**Title:**
***Salmonella* impairs macrophage immunity through effector-independent rapid translational induction in response to membrane puncture by the SPI-1 injectisome**

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**Abstract**

*Salmonella* is a globally important pathogen that actively invades and replicates within host cells, including epithelial cells and macrophages, during infection. Bacterial internalization is predominantly orchestrated by the *Salmonella* SPI-1 Type III secretion system (T3SS). The SPI-1 T3SS transports effectors directly through the host cell membrane and into its cytosol to trigger *Salmonella* uptake. Here, we demonstrate that *Salmonella* rapidly synthesizes SPI-1 components as they encounter potential host macrophages, priming the bacteria for host invasion. Assembly of the T3SS in the host membrane results in transient pore formation. We show that this leads to the surge of translational induction of host transcription factors, including *Egr1*. In macrophages, the rapid synthesis of EGR1 results in the suppression of immune response and pro-apoptotic genes. Thus, SPI-1-mediated membrane damage limits the macrophage immune response to *Salmonella* infection

## Introduction

The gene expression profile of both host[1–5] and pathogen[6] can be altered dramatically upon their interaction. This response is shaped by the co-evolutionary arms race where the host seeks to detect and counter the invading pathogen[3,5], whilst the pathogen aims to evade this response[6] and modify the host to better suit its survival and replication[1,2]. Changes in gene expression during infection are thus the net result of these competing goals, the balance of which can ultimately determine the infection outcome.

Many members of the Gram-negative bacterial genus *Salmonella* are facultative intracellular pathogens that can infect a diverse spectrum of hosts and cause a range of diseases in humans, from typhoid to gastroenteritis. *Salmonella*'s ability to establish an intracellular infection is key to its pathogenesis[7]. During infection, the bacterium invades host cells, including the epithelial cells lining the intestinal tract and immune cells such as macrophages. Once inside, *Salmonella* resides in a specialised membrane-bound compartment, the *Salmonella*-containing vacuole (SCV). The SCV provides a protective niche for the pathogen while giving it access to host cell nutrients to support its replication[8,9].

The intracellular lifestyle of pathogenic *Salmonella* is supported by two Type III secretion systems (T3SS) encoded on *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2) with different substrate specificity. The SPI-1 T3SS, also called the SPI-1 injectisome, is a multi-protein complex that spans the bacterial envelope and transports proteins into target cells along a homomeric needle-like structure that is inserted into the host cell membrane by the bacterial effectors SipB, SipC and SipD[10–12]. PrgJ forms the inner rod that connects the injectisome basal body, embedded in the *Salmonella* envelope, with the needle structure and thus acts as a channel connecting the bacterial and host cytoplasm[10]. The SPI-1 injectisome is used to invade host cells by transporting effectors to modify host behaviour, such as cytoskeletal remodelling of epithelial cells and leads to bacteria internalisation by macropinocytosis, i.e. the trigger mechanism[13–15]. The SPI-2 T3SS is only expressed once the bacterium is intracellular and is used to support the intracellular lifecycle by avoiding the host bacteria-degradation machinery[16] and by adapting the host cell to support *Salmonella* replication[8]. While SPI-1-dependent invasion is the predominant form of entry into host cells, SPI-1-independent mechanisms for invasion of fibroblasts, macrophages and epithelial cell lines have also been reported[15]. Entry into phagocytes can also occur through conventional phagocytosis.

To allow transport of effector proteins *via* the SPI-1 injectisome into the host cell, the *Salmonella* translocon subunits SipB and SipC are secreted via the injectisome and inserted

into the host cell membrane to form a transient pore[11,12,17]. This leads to transient loss of membrane integrity, potentially triggering an osmotic stress response and disruption of ion gradients including $Ca^{2+}$ influx and $K^{+}$ efflux. The fact of such SPI-1-dependent membrane damage is supported by the haemolysin activity of the SPI-1 injectisome in red blood cells, which lyse upon SipBC insertion into the red blood cell membrane, suggesting a loss of cell membrane integrity due to SipBC pore formation[12]. While it is known that loss of membrane integrity can trigger inflammatory response pathways[17], the contribution of this damage to the host response to *Salmonella* remains unclear.

Macrophages are a major host cell during systemic *Salmonella* infection[18] and *Salmonella* survival within macrophages is critical for virulence[7]. However, *Salmonella* factors are detected by the macrophage innate immune machinery, activating the inflammasome and leading to pyroptosis (inflammatory cell death)[19–21]. In tissue culture, it is observed that most macrophages die within the first few hours of infection[22–24], with similar macrophage death possibly occurring *in vivo*[18]. This rapid cytotoxicity is dependent on the SPI-1 injectisome[22,24]. The balance of macrophage survival and death will influence the outcome of infection[25] and as such, understanding the gene expression changes that govern this balance remains an important goal.

Although much research has been carried out on the transcriptional response to *Salmonella* infection[2–4], the analysis of gene expression is incomplete without also exploring the translational response. This is particularly important given the critical nature of events occurring very early in infection, where translational regulation may allow for a more rapid response, either through modulating pre-existing mRNAs and/or enhanced translation on the relatively small amount of newly transcribed mRNAs. In this study, we sought to characterise these rapid changes in both host and *Salmonella* translation that occur during infection of macrophages, and uncouple the general response to Gram-negative bacteria from the response to active, SPI-1-dependent, invasion.

## Results

### *Salmonella* infection triggers rapid host cell responses

*Salmonella* enters immortalised murine bone marrow derived macrophages (iBMDM, hereafter macrophages unless otherwise stated) predominantly by inducing SPI-1 dependent membrane ruffling resulting in bacterial macropinocytosis[13–15,26]. *Salmonella* associated with host membrane ruffles, i.e. those that are actively invading, can be seen as early as 5 min post exposure, with *Salmonella* rapidly internalised within 15 min (Figure 1A-

B). We see little further macrophage infection after 15 min of infection indicating a change in susceptibility (Figure S1A). SPI-1 independent internalisation was assayed with infection with *Salmonella* unable to assemble the SPI-1 injectisome needle, generated by knockout of the SPI-1 inner-rod protein PrgJ[27] and referred to as the injectisome mutant hereafter. Invasion of injectisome mutant *Salmonella*, e.g. through phagocytosis, can also be observed within this timeframe, though to a lower degree than WT (Figure S1B).

As previously described[22–24], infection of macrophages with wildtype (WT) *Salmonella* led to rapid cell death within the first 60 minutes of infection. After this, the rate of cell death considerably reduced despite a large proportion of cells still containing viable bacteria (Figure 1C-D, S1A). In contrast, and similar to previous studies[28], infection with injectisome mutant *Salmonella* showed no greater cell death than mock-infection.

**High resolution ribosome profiling allows identification of rapid changes in translation**

It is clear that macrophages are rapidly altered during *Salmonella* infection (Figure 1A-D). We hypothesised that the plateau in cell death from 60 minutes post-infection with WT *Salmonella* may be a consequence of combined rapid gene expression responses (translational and transcriptional) that occurred during the first 60 minutes of infection to promote macrophage survival. Most previous studies investigating bacterial-induced reprogramming of host gene expression have focused on the transcriptional level[2–4]. Furthermore, few studies have examined the immediate changes that occur within the first few hours of infection. To investigate the rapid changes in gene expression upon infection, we prepared parallel Ribo-Seq and RNA-Seq libraries from macrophages at 60 minutes post-infection with WT and the injectisome mutant (Figure 1E, S6A). RNA-Seq and Ribo-Seq enable us to quantify the transcriptome and translatome respectively, and together can be used to calculate the number of ribosomes per mRNA, providing a measure of translational efficiency (TE) for all transcripts (Figure 1F).

We first observed significant co-transcriptional and translational induction of genes such as *Tnf*, *Zfp36*, *Il10*, *Osm*, *Irf1* and *Cd14* in macrophages infected by either the WT or injectisome mutant. Such behaviour is well established to be triggered by pathogen-associated molecular patterns (PAMP), where the translational response (i.e. protein synthesis) is significantly enhanced over the transcriptional response (i.e. RNA synthesis), with *Tnf* particularly well characterised[29,30] (Figure 1G). However, our data revealed the translational response is greater in macrophages infected by WT *Salmonella* over those infected by the injectisome mutant for some of these genes, including *Il10, Osm, Irf1* and

*Cd14* (Figure 1G), suggesting the translational response of these genes is a consequence of a compounded PAMP-associated response and the interaction with the SPI-1 injectisome. To identify transcripts that are subjected to specific injectisome-dependent translational upregulation, we next selected those where the TE is significantly induced (i.e. $log_2$ fold-change ($log_2$FC) TE is greater than 1.5 in WT-infected cells over cells infected by the injectisome mutant). Early Growth Response factor 1 (*Egr1*), was identified as the most SPI-1 dependent translationally upregulated gene (Figure 1H).

EGR1 is a zinc-finger family transcription factor that binds GC-rich consensus sequences in gene promoters and enhancers, where it can either activate or supress transcription. Targets span diverse biological processes including the immune response, cell growth, differentiation, and apoptosis[31–35]. Due to its highly injectisome-dependent translational upregulation, the biological function of EGR1 in *Salmonella*-macrophage infection is further characterised in this study.

**SPI-1 dependent co-translational and transcriptional induction of EGR1**

Transcriptional upregulation of *Egr1* has previously been shown to be largely dependent on a bacterial secretion system in other infection contexts[2,36–38]. However, EGR1 has not been studied in the context of *Salmonella* infection of macrophages nor has *Egr1* been studied at the translation level. We were particularly interested in EGR1 given its described association with cell survival and importance in macrophage development.

At 60 min post-infection there was a clear increase in both transcription and translation of *Egr1* in WT-infected cells, with translation efficiency of *Egr1* three times higher in WT-infection over injectisome mutant-infection (Figure 2A). In contrast, the level of *Egr1* mRNA and protein synthesis in injectisome mutant infection was similar to that observed in the mock infection. Consistent with our RNA-Seq and Ribo-Seq data, we found higher levels of *Egr1* mRNA (Figure 2B) and protein in WT-infected cells at 60 min post WT *Salmonella* infection (Figure 2C-D). This upregulation was rapid but transient, consistent with previous reports[39,40]. The abundance of mRNA peaked at 60 min and reduced to near baseline levels by 120 min. The increase in protein abundance is, as expected, offset from the increase in mRNA with the protein level peaking at 120 min post-infection and returning to undetectable levels by 240 min, indicating short protein half-life of EGR1 and that the efficacy is rapid and temporal. In contrast, infection with the injectisome mutant led to a slight increase in *Egr1* mRNA abundance at 60 min, followed by detectable protein level at 120 min, although at a considerably lower degree than the corresponding WT *Salmonella* infected cells (Figure 2A-D).

We reasoned that the *Egr1* transcriptional upregulation could be a result of either penetration of the SPI-1 injectisome into the host cell, insertion of the SipB/C translocon complex into the host cell membrane (and subsequent transient pore formation) or due to transport of effector proteins once the T3SS is fully assembled and is in a secretion competent state. To test this, we engineered an injectisome blocking substrate in which the effector protein SptP is fused to GFP (SptP-GFP). This injectisome blocking substrate is targeted to the export machinery before stalling in the injectisome export channel only after needle assembly has completed, thereby blocking transport of effectors and translocon subunits only after SPI-1 injectisomes have assembled on the bacterial cell surface[41] (Figure 2E-F). This allows us to uncouple the effect of penetration of host cells by the injectisome from the transport of effector and translocon subunits on *Egr1* transcriptional upregulation.

Expression of the SptP-GFP injectisome blocking substrate was induced upon dilution of the bacterial cultures approximately 2 hours before infection, 5 min post infection or not induced (Figure 2E). Blocking of the injectisome by SptP-GFP 5 min post infection resulted in only a slight decrease in *Egr1* expression as compared to the unobstructed T3SS, whereas pre-blockage of the T3SS resulted in little *Egr1* upregulation (Figure 2G). This suggests that the trigger for *Egr1* induction occurs very rapidly during infection and is likely a direct result of injectisome penetration or the action of one of the first secreted effectors.

**Upregulation of *Egr1* restrains the immune response to *Salmonella* infection**

To further investigate the role of EGR1 during injectisome dependent infection of macrophages, we generated EGR1 knockout (KO) macrophages using CRISPR-Cas9. We confirmed no detectable levels of EGR1 protein 120 min after infection with WT *Salmonella* (Figure 2H) and targeted sequencing of isolated genomic DNA showed that the cells harboured insertions and deletions at the annotated *Egr1* exonic sequence (Figure S2A). Cytotoxicity assay of EGR1 KO macrophages further revealed an increase in host death in the absence of EGR1, particularly between 30-120 min post infection (Figure 2I).

EGR1 is known to act as a transcription factor and so, to determine the role of EGR1 in infection-dependent transcription, we prepared transcriptomic libraries from EGR1 KO and CRISPR negative control macrophage clones over the course of a WT *Salmonella* infection. Principal component analysis (PCA) of RNA abundance showed a clear separation of samples by cell line and time post-infection. PC1 reflects variation between the EGR1 KO and negative control cells, while PC2 separates the 120 min and 240 min samples from the 15-60 min samples (Figure S2B). We observed an increase in the abundance of many

genes in the EGR1 KO cells compared to the negative control cells, particularly at 240 min post-infection (Figure 2J; Table S1). Gene ontology (GO) term enrichment analysis showed that many of these genes have roles in the immune response, suggesting that EGR1 is an important immune modulator (Figure 2K; Table S2). In addition, Gene ontology enrichment analysis also revealed significant upregulation of known pro-apoptotic genes in infection of the EGR1 KO macrophages, including the FAS death receptor, conferring the role of EGR1 in infection-dependent macrophage death (Figure 2I; Table S1-2).

**Refreshing culture media triggers transcription factor expression akin to *Salmonella* injectisome-dependent infection**

*Egr1* expression is known to be stimulated by a variety of signals including mitogen signalling[35,42], cellular stresses such as oxidative stress[43], DNA damage[44] and hypoxia[45], as well as immune-related signals[46] and other infection contexts including exposure to Enteropathogenic *E. coli*[36], *Yersinia enterocolitica*[37] and *Helicobacter pylori*[38]. We also demonstrated that EGR1 acts as a pro-survival factor in WT *Salmonella* infection between 30-120 min post infection (Figure 2J). *Egr1* clearly plays an important role in the response to both bacterial infection and changing cellular conditions. As such, we sought to explore the relationship between changes in the culture environment and *Egr1* induction further, and to identify commonalities with *Salmonella* infection.

Supplying cells with fresh culture medium is known to provide them with a variety of compounds such as mitogens and nutrients that activate transcription of proliferation genes[47]. To validate this we cultured macrophages overnight before removing the culture supernatant and overlaying them with either fresh medium or exhausted medium (i.e. the original culture supernatant). This resulted in an increase in *Egr1* and *Fosb* (a known mitogen-activated transcription factor[48]) expression only when overlayed with fresh medium (Figure S3A). Analysis of the global transcriptome revealed upregulation of further genes induced by mitogenic and immune-related signals, including *Fos* and *Zfp36*[49,50] (Figure S3B). These genes all showed injectisome-dependent increases during WT *Salmonella* infection, though *Fos* and *Zfp36* were also induced in injectisome mutant infection (Figure S3C). Their upregulation in WT infection is likely a compound effect of Toll-Like-Receptors (TLR) stimulation and injectisome-dependent upregulation (Figure S3C).

**Infection drives temporal regulation of key genes in both host and bacteria**

*Egr1* shows clear temporal regulation at the protein and transcript level. To assess temporal transcription and translation dynamics globally throughout *Salmonella* infection, we prepared RNA-Seq and Ribo-Seq libraries from a time course of primary BMDMs infected with WT

and the injectisome mutant *Salmonella*, with media changes performed on addition of the inoculum and for gentamicin treatment, as is often done during cell culture bacterial infection[51,52] (Figure 3A). This allows us to uncouple the injectisome-dependent responses from PAMP stimulation and compare them to the response to change of exhausted media to fresh media, in a host cell that more closely resembles in vivo infection. The primary macrophages showed similar rates of infection to macrophages for WT *Salmonella*, but a greater proportion were infected with the injectisome mutant (Figure S4A).

Analysis of parallel Ribo-Seq and RNA-Seq throughout infection time course (Figure S6B) enabled a global perspective of the dynamics of gene expression at multiple levels, notably changes in RNA abundance and translation efficiency. At the first instance, we can readily visualise both WT and injectisome mutant *Salmonella* induced rapid translational regulation of host macrophages (Figure S4B). However, further analysis revealed injectisome-specific translational upregulation precedes the transcriptional regulation in terms of the number of genes, which shows minimal changes in mRNA abundance before 60 min, with more genes exhibiting differential TE than mRNA abundance at all timepoints (Figure 3B-D).

Gene Ontology enrichment analysis showed that genes with differential translational efficiency during the first 60 min of WT *Salmonella* infection compared to mock or injectisome mutant infection have important roles in cytokine production and cell signalling (Figure 3E). Interestingly, GO terms related to DNA binding and transcription were also enriched in these comparisons. In contrast, genes with differential transcript abundance are not enriched for transcription factors, but were enriched for cytokines and other cell signalling genes, similar to previous transcriptomic studies[3] (Figure 3F). This reinforces the hypothesis that rapid, injectisome-dependent, translational induction of transcription modulators shapes the transcriptional response to WT *Salmonella* infection of macrophages.

Furthermore, many AP-1 components were among the differentially expressed DNA binding proteins. AP-1 is a group of transcription factors that are predominantly formed of homo- and hetero-dimers of JUN, FOS and ATF family proteins. Altering the abundance of different compositions of AP-1 can allow for the tuning of specific responses. Indeed different AP-1 components can drive cells towards apoptosis or potentiate inflammation[53,54]. The AP-1 components *Fos* and *Fosb* were among the genes we identified as transcriptionally upregulated upon SPI-1 dependent infection and exchange of exhausted to fresh media (Figure S3). As such, we sought to analyse the expression of these proteins further. The behaviour of these genes throughout the infection fell into three clear categories: those which showed increased translation on exposure to either WT *Salmonella* or the injectisome

mutant, those which showed increased expression due to fresh media replacement, and those that exhibit more complicated behaviour. The first category follows the profile of rapid co-transcriptional and translational induction, including mock infection due to change of fresh media, peaking at around 60 min and then returning to baseline. This differential media driven translational upregulation could be further enhanced in a number of these genes by *Salmonella* infection (Figure S5).

Finally, our data not only captured host cell gene expression dynamics but also captured bacterial translational dynamics of highly expressed genes over the course of infection. It has been established that culturing *Salmonella* in Luria Broth (or lysogeny broth, LB) to late exponential phase, as they were prepared for our infection assays, induces the expression of SPI-1 genes[24]. Consistently, we detected abundant SPI-1-derived transcripts and proportional protein synthesis at the start of the infection with a marked increase in the synthesis of injectisome components, such as PrgH/I/J/K, SipB/C, and InvC/J/G/E/A as early as 5-15 min post-infection, which could suggest that *Salmonella* responds to the proximity of host cells by upregulating certain genes that enable invasion (Figure 4) and suggests rapid assembly of injectisomes on the bacterial cell surface during the first 15 minutes of infection. Transcriptional and translational response of non-structural SPI-1 genes that encode effector proteins, structural genes with dual-role as effectors such as SipB and sipC, and other virulence factors also increases during the first 15 minutes of infection whereas some genes occur maximally at 2-4 hours post infection such as for AvrA and IacP (Figure 4). Notably, we observed increases in translation efficiency of many intramacrophage genes such as htrA, *hisG* and *cpxP,* that plays a crucial role in the survival and replication of the bacterium inside macrophages rapidly during early stages of infection (i.e. within the first 30 minutes of infection), whereas maximal transcription occurs later for most known intramacrophage genes. The functions of these genes include roles in magnesium and phosphate transport and the envelope stress response[55,56].

At the 30 min and 60 min time points, when *Salmonella* is intracellular and after SCV acidification[57], the transcript abundance of SPI-2 genes increases while the expression of the SPI-1 genes decreases. This agrees with the recognised switch of *Salmonella* secretion mediated by the transcriptional regulator SsrB. We also observe concurrent decreases in expression of the flagella components, consistent with previously reported SsrB-mediated repression[55,58,59].

## Discussion

Regulation of protein synthesis through modulating TE affects far more genes than regulation of transcript abundance during the initial stages of *Salmonella*-macrophage interaction and changes in TE precede changes in RNA abundance. Much of the regulation in TE is SPI-1 injectisome dependent and these differentially expressed genes show transcriptional upregulation with greater translational upregulation. The increase in TE thus amplifies the increase in transcript abundance to yield more protein.

On the *Salmonella* side, despite using *Salmonella* cultures at late growth phase when SPI-1 injectisome expression is abundant[24], we observed further increases in transcription and translation of both structural and non-structural SPI-1 injectisome components immediately following infection. This suggests proximity to potential host cells drives expression of the machinery to invade them. Consistent with previous studies looking at gene expression of intramacrophage *Salmonella*[55,58,59], we observed the expected switch from SPI-1 to SPI-2 gene expression, increased expression of intramacrophage markers and a decrease in synthesis of flagella components (Figure 3H).

The translationally upregulated host genes are enriched for immune processes, which is distinct from the well characterised transcriptional response of macrophages to Salmonella infection[2–4]. Alongside immune process genes, the transcripts with increased TE throughout *Salmonella* infection showed an enrichment for mRNAs encoding transcription factors (Figure 1H and S5). *Egr1* was the most SPI-1-dependently translationally enhanced gene at 60 min post infection. EGR1 has been assigned a diverse range of roles in response to various stimuli, including activating the immune response and regulating replication and cell death[35,45]. Knockout of EGR1 led to infection-dependent increases in expression of a subset of transcripts to a much greater degree than in WT macrophages, indicative of the role of EGR1 in limiting their upregulation. Many of these genes encode proteins related to immune processes, indicating the critical role of EGR1 in restraining the immune response in *Salmonella* infection. Pro-apoptotic genes were also upregulated in *Salmonella* infected macrophages lacking EGR1. Their increased expression may contribute to the observed increases in both the rate and amount of cell death observed in infection of macrophages lacking EGR1. While the rapid death on *Salmonella* infection of macrophages is typically attributed to induction of pyroptosis[19,20], this suggests there are also pro-apoptotic signals that are usually restrained by EGR1.

*Egr1* expression has previously been shown to be induced downstream of a variety of signalling pathways[35–38,42–46], as we also show here. It is therefore unsurprising that we also

see *Egr1* upregulation upon replacement of exhausted media with fresh media. This effect is compounded upon *Salmonella* infection in a SPI-1-dependent manner. This indicates that either signalling through these pathways is not saturated and they can be further activated by the actions of the injectisome, or *Salmonella* injectisome-specific induction acts through an alternative pathway. The upregulation of *Egr1* has been shown to be dependent on T3SS or T4SS in other bacterial infection contexts[2,36–38]. In these infections T3SS and T4SS deliver bacterial effectors into the host cytosol and therefore a transient pore in the host membrane must be formed during their assembly[17,60]. Membrane damage by pore-forming toxins is also known to increase *Egr1* expression[61,62]. It is therefore likely that membrane damage by these secretions systems drives *Egr1* upregulation. Recently, a model has been proposed that describes transcriptional induction following exposure to the *Candida albicans* pore forming toxin candidalysin[63]. It is likely that the secretion system dependent transcription of *Egr1* is through this same mechanism, supported by previous observations showing the requirement of EGFR and ERK kinases[36,38]. Supporting this, we show that activation of SPI-1-dependent *Egr1* expression occurs rapidly as *Salmonella* establishes its infection, in the same timeframe as T3SS pore formation. We show that the ability of the injectisome to form pores is required for this response, but secretion of subsequent effectors is not.

EGR1 has recently been shown to be involved in macrophage development where it limits the accessibility of inflammatory enhancers through recruitment of the NuRD chromatin remodelling machinery[34]. Here we show this is a dynamic process that is rapidly activated in differentiated macrophages through rapid translational and transcriptional induction in response to bacterial secretion system puncture of the host membrane. In summary, during *Salmonella* infection of macrophages, assembly of the SPI-1 injectisome causes a transient loss of host membrane integrity resulting in *Egr1* upregulation and hence suppression of inflammatory gene expression, thus weakening the immune response (Figure 4).

In addition to *Egr1*, fresh media and SPI-1 dependent *Salmonella* infection both drive the expression of other transcription factors, notably AP-1 components. We show that this upregulation is driven by increases in both transcription and translational efficiency. Other AP-1 components are not upregulated by this fresh media- or SPI-1-dependent mechanism but rather are upregulated on *Salmonella* exposure, independently of SPI-1. The balance of AP-1 transcription factor components can drive cells towards different fates, including the expression of pro-apoptotic and pro-inflammatory genes[53,54]. As such, the AP-1 complex acts as a nexus which can integrate the general response to *Salmonella* and the response to SPI-1 membrane puncture.

## Methods

### Macrophage cell culture

Primary bone marrow derived macrophages (BMDM) were harvested as previously described[27]. Briefly, bone marrow from the rear legs of C57BL/6 mice was extracted, suspended in culture media, and plated at a density of $10^6$ cells/ml supplemented with 20 ng/ml M-CSF (Peprotech). Cells were differentiated for 7 days, with additional M-CSF supplementation 4 days post-extraction. Immortalised BMDM (iBMDM) were generated by retroviral transformation of BMDMs as previously described[64]. Cells were routinely grown in DMEM supplemented with 10% Foetal Bovine Serum at 37°C, 5% $CO_2$. For TLR4 stimulation, cell culture was spiked with LPS from *Escherichia coli* O127:B8 (Sigma-Aldrich) at the indicated concentration.

### *Salmonella* infection

*Salmonella* Typhimurium SL1344 WT and Δ*prgJ* were sub-cultured in LB from a stationary phase culture and grown at 37°C, 200 rpm, until late exponential phase. Bacteria were washed and diluted in culture media and added to cells at a multiplicity of infection (MOI) of 10. For infections using BMDMs, all media was supplemented with 20 ng/ml M-CSF. Unless otherwise indicated, after 15 min media was supplemented with 100 µg/ml gentamicin.

### Microscopy

At the indicated timepoint, cells were fixed in 4% paraformaldehyde, permeabilised and stained with Phalloidin-CF594 conjugate (Biotium), and goat anti-*Salmonella* CSA-1 (Insight Biotechnology) followed by anti-goat IgG conjugated to Alexa488 (Abcam). Cells were imaged and the proportion infected determined. Macrophages with intracellular bacteria, as determined by intensity of CSA-1 staining and height of *Salmonella*, were considered infected and uninfected macrophages but with *Salmonella* associated with membrane ruffles were considered as becoming infected.

### Gentamicin protection assay

The use of gentamicin protection assay to assess the number of intracellular bacterial has been described previously[2]. This protocol was modified to account for host cell death. Briefly, 1 h post gentamicin treatment (i.e. 75 min post infection) macrophages were trypsinized, counted, and lysed in 0.09% Triton X-100. Serial dilutions of lysates were plated on LB-agar and grown at 37°C overnight. The *Salmonella* colonies were counted and divided by the number of counted host macrophages.

### Cytotoxicity assay

Cytotoxicity was measured by Lactate Dehydrogenase (LDH) release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. Cytotoxicity was determined relative to total cell lysis by 0.9% TritonX-100.

**Ribosome profiling with parallel RNA sequencing**

Ribosome profiling was performed as previously described[65], a schematic of which is presented in Figure 1E. Briefly, at the indicated timepoint, culture supernatant was removed from cells and flash frozen. Cells were lysed in buffer containing cycloheximide and chloramphenicol, and lysates were split for RNA-Seq and Ribo-Seq. For Ribo-Seq, lysates were treated with RNase I and fragments protected from digestion by the ribosome were purified. For RNA-Seq, total cellular RNA was fragmented by alkaline hydrolysis. This was followed by library generation as previously described[65–68] . Sequencing was performed using NextSeq-500 or 2000 (Illumina).

Reads were aligned sequentially to mouse rRNA, mouse mRNA, *Salmonella* rRNA and *Salmonella* mRNA. Mouse reference sequences were based on NCBI release mm10, and *Salmonella* reference sequences were based on GenBank sequences FQ312003.1, HE654725.1, HE654726.1 and HE654724.1. RiboSeqR[65] was used to confirm the quality of libraries and to count reads aligning to coding sequences. Xtail[69] was used to determine differential translational efficiency of these coding sequences and edgeR[70] was used for normalisation and to filter *Salmonella* genes by expression for retention in further analysis. Gene set enrichment analysis was performed using g:Profiler[71].

**Immunoblotting**

Protein was harvested from cells with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% IGEPAL-CA630) containing protease (cOmplete Mini, Roche) and phosphatase inhibitors (PhosSTOP, Roche). 30 µg protein was separated by SDS-PAGE and transferred to nitrocellulose membrane. EGR1 was detected using rabbit anti-EGR1 (Cell Signaling) followed by anti-rabbit conjugated to HRP (Cell Signaling). The HRP signal was assayed using SuperSignal West Pico PLUS substrate (Thermo Scientific). GAPDH was detected using mouse anti-GAPDH (Sigma Aldrich) followed by anti-mouse conjugated to IRDye 800 CW (Licor). SipB, SipC[72] and GroEL were detected using primary antibodies raised in rabbit followed by anti-rabbit antibody conjugated to HRP. Myc tagged SptP-GFP blocking substrates were detected with anti-Myc-HRP conjugate mouse antibody (Cell signalling).

**Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)**

RNA was extracted from cells using TRIzol (Invitrogen) per the manufacturer's instructions. Reverse transcription was performed using M-MLV reverse transcriptase (Promega) with random hexamer primers (Promega) per the manufacturer's instructions. Realtime qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and assayed on ViiA 7 system (Applied Biosystems) per the manufacturer's instructions. Primers were designed using PrimerBLAST (Table S4).

**Knockout of EGR1**

The Alt-R CRISPR-Cas system (IDT) was used per the manufactures instructions to edit the *Egr1* coding sequence using guide RNAs targeting *Egr1* or no genes as a negative control (Table S5)[73]. The system was delivered by lipofection into macrophages using Lipofectamine CRISPRMAX (Invitrogen). A clonal population was generated and targeted Sanger sequencing at the *Egr1* locus was performed (Genewiz) to confirm mutation of *Egr1*.

**mRNA 3' end sequencing**

Preparation of mRNA 3' end sequencing libraries was performed using QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen) with TRIzol extracted RNA and libraries were sequenced by Novogene Ltd. Reads were aligned to the mouse genome (mm10) and those aligning to genes counted. Read count normalisation and differential expression analysis was performed using edgeR.

**Protein export assays**

Export assays were performed as previously described[74–76]. Briefly, *Salmonella* strains were cultured at 37 °C in LB broth to mid-log phase (OD600nm 1.5). Cells were centrifuged (6000 x g, 3 min) and resuspended in fresh media and grown for a further 60 min at 37 °C. The cells were pelleted by centrifugation (16,000 x g, 5 min) and the supernatant passed through a 0.2 µm nitrocellulose filter. Proteins were precipitated with 10% trichloroacetic acid (TCA) and 1% Triton X-100 on ice for 1 hr, pelleted by centrifugation (16,000 x g, 10 min), washed with ice-cold acetone, and resuspended in SDS-PAGE loading buffer (volumes calibrated according to cell densities). Fractions were analysed by immunoblotting with anti-SipB, anti-SipC[72], anti-Myc (Cell Signaling) and anti-GroEL (Abcam) anti-sera.

**Conflict of interest:**

The authors declare no conflict of interest

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**Authors contribution:**

B.Y.W.C., conceived the research. B.Y.W.C., G.W., R.J., J.P., O.J.B., F.L., J.C. and C.B. designed experiments. R.J., J.P. and B.Y.W.C. generated RiboSeq, RNA-Seq and QuantSeq libraries, G.W., R.J. and J.P. performed molecular and cell biology experiments, O.J.B. generated *Salmonella* mutants, F.L. performed bacterial bioinformatics, B.Y.W.C., G.W., R.J., J.P., F.L. and M.B. performed the bioinformatic analysis. C.B. provided macrophages and *prgJ* mutant. P.T. provided training for extracting BMDM, B.Y.W.C., G.W., O.J.B., and J.C. wrote the manuscript.

**Figure Legends**

**Fig 1:** *Salmonella* SPI-1 dependent infection rapidly alters macrophage translation. (**A**) WT *Salmonella* infected macrophages 5 min post infection. Phalloidin-actin is shown in red, and *Salmonella* are shown in green. Arrows indicate *Salmonella* associated with host membrane ruffles. (**B**) Percentage of macrophages affected by WT *Salmonella* during the first 15 min of infection determined by immunofluorescent microscopy. Infected cells contain intracellular *Salmonella*, infecting cells do not but have *Salmonella* associated with membrane ruffles, n=3. (**C**) *Salmonella* colony forming units (CFU) recovered 75 min post WT and SPI-1 deficient (Δ*prgJ*) *Salmonella* infection per host macrophage, n=2. (**D**) Cytotoxicity of WT or Δ*prgJ Salmonella* or mock infection on host macrophages as determined by LDH release

into culture supernatant, n=2, representative of 2 independent experiments. (**E**) Experimental outline detailing the preparation of ribosome profiling and RNA-Seq libraries from infected macrophages. Details are provided in the materials and methods. (**F**) Diagram illustrating the relationship between mRNA abundance (measured by RNA-Seq), protein synthesis (measured by Ribo-Seq) and translational efficiency (TE) (**G**) mRNA abundance, protein synthesis and TE of genes known to be translationally activated by bacterial pathogen associated molecular patterns at 60 min post infection. Genes with low read counts (i.e. those with normalised RNA-Seq and Ribo-Seq counts less than 20) are considered not expressed and therefore TE cannot be reliably calculated (grey bars). (**H**) Relative mRNA abundance, protein synthesis and TE of genes with $\log_2$FC in TE of WT over Δ*prgJ* infected macrophages greater than 1.5 and at least 50 normalised Ribo-Seq counts in WT infection.

**Fig 2**: *Egr1* is rapidly induced in *Salmonella* infection to restrict expression of immune response genes. (**A**) Relative RPF and RNA-Seq counts and relative translational efficiency of *Egr1* from **Fig 1G**. Conditions with low read counts are considered not expressing *Egr1* and therefore TE cannot be reliably calculated (grey bar). (**B**) *Egr1* transcript abundance over WT and Δ*prgJ* infection, normalised to the housekeeping gene Supt16 and relative to cells prior to infection, n=2. (**C**) Quantification of EGR1 abundance from **D** normalised to GAPDH. (**D**) Western blot following EGR1 protein abundance across WT and Δ*prgJ* infection. (**E**) Experimental outline illustrating the effect of the SptP-GFP injectisome blocking subunit on protein export *via* the injectisome. *Salmonella* cells not producing the blocking subunit can transport effector proteins into host cells (Unobstructed injectisome, left). *Salmonella* cells expressing the injectisome blocking subunit before infection are unable to transport effector subunits *via* the injectisome (Pre-blocked injectisome, middle). *Salmonella* cells were incubated with macrophage for 5 minutes before inducing expression of the injectisome blocking substrate such that injectisomes can engage with macrophage cells but secretion of effectors proteins from *Salmonella* into macrophage are blocked at 5 minutes. (Injectisome blocked at 5 min, right). (**F**) Secretion analysis of wildtype *Salmonella* either expressing the SptP-GFP injectisome blocking subunit or carrying empty vector. Whole cell (cell) and secreted proteins (sec) from late-exponential-phase cultures were separated by SDS-PAGE and immunoblotted with anti-Myc tag (SptP-GFP), anti-SipC, anti-SipB or anti-GroEL antisera. (**G**) *Egr1* transcript abundance over *Salmonella* infection with blockade of the SPI-1 injectisome induced at various timepoints, n=2. (**H**) Western blot showing EGR1 expression of negative control and EGR1 knockout (KO) macrophages infected with WT *Salmonella* at 120 min post infection, the time of peak EGR1 abundance in WT macrophages. (**I**) Cytotoxicity of WT *Salmonella* or mock infection on infected control and

EGR1 KO macrophages. (**J**) Relative abundance of genes upregulated ($log_2FC > 2$) at any timepoint in WT *Salmonella* infection of EGR1 KO over control macrophages. (**K**) 10 most significant enriched gene ontology biological process terms in genes identified in J; term recall is the proportion of genes covered by the term in the query.

**Fig 3**: Time resolved RiboSeq reveals dynamic changes in host translation. (**A**) Outline of infection timecourse. Culture supernatant was replaced at 0 min to add the inoculum, 15 min to add 50 µg/ml gentamicin, and 60 min to reduce this to 10 µg/ml gentamicin. (**B**) $Log_2FC$ in TE vs $log_2FC$ mRNA abundance of WT *Salmonella* infection over Δ*prgJ* infection, across the timecourse. Dashed orange lines show the $log_2FC$ cutoffs (±2) used to select differentially expressed genes, genes that pass these thresholds are coloured accordingly. Only genes with $log_2FC$ in mRNA and TE between -5 and 5 are shown here, see Fig S4B for uncropped plots (**C** and **D**) Number of genes differentially expressed between WT and Δ*prgJ* *Salmonella* infection at both the TE (**C**) and mRNA (**D**) level. (**E** and **F**) Top 10 enriched GO molecular function terms in differentially expressed genes at or before 60 min post WT and Δ*prgJ Salmonella* infection at both the TE (**E**) and mRNA (**F**) level. Terms are grouped and coloured by related functions; term recall is the proportion of genes covered by the term in the query.

**Fig 4**: Temporal dynamics of *Salmonella* gene expression over 4 hours of macrophage infection (**A**) Relative mRNA abundance, protein synthesis and TE of selected groups of *Salmonella* genes throughout WT *Salmonella* infection of BMDM. Only genes that have sufficiently large mRNA or RPF read counts are included. (**B**) Diagram illustrating the temporal regulation of specific classes of *Salmonella* genes as infection is established.

**Fig 5**: *Salmonella* SPI-1 injectisome assembly forms transient pores in the host membrane which activates host signalling pathways, leading to increased *Egr1* transcription and enhanced translation. EGR1 negatively modulates immune gene transcription and, in doing so, inflammatory cytokine production in response to *Salmonella* infection is restrained, as is host cell death.

**Fig S1**: (**A**) Percentage of macrophages infected by WT *Salmonella* determined by microscopy at 15 min and 60 minutes, with and without addition of gentamicin at 15 min, n=2. (**B**) Percentage of macrophages infected by WT or Δ*prgJ Salmonella* determined by microscopy at 15 min, n=2.

**Fig S2**: (**A**) Example section of the *Egr1* coding sequence with an alignment from targeted sequencing of the Egr1 locus in the EGR1 KO macrophages. Mismatched bases and deletions are highlighted in red. (**B**) Principal component analysis of the transcriptomes of WT *Salmonella* infected or mock infected EGR1 KO or control macrophages over an infection timecourse.

**Fig S3**: (**A**) Transcript abundance of *Egr1* and *Fosb* 60 min post replacement of culture supernatant with fresh or exhausted (i.e. the original culture supernatant) media, normalised to *Supt16* and relative to 0 min, n=2. (**B**) Genes transcriptionally up- or down- regulated ($|\log_2 FC| > 1$) at 60 min post replacement of culture supernatant with fresh or exhausted media. (**C**) Transcript abundance of genes upregulated by fresh media 60 min post WT or Δ*prgJ Salmonella* infection, or TLR4 stimulation with LPS. Values are normalised to *Supt16* and relative to mock infection or 0 ng/ml LPS.

**Fig S4**: (**A**) Percentage of primary macrophages infected by WT or Δ*prgJ Salmonella* determined by microscopy at 15 min (**B**) $\text{Log}_2$FC in TE vs $\log_2$FC mRNA abundance between the indicated infections, across the timecourse. Dashed orange lines show the $\log_2$FC cutoffs (±2) used to select differentially expressed genes, genes that pass these thresholds are coloured accordingly.

**Fig S5**: Normalised mRNA and RPF abundance of AP-1 transcription factors across mock, WT or Δ*prgJ Salmonella* infection, with exchange of culture supernatant with fresh media as shown in Fig 3A.

**Fig S6**: Meta-gene translatome from ribosome profiling of *Salmonella* infected macrophages at 60 min (**A**) and across a *Salmonella* primary BMDM infection time course (**B**). Histograms of RPF 5′ ends relative to start and stop codons coloured by their reading frame relative to the coding sequence.

## References


1. Denzer, L., Schroten, H., and Schwerk, C. (2020). From gene to protein - how bacterial virulence factors manipulate host gene expression during infection. Int. J. Mol. Sci. *21*, 3730. 10.3390/ijms21103730.

2. Hannemann, S., Gao, B., and Galán, J.E. (2013). Salmonella modulation of host cell gene expression promotes its intracellular growth. PLoS Pathog. *9*, e1003668. 10.1371/journal.ppat.1003668.

3. Jensen, K., Gallagher, I.J., Kaliszewska, A., Zhang, C., Abejide, O., Gallagher, M.P., Werling, D., and Glass, E.J. (2016). Live and inactivated Salmonella enterica serovar Typhimurium stimulate similar but distinct transcriptome profiles in bovine macrophages and dendritic cells. Vet. Res. *47*. 10.1186/s13567-016-0328-y.

4. Ordas, A., Hegedus, Z., Henkel, C. V, Stockhammer, O.W., Butler, D., Jansen, H.J., Racz, P., Mink, M., Spaink, H.P., and Meijer, A.H. (2011). Deep sequencing of the innate immune transcriptomic response of zebrafish embryos to Salmonella infection. Fish Shellfish Immunol. *31*, 716–724. 10.1016/j.fsi.2010.08.022.

5. Maekawa, S., Wang, P.C., and Chen, S.C. (2019). Comparative study of immune reaction against bacterial infection from transcriptome analysis. Front. Immunol. *10*. 10.3389/fimmu.2019.00153.

6. Westermann, A.J., Venturini, E., Sellin, M.E., Förstner, K.U., Hardt, W.D., and Vogel, J. (2019). The major RNA-binding protein ProQ impacts virulence gene expression in Salmonella enterica serovar Typhimurium. MBio *10*, e02504-2518. 10.1128/mBio.02504-18.

7. Fields, P.I., Swanson, R. V., Haidaris, C.G., and Heffron, F. (1986). Mutants of Salmonella Typhimurium that cannot survive within the macrophage are avirulent. PNAS *83*, 5189–5193. 10.1073/pnas.83.14.5189.

8. Liss, V., Swart, A.L., Kehl, A., Hermanns, N., Zhang, Y., Chikkaballi, D., Böhles, N., Deiwick, J., and Hensel, M. (2017). Salmonella enterica remodels the host cell endosomal system for efficient intravacuolar nutrition. Cell Host Microbe *21*, 390–402. 10.1016/j.chom.2017.02.005.

9. Jiang, L., Wang, P., Song, X., Zhang, H., Ma, S., Wang, J., Li, W., Lv, R., Liu, X., Ma, S., et al. (2021). Salmonella Typhimurium reprograms macrophage metabolism via T3SS effector SopE2 to promote intracellular replication and virulence. Nat. Commun. *12*, 1–18. 10.1038/s41467-021-21186-4.

10. Miletic, S., Fahrenkamp, D., Goessweiner-Mohr, N., Wald, J., Pantel, M., Vesper, O., Kotov, V., and Marlovits, T.C. (2021). Substrate-engaged type III secretion system structures reveal gating mechanism for unfolded protein translocation. Nat. Commun. *12*, 1546. 10.1038/s41467-021-21143-1.

11. Myeni, S.K., Wang, L., and Zhou, D. (2013). SipB-SipC complex is essential for translocon formation. PLoS One *8*, e60499. 10.1371/journal.pone.0060499.

12. Miki, T., Okada, N., Shimada, Y., and Danbara, H. (2004). Characterization of Salmonella pathogenicity island 1 type III secretion-dependent hemolytic activity in

Salmonella enterica serovar Typhimurium. Microb. Pathog. *37*, 65–72. 10.1016/j.micpath.2004.04.006.

13. Ly, K.T., and Casanova, J.E. (2007). Mechanisms of Salmonella entry into host cells. Cell. Microbiol. *9*, 2103–2111. 10.1111/j.1462-5822.2007.00992.x.

14. Galán, J.E. (1998). Interactions of Salmonella with host cells: Encounters of the closest kind. Proc. Natl. Acad. Sci. *95*, 14006–14008. 10.1073/pnas.95.24.14006.

15. Hume, P.J., Singh, V., Davidson, A.C., and Koronakis, V. (2017). Swiss army pathogen: The Salmonella entry toolkit. Front. Cell. Infect. Microbiol. *7*, 348. 10.3389/fcimb.2017.00348.

16. Vazquez-Torres, A., Xu, Y., Jones-Carson, J., Holden, D.W., Lucia, S.M., Dinauer, M.C., Mastroeni, P., and Fang, F.C. (2000). Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science (80-. ). *287*, 1655–1658. 10.1126/science.287.5458.1655.

17. Guignot, J., and Tran Van Nhieu, G. (2016). Bacterial control of pores induced by the type III secretion system: mind the gap. Front. Immunol. *7*, 84. 10.3389/fimmu.2016.00084.

18. Richter-Dahlfors, A., Buchan, A.M.J., and Finlay, B.B. (1997). Murine salmonellosis studied by confocal microscopy: Salmonella Typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. J. Exp. Med. *186*, 569–580. 10.1084/jem.186.4.569.

19. Grant, A.J., Sheppard, M., Deardon, R., Brown, S.P., Foster, G., Bryant, C.E., Maskell, D.J., and Mastroeni, P. (2008). Caspase-3-dependent phagocyte death during systemic Salmonella enterica serovar Typhimurium infection of mice. Immunology *125*, 28–37. 10.1111/j.1365-2567.2008.02814.x.

20. Gram, A.M., Wright, J.A., Pickering, R.J., Lam, N.L., Booty, L.M., Webster, S.J., and Bryant, C.E. (2021). Salmonella flagellin activates NAIP/NLRC4 and canonical NLRP3 inflammasomes in human macrophages. J. Immunol. *206*, 631–640. 10.4049/jimmunol.2000382.

21. Bergsbaken, T., Fink, S.L., and Cookson, B.T. (2009). Pyroptosis: host cell death and inflammation. Nat. Rev. Microbiol. *7*, 99–109. 10.1038/nrmicro2070.

22. Chen, L.M., Kaniga, K., and Galán, J.E. (1996). Salmonella spp. are cytotoxic for cultured macrophages. Mol. Microbiol. *21*, 1101–1115. 10.1046/j.1365-2958.1996.471410.x.

23. Monack, D.M., Raupach, B., Hromockyj, A.E., and Falkow, S. (1996). Salmonella Typhimurium invasion induces apoptosis in infected macrophages. PNAS *93*, 9833–9838. 10.1073/pnas.93.18.9833.

24. Lundberg, U., Vinatzer, U., Berdnik, D., von Gabain, A., and Baccarini, M. (1999). Growth phase-regulated induction of Salmonella-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. J. Bacteriol. *181*, 3433–3437. 10.1128/JB.181.11.3433-3437.1999.

25. Price, J. V., and Vance, R.E. (2014). The macrophage paradox. Immunity *41*, 685–693. 10.1016/j.immuni.2014.10.015.

26. Drecktrah, D., Knodler, L.A., Ireland, R., and Steele-Mortimer, O. (2006). The mechanism of Salmonella entry determines the vacuolar environment and intracellular gene expression. Traffic *7*, 39–51. 10.1111/J.1600-0854.2005.00360.X.

27. Man, S.M., Hopkins, L.J., Nugent, E., Cox, S., Glück, I.M., Tourlomousis, P., Wright, J.A., Cicuta, P., Monie, T.P., and Bryant, C.E. (2014). Inflammasome activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular complex. Proc. Natl. Acad. Sci. *111*, 7403–7408. 10.1073/PNAS.1402911111.

28. Fink, S.L., and Cookson, B.T. (2007). Pyroptosis and host cell death responses during Salmonella infection. Cell. Microbiol. *9*, 2562–2570. 10.1111/J.1462-5822.2007.01036.X.

29. Dumitru, C.D., Ceci, J.D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J.-H., Patriotis, C., Jenkins, N.A., Copeland, N.G., Kollias, G., et al. (2000). TNF-α induction by LPS Is tegulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell *103*, 1071–1083. 10.1016/S0092-8674(00)00210-5.

30. Wang, L., Trebicka, E., Fu, Y., Waggoner, L., Akira, S., Fitzgerald, K.A., Kagan, J.C., and Cherayil, B.J. (2011). Regulation of lipopolysaccharide-induced translation of tumor necrosis factor-alpha by the toll-like receptor 4 adaptor protein TRAM. J. Innate Immun. *3*, 437–446. 10.1159/000324833.

31. Thiel, G., and Cibelli, G. (2002). Regulation of life and death by the zinc finger transcription factor Egr-1. J. Cell. Physiol. *193*, 287–292. 10.1002/jcp.10178.

32. Chbicheb, S., Yao, X., Rodeau, J.-L., Salamone, S., Boisbrun, M., Thiel, G., Spohn, D., Grillier-Vuissoz, I., Chapleur, Y., Flament, S., et al. (2011). EGR1 expression: A calcium and ERK1/2 mediated PPARγ-independent event involved in the antiproliferative effect of 15-deoxy-Δ12,14-prostaglandin J2 and thiazolidinediones in breast cancer cells. Biochem. Pharmacol. *81*, 1087–1097. 10.1016/j.bcp.2011.02.006.

33. Banerji, R., and Saroj, S.D. (2021). Early growth response 1 (EGR1) activation in initial stages of host–pathogen interactions. Mol. Biol. Rep. *48*, 2935–2943. 10.1007/s11033-021-06305-0.

34. Trizzino, M., Zucco, A., Deliard, S., Wang, F., Barbieri, E., Veglia, F., Gabrilovich, D., and Gardini, A. (2021). EGR1 is a gatekeeper of inflammatory enhancers in human macrophages. Sci. Adv. *7*. 10.1126/sciadv.aaz8836.

35. Kimura, T.E., Duggirala, A., Hindmarch, C.C.T., Hewer, R.C., Cui, M.Z., Newby, A.C., and Bond, M. (2014). Inhibition of Egr1 expression underlies the anti-mitogenic effects of cAMP in vascular smooth muscle cells. J. Mol. Cell. Cardiol. *72*, 9–19. 10.1016/j.yjmcc.2014.02.001.

36. de Grado, M., Rosenberger, C.M., Gauthier, A., Vallance, B.A., and Finlay, B.B. (2001). Enteropathogenic Escherichia coli infection induces expression of the early growth response factor by activating mitogen-activated protein kinase cascades in epithelial cells. Infect. Immun. *69*, 6217–6224. 10.1128/IAI.69.10.6217-6224.2001.

37. Shin, H., and Cornelis, G.R. (2007). Type III secretion translocation pores of Yersinia enterocolitica trigger maturation and release of pro-inflammatory IL-1β. Cell. Microbiol. *9*, 2893–2902. 10.1111/j.1462-5822.2007.01004.x.

38. Keates, S., Keates, A.C., Nath, S., Peek, R.M., and Kelly, C.P. (2005). Transactivation of the epidermal growth factor receptor by cag+ Helicobacter pylori induces upregulation of the early growth response gene Egr-1 in gastric epithelial cells. Gut *54*, 1363–1369. 10.1136/gut.2005.066977.

39. McMullen, M.R., Pritchard, M.T., Wang, Q., Millward, C.A., Croniger, C.M., and Nagy, L.E. (2005). Early growth response-1 transcription factor is essential for ethanol-induced fatty liver injury in mice. Gastroenterology *128*, 2066–2076. 10.1053/j.gastro.2005.02.065.

40. Pang, Z., Xu, Y., and Zhu, Q. (2022). Early growth response 1 suppresses macrophage phagocytosis by inhibiting NRF2 activation through upregulation of autophagy during Pseudomonas aeruginosa infection. Front. Cell. Infect. Microbiol. *11*, 1. 10.3389/fcimb.2021.773665.

41. Radics, J., Königsmaier, L., and Marlovits, T.C. (2013). Structure of a pathogenic type 3 secretion system in action. Nat. Struct. Mol. Biol. 2013 211 *21*, 82–87. 10.1038/nsmb.2722.

42. Maegawa, M., Arao, T., Yokote, H., Matsumoto, K., Kudo, K., Tanaka, K., Kaneda, H., Fujita, Y., Ito, F., and Nishio, K. (2009). EGFR mutation up-regulates EGR1

expression through the ERK pathway. Anticancer Res. *29*, 1111–1117.

43. Nose, K., and Ohba, M. (1996). Functional activation of the egr-1 (early growth response-1) gene by hydrogen peroxide. Biochem. J. *316*, 381. 10.1042/BJ3160381.

44. Quiñones, A., Dobberstein, K.U., and Rainov, N.G. (2003). The egr-1 gene is induced by DNA-damaging agents and non-genotoxic drugs in both normal and neoplastic human cells. Life Sci. *72*, 2975–2992. 10.1016/S0024-3205(03)00230-3.

45. Yan, S.F., Fujita, T., Lu, J., Okada, K., Shan Zou, Y., Mackman, N., Pinsky, D.J., and Stern, D.M. (2000). Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. Nat. Med. *6*, 1355–1361. 10.1038/82168.

46. Cai, Y., Liu, Y., and Zhang, X. (2006). Induction of transcription factor Egr-1 gene expression in astrocytoma cells by Murine coronavirus infection. Virology *355*, 152–163. 10.1016/j.virol.2006.07.012.

47. Mahat, D.B., and Lis, J.T. (2017). Use of conditioned media is critical for studies of regulation in response to rapid heat shock. Cell Stress Chaperones *22*, 155. 10.1007/S12192-016-0737-X.

48. Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Müller, R., and Bravo, R. (1989). The product of a novel growth factor activated gene, fosB, interacts with JUN proteins enhancing their DNA binding activity. EMBO J. *8*, 805–813. 10.1002/j.1460-2075.1989.tb03441.x.

49. Lai, W.S., Thompson, M.J., Taylor, G.A., Liu, Y., and Blackshear, P.J. (1995). Promoter analysis of Zfp-36, the mitogen-inducible gene encoding the zinc finger protein tristetraprolin. J. Biol. Chem. *270*, 25266–25272. 10.1074/jbc.270.42.25266.

50. Okazaki, R., Ikeda, K., Sakamoto, A., Nakano, T., Morimoto, K., Kikuchi, T., Urakawa, K., Ogata, E., and Matsumoto, T. (2009). Transcriptional activation of c-fos and c-jun protooncogenes by serum growth factors in osteoblast-like MC3T3-E1 cells. J. Bone Miner. Res. *7*, 1149–1155. 10.1002/jbmr.5650071006.

51. Broz, P., and Monack, D.M. (2013). Measuring inflammasome activation in response to bacterial infection. In The Inflammasome: Methods and Protocols, C. M. De Nardo and E. Latz, eds. (Humana Press), pp. 65–84. 10.1007/978-1-62703-523-1_6.

52. Lathrop, S.K., Cooper, K.G., Binder, K.A., Starr, T., Mampilli, V., Detweiler, C.S., and Steele-Mortimer, O. (2018). Salmonella Typhimurium infection of human monocyte-derived macrophages. Curr. Protoc. Microbiol. *50*, e56. 10.1002/cpmc.56.

53. Hess, J., Angel, P., and Schorpp-Kistner, M. (2004). AP-1 subunits: quarrel and

harmony among siblings. J. Cell Sci. *117*, 5965–5973. 10.1242/JCS.01589.

54. Atsaves, V., Leventaki, V., Rassidakis, G.Z., and Claret, F.X. (2019). AP-1 Transcription Factors as Regulators of Immune Responses in Cancer. Cancers (Basel). *11*. 10.3390/CANCERS11071037.

55. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J.C.D. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. Mol. Microbiol. *47*, 103–118. 10.1046/j.1365-2958.2003.03313.x.

56. Srikumar, S., Kröger, C., Hébrard, M., Colgan, A., Owen, S. V., Sivasankaran, S.K., Cameron, A.D.S., Hokamp, K., and Hinton, J.C.D. (2015). RNA-seq brings new insights to the intra-macrophage transcriptome of Salmonella Typhimurium. PLOS Pathog. *11*, e1005262. 10.1371/journal.ppat.1005262.

57. Chakraborty, S., Mizusaki, H., and Kenney, L.J. (2015). A FRET-based DNA biosensor tracks OmpR-dependent acidification of Salmonella during macrophage infection. PLOS Biol. *13*, e1002116. 10.1371/journal.pbio.1002116.

58. Pérez-Morales, D., Banda, M.M., Chau, N.Y.E., Salgado, H., Martínez-Flores, I., Ibarra, J.A., Ilyas, B., Coombes, B.K., and Bustamante, V.H. (2017). The transcriptional regulator SsrB is involved in a molecular switch controlling virulence lifestyles of Salmonella. PLOS Pathog. *13*, e1006497. 10.1371/journal.ppat.1006497.

59. Brown, N.F., Rogers, L.D., Sanderson, K.L., Gouw, J.W., Hartland, E.L., and Foster, L.J. (2014). A horizontally acquired transcription factor coordinates Salmonella adaptations to host microenvironments. MBio *5*, 1727–1741. 10.1128/mBio.01727-14.

60. Chang, Y.-W., Shaffer, C.L., Rettberg, L.A., Ghosal, D., and Jensen, G.J. (2018). In vivo structures of the Helicobacter pylori cag Type IV secretion system. Cell Rep. *23*, 673–681. 10.1016/j.celrep.2018.03.085.

61. Haugwitz, U., Bobkiewicz, W., Han, S.R., Beckmann, E., Veerachato, G., Shaid, S., Biehl, S., Dersch, K., Bhakdi, S., and Husmann, M. (2006). Pore-forming Staphylococcus aureus α-toxin triggers epidermal growth factor receptor-dependent proliferation. Cell. Microbiol. *8*, 1591–1600. 10.1111/j.1462-5822.2006.00733.x.

62. Kao, C.Y., Los, F.C.O., Huffman, D.L., Wachi, S., Kloft, N., Husmann, M., Karabrahimi, V., Schwartz, J.L., Bellier, A., Ha, C., et al. (2011). Global functional analyses of cellular responses to pore-forming toxins. PLoS Pathog. *7*, 1001314. 10.1371/journal.ppat.1001314.

63. Ponde, N.O., Lortal, L., Tsavou, A., Hepworth, O.W., Wickramasinghe, D.N., Ho, J., Richardson, J.P., Moyes, D.L., Gaffen, S.L., and Naglik, J.R. (2022). Receptor-kinase EGFR-MAPK adaptor proteins mediate the epithelial response to Candida albicans via the cytolytic peptide toxin, candidalysin. J. Biol. Chem. *298*. 10.1016/j.jbc.2022.102419.

64. De Nardo, D., Kalvakolanu, D. V., and Latz, E. (2018). Immortalization of murine bone marrow-derived macrophages. Methods Mol. Biol. *1784*, 35–49. 10.1007/978-1-4939-7837-3_4/FIGURES/3.

65. Chung, B.Y., Hardcastle, T.J., Jones, J.D., Irigoyen, N., Firth, A.E., Baulcombe, D.C., and Brierley, I. (2015). The use of duplex-specific nuclease in ribosome profiling and a user-friendly software package for Ribo-seq data analysis. RNA *21*, 1731. 10.1261/RNA.052548.115.

66. Irigoyen, N., Firth, A.E., Jones, J.D., Chung, B.Y.W., Siddell, S.G., and Brierley, I. (2016). High-resolution analysis of Coronavirus gene expression by RNA sequencing and ribosome profiling. PLOS Pathog. *12*, e1005473. 10.1371/journal.ppat.1005473.

67. Chung, B.Y.W., Deery, M.J., Groen, A.J., Howard, J., and Baulcombe, D.C. (2017). Endogenous miRNA in the green alga Chlamydomonas regulates gene expression through CDS-targeting. Nat. Plants 2017 310 *3*, 787–794. 10.1038/s41477-017-0024-6.

68. Chung, B.Y.W., Balcerowicz, M., Di Antonio, M., Jaeger, K.E., Geng, F., Franaszek, K., Marriott, P., Brierley, I., Firth, A.E., and Wigge, P.A. (2020). An RNA thermoswitch regulates daytime growth in Arabidopsis. Nat. Plants 2020 65 *6*, 522–532. 10.1038/s41477-020-0633-3.

69. Xiao, Z., Zou, Q., Liu, Y., and Yang, X. (2016). Genome-wide assessment of differential translations with ribosome profiling data. Nat. Commun. *7*. 10.1038/ncomms11194.

70. Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139–140. 10.1093/BIOINFORMATICS/BTP616.

71. Peterson, H., Kolberg, L., Raudvere, U., Kuzmin, I., and Vilo, J. (2020). gprofiler2 - an R package for gene list functional enrichment analysis and namespace conversion toolset g:Profiler. F1000Research *9*, 709. 10.12688/f1000research.24956.2.

72. Hayward, R.D., and Koronakis, V. (1999). Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella. EMBO J. *18*, 4926–4934.

10.1093/EMBOJ/18.18.4926.

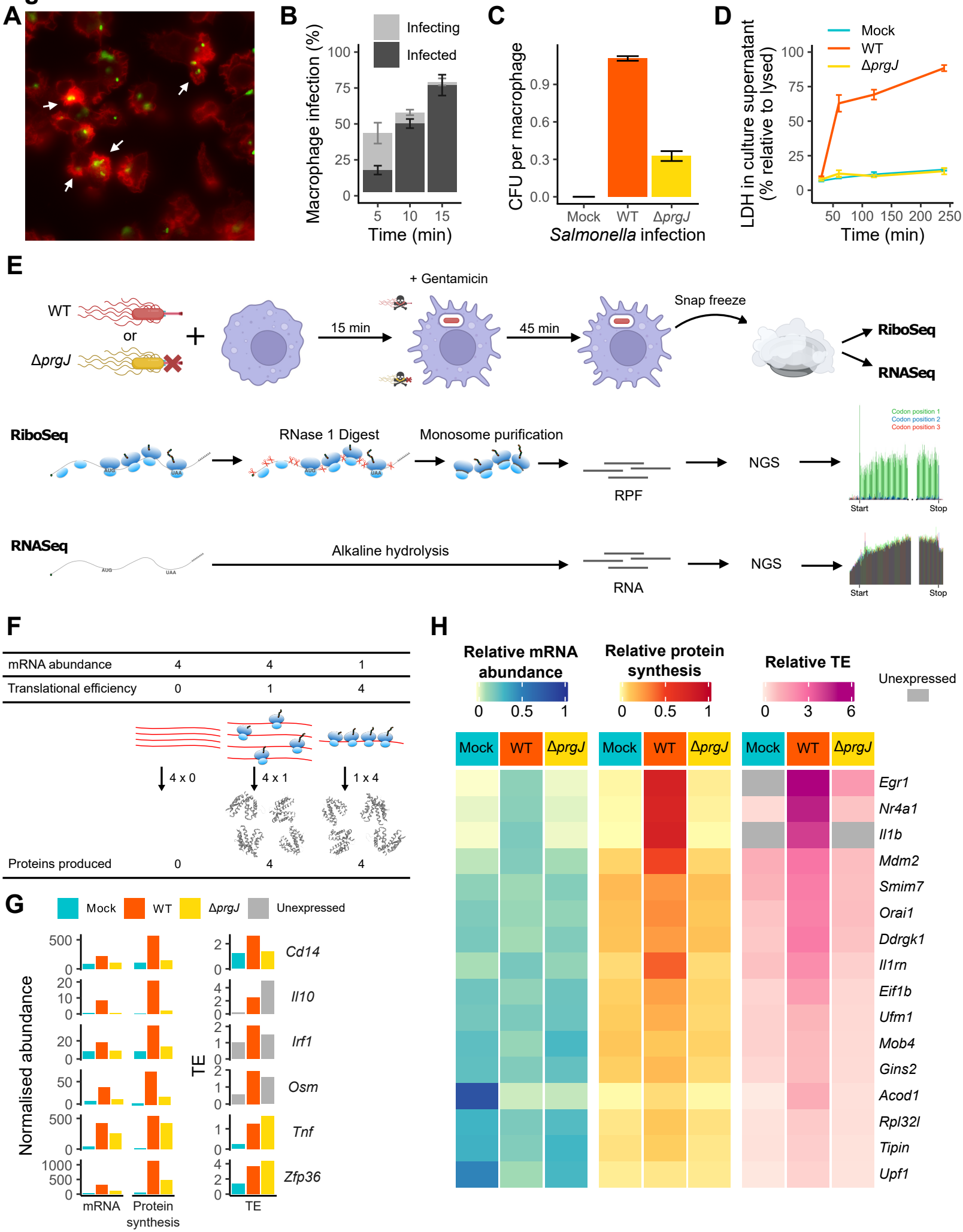
73. Wise, D. (2019). Understanding antigen processing in chickens using genome editing technology. 10.17863/CAM.40666.

74. Bryant, O.J., Chung, B.Y. -W., and Fraser, G.M. (2021). Chaperone-mediated coupling of subunit availability to activation of flagellar Type III secretion. Mol. Microbiol. *116*, 538–549. 10.1111/mmi.14731.

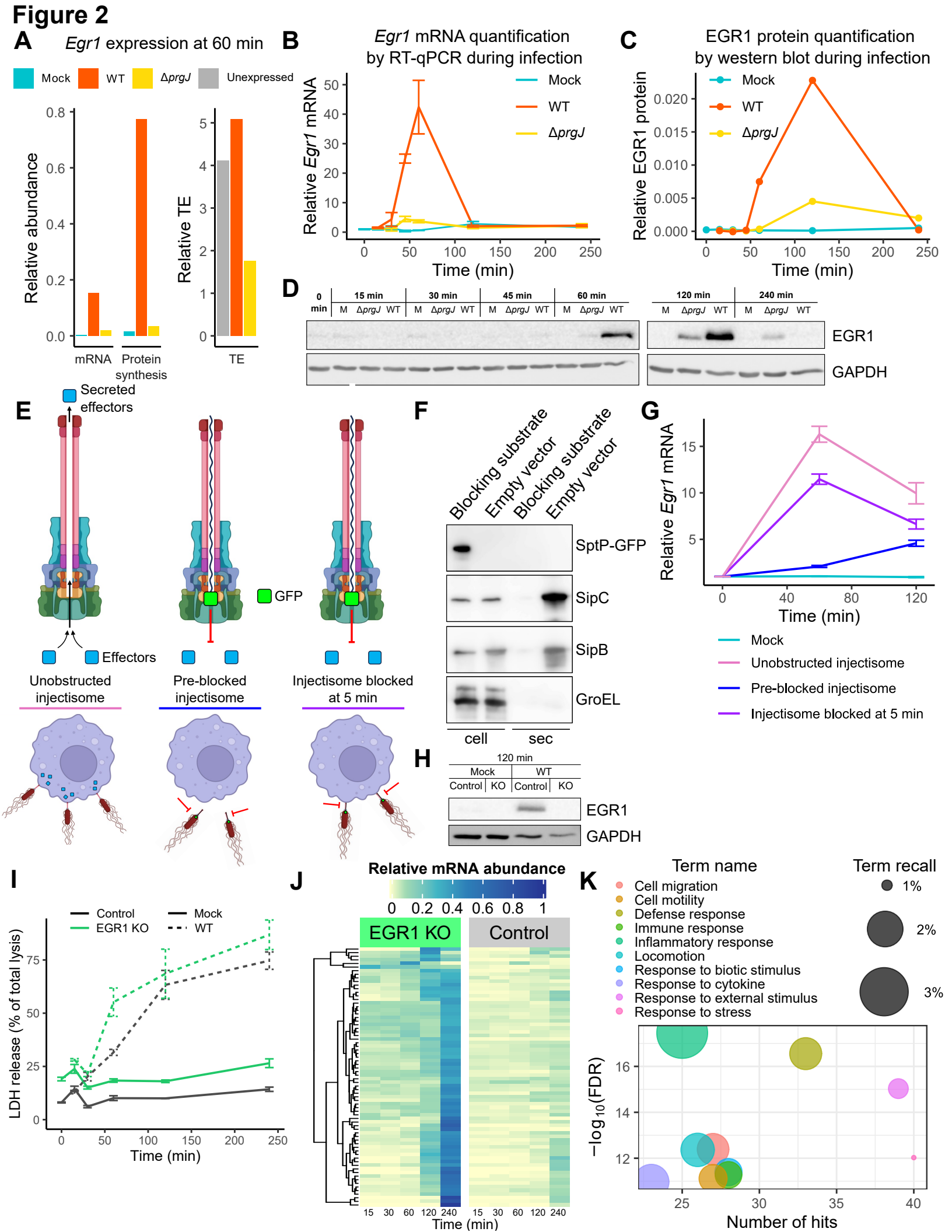
75. Bryant, O.J., and Fraser, G.M. (2022). Regulation of bacterial Type III Secretion System export gate opening by substrates and the FliJ stalk of the flagellar ATPase. FEBS *289*, 2628–2641. 10.1111/febs.16294.

76. Bryant, O.J., Dhillon, P., Hughes, C., and Fraser, G.M. (2022). Recognition of discrete export signals in early flagellar subunits during bacterial type III secretion. Elife *11*. 10.7554/eLife.66264.

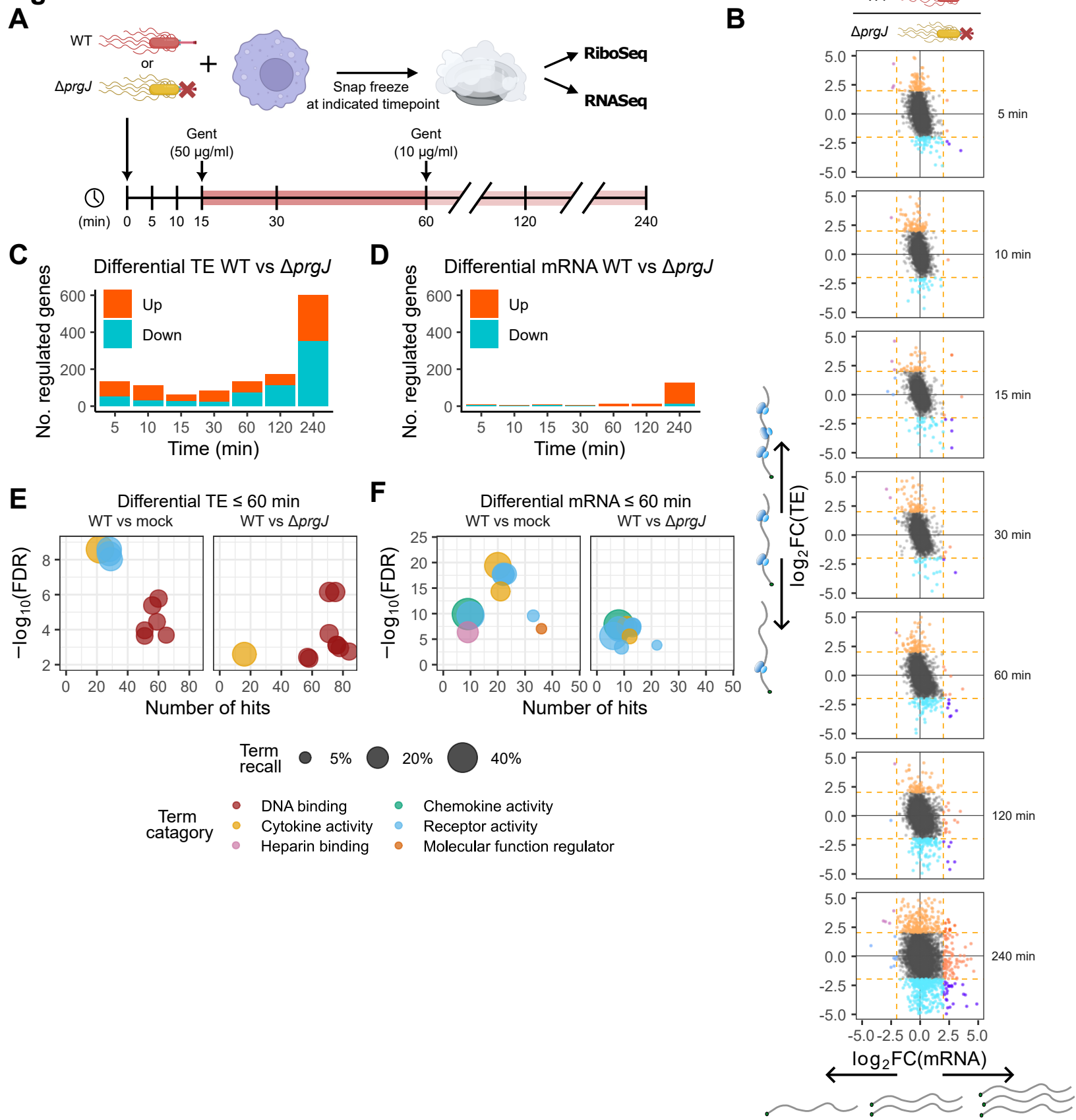
# Figure 1


A
B
Infecting
Infected
Macrophage infection (%)
100
75
50
25
0
5
10
15
Time (min)
C
CFU per macrophage
0.9
0.6
0.3
0.0
Mock
WT
ΔprgJ
Salmonella infection
D
Mock
WT
ΔprgJ
LDH in culture supernatant (% relative to lysed)
100
75
50
25
0
50
100
150
200
250
Time (min)
E
WT
or
ΔprgJ
+ Gentamicin
15 min
45 min
Snap freeze
RiboSeq
RNASeq
RiboSeq
RNase 1 Digest
Monosome purification
RPF
NGS
Codon position 1
Codon position 2
Codon position 3
Start
Stop
RNASeq
Alkaline hydrolysis
RNA
NGS
Start
Stop
F
mRNA abundance 4 4 1
Translational efficiency 0 1 4
4 x 0
4 x 1
1 x 4
Proteins produced 0 4 4
G
Mock
WT
ΔprgJ
Unexpressed
Normalised abundance
TE
Cd14
Il10
Irf1
Osm
Tnf
Zfp36
mRNA
Protein synthesis
TE
H
Relative mRNA abundance
0
0.5
1
Relative protein synthesis
0
0.5
1
Relative TE
0
3
6
Unexpressed
Mock
WT
ΔprgJ
Egr1
Nr4a1
Il1b
Mdm2
Smim7
Orai1
Ddrgk1
Il1rn
Eif1b
Ufm1
Mob4
Gins2
Acod1
Rpl32l
Tipin
Upf1

# Figure 2

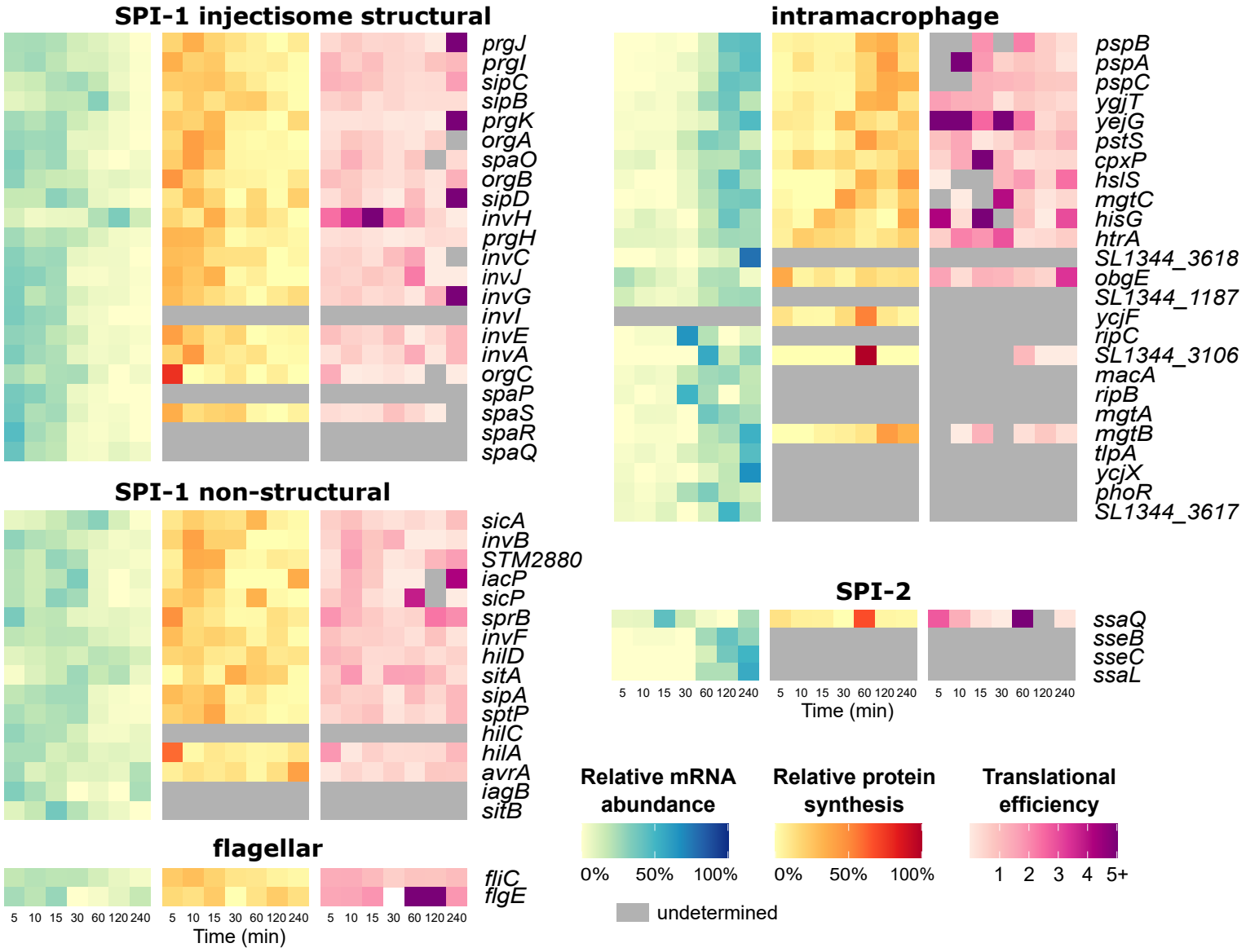

A
Egr1 expression at 60 min
Mock
WT
ΔprgJ
Unexpressed
Relative abundance
mRNA
Protein synthesis
Relative TE
TE
B
Egr1 mRNA quantification by RT-qPCR during infection
Relative Egr1 mRNA
Time (min)
C
EGR1 protein quantification by western blot during infection
Relative EGR1 protein
D
15 min
30 min
45 min
60 min
120 min
240 min
EGR1
GAPDH
E
Secreted effectors
GFP
Effectors
Unobstructed injectisome
Pre-blocked injectisome
Injectisome blocked at 5 min
F
Blocking substrate
Empty vector
SptP-GFP
SipC
SipB
GroEL
cell
sec
G
Relative Egr1 mRNA
Time (min)
Mock
Unobstructed injectisome
Pre-blocked injectisome
Injectisome blocked at 5 min
H
120 min
Control
KO
EGR1
GAPDH
I
Control
EGR1 KO
Mock
WT
LDH release (% of total lysis)
Time (min)
J
Relative mRNA abundance
EGR1 KO
Control
Time (min)
K
Term name
Term recall
Cell migration
Cell motility
Defense response
Immune response
Inflammatory response
Locomotion
Response to biotic stimulus
Response to cytokine
Response to external stimulus
Response to stress
1%
2%
3%
−log10(FDR)
Number of hits

# Figure 3


A
WT
or
ΔprgJ
Snap freeze
at indicated timepoint
RiboSeq
RNASeq
Gent
(50 µg/ml)
Gent
(10 µg/ml)
(min) 0 5 10 15 30 60 120 240
B
WT
ΔprgJ
5 min
10 min
15 min
30 min
60 min
120 min
240 min
log2FC(TE)
log2FC(mRNA)
C
Differential TE WT vs ΔprgJ
No. regulated genes
Up
Down
Time (min)
D
Differential mRNA WT vs ΔprgJ
No. regulated genes
Up
Down
Time (min)
E
Differential TE ≤ 60 min
WT vs mock
WT vs ΔprgJ
−log10(FDR)
Number of hits
F
Differential mRNA ≤ 60 min
WT vs mock
WT vs ΔprgJ
−log10(FDR)
Number of hits
Term recall
5%
20%
40%
Term catagory
DNA binding
Cytokine activity
Heparin binding
Chemokine activity
Receptor activity
Molecular function regulator

# Figure 4

## A


SPI-1 injectisome structural
prgJ
prgI
sipC
sipB
prgK
orgA
spaO
orgB
sipD
invH
prgH
invC
invJ
invG
invI
invE
invA
orgC
spaP
spaS
spaR
spaQ
SPI-1 non-structural
sicA
invB
STM2880
iacP
sicP
sprB
invF
hilD
sitA
sipA
sptP
hilC
hilA
avrA
iagB
sitB
flagellar
fliC
flgE
5 10 15 30 60 120 240
Time (min)
intramacrophage
pspB
pspA
pspC
ygjT
yejG
pstS
cpxP
hslS
mgtC
hisG
htrA
SL1344_3618
obgE
SL1344_1187
ycjF
ripC
SL1344_3106
macA
ripB
mgtA
mgtB
tlpA
ycjX
phoR
SL1344_3617
SPI-2
ssaQ
sseB
sseC
ssaL
Relative mRNA abundance
0% 50% 100%
Relative protein synthesis
0% 50% 100%
Translational efficiency
1 2 3 4 5+
undetermined

## B

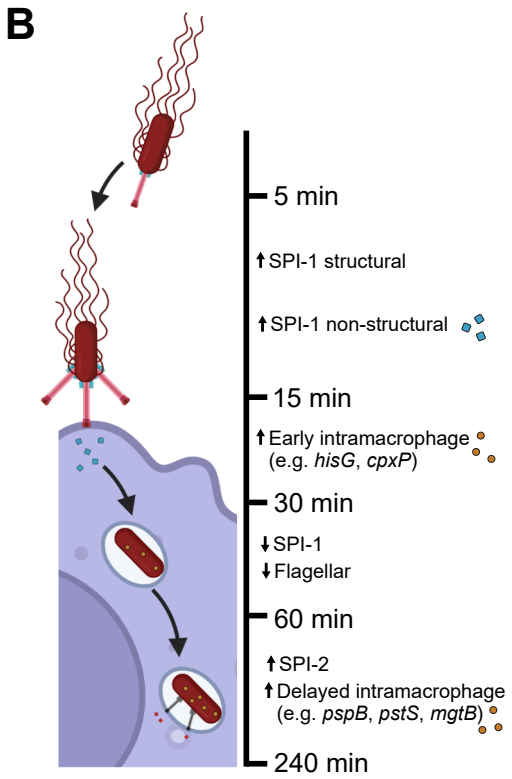
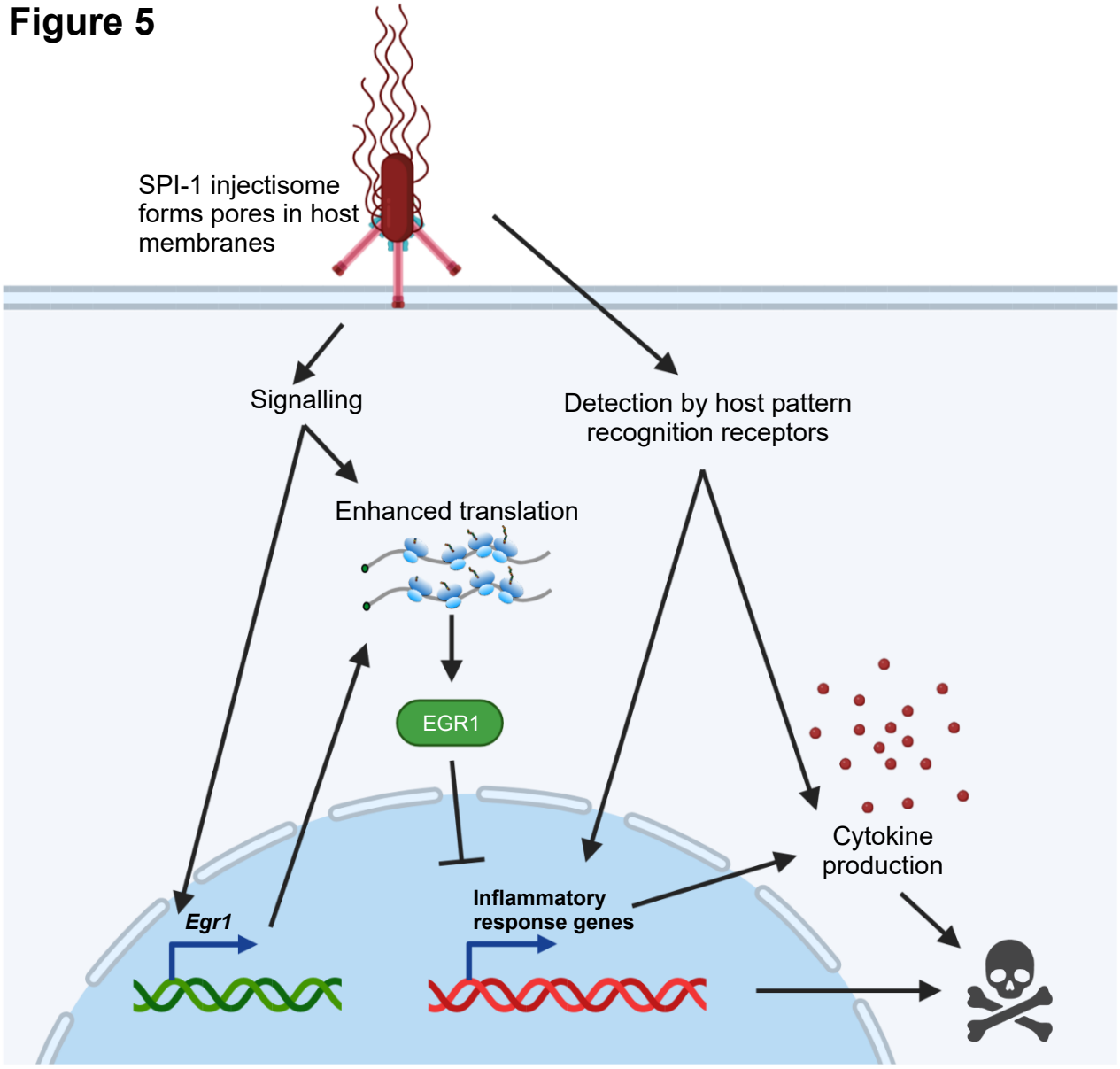

5 min
SPI-1 structural
SPI-1 non-structural
15 min
Early intramacrophage
(e.g. hisG, cpxP)
30 min
SPI-1
Flagellar
60 min
SPI-2
Delayed intramacrophage
(e.g. pspB, pstS, mgtB)
240 min

**Figure 5**


SPI-1 injectisome forms pores in host membranes
Signalling
Detection by host pattern recognition receptors
Enhanced translation
EGR1
Cytokine production
Egr1
Inflammatory response genes