was a reduction in ISG15, B18R did not abolish WT-toxin dependent ISG15 upregulation, suggesting that the toxin was upregulating ISG15 via both type-I IFN-dependent and -independent pathways.
Fig. 4.9 Examining the role of IFN in the toxin ISG response

(A) Heatmap shows all IFN genes detected in the microarray presented in fig. 3.4 and represents fold change between WT- and HQ-toxin treated cells. (B-C) Quantification of mRNA levels in HT1080s of IFNa and β compared between untreated and 24h WT- and HQ-toxin treated samples. Bars represent mean fold change of three samples (three technical replicates run for each sample). Each circle is an individual technical replicate. One way ANOVA was used with Šidák’s multiple comparison test. Asterisks indicate significance. Error bars indicate SD. (D) Immunoblot of protein levels in HT1080s of ISG15 72 h after treatment with WT-toxin or 100 ng/ml purified IFNa in the presence and absence of B18R. (E) Densitometry analysis (ImageStudio) of 15 kDa free ISG15 band in (D) relative to tubulin (one independent replicate).

4.3 Discussion

The host response to the typhoid toxin is key to understanding mechanistic links between the toxin and systemic Salmonella dissemination, chronic carriage, bacteraemia, and cancer. For Salmonella to establish chronic infection it must evade or manipulate host immune responses, and indeed data in this chapter has shown that the typhoid toxin induced upregulation of genes involved in the type-I IFN pathway.
Beyond responses to pathogen invasion, which were discussed in section 3.5, the type-I IFN response has been observed in response to inducers of replication stress, such as progerin or knockdown of RPA. Replication stress leads to DNA leakage into the cytosol, activation of cGAS and a type-I IFN response (Wolf et al., 2016; Kreienkamp et al., 2018). In these contexts, the IFN response regulates cell fate decisions, such as inducing senescence in order to prevent escalation of genomic instability into oncogenic phenotypes. It is possible that the toxin IFN response links toxin-induced replication stress to the senescence phenotype observed in Ibler et al. However, the IFN response was observed predominantly in G0/G1 phase rather than with replication stress in G2/S phase. Further work will be needed to determine whether the toxin IFN response is part of a distinct host response to damage in G0/G1, including further replicates of immunoblotting and immunofluorescence experiments to examine toxin induced ISG responses following serum starvation.

Of the ISGs identified from the microarray data, ISG15 showed the greatest significant upregulation in response to the toxin when using qRT-PCR, immunoblotting and immunofluorescence. Because of this, ISG15 was used as a marker for the toxin ISG response going forward. However, the myriad functions of ISG15 prompted interest in determining its specific role in the host response to the toxin, which I will discuss in the following chapters.

Although ISG15 upregulation was validated by other methods, this was not the case for other ISGs detected. For example, significant differences were not seen in OAS1, OAS3, STAT1 and IFI6 mRNA levels between WT- and HQ-toxin treated cells. It is possible that the 24h timepoint at which RNA samples were prepared post-intoxication was not optimal for seeing toxin-dependent changes in ISG mRNA levels. RNA samples for the microarray were taken 48h post-intoxication whereas qRT-PCR samples were prepared 24h post-intoxication. Furthermore, increases in ISG15 and IFIT1 protein levels were not seen until 48h post-intoxication and were strongest at 72h post-intoxication. It would be interesting to repeat the qRT-PCR experiments at different timepoints, including shorter timepoints (approx. 2h) and longer timepoints (48h-72h).
Initial data suggested that the toxin ISG response was partially independent of type-I IFNs. IFN upregulation was not seen in the microarray, and mRNA levels of IFNα and IFNβ did not change in a toxin-dependent manner. It was interesting to observe that IFNα was significantly upregulated in response to both WT- and HQ-toxin compared to untreated. This could be because *E. coli* contaminants in the toxin preparations were acting as PAMPs and inducing IFN production in a canonical manner. However, based on the observed CT values, these levels of IFN mRNA were low, especially when compared to toxin dependent induction of ISGs.

The type-I IFN inhibitor B18R did not abolish toxin induced ISG15. It is possible that some of the ISGs seen to be upregulated in the microarray may be upregulated in an IFN-independent manner. For example, ISG15 has been shown to have a P53-response element and is induced in response to P53 activation following DNA damage (Park *et al.*, 2016). However, further information will be needed to confirm whether the toxin ISG response in IFN-independent. The experiment with B18R was a single immunoblot and should be repeated. Furthermore, as B18R is an inhibitor of type-I IFNs only, it would be interesting to use inhibitors of other types of IFN. Alternatively, toxin responses could be examined in genetically engineered cells lacking IFN receptors.

In order to greater understand the toxin ISG response, an understanding of how the response was regulated, and how it was linked to the DDR induced by the toxin, was required. The next chapter presents efforts to elucidate the link between toxin-induced DNA damage and the ISG response.
5 Regulation of the toxin ISG response

5.1 Introduction

The toxin is thought to functionally mimic mammalian DNase-1 by introducing SSBs before their accumulation on complementary strands generate DSBs (Bezine, Vignard and Mirey, 2014). This causes replication stress which can lead to DNA leakage into the cytosol, which acts as a DAMP and triggers activation of DNA sensing PRRs (MacKenzie et al., 2017; Bakhoum et al., 2018). PRRs signal to downstream kinases that induce immune responses including the type-I IFN pathway. It was hypothesised that the toxin was inducing an ISG response by triggering cytosolic leakage of DNA.

5.2 Results

5.2.1 The toxin induces single strand breaks, micronuclei and cytosolic DNA leakage

Previous work in the Humphreys lab has established that the typhoid toxin creates SSBs in DNA, which can be shown by assaying RPA32 pT21 (Ibler et al., 2019). In order to further confirm these findings, it was necessary to find a method to identify SSBs.

To detect SSBs a novel in vitro polymerase assay was used, whereby ssDNA within fixed nuclei acts as a template for a DNA polymerase reaction, which can incorporate complementary nucleotide analogues to these sites. Using antibodies targeting these nucleotide analogues, in this case CldU, sites of ssDNA were identified (Fig. 5.1A). 100% of nuclei treated with WT-toxin were positive for CldU foci when using the upper quartile of untreated cell intensity as a threshold (Fig. 5.1B). In comparison, only 25% of nuclei were positive for CldU in HQ-toxin treated cells. CldU in intoxicated nuclei was coincident with a DDR characterised by both RPA32 pT21 and gH2AX.
Fig. 5.1 Toxin activity results in ssDNA formation and DNA leakage into the cytosol

(A) Immunofluorescence of γH2AX (magenta) and RPA32 pT21 (grey) 24h post-intoxication by WT- and HQ-toxin in HT1080 cells. Sites of exposed ssDNA are detected using the in vitro polymerase assay and are shown by CldU foci (red). Scale bars indicate 10 μm. (B) Quantification of nuclei positive for the markers indicated in (A). Bars represent percentages of approximately 40-100 nuclei in one independent replicate. (C) Immunofluorescence of Lamin B1 (green) and γH2AX (red) 24h post-intoxication by WT- and HQ-toxin in HT1080 cells. Micronucleus indicated with white arrow. Scale bars indicate 10 μm. (D) Quantification of cells containing micronuclei. Each circle is a technical replicate from a single independent replicate. One way ANOVA was used with Tukey’s multiple comparison test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD. (E) Immunoblot of protein levels of tubulin, histone H3, Lamin B1, γH2AX and RPA32 in HT1080s 24h after treatment with WT-toxin, HQ-toxin, etoposide, aphidicolin or transfection with 20 nM immunostimulatory DNA. Cells were fractionated into cytosolic, soluble nuclear and chromosomal fractions. (F) DNA gel of the cytosolic fraction in (E), showing relative levels of DNA in the soluble cytosolic fractions from (E). (G) Immunoblot of protein levels in HT1080s of ISG15 48h after transfection with empty vector pcDNA. Lipo. is lipofectamine.
Micronuclei are small membrane-bound fragments of chromosomes which are associated with genomic instability and are indicative of cytosolic DNA leakage. To investigate whether the toxin was inducing micronuclei formation, micronuclei were visualised by immunofluorescence using a combination of DAPI and lamin B1. Micronuclei marked by γH2AX were observed (Fig. 5.1C, marked by white arrow). Only 10-15% of cells contained micronuclei following intoxication, however this was significantly greater than untreated or HQ-intoxicaited cells where less than 1% of cells contained micronuclei (Fig. 5.1D).

Visualisation of unprotected cytosolic ssDNA was attempted by labelling DNA with EdU and measuring cytosolic foci. However, foci could not be identified, possibly because the signal was overwhelmed by nuclear EdU, and possibly because the image resolution was not sufficient to identify such small DNA fragments (data not shown). Instead, whole cell lysates were fractionated into soluble cytosolic, soluble nuclear and chromosomal fractions following a standard intoxication assay. As positive controls, cells were fractionated following treatment with the DNA damage inducers aphidicolin and etoposide, and cells transfected with 20 nM of immunostimulatory DNA which had been shown to mimic cytosolic DNA in other studies (Fu et al., 2019; Lama et al., 2019).

The reliability of the fractionation was confirmed by the presence of tubulin in the cytosolic fraction and histone H3 and lamin B1 in the chromosomal fraction using immunoblotting (Fig. 5.1E). A reliable marker for the soluble nuclear fraction could not be found. γH2AX was also assayed, which as expected was predominantly found in the chromosomal fraction and was particularly evident in WT-intoxicaited and aphidicolin- and etoposide -treated cells. Finally, RPA32 was assayed, which was found predominantly in the soluble cytosolic fraction. This makes sense as in RPA32 immunofluorescence protocols, cultured cells must be treated briefly with detergent prior to fixation that extracts cytosolic RPA whilst retaining nuclear RPA (Ibler et al., 2019).

In order to compare levels of cytosolic DNA, the soluble cytosolic fraction was run on an agarose gel to visualise relative amounts of DNA. DNA was visible in each fraction but was more intense in WT-intoxicaited and aphidicolin- and etoposide -
treated cells compared to untreated and HQ-intoxicated (Fig. 5.1F). The DNA-transfected cells did not show evidence of any increased DNA in the cytosolic fraction (Fig. 5.1F) despite confirming that transfection with empty pcDNA vector induced ISG15 expression (Fig. 5.1G). Taken together, this suggests that the toxin was inducing an ISG response by triggering leakage of damaged DNA into the cytosol, however further experiments would be needed for definitive evidence.

5.2.2 The role of ssDNA-binding proteins in regulation of the toxin ISG response

SsDNA binding proteins (SBPs) are essential for prevention of the accumulation of cytosolic self-DNA. For example, the SBP RPA was found to sequester fragments of damaged DNA at nuclear pores to prevent leakage (Wolf et al., 2016). RPA knockdown was shown to amplify levels of cytosolic DNA, cGAS-STING activity and a type-I IFN response including upregulation of ISG15. The Humphreys lab recently established that the RPA binds toxin induced SSBs in S/G2 (Ibler et al., 2019). RPA plays an important role in the cellular response to the toxin. Toxin-induced DNA damage creates an excess of ssDNA substrate that overwhelms the pool of RPA, resulting in the build-up of unprotected ssDNA that ultimately results in DSBs (Ibler et al., 2019). However, the phenomenon of RPA exhaustion was restricted to S/G2 of the cell cycle and whether the toxin induces SSBs in G1 is not known. As the RPA response was restricted to G2/S phase, and data presented in Fig. 4.5D suggested that the ISG response was occurring in G0/G1 phase, the possibility that different SBPs were detecting toxin induced SSBs in G0/G1 phase was explored.

Global changes in expression of genes labelled as ssDNA binding were examined on STRING v11 within the transcriptome of toxin-treated cells. Of particular interest were those SBPs that were co-incident with the ISG response in G1. Interestingly, most SBPs were downregulated in response to the toxin including all three components of the RPA complex (Fig. 5.2A). Identified hits were involved in DNA metabolism, the DDR or telomere function, with SSBP2, SSBP3 and SSBP4 being of unknown function (Fig. 5.2B). Of the SBPs analysed, HMGB2 and NABP1 were chosen for further analysis.
Fig. 5.2 Regulation of ssDNA-binding proteins in response to the toxin
(A) Heatmap shows 19 genes for ssDNA-binding proteins detected in the microarray presented in fig. 3.4 and represents fold change 24h post-intoxication in WT- and HQ-toxin treated cells due to replication stress in S/G2M phase or damage in G0/G1 phase and compared to asynchronous responses. (B) STRING network highlighting functional and annotated relationships between the genes presented in (A).

HMGB2 (high mobility group box 2) is a master regulator of senescence that regulates transcription of SASP and formation of senescence-associated heterochromatin foci (Aird et al., 2016). HMGB2 is also a DNA sensor and a regulator of innate immune responses (Yanai et al., 2009; Kawasaki, Kawai and Akira, 2011). HMGB2 is typically downregulated upon entry into senescence and its knockdown is sufficient to trigger the senescent program (Zirkel et al., 2018). Indeed, HMGB2 was downregulated two-fold by the toxin compared to HQ (Fig. 5.2A). It was hypothesised that the toxin may be inducing downregulation of HMGB2 resulting in induction of the senescent program, which may include upregulation of the toxin induced ISG response.
**Fig. 5.3** Regulation of HMGB2 and the SOSS complex in response to the toxin

(A) Immunoblots of protein levels in HT1080s of HMGB2 24 h, 48 h and 72 h after treatment with either WT- or HQ-toxin, 24h after treatment with aphidicolin or after 72h of treatment with purified IFNα. (B) Graph shows densitometry analysis (ImageStudio) of HMGB2 relative to tubulin and normalised to untreated (3 independent immunoblots). (C) Immunoblots of protein levels in HT1080s of NABP1, NABP2 and INT3 24 h, 48 h and 72 h after treatment with either WT- or HQ-toxin, 24h after treatment with aphidicolin or after 72h of treatment with purified IFNα. (D - F) Graph shows densitometry analysis (ImageStudio) of NABP1, NABP2 and INT3 relative to tubulin and normalised to untreated (2 independent immunoblots).
Analysis of HMGB2 protein levels by immunoblotting revealed a downregulatory trend between 24-72h post-intoxication (Fig. 5.3A). HMGB2 was upregulated 3-fold by the toxin 24h after intoxication but by 72h had returned to untreated levels (Fig. 5.3B). However, this phenotype was also seen with HQ-toxin, suggesting that HMGB2 regulation was not toxin dependent though later timepoints may be required to assess any role in toxin-induced senescence.

NABP1, together with INT3 and C9Orf80, forms a heterotrimeric SBP known as the SOSS complex, with similar roles to RPA in DNA replication and repair (Huang et al., 2009; Kar et al., 2015). It was one of only three SBPs that were upregulated in a toxin-dependent manner including the mitochondrial helicase C10Orf2 and the telomere regulator TERF2IP. NABP1 was also upregulated two-fold in serum-starved conditions, showing it was possibly sensing SSBs in G0/G1 (Fig. 5.2A). A complementary SOSS complex can be formed with the NABP1 homologue NABP2, INT3 and C9Orf80, although interestingly neither NABP2 nor INT3 were significantly differentially regulated between any conditions in the microarray data (data not shown).

Protein levels of all three components of the SOSS complex were analysed 24-72 h post-intoxication. More intense protein bands were observed for NABP1, NABP2 and INT3 48h post-intoxication compared to HQ (Fig. 5.3C). INT3 and NABP2 were also upregulated by 24 h of continuous treatment with aphidicolin, but interestingly not NABP1. However, quantification revealed that these observable increases were not significant and that there was a high level of variation between replicates (Fig. 5.3E-F). In the case of NABP1, the antibody used was not specifically designed for immunoblotting and the data may be unreliable. NABP1, NABP2 and INT3 were assayed using immunofluorescence following a standard 24 h intoxication assay and with 24 h continuous treatment by aphidicolin as a positive control. Levels of all three proteins increased following treatment with either WT-toxin or aphidicolin compared to untreated and HQ-toxin (Fig. 5.4A). However, only NABP1 showed a significant increase in positive nuclei between untreated and WT-intoxication, and an increase between WT-toxin and HQ-toxin that was not significant (Fig. 5.4B).
There were no significant differences in NABP2 and INT3 levels between any treatments (Fig. 5.4C-D).

**A**

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**B**

- **NABP1**
- **NABP2**
- **INT3**

**Fig. 5.4** NABP1 is upregulated in response to the toxin

(A) Immunofluorescence of NABP1, NABP2 and INT3 24h after treatment with WT-toxin, HQ-toxin and aphidicolin in HT1080 cells. Scale bars indicate 20 μm. (B - D) Quantification of nuclei positive for the proteins in (A). Each circle is the percentage of nuclei from three technical replicates of an independent replicate. One way ANOVA was used with Tukey’s multiple comparison test. Nuclei were counted as positive if greater than the upper quartile of untreated intensity. Asterisks indicate significance. Bars indicate mean and error bars indicate SD. Nuclei indicated by DAPI outline (grey).

It was investigated whether the SOSS complex was performing a similar role to that of RPA in sequestering fragments of damaged DNA and preventing leakage, as
shown in Wolf et al 2016. Cells were transfected with siRNA for each component of the SOSS complex before performing a standard intoxication assay with a 72h chase. The combination of NABP1 or NABP2 siRNA transfection with WT-toxin increased ISG15 expression compared to toxin alone or toxin with control siRNA (Fig. 5.5A). Whereas the increase in ISG15 expression following WT-intoxication was approximately 5-fold with control siRNA, it was 8-fold for NABP1 siRNA and 6-fold for NABP2 siRNA (Fig. 5.5B). However, these differences were not found to significant, although only WT-toxin with NABP1 siRNA was found to be significantly increased compared to untreated.

ISG15 was assayed following SOSS siRNA transfection using immunofluorescence. As seen with immunoblotting, transfection with siRNA for NABP1, NABP2 and INT3 followed by intoxication caused an observable increase in ISG15 levels compared to control siRNA (Fig. 5.5C). High levels of variability were seen between repeats, possibly because the transfection process was inducing ISG15 expression as seen in Fig. 5.1G. It was found that transfection with siRNA for SOSS components caused approximately 60% of nuclei to be positive for ISG15 when compared to the upper quartile of control siRNA intensity in the presence of WT-toxin (Fig. 5.5D). However, this increase was not significant. Together, the data show that SOSS complex components play no significant role in the toxin induced ISG response.
Fig. 5.5 **NABP1 knockdown enhances the toxin dependent ISG15 response**

(A) Immunoblots of protein levels in HT1080s of ISG15 and γH2AX following 48h transfection with siRNA for NABP1, NABP2, INT3 or control, and 72h treatment with WT- or HQ-toxin. (B) Graph shows densitometry analysis (ImageStudio) of 15 kDa free ISG15 band relative to tubulin and normalised to untreated (minimum 2 independent immunoblots). (C) Immunofluorescence of ISG15 (red) 48h after transfection with siRNA for NABP1, NABP2, INT3 or control, and 72h treatment with WT- or HQ-toxin in HT1080 cells. (D) Quantification of ISG15-positive nuclei in (C). Nuclei were counted as positive if greater than the upper quartile of control siRNA-transfected and WT-toxin treated ISG15 intensity. Nuclei indicated by DAPI outline (grey). Scale bars indicate 20μm. Circles indicate technical replicates from two independent replicates. One way ANOVA was used with Tukey’s multiple comparison test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD.
5.2.3 Toxin induced ISG15 is dependent on STING

It was investigated whether the toxin was activating cytosolic DNA sensing pathways. Further analysis of the microarray data, using a list of known DNA sensors summarised in the literature (Dempsey and Bowie, 2015), revealed that only two DNA sensors, IFIH1/MDA5 and PQBP1, were significantly upregulated in response to the toxin (Fig. 5.6A). Of particular interest was IFIH1 (IFN induced with helicase C domain 1) as it is a type-I ISG. However, there was no significant difference in IFIH1 mRNA levels in HT1080 cells between treatment for 24 h with WT- or HQ-toxin (Fig. 5.6B). qRT-PCR was also performed for cGAS, as it was seen to be responsible for detecting cytosolic DNA following RPA knockdown in Wolf et al. However, cGAS mRNA was not upregulated in a toxin-dependent manner and indeed HQ-toxin treatment was shown to significantly induce higher mRNA levels compared to WT-toxin or untreated (Fig. 5.6C). Furthermore, analysis of cGAS protein levels using immunoblotting following a standard intoxication assay revealed no difference between untreated, WT- and HQ-intoxicated cells (Fig. 5.6D).
Fig. 5.6 **The toxin does not directly regulate expression of cytosolic DNA sensors**

(A) Heatmap shows all genes for cytosolic DNA sensing proteins detected in the microarray presented in fig. 3.4C and represents fold change 24h post-intoxication in WT- and HQ-toxin treated cells. (B - C) Quantification of mRNA levels in HT1080s of IFIH1 and cGAS compared between untreated and 24h WT- and HQ-toxin treated samples. Bars represent mean fold change of three samples (three technical replicates run for each sample). Each circle is an individual technical replicate. One way ANOVA was used with Šidák’s multiple comparison test. Asterisks indicate significance. Error bars indicate SD. (D) Immunoblot of protein levels in HT1080s of cGAS 24 h after treatment with either WT- or HQ-toxin. 2 technical replicates per treatment.
However, DNA sensors would not necessarily be differentially expressed at the mRNA or protein level and could instead be constitutively expressed and post-translationally modified in response to stimulus. Therefore, activation of downstream effectors was investigated instead. STING, IRF3 and TBK1 were assayed, all of which are phosphorylated following activation of multiple DNA sensors including cGAS (Fig. 5.7A). Phosphorylated IRF3 could not be detected using immunoblotting and no difference was detected in levels of phosphorylated STING in response to the toxin (Fig. 5.7B). A slight increase of phosphorylated TBK1 (pTBK1) was observed 24, 48 and 72 h post-intoxication with WT-toxin compared to HQ-toxin, although this increase was not found to be significant at any timepoint (Fig. 5.7C).
Fig. 5.7 The toxin dependent ISG15 response is dependent on STING and TBK1
ISG15 was assayed following inhibition of STING and TBK1 by small molecule inhibitors H151 and BX795 respectively. Continual inhibition by both during a standard 72h intoxication assay reduced toxin induced ISG15 (Fig. 5.7D). This effect was significant in the case of both inhibitors, and indeed TBK1 inhibition by BX795 abrogated the toxin induced ISG15 response entirely (Fig. 5.7E). BX795-treated cells displayed stressed morphology characterised by extreme cellular elongation (data not shown), suggesting that the inhibitor may have been affecting other cell processes as well. Regardless, the effects of H151 STING inhibition showed that the toxin ISG response was dependent on STING.

5.3 Discussion

This chapter explored the mechanisms by which the toxin may induce an ISG response. It was hypothesised that the toxin was causing cytosolic leakage of DNA that was activating PRRs and stimulating ISG expression. Using a novel method, the *in vitro* polymerase assay, it was confirmed that the toxin was inducing SSBs in DNA. Furthermore, it was confirmed that there was a significant increase in micronuclei in intoxicated cells, indicative of genomic instability and cytosolic leakage. However, it proved too challenging to find a reliable and direct method of identifying cytosolic DNA. It was possible to show that more DNA was detected in the soluble cytosolic fraction of intoxicated cell lysates, however it is not possible to be certain that this was due to the toxin causing DNA leakage. For example, DNA damage may have weakened the integrity of the nuclear envelope, thus reducing the reliability of the fractionation process. DNA was labelled with EdU in an attempt to detect cytosolic foci, but fragments of cytosolic DNA were likely too small and
thus beyond the limits of detection, especially when the nuclear EdU signal was high enough to overwhelm less intense signals. In future, immuno-FISH using a telomere probe may be a good method to identify cytosolic DNA (Kreienkamp et al., 2018).

The SBP NABP1 was found to be upregulated by the toxin. Most studies in the literature regarding NABP1 focus on its role in the heterotrimeric SOSS complex, formed of NABP1 or NABP2, INT3 and C90rf80 (Huang et al., 2009). The NABP1/NABP2 complexes are complementary and the distinction between their functions remains uncertain. It has been suggested that the SOSS complex is a redundancy measure to RPA at the replication fork due to its ssDNA binding activity and ability to recruit and activate ATR (Kar et al., 2015). Both NABP1 and NABP2 have been shown to cooperate to resolve replication stress, and loss of both was shown to activate P53 and IFN pathways in hematopoietic stem cells (Shi et al., 2017). Interestingly, only NABP1 was seen to be significantly upregulated by the toxin in the microarray, suggesting that it had a distinct role rather than as a component of the SOSS complex. Whereas NABP2 has been shown to directly interact with the MRN complex at DSBs where it initiates homologous recombination, and to localise to stalled replication forks where it has roles in stabilisation and repair, NABP1 remains uncharacterised in comparison (Richard et al., 2011; Bolderson et al., 2014). Recent data has revealed a direct role for NABP1 in RPA recruitment to sites of UV-induced DNA damage (Boucher et al., 2021), and it is possible that NABP1 is performing a similar cooperative role with RPA in response to the toxin.

Knockdown of components of the SOSS complex, especially NABP1, resulted in amplification of the toxin ISG15 response following intoxication. Studies have shown that RPA sequesters fragments of damaged DNA and prevents leakage into the cytosol where they act an IFN response (Wolf et al., 2016). As SOSS has been suggested to have redundant functions to RPA, it is possible that SOSS, or NABP1 alone, may be preventing cytosolic DNA leakage in a similar manner. It is important to note that the increase in ISG15 was not found to be statistically significant following knockdown of NABP2 and INT3, nor was it found to be significant between WT-toxin and untreated in this experiment. Further replicates of both the
immunoblotting and immunofluorescence experiments will be needed to increase the statistical power of the analysis.

Inhibitors of STING and TBK1, which are key regulators of innate immunity, were found to abrogate the toxin ISG response. STING is both a cytosolic DNA sensor itself as well as an adaptor protein regulating signalling via other DNA sensors such as cGAS (Dempsey and Bowie, 2015). TBK1 has numerous roles beyond innate immunity, including in apoptosis, autophagy and cell proliferation (Louis, Burns and Wicks, 2018). Consistent with this, inhibition of TBK1 appeared to have other effects beyond abrogation of the toxin ISG response, as inhibited cells displayed a stressed and abnormal morphology. Furthermore, whereas inhibition of TBK1 abrogated the toxin ISG15 response, significant increases in pTBK1 were not seen between WT- and HQ-toxin treatment. The role of STING and TBK1 in response to the toxin will need further study, for example by examining toxin dependent ISG responses following siRNA mediated knockdown of STING, TBK1 or other components of DNA sensing pathways.

In summary, the toxin induces SSBs in DNA and triggers release of micronuclei. Furthermore, the toxin ISG response is dependent on STING, suggesting that cytosolic DNA sensing pathways are being activated by the toxin. Finally, the toxin ISG response can be amplified by interruption of SBP complexes which could be preventing cytosolic leakage of DNA. Having examined regulation of the toxin ISG response, the role it was playing in the wider host response was explored. As ISG15 was the most reliably and consistently expressed component of the toxin ISG response, the specific role of ISG15 was explored in greater detail.
6 The role of ISG15 in the host response to the typhoid toxin

6.1 Introduction

IFN-stimulated gene 15 (ISG15) was first identified in 1979 when researchers found that treating mouse Ehrlich ascites tumour cells with purified IFN stimulated production of mRNA for a 15 kDa protein, which they translated in vitro (Farrell, Broeze and Lengyel, 1979). It was not described again until five years later when the human and bovine forms were identified and correlated with a level of IFNα and IFNβ stimulation sufficient to induce an antiviral state (Korant et al., 1984). ISG15 expression was shown to be dose dependent on IFN concentration and to inversely correlate with vesicular stomatitis virus yield (Haas et al., 1987).

Attempts to sequence the protein showed that cDNA clones for the protein could be prepared from IFN-treated human lymphoblastic cells but not untreated cells (Blomstrom et al., 1986). Whilst the initial sequence was erroneous, the correct sequence was found the following year and showed that ISG15 was 168 amino acids long and had a molecular weight of 17,890 Da (Reich et al., 1987). This full-length protein was found to be a precursor to the mature form of ISG15, which is formed by cleavage of 8 C-terminus amino acids to form a 17,145 Da protein (Knight et al., 1988).

ISG15 was initially named ubiquitin cross-reactive protein (UCRP) as it was of sufficient sequence homology to ubiquitin to be detected by mono-ubiquitin antibodies at the time (Haas et al., 1987). ISG15 is formed of two ubiquitin-like domains and has an identical C-terminus motif of LRLRGG to ubiquitin, leading to initial hypotheses that it was a ubiquitin isoform (Fig. 6.1A-B) (Knight et al., 1988). Interestingly however, whereas ubiquitin is highly conserved between different species, ISG15 is only found in vertebrates, unlike ubiquitin which is found in most eukaryotic cells (Zhang and Zhang, 2011).
6.1.1 The ISGylation pathway

Much like ubiquitin, ISG15 was found to conjugate to other intracellular proteins in a process now known as ISGylation (Fig. 6.1C) (Loeb and Haas, 1992). Ubiquitination is a three-step process involving ubiquitin activation, conjugation and ligation using E1, E2 and E3 enzymes respectively (Pickart and Eddins, 2004). It was hypothesised by Loeb and Haas that ISGylation would be a ‘parallel pathway’ to ubiquitination, and a proteomics study in 2005 revealed that known E1, E2 and E3 ubiquitination enzymes were pulled down with ISG15 (Zhao et al., 2005).

Ubiquitin E1-like protein 1 (UBE1L) is an E1 activating enzyme for ISG15 and was found to be inhibited by the NS1B protein of influenza B (Yuan and Krug, 2001).
UbcH8 and UbcH6 were identified as ISGylation E2 enzymes (Kim et al., 2004; Zhao et al., 2004; Takeuchi et al., 2005). E3 ligases include HERC5 (HECT E3 ligase) (Dastur et al., 2006; Takeuchi, Inoue and Yokosawa, 2006; Wong et al., 2006) and EFP (oestrogen-responsive finger protein) (Zou and Zhang, 2006) in humans, as well as HERC6 in mice (Ketscher et al., 2012; Oudshoorn et al., 2012). Several of these studies found that the ISGylation machinery was also induced by IFN.

Surprisingly, it was not until 10 years after the process of ISGylation was discovered that the first ISGylated substrate was found. Serpin 2A was shown to be upregulated in macrophages following infection with Mycobacterium bovis bacillus Calmette-Guérin (BCG), Salmonella Typhimurium and Listeria monocytogenes and indeed ISGylated conjugates were found following incubation with bacterial products (Hamerman et al., 2002). This was followed by the discovery of ISGylation of signal transducers such as Cγ1, JAK1, ERK1 and STAT1, in the context of which ISGylation appeared to have a regulatory role rather than marking proteins for proteasomal degradation like ubiquitin (Malakhov et al., 2003). The use of high throughput proteomics massively increased the known pool of ISGylation substrates. Mass spectrometry of NiNTA purified His-tagged ISG15 revealed 158 target proteins with a wide range of functions including antiviral activity, chromatin remodelling, mRNA splicing, translation, cytoskeletal organisation and stress responses (Zhao et al., 2005). More recent proteomic analysis revealed 930 ISGylated sites on 434 proteins in response to L. monocytogenes infection, including modification of factors involved in autophagy (Y. Zhang et al., 2019).

The process of ISGylation is carefully regulated. Ubiquitin specific protease 18 (USP18) was shown to be an ISG15-specific protease and found to be IFN-inducible (Malakhov et al., 2002). USP18 is specific to ISG15 based on a small hydrophobic patch within the interaction site (Basters et al., 2017). Interestingly, USP18 does not solely act as a ISG15-protease but also directly binds to the IFN receptor and negatively regulates IFN signalling in a STAT2 dependent process (Malakhova et al., 2006; Arimoto et al., 2017). Furthermore, USP18-deficient mice die and are not rescued by excess ISG15, showing that USP18 has roles beyond ISG15 deconjugation (Knobeloch et al., 2005).
ISG15 does not appear to mark proteins for degradation like ubiquitin, but it does competitively conjugate to ubiquitin-binding sites and in the process interfere with ubiquitin-dependent proteasomal degradation (Liu, Xiao-Ling and Hassel, 2003; Desai et al., 2006). Furthermore, ISG15 can form mixed chains with ubiquitin, which also negatively regulates ubiquitin-dependent proteasomal degradation (Fan et al., 2015).

6.1.2 Free ISG15

As well as functioning as an adduct for regulation of other proteins, ISG15 has also been shown to act freely and is secreted as a cytokine. ISG15 was detected in conditioned media from IFNβ-treated monocytes and lymphocytes (Knight and Cordova, 1991), and detected in healthy human serum following IFN treatment (D'Cunha, Ramanujam, et al., 1996). Free ISG15 was seen to induce IFNγ secretion in CD3+ cells (Recht, Borden and Knight, 1991) and has been linked to natural killer T cell proliferation (D'Cunha, Knight, et al., 1996) dendritic cell maturation (Neves et al., 2005) and as a chemotactic factor for neutrophils (Ohashi et al., 2003). Regulation of the ratio of free ISG15 to ISGylated ISG15 is cell-type dependent (Tecalco Cruz and Mejía-Barreto, 2017).

6.1.3 Antiviral Functions of ISG15

The function of ISG15 as an antiviral molecule is well established in the literature and has been extensively reviewed (Morales and Lenschow, 2013; Hermann and Bogunovic, 2017; Perng and Lenschow, 2018). HERC5, the ISG15 E3 ligase, is associated with the polyribosome, meaning that it is primarily newly synthesised proteins that are targeted for ISGylation. In a virally infected cell the majority of newly synthesised proteins will be viral proteins, meaning that ISG15 is able to preferentially ISGylate viral proteins (Durfee et al., 2010). ISGylation of viral proteins can disrupt interaction of viral proteins with host pathways or prevent formation of oligomeric viral protein structures, therefore interrupting viral replication (Perng and Lenschow, 2018). Examples of the antiviral effects of ISG15 include activity against
influenza, herpes and Sindbis virus (Lenschow et al., 2005, 2007; Giannakopoulos et al., 2009).

ISG15 can regulate host responses and induce an antiviral state in cells. For example, ISGylation has been shown to have a role in mitochondrial dysfunction and oxidative phosphorylation in macrophages infected with vaccinia virus, reducing viral titre (Baldanta et al., 2017). ISG15 enhances viral antigen processing and MHC class I antigen presentation (Held et al., 2021). ISG15 can also inhibit translation of viral proteins. ISGylation was shown to activate the ds-RNA dependent protein kinase (PKR), which inhibits translation by phosphorylation of eIF2α in response to viral infection (Okumura et al., 2013). ISGylation of mRNA-binding 4EHP allows it outcompete the translation initiation factor eIF4E when binding 5’ mRNA caps, thus inhibiting translation (Okumura, Zou and Zhang, 2007).

Many of the antiviral roles of ISG15 are dependent on its role ISGylating other proteins, and indeed mice lacking the conjugating UBE1L enzyme showed increased susceptibility to influenza B infection (Lai et al., 2009). However, conjugation is not essential for viral protection in some contexts, as ISG15 was found to be essential to the control of Chikungunya virus infection in both the presence and absence of UBE1L (Werneke et al., 2011). Interestingly, clinical studies of patients with ISG15 deficiency revealed that ISG15 plays a redundant role in antiviral immunity in humans (Bogunovic et al., 2012).

6.1.4 Role of ISG15 in non-viral infections

Upregulation of ISG15 by bacterial PAMPs was found several decades ago (Li et al., 2001), but until recently the role of ISG15 in response to non-viral pathogens was not clear. Recent studies have shed light on the roles of ISG15 in response to bacterial pathogens including S. Typhimurium, L. monocytogenes, M. tuberculosis and fungal pathogens including Candida albicans (Perng and Lenschow, 2018).

Mice with a loss of function mutation in the deconjugating enzyme USP18 showed increased susceptibility to S. Typhimurium infection. However, this found to be due to the role of USP18 as an inhibitor of IFN regulation, rather than its role as a
delISGylase, as ISG15 knockout had no effect on survival or bacterial burden either *in vivo* or *in vitro* (Dauphinee et al., 2014; Radoshevich et al., 2015).

Data on the role of ISG15 in *M. tuberculosis* infection is conflicted. One study reported that ISG15-deficient mice were susceptible to infection, suffering from increased lethality after 150 days (Bogunovic et al., 2012). The study went on to show clinical data revealing that ISG15 deficient patients are susceptible to mycobacterial infection. However, a separate study found no difference in lethality in mice and showed that ISG15 knockout mice had reduced bacterial burden in their spleens and lungs at acute (7 day) and chronic (77 day) phases of infection, suggesting that ISG15 was having a detrimental effect (Kimmey et al., 2017).

ISG15 was shown to have a host defensive role against *L. monocytogenes* infection *in vivo* and *in vitro*, with ISG15 deficiency resulting in increased bacterial burden. The role of ISG15 was protective in a conjugation-dependent manner that required activation of cytosolic DNA sensing processes involving STING, TBK1 and IRF-3 and -7, but was type-I IFN independent. The study further revealed that infection triggered ISGylation of components of secretory pathways and modulation of cytokine release (Radoshevich et al., 2015).

ISG15 was found to be upregulated, along with the ISGylation machinery, by *C. albicans* infection. Knockdown of ISG15 increased the severity of keratitis in mice, suggesting that ISG15 again had a defensive role (Dong et al., 2017).

### 6.1.5 ISG15 in the DDR

ISG15 has been shown to be induced in response to different genotoxic agents such as camptothecin, doxorubicin and UV radiation. Recent studies have shown that ISG15 plays a role in response to genotoxic stress by ISGylating transcription factors controlling cell fate such as P53 and the P53-related p63 isotype ΔNp63α, as well as key components of the replisome such as PCNA (Jeon, Park and Chung, 2017).

ISGylation of PCNA was found to prevent translesion DNA synthesis (TLS) and hence reduce mutagenesis. Polymerase-η is recruited by ubiquitinated PCNA
following DNA damage, which can continue DNA synthesis despite the presence of breaks. ISGylation of PCNA stimulated the release Polymerase-\(\eta\), terminating TLS and thus preventing error-prone DNA replication (Park et al., 2014).

Another study indicated that non-covalent localisation of ISG15 at replication forks in complex with PCNA leads to conjugation-independent interaction with the helicase RECQ1, promoting replication fork restart after stalling and causing replication stress and chromosomal breakage. This led to sensitisation of cells treated with chemotherapeutic drugs (Raso et al., 2020).

ISGylation of \(\Delta Np63\alpha\), which inhibits P53 transcription, triggers its cleavage by caspase-2 and hence prevents tumorigenesis (Jeon et al., 2012). ISG15 and indeed the ISGylation machinery components UBE1L, UBCH8 and EFP were shown to have P53-response elements and to be activated in a DNA damage dependent manner by P53. Furthermore, ISG15 was shown to ISGylate P53 itself and enhance P53 binding to its target promoters, including its own promoter. This positive feedback loop was necessary for prevention of excessive cell growth and tumorigenesis (Park et al., 2016). A further role of ISG15 in tumorigenesis was shown by ISGylation of the tumour suppressor PTEN, and that regulation and stability of PTEN was dependent upon USP18 activity (Mustachio et al., 2017). ISG15 and USP18 have been shown to induce apoptosis in cancer cells by suppression of the NF-\(\kappa\)B pathway (Mao et al., 2016).

### 6.2 Results

The toxin has been shown to promote Salmonella infection (Ibler et al., 2019), and S. Typhi infection has also been shown to increase the likelihood of gallbladder cancer (Scanu et al., 2015). ISG15 has been shown to have roles in response to bacterial infection and regulation of oncogenesis. Therefore, potential roles for ISG15 in response to the toxin were investigated in the context of both Salmonella infection and development of oncogenic phenotypes.
6.2.1 ISG15 is upregulated by *Salmonella* Javiana infection

It was first investigated whether ISG15 was upregulated by infection with *Salmonella*. For this, four strains of *Salmonella* were used including the NTS serovar S. Typhimurium 19 (ST19), iNTS serovar S. Typhimurium 313 (ST313), toxigenic wild-type S. Javiana (S. J. \(^{WT}\)) and mutant non-toxigenic S. Javiana (S. J. \(^{ΔCdtB}\)). S. Javiana encodes the Javiana toxin with an identical CdtB subunit to the typhoid toxin, and thus was used as a hazard group 2 (HG2) model for S. Typhi, which is HG3 and requires level 3 containment. Javiana toxin has been shown to cause a similar DNA damage phenotype to the typhoid toxin of S. Typhi (R. A. Miller and Wiedmann, 2016). As ISG15 was strongly upregulated 72 h post-intoxication with purified toxin, ISG15 was assayed using immunofluorescence in HT1080s 72 h after infection with an MOI of 10. The intracellular presence of all four serovars of *Salmonella* was confirmed (Fig. 6.2A). ISG15 was not significantly upregulated by infection with any of the four serovars after 72 h, although it was slightly elevated by Javiana\(^{WT}\), Javiana\(^{ΔCdtB}\) and ST19 compared to untreated (Fig. 6.2B). However, Javiana\(^{WT}\) infection did cause a significant reduction in EdU positive cells compared to untreated (Fig. 6.2C). None of the other serovars caused a significant reduction in EdU, showing that Javiana\(^{WT}\) was likely causing cell cycle arrest through the action of the toxin.
To confirm that Javiana<sup>WT</sup> was secreting the toxin and inducing a DDR, γH2AX was assayed using immunoblotting 24h post-infection. Infection with Javiana<sup>WT</sup> caused a strong observable increase in γH2AX protein levels unlike ST19, ST313 and
Javiana$^{\Delta \text{CdlB}}$, showing that the response was toxin dependent (Fig. 6.3A). This increase was $\sim$12-fold compared to untreated, although this was not found to be significant to the $\sim$5-fold increase observed with purified toxin or the $\sim$4-fold increase with Javiana$^{\Delta \text{CdlB}}$ (Fig. 6.3B). However, only Javiana$^{\text{WT}}$ was significant to untreated, unlike Javiana$^{\Delta \text{CdlB}}$.

ISG15 was assayed at 48h and 72h post-infection and saw faint bands for ISG15 following Javiana$^{\text{WT}}$ infection at both timepoints, however this was not comparable to the ISG15 levels observed following intoxication with purified toxin (Fig. 6.3C-D). Javiana$^{\text{WT}}$ infection did appear to induce slightly more ISG15 expression compared to ST19, ST313 and Javiana$^{\Delta \text{CdlB}}$. It was investigated whether higher MOIs or longer timepoints would affect the ISG15 response. ISG15 was assayed 6 days (144 h) post-infection with Javiana$^{\text{WT}}$ and Javiana$^{\Delta \text{CdlB}}$ with MOIs of 10, 20 and 50. A strong ISG15 upregulation was observed with Javiana$^{\text{WT}}$ at an MOI of 10 (Fig. 6.3E). This was significantly higher than the response to Javiana$^{\Delta \text{CdlB}}$ which was not significant to untreated, showing that the response was toxin dependent (Fig. 6.3F). Interestingly the Javiana$^{\text{WT}}$ ISG15 response was reduced at MOIs of 20 and 50, where it was not significant to untreated. This was possibly due to higher MOIs causing cell death, which can be seen in the reduction in intensity of the tubulin bands at higher MOIs of Javiana$^{\text{WT}}$ but not with Javiana$^{\Delta \text{CdlB}}$. Consistent with earlier findings with WT-Tox (Fig. 4.7A), ISGylation was observed with IFN but not Javiana$^{\text{WT}}$ (Fig. 6.3E).
Fig. 6.3 *Salmonella* Javiana infection induces a DNA damage response and upregulates ISG15
(A) Immunoblot of protein levels of γH2AX in HT1080s 24 h after treatment with either WT- or HQ-toxin, after 24 h continuous treatment with purified IFNa, or 24 h after infection with ST19, ST313, S. Javiana\textsuperscript{WT} or S. Javiana\textsuperscript{ΔCdtB}. (B) Densitometry analysis (ImageStudio) of γH2AX relative to tubulin from (A) and normalised to untreated (circles represent independent immunoblots). One way ANOVA with Tukey’s multiple comparisons test. (C) Immunoblot of protein levels in HT1080s of ISG15 48 h after treatment with either WT- or HQ-toxin, after 48 h continuous treatment with purified IFNa, or 48 h after infection with ST19, ST313, S. Javiana\textsuperscript{WT} or S. Javiana\textsuperscript{ΔCdtB}. (D) as (C) but 72 h after treatment or infection. (E) Immunoblot of protein levels in HT1080s of ISG15 144 h after treatment with 2, 20 and 100 ng/ml WT-toxin, 20 ng/ml HQ-toxin, 144 h continuous treatment with purified IFNa or 144 h after infection with MOI 10, 20 or 50 of S. Javiana\textsuperscript{WT} or S. Javiana\textsuperscript{ΔCdtB}. (F) Densitometry analysis (ImageStudio) of 15 kDa free ISG15 band relative to tubulin from (E) and normalised to untreated (circles represent independent immunoblots). One way ANOVA with Šidák’s multiple comparisons test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD.

### 6.2.2 ISG15 overexpression reduces *Salmonella* CFUs

Having established that ISG15 was upregulated by *Salmonella* infection in a toxin-dependent manner, role that it was playing in the host response was explored. HT1080s were pre-treated with 72 h of continuous IFNa treatment to strongly upregulate ISG15. Both pre-treated and untreated cells were infected with Javiana\textsuperscript{WT} and Javiana\textsuperscript{ΔCdtB} and *Salmonella* invasion assayed 24 h post-infection by culturing whole cell lysates on LB agar. At 24h, intracellular Javiana\textsuperscript{WT} had approximately doubled from 2 h post-infection, but interestingly there was no significant difference between CFUs from pre-treated and untreated cells (Fig. 6.4A). Intracellular Javiana\textsuperscript{ΔCdtB} however approximately quadrupled in untreated cells, a significant increase compared to IFNa pre-treated cells where they only doubled (Fig. 6.4B). At 48 h post-infection, there was significantly more Javiana\textsuperscript{WT} CFUs in IFN pre-treated cells compared to untreated cells, but there was no significant difference in Javiana\textsuperscript{ΔCdtB} CFUs between treatments.
Fig. 6.4 IFN pre-treatment reduces Salmonella burden 24 h post-infection
(A) S. JavianaWT CFUs 2, 24 and 48 h after infection in HT1080s with and without 72 h continuous pre-treatment with purified IFNα. CFUs are a percentage of CFUs at 2h. (B) as (A) but for S. JavianaΔcdtB. Circles indicate individual CFU counts from one independent replicate. One way ANOVA with Šidák’s multiple comparisons test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD.

IFN upregulates a large array of different ISGs, meaning that the differences seen in Fig. 6.4 cannot be attributed to ISG15 alone. To uncouple the role of ISG15 from other ISGs, HT1080s were transfected with a mammalian expression vector encoding a 5HA-tagged mouse ISG15 (pCAGGs-5HA-mISG15). It was first confirmed that the transfection did induce increased expression of 5HA-ISG15 by detecting a 20 kDa band using both ISG15 and HA antibodies (Fig. 6.5A). It was determined that the optimal transfection reagent was FuGENE as opposed to lipofectamine, which caused ISG15 expression. A pcDNA empty vector was transfected into cells as a control and did not lead to ISG15 induction. However, a non-specific band from the HA antibody was visible at approximately 20-25 kDa.
**ISG15** over-expression reduces *Salmonella* burden 24 h post-infection

(A) Immunoblot of protein levels of ISG15 and HA in HT1080s 24 h after transfection with either pCAGGS-5HA-mISG15 or empty vector pcDNA using lipofectamine or FuGENE as a transfection reagent. Non-specific band indicated by asterisk. (B) Immunoblot of protein levels of ISG15 and HA in HT1080s 24, 48, 72 and 144 h after transfection with either pCAGGS-5HA-mISG15 or empty vector pcDNA using FuGENE as a transfection reagent. (C - E) Densitometry analysis (ImageStudio) of one immunoblot showing free ISG15 (15 kDa), free 5HA-ISG15 (20 kDa) and HA (20 kDa) relative to tubulin from (B). (F) *S. Javiana*WT CFUs 2, 24, 48 and 72 h after infection in HT1080s with and without 24 h transfection with pCAGGS-5HA-mISG15 or empty vector pcDNA. CFUs are a percentage of CFUs at 2h. Circles indicate individual CFU counts from one independent replicate. (G) as (F) but for *S. Javiana*ΔcdtB.

One way ANOVA with Šidák’s multiple comparisons test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD.

ISG15 and HA were assayed using immunoblotting every 24 h over a 6-day time course to determine the timepoint at which ISG15 was best expressed (**Fig. 6.5B**).
Endogenous ISG15 (15 kDa) and 5HA-ISG15 (20 kDa) were most strongly detected at 24 h and decreased steadily to low levels at 6-days post-transfection (Fig. 6.5C-E). Interestingly, it was also apparent that transfection with the pcDNA empty vector at later timepoints (48 h onwards) induced endogenous ISG15 expression, with bands visible at 15 kDa in the control lanes. Approximately 60% of ISG15 induced by the HA-ISG15 transfection was not HA-tagged, suggesting that the HA tags were being cleaved or not expressed.

Cells were transfected with 5HA-ISG15 and empty vector before infection with Javiana\textsuperscript{WT} 24 h post-transfection, when ISG15 was highest expressed. Invasion was assayed by culturing whole cell lysates on LB agar 24 h post-infection. Like previous results, there was no significant difference between 5HA-ISG15 and empty vector transfection in Javiana\textsuperscript{WT} CFU counts at any timepoint (Fig. 6.5F), presumably because ISG15 is present in all conditions due to the toxin. Thus, to avoid ISG15-induction by the toxin, the experiment was repeated with Javiana\textsuperscript{ΔCdtB}. ISG15 overexpression resulted in significantly less CFUs of Javiana\textsuperscript{ΔCdtB} 24h and 48h post-infection (Fig. 6.5G). Taken together, ISG15 overexpression caused a decrease in Salmonella CFUs suggesting ISG15 counteracts infection.

### 6.2.3 ISG15 knockout promotes oncogenic phenotypes

In order to further examine the role of ISG15 in the host response to the toxin, responses to the toxin in wild-type mouse embryonic fibroblasts (MEF\textsuperscript{WT}), ISG15 knockout MEFs (MEF\textsuperscript{ISG15\textsuperscript{-/-}}) and USP18 C61A MEFs (MEF\textsuperscript{USP18\textsuperscript{CA}}) were investigated, all of which were kindly donated by Liliana Radoshevich (University of Iowa). USP18 C61A is a constitutive inactive mutant of USP18, meaning that ISGylated proteins cannot be deISGylated and build up within the cell (Fig. 6.6A).
**Fig. 6.6 Validation of MEF<sup>ISG15<sup>-/-</sup></sup> and MEF<sup>USP18 CA</sup> cell lines**

(A) Schematic of the ISGylation process and how it is modified in MEF<sup>WT</sup>, MEF<sup>ISG15<sup>-/-</sup></sup> and MEF<sup>USP18 CA</sup> cell lines. (B) Immunoblot of protein levels of ISG15 and phospho-P53 in MEF<sup>WT</sup>, MEF<sup>ISG15<sup>-/-</sup></sup> and MEF<sup>USP18 CA</sup> cells 72 h after treatment with either WT- or HQ-toxin. (C) Densitometry analysis (ImageStudio) of 15 kDa free ISG15 band relative to tubulin from (B) and normalised to untreated (circles represent independent immunoblots). One way ANOVA with Tukey’s multiple comparisons test. Asterisks indicate significance. Bars indicate mean and error bars indicate SD. (D) Densitometry analysis (ImageStudio) of phospho-P53 relative to tubulin from (B) and normalised to untreated (one immunoblot).

ISG15 levels were tested within these cell lines in response to a standard intoxication assay with WT- and HQ-toxin. WT-toxin significantly induced ISG15 72 h post-intoxication compared to untreated and HQ (Fig. 6.6B-C). ISG15 was not detectable in MEF<sup>ISG15<sup>-/-</sup></sup> cells aside from a faint background signal. Only low levels of ISG15 were visible in MEF<sup>USP18 CA</sup> cells. Interestingly, WT-toxin caused high levels of
cell death in \text{MEF}^{\text{USP18CA}} \text{cells}, which is reflected by the fainter tubulin signal in the WT-toxin lane.

As ISG15 has been shown to be induced by P53, and to promote activation of P53, levels of phosphorylated P53 (p-P53) were assayed in the MEF lines. There was no observable difference in p-P53 levels in untreated or HQ-intoxicated cells of any of the three cell lines (Fig. 6.6B). However, there was approximately three-fold more p-P53 in WT-intoxicated MEF\textsuperscript{WT} cells compared to MEF\textsuperscript{ISG15\text{-/-}} (Fig. 6.6D). P-P53 also increased approximately two-fold in WT-intoxicated MEF\textsuperscript{USP18CA} cells, although the reliability of this figure is uncertain due to the high levels of cell death and comparatively low sample amount. This experiment will need to be repeated before P53 activation can be reliably determined, however it does provide an initial indicator that ISG15 is involved in P53-activation in response to the toxin.

EdU was assayed using immunofluorescence to determine cell fate responses in the MEF lines 72 h after intoxication. EdU was added for 2 h pre-fixation. Untreated and HQ-intoxicated cells of all three lines appeared to be replicating normally (Fig. 6.7A). However, WT-toxin caused high levels of cell death in both MEF\textsuperscript{WT} and MEF\textsuperscript{USP18CA} cells, with only approximately 5% cells per field of view (FOV) compared to untreated (Fig. 6.7B). Of the MEF\textsuperscript{WT} and MEF\textsuperscript{USP18CA} cells that remained, there was a significant reduction in EdU positive nuclei, with approximately 20% of nuclei EdU positive following WT-intoxication compared to 40-50% in untreated and HQ-intoxicated cells (Fig. 6.7C). This showed that in MEF\textsuperscript{WT} and MEF\textsuperscript{USP18CA} cells the toxin was causing both cell death and cell cycle arrest.
Fig. 6.7 ISG15 knockout promotes tumorigenic phenotypes in response to the toxin (A) Immunofluorescence of DAPI (blue) and EdU (magenta) in response to WT- and HQ-toxin in MEF<sup>WT</sup>, MEF<sup>ISG15−/−</sup> and MEF<sup>USP18 CA</sup> cells 72 h post-intoxication. Scale bars indicate 10 μm. Micronuclei indicated with white arrows. (B) Quantification of cells per field of view (FOV) in (A). Cell count is normalised to untreated in each cell line. (C) Quantification of nuclei positive for EdU in (A). Each circle is a technical replicate from two independent replicates. One way ANOVA was used with Tukey’s multiple comparison test. Bars indicate mean and error bars indicate SD.

However, in MEF<sup>ISG15−/−</sup> cells, less cell death and less cell cycle arrest were observed in response to the toxin. There were approximately 3-fold more cells observed per FOV in WT-intoxicated MEF<sup>ISG15−/−</sup> cells compared to MEF<sup>WT</sup> and MEF<sup>USP18 CA</sup> cells (Fig. 6.7B). Furthermore, WT-intoxicated MEF<sup>ISG15−/−</sup> cells showed no significant difference in EdU-labelling compared to untreated and HQ-intoxicated cells (Fig. 6.7C). This showed that cell death and cell-cycle arrest by the toxin was impaired in MEF<sup>ISG15−/−</sup> cells relative to MEF<sup>WT</sup>. Notably, several of the nuclei positive for EdU in WT-intoxicated MEF<sup>ISG15−/−</sup> cells contained micronuclei (Fig. 6.7A, indicated with
white arrows), hallmarks of genomic instability. Thus, WT-intoxicated MEF\textsuperscript{ISG15\textasciitilde} cells appear to replicate despite DNA damage. It was hypothesised that ISG15 was protecting against development of oncogenic phenotypes by causing cell death in damaged MEF\textsuperscript{WT} cells, and ISG15 knockout was allowing survival of these potentially cancerous cells.

In order to examine this further, a clonogenic assay was used to determine the ability of each cell line to replicate and form colonies in the presence of the toxin. None of the three MEF lines were able to form colonies in response to 20 ng/ml of WT-toxin (data not shown), so the toxin concentration was reduced to 0.2 ng/ml. WT-toxin caused a 30% reduction in MEF\textsuperscript{WT} and MEF\textsuperscript{USP18\textasciitilde} colonies, but only a 15% reduction in MEF\textsuperscript{ISG15\textasciitilde} colonies, again showing that ISG15 knockout enabled increased survival and replication in the presence of the toxin (Fig. 6.8A-B).

![Fig. 6.8 ISG15 knockout permits greater MEF colony formation in the presence of the toxin
(A) Images of all MEF\textsuperscript{WT}, MEF\textsuperscript{ISG15\textasciitilde} and MEF\textsuperscript{USP18\textasciitilde} colonies in 10cm dishes 8 days after treatment with 0.2 ng/ml of WT-toxin. (B) Quantification of colony count in (A) 8 d after treatment with 0.2 ng/ml of WT-toxin or HQ-toxin. Colony counts are normalised to untreated of each cell line. Circles are technical replicates from one independent replicate.](image-url)
6.3 Discussion

Both intoxication with purified toxin and infection with toxigenic \textit{S. Javiana} was found to induce ISG15 expression. \textit{Salmonella} infection in the absence of toxin (\textit{S. Javiana}^{\text{CDT}}) did not induce ISG15, indicating that other PAMPs such as LPS are not responsible for the response. Whereas the concentration of purified toxin was controlled (20 ng/ml), the concentration of toxin secreted during infection was unknown. However, the ISG15 response at 144 h post-infection was comparable to the responses seen at earlier timepoints with purified toxin. The difference in time could be attributed to the time taken for \textit{Salmonella} to replicate intracellularly and to express and secrete the toxin. However, higher \(\gamma\)H2AX expression was observed 24h post infection compared to 24h following intoxication, indicative of higher toxin activity even at this early timepoint. However, significant \(\gamma\)H2AX responses to the toxin have been seen with as low as 0.05 ng/ml of toxin (Ibler \textit{et al.}, 2019). It is possible that \textit{S. Javiana} infection was able to induce a higher \(\gamma\)H2AX response than purified toxin alone in combination of other PAMPs such as LPS. To conclusively determine the regulation of ISG15 following \textit{S. Javiana} infection, experiments examining the ISG15 response at 24h, 48h and 72h will need to be repeated. Furthermore, it would be interesting to perform similar infection time courses with \textit{S. Typhi}.

The timeline of ISG15 expression differs greatly from that observed in other studies, where ISGs including ISG15 are not seen until 55 days after intoxication with \textit{E. coli} CDT (Pons \textit{et al.}, 2021). However, Pons \textit{et al} used a considerably lower concentration of CDT (0.25 ng/ml) than this study (20 ng/ml) and chronically exposed cells to the toxin as opposed to a 2 h pulse followed by fresh media chase. It would be interesting to replicate their experiments using the typhoid toxin, as this would possibly be a more suitable method of analysing chronic exposure to \textit{Salmonella} and the toxin.

Interestingly, ISG15 was the strongest expressed when infecting cells with lower MOIs (10 compared to 20 or 50). It is possible that at higher MOIs, ISG15-expressing cells were killed by higher bacterial loads. Indeed, higher amounts of cell death were observed at higher MOIs.
Overexpression of ISG15 reduced *Salmonella* burden 24 h after infection, whether overexpression was caused by pre-treatment with purified IFN or transfection with a mammalian expression vector encoding ISG15. ISG15 has previously been shown to have no effect on *Salmonella* Typhimurium invasion (Radoshevich *et al.*, 2015), with no significant difference observed in *Salmonella* invasion between ISG15/− MEFs and WT MEFs. However, it is important to note that CFU measurements were taken at 4 h post-infection in Radoshevich *et al.*, as opposed to 24 h in this study. It may take a longer period of time for ISG15 to exert its role.

Interestingly, a decrease in *Salmonella* burden was only seen in ΔCdtB *Salmonella*. This could be because wild-type Javiana encodes the toxin, which would have been inducing ISG15 even in the absence of purified IFN or ISG15 transfection, albeit at lower levels. At later timepoints (48 h and 72 h), *Salmonella* burden reduced to almost zero, probably because intracellular *Salmonella* replication caused infected cells to lyse and spill *Salmonella* into media containing gentamicin where they were killed.

Beyond a function of ISG15 in protecting against infection, ISG15 knockout promotes survival of intoxicated cells with signs of genomic instability, suggesting that ISG15 could be protecting the host against tumorigenic effects of the toxin. Initial data also showed that ISG15 knockout was concomitant with a reduction in phosphorylated P53. However, further experiments will be needed to confirm these findings regarding suppression of P53-activation in ISG15/− cells and thus determine if ISG15 is indeed acting as a tumour suppressor.
Part 3: Discussion

7 Discussion

My thesis explored the host responses to the toxin with an aim of understanding the role of the toxin in Salmonella infection and potential links to cancer. I found that the toxin upregulates genes linked to the type-I IFN pathway, which has links to infection, senescence and cancer. One of these genes, ISG15, plays a role in the response to the toxin by both reducing the burden of Salmonella infection and potentially acting as a suppressor of genomic instability.

7.1 The effect of the toxin ISG response on Salmonella infection

The typhoid toxin facilitates chronic carriage and systemic Salmonella infection in mice (Del Bel Belluz et al., 2016; Miller et al., 2018). In a human infection challenge study, the toxin was shown to have no effect on initiating typhoid, in fact, toxin-negative S. Typhi increased pathology and significantly prolonged bacteraemia (Gibani et al., 2019). This is consistent with mouse infection models where toxigenic Salmonella reduced inflammation (Miller et al., 2018) and promoted host survival relative to the lethal effects of the toxin-negative strain (Del Bel Belluz et al., 2016). How does this correlate with findings in this study that the toxin induces an innate immune response characterised by expression of ISGs, which typically are associated with anti-infective roles?

It is possible that the toxin ISG response is anti-inflammatory and thus protects Salmonella. Type-I IFN responses have been shown to have anti-inflammatory roles (Benveniste and Qin, 2007). For example, ISG15 protects hosts from excessive inflammation by negatively regulating IFN signalling (Zhang et al., 2015). A recent in vivo study in mice showed that the typhoid toxin induced an anti-inflammatory senescent state characterised by NFκB and P16 activation following DNA damage (Martin et al., 2021). Furthermore, senescent cells are more susceptible to
Salmonella infection (Ibler et al., 2019). It is interesting to speculate whether the toxin ISG response is involved in establishing an anti-inflammatory state.

### 7.2 What regulates the toxin ISG response?

In chapter 4, it was shown that the toxin led to upregulation of ISGs but not IFNs themselves. Canonically, ISG expression is triggered by expression and secretion of IFNs. However, no significant differential IFN expression was seen between WT and HQ-intoxicated cells, and qRT-PCR revealed that IFNα was significantly upregulated by both WT- and HQ-toxin compared to untreated cells. It is possible that other PAMPs in the toxin preparations such as *E. coli* LPS were inducing IFN expression. Further information will be needed to confirm the role of IFNs in response to the toxin.

In chapter 6, initial data suggested that the toxin was also causing P53 activation in MEFs. ISG15 and the ISGylation machinery contain P53 response elements (Park et al., 2016), and thus it is possible that ISG15 is being directly induced by P53 in an IFN independent manner. ISG15 has also been shown to ISGylate and stabilise P53, creating a positive feedback loop whereby it can be phosphorylated and induce expression of more ISG15. Other ISGs detected in the microarray, such as IRF9, have also been found to be direct P53 target genes (Rivas, Aaronson and Munoz-Fontela, 2010).

It was found that STING inhibition reduced toxin dependent ISG15 induction. This is consistent with findings with other CDTs, which showed activation of a type-I IFN response in a cGAS-STING dependent manner (Pons et al., 2021). As STING inhibition did not completely abrogate ISG15, it was hypothesised that the toxin was inducing ISG15 in both STING dependent and independent ways. Activation of STING canonically leads to upregulation of type-I IFNs via phosphorylation of TBK1 and IRF3, so the role of STING in the IFN independent toxin ISG response is uncertain. However, studies have shown that STING can be non-canonically activated by a signalling process involving ATM, P53 and IFI16, leading to NFκB signalling in DNA damage conditions (Dunphy et al., 2018). STING has been shown to have IFN independent roles in restricting viral infection, tumour immune evasion.
and adaptive immunity (Wu et al., 2020; Yamashiro et al., 2020). These findings merit further investigation, including examining ISG15 levels in intoxicated cells following siRNA mediated STING depletion.

7.3 Is toxin induced ISG15 in free or conjugated form?

When assaying ISG15 using immunoblotting in both HT1080s and MEFs, the toxin was observed to induce ISG15 but did not appear to be ISGylating other proteins. Whereas IFN-treatment resulted in an intense smear of ISGylated proteins in HT1080 lysates, the same was not seen in intoxicated cells. Furthermore, intoxication did not induce ISGylation in MEFs, unlike what has been shown following IFN treatment or Listeria infection (Radoshevich et al., 2015). ISGylation machinery (UBE1L, UbcH6/8 and HERC5) was not upregulated in a toxin dependent manner in the microarray data (Chapter 4). This suggests that toxin induced ISG15 is free and performing a role that does not involve conjugation to other proteins, although validation of the microarray data regarding the ISGylation machinery in response to the toxin will be necessary to confirm this.

It was hypothesised in Chapter 6 that ISG15 promotes P53 activation by conjugation, but how does this correlate with the fact that ISGylation was not observed? It is possible that in response to the toxin, ISG15 is only targeted to a small number of specific proteins including P53. However, there is little evidence of such targeted ISGylation in the literature, and indeed studies have suggested that ISGylation is a broad process targeting all newly synthesised proteins (Durfee et al., 2010). This question will need to be explored further and could be addressed by immunoprecipitation of either ISG15 or P53 following intoxication.

Free ISG15 can act as an extracellular cytokine and can be secreted from both fibroblasts and certain immune cells including neutrophils, monocytes and lymphocytes. Secreted ISG15 binds to leukocyte function-associated antigen 1 (LFA-1) and triggers IFNγ expression in NK cells and T lymphocytes (Dzimianski et al., 2019). Interestingly, ISG15 was a hit in a mass spectrometry analysis within the Humphreys lab that analysed the secretome of cells treated with typhoid toxin.
(ElGhazaly, unpublished). Further analysis will be needed to confirm whether ISG15 is acting as an extracellular cytokine in response to the toxin.

Free intracellular ISG15 was also shown to stabilise USP18 and prevent its degradation by S-phase kinase-associated protein 2 (SKP2) (Zhang et al., 2015). This allows for accumulation of USP18 that prevents over-amplification of immune responses and autoinflammation. Human patients deficient in ISG15 display symptoms similar to interferonopathies including Aicardi-Goutières syndrome and spondyloenchondrodysplasia, characterised by autoinflammation and higher steady states of other ISG transcripts in blood samples (Zhang et al., 2015). ISG15 may be activated by the host as a countermeasure to dampen inflammatory responses to the toxin.

ISG15 and USP18 have been shown to exist in a dynamic equilibrium with SKP2, with deregulation of either resulting in cell cycle arrest (Vuillier et al., 2019). It is therefore possible that increased expression of ISG15 is inducing toxin dependent cell cycle arrest.

Free ISG15 has also been shown to interact non-covalently with DNA replication machinery. ISG15 forms a complex with PCNA and DNA at the replication fork, where it accelerates fork progression and induces chromosomal breakage (Raso et al., 2020). The authors suggested that this process is controlled by the dynamic ratio of free to conjugated ISG15. The pool of intracellular ISG15 is limited by its constant conjugation by the ISGylation machinery, and it is only when this process is uncoupled, for example by infection, that such detrimental effects occur. It is possible that increased levels of ISG15 triggered by toxin activity exacerbate the replication stress response initiated by the toxin, leading to increased genomic instability and senescence. This could explain how the typhoid toxin can trigger a potent DDR despite having attenuated nuclease activity compared to bovine DNase I (Elwell et al., 2001).
7.4 The role of ISG15 in *Salmonella* infection

Overexpression of ISG15 reduced *Salmonella* CFUs 24h post-infection, suggesting that ISG15 is performing a host defensive role. However, the exact mechanism by which ISG15 is protecting the host against *Salmonella* infection is uncertain. In response to viral infection, ISG15 directly ISGylates viral proteins to interrupt the viral life cycle (Perng and Lenschow, 2018). However, a recent review of proteomic studies that have identified ISGylation targets in innate immune responses did not find evidence of direct ISGylation of bacterial effectors (Thery, Eggermont and Impens, 2021). Nonetheless, it remains possible that ISG15 is modulating the functions of *Salmonella* effectors in a similar manner to ubiquitin. For example, the Salmonella Type III effector SopB has been shown to be ubiquitinated by the host, thus modulating its enzymatic function (Knodler et al., 2009). It would be interesting to pull down ISG15 and analyse, by specific immunoblotting or a more non-biased approach such as mass spectrometry, whether there was evidence of ISGylation of *Salmonella* effectors.

ISG15 may be ISGylating other host proteins and promoting expression of antibacterial effectors. For example, in the case of *Listeria* infection, increased ISGylation of Golgi and ER proteins promotes secretion of cytokines that counteract infection (Radoshevich et al., 2015; Y. Zhang et al., 2019). However as previously discussed, there is little evidence of toxin induced ISGylation. Alternatively, ISG15 could be being secreted as a cytokine, which has been shown to trigger IFNγ release and counteract *Mycobacterium* infection (Bogunovic et al., 2012). However, IFNγ is predominantly secreted by NK cells and T lymphocytes, so it unlikely an antibacterial effect via IFNγ secretion would be seen in the context of HT1080 fibrosarcoma cells (Schoenborn and Wilson, 2007). Finally, it is possible that ISG15 may be preventing bacterial growth via modulation of P53. Data in chapter 6 showed that levels of P53 were increased in WT MEFs compared to ISG15−/− MEFs. P53 has been shown to suppress cell metabolism and thus inhibit growth of *Chlamydia*, another intracellular bacteria (Siegl et al., 2014). It is possible that ISG15 is increasing P53 levels and thus preventing *Salmonella* replication in a similar
manner. However, further work will be needed to determine how ISG15 is exerting an antibacterial role in response to *Salmonella* infection.

![Diagram](Fig. 7.1 Proposed model for the role of ISG15 in response to the toxin. The toxin-induced DDR results in cytosolic leakage of DNA and activation of an ISG response in a STING-dependent manner. Toxin-induced damage also activates P53, which may upregulate ISG15 directly. ISG15 inhibits *Salmonella* infection and may stabilise P53, thus influencing cell fate decisions.)

### 7.5 The role of ISG15 in cancer

*S. Typhi* infection has been shown to positively correlate with incidence of gallbladder cancer. As the typhoid toxin causes DNA damage, which is commonly associated with cancer, it was hypothesised that toxin activity may be linked to this cancerous phenotype. The finding that ISG15 was induced by the toxin was interesting, as ISG15 expression has been shown to be elevated in many types of cancer, including bladder, breast and colon (Andersen *et al.*, 2006; Talvinen *et al.*, 2006; Bektas *et al.*, 2008). Indeed, several studies suggest that ISG15 is a tumour promoter (Desai *et al.*, 2012; Burks, Reed and Desai, 2014; Forys *et al.*, 2014). Interestingly in these studies, malignant transformation appeared to be dependent on conjugated but not free ISG15. However, addition of extracellular free ISG15 to tumours was seen to inhibit growth, and intracellular free ISG15 triggered expression of MHC complexes which are critical for activation of adaptative
immunity (Burks, Reed and Desai, 2014, 2015). This suggests that ISG15 broadly has pro-tumour effects when conjugated and anti-tumour effects when free.

Data in chapter 6 showed that ISG15 had a tumour suppressive role in response to the toxin. It was found that wild-type MEFs treated with the typhoid toxin died, but ISG15−/− MEFs survived and continued to proliferate, even with evidence of genomic instability. These ISG15−/− MEFs also showed reduced levels of P53, and indeed one of the determining factors for oncogenic transformation of MEFs in response to S. Typhi was found to be mutated P53 (Scanu et al., 2015). ISGylation of P53 in response to DNA damage was shown to promote its activation and binding to enhancer regions of pro-apoptotic target genes including CDKN1 and BAX (Park et al., 2016). It is possible that ISG15 is performing a similar tumour suppressor function in response to the DNA damaging activity of the toxin. However, the role of ISG15 in tumorigenesis remains uncertain and further work will be needed to determine its role in response to the toxin.

7.6 Conclusion

This thesis provided an unbiased analysis of the host response to the typhoid toxin by identifying the toxin-dependent transcriptome. By doing this, it was found that a type-I IFN like response is induced in a toxin dependent manner. Through validation of the microarray data, ISG15 was identified as a regulator of Salmonella infection and a potential suppressor of bacterial induced genomic instability. Recent studies have determined that other CDTs trigger a type-I IFN response, and therefore it is possible that the findings of this thesis will be of relevance to other CDT-secreting bacterial pathogens. This thesis contributes to elucidating the functional role of the toxin and opens new avenues of research into links between the toxin, chronic carriage and cancer.
8 Biochemical assays

8.1 Bacterial transformation

8.1.1 Creation of chemically competent cells

*E. coli* (i.e., DH5α, C41 Rosetta) was cultured in 500 ml sterile LB broth in a shaking incubator at 37°C and 200 rpm to an OD of 0.5. Cultures were centrifuged at 3000 rpm for 10 mins, supernatant discarded, and pellets resuspended in 40 ml of sterile 0.1M CaCl$_2$. Centrifugation was repeated, pellets resuspended in 2-3 ml of sterile CaCl$_2$ with glycerol and stored at -80°C in 50 µl aliquots.

8.1.2 Transformation in chemically competent cells

Frozen plasmid and bacterial stocks were thawed on ice. Approximately 200 ng of plasmid DNA was added to 50 µl of chemically competent *E. coli* and incubated on ice for 30 mins. Bacteria were heat shocked at 42°C for 30 seconds and immediately placed on ice for 5 mins, before incubation in 900 µl of SOC broth (BioBasic, SD7009) at 37°C for 1h. Cultures were centrifuged at 13000 rpm for 1 min and resuspended in 200 µl of SOC broth. Bacteria were spread onto LB-agar with relevant antibiotic(s) and incubated overnight at 37°C.

8.1.3 Purification of plasmid DNA by midiprep

A single colony of freshly transformed bacteria was added to 100 ml of LB broth supplemented with appropriate antibiotic(s) and grown overnight in a shaking incubator at 37 °C and 180 rpm. Midipreps were carried out using the NucleoBond Xtra Midi (Macherey-Nagel, 740410) kit according to manufacturer’s instructions. Concentration was determined using a Nanodrop Lite spectrophotometer (Thermo Fisher) and plasmid preparations were stored at −20 °C.
8.2 Purification of recombinant typhoid toxin

The T7 expression vector encoding pETDuet1-pltB-HIS/pltA-MYC/cdtB-FLAG was transformed into chemically competent C41 Rosetta E. coli. Transformed colonies were used to inoculate 3x 10 ml starter cultures, which were incubated overnight at 37°C and 200 rpm and used to seed 3x 1000 ml cultures the following day. Day cultures were incubated at 37°C and 200 rpm to an OD of 0.8-1.0. Toxin expression was induced by addition of 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 30°C overnight. Cultures were pelleted for 10 mins at 6000 xg and supernatant discarded. All pellets were resuspended, combined and homogenised in 50 ml TBS (tris-buffered saline, 20 mM tris, 150 mM NaCl, pH 7.4) with two tablets of cOmplete™ protease inhibitor cocktail (Roche). Bacteria were lysed using a French press at 40 ksi and lysate centrifuged at 70000 xg for 40 mins at 4°C. Supernatant was added to 1 ml of NiNTA agarose beads (Jena Bioscience, AC-501-25), with 5 mM imidazole and incubated overnight at 4°C. Beads were immobilised on a column and washed with wash buffer (20 mM tris, 500 mM NaCl, pH 7.4) before bound protein was eluted in elution buffer (20 mM tris, 50 mM NaCl, 250 mM imidazole, pH 7.4). The elution was dialysed in TBS at 4°C overnight using cellulose dialysis tubing (Spectrum Labs™ 128058). Toxin concentration was estimated by relative densitometry to an ~80% pure preparation of GST-CdtB. Toxin preparations were kept at -80°C with 20% glycerol.

8.3 Cell fractionation

Cells were trypsinised and spun down at 1000 rpm for 5 mins. Supernatant was removed and pellets resuspended in buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, one tablet Roche cOmplete™ protease inhibitor cocktail). A 0.1% final volume of 10% Triton X-100 stock was added to the suspension and mixed by inversion. The suspension was incubated on ice for 5 mins and spun down at 1300xg for 5 mins at 4°C, leaving the supernatant (S1) and pellet (P1). Residual debris in S1 was removed by centrifugation at 20,000xg for 15 mins at 4°C and supernatant retrieved (soluble
cytosolic fraction). P1 was resuspended in Buffer A, incubated on ice for 5 mins and centrifuged at 1300g for 5 mins at 4°C. Supernatant was discarded and remaining pellet resuspended in buffer B (3 mM EDTA, 0.1 mM EGTA, 1 mM DTT, one tablet Roche cOmplete™ protease inhibitor cocktail). The suspension was incubated on ice for 30 mins to lyse, before spinning at 1700g for 5 mins at 4°C. The supernatant was retrieved (soluble nuclear fraction). Buffer B was added to the pellet and incubated on ice for 5 mins, before centrifugation again at 1700g for 5 mins at 4°C. The supernatant was discarded, and remaining pellet retrieved (insoluble nuclear fraction).

8.4 Protein gels

8.4.1 Cell lysate preparation

Cells were either seeded in 10 cm dishes or 6-well plates. Generally, for 24 h experiments, cells were seeded at 1x10⁶ cells/well or 2x10⁵ cells/well respectively, with these values halved for every additional 24 h.

At the end of the experiment, cells were washed up to 3 times with PBS, and 1 ml of PBS added. Cells were scraped across the whole plate using a cell scraper and their OD₆₀₀ measured using a spectrophotometer. The cell suspension was then centrifuged at 2000 rpm for 5 mins and supernatant discarded. The pellet was resuspended in sample buffer (50 mM Tris pH 6.8, 8 M Urea, 2% SDS, 0.3% Bromo blue, 1% β-mercaptoethanol). Volume of sample buffer in μl was determined by multiplying the OD₆₀₀ value by 250. Samples were stored at -20°C.

8.4.2 SDS-PAGE protein gel preparation

SDS-PAGE was performed using 9% Bis-tris acrylamide gels cast in BioRad Mini PROTEAN Tetra Cell Casting Stand Clamps (1658050). Resolving gel was formed of 9% 37.5:1 acrylamide/bis solution (1610148), 356 mM Bis-Tris (pH 6.5), 0.1% SDS and MQ water. Stacking gel was formed of 29:1 acrylamide/bis solution (1610156), 356 mM Bis-Tris (pH 6.5), 0.1% SDS and MQ water.
In both cases, 0.1% Ammonium Persulfate (APS, Melford, A1512), and 0.1% TEMED (Sigma Aldrich, T9281) were added for polymerisation. Gels were run at 40 mA/gel in Mini-PROTEAN Tetra System (Bio-Rad) in MES buffer (Life Technologies, NP0001). PageRuler Plus Prestained Protein Ladder (Thermo Scientific, #26619) was used as a protein size standard. Gels were analysed with either Coomassie staining or immunoblotting.

8.4.3 Coomassie staining

Gels were incubated in a petri dish containing Coomassie (50% methanol, 10% acetic acid, 2.5 g/L Blue R250) until protein bands appear. The stain was then discarded, and gels washed with destain (40% methanol, 10% acetic acid, MQ water) overnight with agitation. Gels were imaged using a Gel Doc EZ Imaging System.

8.4.4 Immunoblotting

Prior to transfer, PVDF membrane (Thermo Scientific, 88518) was cut to size and activated by 100% methanol (Sigma, 900658) for 10 seconds. Transfer was performed using either wet or semi-dry methods.

**Wet Transfer**: Activated PVDF membranes were submerged into fresh transfer buffer (20mM Tris-base, 150 mM Glycine, 20% methanol v/v). Membrane and gel were sandwiched in filter paper and sponge and immersed fully in the transfer tank. Transfer was performed at 400 mA for 80 min on ice or 20 mV at room temperature overnight.

**Semi-dry transfer**: The Trans-Blot Turbo Transfer System (Bio-Rad, 1704150), or iBlot 2 (ThermoFisher Scientific, IB21001) were used according to manufacturer’s instructions using the default programs.

Membranes were blocked with 5% non-fat dried milk in TBS for 1 h at room temperature with agitation. Membranes were then washed 3 times in TBS with 0.1% Tween for 5 mins each before incubation with primary antibody (Table 8-1). Primary antibody diluted in TBS with 0.1% Tween was added overnight at 4°C with
agitation. The following day, membranes were washed 3 times in TBS with 0.1% Tween for 5 mins each before addition of secondary antibody. IRDye 800CW anti-mouse (926-32212, Li-Corr) and IRDye 680CW anti-rabbit (926-68073, Li-Corr) were diluted in TBS with 0.1% Tween at 1:10000 for 1 hour at room temperature with agitation. Membranes were imaged at 200 μm resolution using an OdysseySa Li-Cor scanner and images processed using ImageStudioLite v5.2.5.

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Table 8.1 Primary antibodies used for immunoblotting

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8.5 Flow cytometry

Flow cytometry was used to measure DNA content and thus cell cycle phase using propidium iodide (PI). A standard intoxication assay was performed with samples prepared every 2h after intoxication over the 24h media chase. Cells were detached using trypsin and fixed with 1 ml ice-cold 70% ethanol in PBS. Cell pellets were resuspended in 300 μl PBS containing 100 μg/ml RNase and 40 μg/ml PI, incubated at 37 °C for 30 min and analysed using a FACSCalibur Flow Cytometer (Beckman Coulter). At least 10,000 cells were counted per sample.

Analysis was performed with FlowJo. The percentage of cells in G1 phase was determined by calculating the integral of the lowest PI value of the G1 curve to the peak of G1, whereas the percentage of cells in G2 phase was determined by calculating the integral of the highest PI value of the G2 curve to the peak of G2. S phase percentage was calculated as the remainder after G1 and G2 values were subtracted from 100.

8.6 Microarray

Microarray samples were prepared by Angela Ibler, who performed a standard intoxication assay with 5 ng/ml of WT- and HQ-toxin and a 48h chase and isolated RNA. Samples were analysed in the Paul Heath lab at SITraN, University of Sheffield, using a human Clariom™S assay (ThermoFisher Scientific, 902927). Analysis was performed with Transcriptome Analysis Console 4.0 software (Applied Biosystems, Thermo Fisher Scientific), which uses the LIMMA statistical analysis framework (Ritchie et al., 2015). The microarray data comparing WT- and HQ-toxin...
treated HT1080s used in Fig. 4.4 was uploaded to the EBI ArrayExpress database under accession number E-MTAB-12333.

The gene list was analysed by me using the ‘proteins with values/ranks’ function of the online tool STRING v11.0 (https://string-db.org/) (Szklarczyk et al., 2019). The gene list was clustered using gene ontology terms for biological processes. STRING v11.0 was also used to visualise protein interactions in the clusters found.

**8.7 qRT-PCR**

Standard intoxication assays were performed in HT1080s with a 24 h media chase. Cells were washed in PBS, scraped in 5ml PBS, aliquoted and spun down. Cell pellets were stored at -80°C.

RNA was isolated using the illustra RNAspin Mini kit (GE healthcare) according to manufacturer’s instructions with 1 unit (0.5 μl) of DNase 1 (M0303S, New England BioLabs). RNA concentration was measured using a Nanodrop Lite spectrophotometer (ND-LITE-PR, Thermo Fisher Scientific) and RNA integrity tested by running the sample on a TAE agarose gel.

cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA synthesis kit (Thermo scientific). qRT-PCR was performed using Maxima SYBR Green/ROX qPCR Master mix (Thermo Scientific) on the CFX96 Real-Time System. 10 μl of master mix were used per sample, and primers were added at a concentration of 0.25 μM (Table 8-2). Analysis was performed using the comparative C\textsuperscript{T} Method (ΔΔ C\textsuperscript{T} Method) according to guidelines by Thermo Fisher (Applied Biosystems, 2008).

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Table 8-2 Primers used for qRT-PCR
9 Cell biology

9.1 Mammalian cell culture

This thesis used both HT1080 human fibrosarcoma cells and mouse embryonic fibroblasts (MEFs, kindly donated by Lilliana Radoshevich, University of Iowa), both of which were maintained in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma Aldrich, D6546) with 10% foetal bovine serum (FBS) (Sigma Aldrich, F7524), 10 U/ml Penicillin/Streptomycin (Gibco, 11548876), 50 μg/ml Kanamycin sulphate (BioBasic, KB0286) and 2 mM L-glutamine (ThermoFisher Scientific, #25030024).

Cells were kept frozen in 10% sterile DMSO (dimethyl sulfoxide, Sigma-Aldrich, D2438) and 90% complete media at -80°C. Cells were thawed at 37°C, diluted 1:10 with complete growth media and transferred to flasks. Cells were maintained in a humidified incubator (Panasonic) at 37°C and 5% CO₂. Cells were passaged when 80% confluent (approximately every 2 days).

For seeding cells for experiments, media was aspirated, and cells washed with sterile PBS (Sigma Aldrich, D8537). Cells were detached using sterile trypsin at 37°C for 5 mins (Sigma Aldrich, T4049). Trypsin was neutralised using a 1:1 volume of complete growth media. Cells were counted in a glass haemocytometer.

9.2 Mammalian cell treatment

9.2.1 Standard intoxication assay

Culture media was replaced with media containing 20 ng/ml (unless otherwise stated) WT- or HQ-toxin for 2h. Cells were then washed with sterile PBS and chased with fresh complete growth media.
9.2.2 Cell cycle arrest by serum-starvation

Cells were serum-starved in media containing all components apart from 10% FBS for the full duration of one replication cycle (24 h in HT1080s).

9.2.3 Drug and recombinant protein treatment

Cells were treated with drugs and recombinant proteins listed in Table 9-1 with the indicated incubation times.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Summary</th>
<th>Working concentration</th>
<th>Incubation time</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aphidicolin</td>
<td>DNA polymerase α inhibitor</td>
<td>20 μM</td>
<td>24 h</td>
<td>Sigma-Aldrich (A0781)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase inhibitor</td>
<td>10 μM</td>
<td>24 h</td>
<td>Cayman Chemicals (12092)</td>
</tr>
<tr>
<td>BX795</td>
<td>TBK1 inhibitor</td>
<td>10 μM</td>
<td>Duration of experiment</td>
<td>Invivogen tirl-bx7</td>
</tr>
<tr>
<td>H151</td>
<td>STING inhibitor</td>
<td>4 μg/ml</td>
<td>Duration of experiment</td>
<td>Invivogen inh-h151</td>
</tr>
<tr>
<td>B18R</td>
<td>Type-I IFN inhibitor</td>
<td>0.1 μg/ml</td>
<td>Duration of experiment</td>
<td>Invitrogen 14-8185-62</td>
</tr>
<tr>
<td>IFNα</td>
<td>Recombinant interferon α</td>
<td>0.1 μg/ml</td>
<td>Duration of experiment</td>
<td>Novus Biologicals NBP2-34971</td>
</tr>
</tbody>
</table>

Table 9-1 Drugs and recombinant proteins used to treat mammalian cells

### 9.2.4 Transfection of mammalian cells

For siRNA transfection, cells were transfected in 6 well plates by addition of 3 μl/well Lipofectamine RNAiMax (Invitrogen, 13778-150) and 20 nM siRNA (Table 9-2) in 100 μl of serum-free, antibiotic-free DMEM. Cells were incubated for 48h before any further treatments.
<table>
<thead>
<tr>
<th>siRNA</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-targeting Control</td>
<td>Horizon D-001810-01-20</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Ambion AM4605</td>
</tr>
<tr>
<td>NABP1</td>
<td>Horizon L-014224-01-0005</td>
</tr>
<tr>
<td>NABP2</td>
<td>Horizon L-014288-01-0005</td>
</tr>
<tr>
<td>INT3</td>
<td>Horizon L-018360-01-0005</td>
</tr>
</tbody>
</table>

Table 9-2 siRNAs used for transfection of mammalian cells
Horizon siRNAs are ON-TARGETplus SMARTpool.

For transfection with immunostimulatory DNA, cells were transfected by addition of 3 μl/well Lipofectamine RNAiMax (Invitrogen, 13778-150) and 5 μl each of two 100 μM oligonucleotide stocks (TACAGATCTACTAGTGATCTATGACTGATCTGTACA-TGATCTACA, TGTAGATCATGTACAGATCAGTCATAGATCACTAGTAGATCTGTA) (Fu et al., 2019; Lama et al., 2019) in 100 μl of serum-free, antibiotic-free DMEM.

For transfection with mammalian expression vectors, 3 μl/well Lipofectamine RNAiMax (Invitrogen, 13778-150) or 6 μl/well FuGENE transfection reagent (Promega) was used. pCAGGS-5HA-mtSG15 was a gift from Dong-Er Zhang (Addgene plasmid # 12444) (Kim et al., 2004). As a control, an empty pcDNA™3.1 (+) mammalian expression vector (Thermo Fisher, V79020) was used.

9.3 Clonogenic Assay

2000 cells were seeded into a 10 cm dish, treated, and incubated in fresh media for 8 days. Media was aspirated and cells were washed with PBS. Next, 80% ethanol was applied to cells for 15 minutes, then removed and cells were left to air-dry. 1% methylene blue was added until colonies were visible, washed off with distilled water and then air-dried before imaging with an ChemiDoc XRS+ imager (BioRad). Images were thresholded and colonies counted using Fiji v2.3.
9.4 Cell staining

9.4.1 Immunofluorescence

Cells were seeded in 24 well plates on glass coverslips. At the end of the experiment, media was aspirated, and cells washed three times with PBS. For staining of RPA32 pT21 and components of the SOSS complex, cells were pre-permeabilised with PBS + 0.1% Tween for 1 minute on ice. Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10-15 mins at room temperature. PFA was removed and cells washed twice more with PBS before being stored in PBS at 4°C until staining.

Cells were blocked using 3% BSA (Sigma-Aldrich, 1073508600) and 0.2% Triton X-100 (VWR, 28817.295) in PBS at room temperature for 1h. Coverslips were washed by dipping in PBS and 0.2% Triton X-100. Primary and secondary antibody dilutions were prepared in PBS 0.2% Triton X-100. Primary antibodies were diluted as appropriate (Table 9-3) and added for an hour at room temperature.

Secondary antibodies (Table 9-4) were added at a 1:500 dilution in PBS with 0.2% Triton X-100 for 30 mins at room temperature. Coverslips were then mounted on 6 μl of VectaShield mounting agent including DAPI (Vector Lab, H1200), sealed and left to dry before being imaged.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Product code</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>rat monoclonal</td>
<td>Abcam ab6326</td>
<td>1:400</td>
</tr>
<tr>
<td>ISG15</td>
<td>mouse monoclonal</td>
<td>Santa Cruz sc-166755</td>
<td>1:100</td>
</tr>
<tr>
<td>Lamin B1</td>
<td>rabbit polyclonal</td>
<td>Abcam ab16048</td>
<td>1:1000</td>
</tr>
<tr>
<td>γH2AX</td>
<td>mouse monoclonal</td>
<td>Merck/Sigma 05-636</td>
<td>1:1000</td>
</tr>
<tr>
<td>RPA32 pT21</td>
<td>rabbit polyclonal</td>
<td>Abcam ab61065</td>
<td>1:1000</td>
</tr>
<tr>
<td>NABP1</td>
<td>rabbit polyclonal</td>
<td>Genetex GTX12092</td>
<td>1:1000</td>
</tr>
<tr>
<td>NABP2</td>
<td>rabbit polyclonal</td>
<td>Bethyl A301-938A-M</td>
<td>1:1000</td>
</tr>
<tr>
<td>INT3</td>
<td>rabbit polyclonal</td>
<td>Bethyl A302-050A-M</td>
<td>1:1000</td>
</tr>
<tr>
<td>53BP1</td>
<td>mouse monoclonal</td>
<td>Merck MAB3802</td>
<td>1:500</td>
</tr>
<tr>
<td>Salmonella</td>
<td>rabbit polyclonal</td>
<td>Abcam ab35156</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 9-3 Primary antibodies used for immunofluorescence

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse 488</td>
<td>Alexa-Fluor A21202</td>
</tr>
<tr>
<td>Anti-rabbit 488</td>
<td>Alexa-Fluor A110008</td>
</tr>
<tr>
<td>Anti-rat 555</td>
<td>Alexa-Fluor A21434</td>
</tr>
<tr>
<td>Anti-rabbit 568</td>
<td>Alexa-Fluor A11036</td>
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<tr>
<td>Anti-rabbit 568</td>
<td>Alexa-Fluor A11036</td>
</tr>
<tr>
<td>Anti-mouse 594</td>
<td>Alexa-Fluor A21203</td>
</tr>
</tbody>
</table>

Table 9-4 Secondary antibodies used for immunofluorescence
9.4.2 EdU staining

Cells were incubated for 2h with 10 μM EdU prior to fixation. Cells were stained for EdU using a Click-iT™ EdU Cell Proliferation kit with Alexa Fluor™ 647 dye (ThermoFisher, C10340) as per the manufacturer’s instruction. Cells were then blocked for 20 mins using 3% BSA (Sigma-Aldrich, 1073508600) and 0.2% Triton X-100 (VWR, 28817.295) in PBS before proceeding with primary and secondary antibodies as detailed in section 9.4.1.

9.4.3 In vitro polymerase assay

A standard intoxication assay was performed in HT1080s in 24 well plate format with a 24 h media chase. The in vitro polymerase mixture (0.4 mM CldU, 0.1 mM dNTP mix, Dream Taq buffer to final concentration 1x, 1 μl/ml Dream Taq polymerase, MQ water) was added to a fresh 24-well plate on ice. The glass cover slips with samples were placed top down onto the in vitro polymerase reaction mixture. The plate was placed in a pre-heated water bath at 72°C for 5 min to allow the polymerase reaction. The reaction was interrupted by placing the plate on ice and washing coverslips with PBS three times. A standard immunofluorescence assay was performed using anti-BrdU (which also recognises CldU) and anti-RPA32pT21 (Table 9-3).

9.5 Infection assay

9.5.1 Preparation of cells and bacterial culture

Cells were seeded in a 24-well plate at an appropriate density to reach approximately 80% confluency by the end of the experiment.

A Salmonella colony was cultured in 5 ml LB broth with the relevant antibiotic overnight at 37°C in a shaking incubator. Next day, a 1:100 dilution of the overnight culture was performed into 10 ml of fresh LB broth with the relevant antibiotic and incubated at 37°C in a shaking incubator until OD\textsubscript{600} was approximately 1.0. The number of Salmonella at an OD\textsubscript{600} of 1.0 was estimated to be 8×10\textsuperscript{8} bacteria/ml. Unless otherwise stated, infections were performed with an MOI of 10.
Bacteria were spun down at 13,000 rpm for 1 minute and supernatant discarded. The pellet was resuspended in 1 ml of sterile PBS before adding to cells. The plate was centrifuged for 1 min at 1000 × g and incubated for 30 min at 37°C 5% CO₂. Infection media was removed, cells were washed with PBS, and fresh media added containing 50 μg/ml gentamicin (Chem Cruz, sc203334) for 90 mins. This media was then replaced with fresh media containing 10 μg/ml gentamicin for the duration of the experiment.

9.5.2 CFU assay

HT1080s were seeded at 2×10⁴ cells per well in a 24-well plate before infection with *Salmonella* as described previously. Cells were lysed 2h, 24h, 48h and 72h cells post-infection. Cells were washed 3x with PBS, lysed with 1% Triton X-100 in deionised sterile water for 10 mins, and pipetted vigorously. The supernatant was transferred to a 96-well plate, and serially diluted 10-fold using a multichannel pipette. 5μl of each *Salmonella* dilution was then cultured on agar overnight in a dry incubator at 37°C. Colonies at the highest countable dilution were counted manually and % CFU calculated as a percentage of *Salmonella* colony forming units (CFUs) at the 2h timepoint.

9.6 Microscopy

Immunostained cells were imaged using a Nikon Widefield microscope equipped with an sCMOS Andor Zyla camera. NIS elements software was used for imaging. Images were processed using Fiji v2.3. For chosen representative images, brightness and contrast were normalised, scalebar added and images cropped if necessary. The DAPI channel was converted into a binary image to obtain DAPI outlines, which were overlaid with other channels.

Quantitative analysis of image intensity was generally carried out using the RING-tracking MATLAB code published in Ibler et al., 2019. For images of MEFs (Fig. 6.7), images were analysed using Fiji v.2.3. Briefly, nuclei were identified by manual thresholding of the DAPI channel. EdU was auto-thresholded using default settings and the percentage of positive pixels within nuclei measured.
Adobe Illustrator was used to prepare illustrations and assemble all results figures.

9.7 Statistical analysis

Graphs and statistical analysis were carried out using GraphPad Prism 9. Generally, one-way ANOVAs were used with Šidák’s or Tukey’s multiple comparisons tests as appropriate. Significance is denoted by asterisks where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
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