Crosstalk between host stress-induced translational control and infection by *Porphyromonas gingivalis*

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January 2023

A.   Candidate Declaration

I hereby declare that:

1. I have not been enrolled for another award of the University, or other academic or professional organisation, whilst undertaking my research degree.
2. None of the material contained in the thesis has been used in any other submission for an academic award.
3. I am aware and understand the University's policy on plagiarism and certify this thesis is my own work. The use of all published or other sources of material consulted have been properly and fully acknowledged.
4. The work undertaken towards the thesis has been conducted in accordance with the SHU Principles of Integrity in Research and the SHU Research Ethics Policy.
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B. Abstract

Periodontitis, a chronic inflammatory gum disease, is caused in part by the periodontopathogen *Porphyromonas gingivalis*. Infection triggers activation of host inflammatory responses which induce stresses such as oxidative stress. Under such conditions, cells can activate the Integrated Stress Response (ISR), a signalling cascade which functions to determine cellular fate, by downregulating protein synthesis and either initiating a stress-response gene expression program, or if stress cannot be overcome, initiating programmed cell death. Recent studies have implicated the ISR signalling in both host antimicrobial defences and within the pathomechanism of certain microbes.

In this study, we investigated how *P. gingivalis* infection alters translation attenuation during oxidative stress-induced activation of the ISR pathway in oral epithelial cells. *P. gingivalis* infection alone did not result in ISR activation. In contrast, infection coupled with stress led to differential stress granule formation and composition, along with dysregulation of the microtubule network. Infection also heightened stress-induced translational repression, a response which could not be rescued by ISRIB, a potent ISR inhibitor. Heightened translational repression during stress was observed with both *P. gingivalis* conditioned media and outer membrane vesicles, implicating the role of a secretory factor, probably proteases known as gingipains, in this exacerbated translational repression. The effects of gingipain inhibitors and gingipains-deficient *P. gingivalis* mutants further confirmed these pathogen-specific proteases as the effector.

Gingipains are known to degrade the mammalian target of rapamycin (mTOR) and these studies implicate the gingipain-mTOR axis as the effector of host translational dysregulation during stress.

C. Acknowledgements

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F. Abbreviations

α-tubulin -   alpha tubulin

Aβ -   Amyloid beta

AA –   Amino acid

AD -   Alzheimer's disease

AKT -   Protein kinase B

ALS -   Amyotrophic lateral sclerosis

ATAT1 -   α-tubulin acetyltransferase 1

ATF –   Activating transcription factor

ArpA –   Alkaline protease A

BiP –   Binding immunoglobulin protein

BSA -   Bovine serum albumin

CD14 -   Cluster of differentiation 14

CHOP -   C/EBP homologous protein

CPS -   Capsular polysaccharide

CR3 -   Complement receptor 3

cUPR –   Cytosolic unfolded protein response

CXCR -   CXC chemokine receptor

CYT1 -   Cytohesin 1

C5aR -   C5a-receptor

DC-SIGN -   Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DMEM -   Dulbecco's modified Eagle's medium

DMSO -   Dimethyl sulphoxide

eIF -   eukaryotic initiation factor

ER –   Endoplasmic reticulum

ERK -   Extracellular signal-regulated protein kinase

FBS -   Foetal bovine serum

FimA -   Fimbriae A

GADD34 -   Growth arrest and DNA damage-inducible protein 34

GAPDH -   Glyceraldehyde-3-phosphate dehydrogenase

GCN2 -   General control non-depressible 2

GDI –   GDP dissociation inhibitor

GEF –   Guanine nucleotide exchange factor

Glu -   L-glutamine

GSK3β -   Glycogen synthase kinase- 3 beta

G3BP -   Ras GTPase-activating protein-binding protein 1

HDAC6 -   Histone deacetylase 6

HLA –   Human leukocyte antigen

HRI -   Heme-regulated inhibitor

HSL -   N-(3-oxododecanoyl)-homoserine lactone

HSPB8 –   Heat shock protein beta-r 8

IL -   Interleukin

iNOS -   Inducible nitric oxidase synthase

IDR –   Intrinsically disordered region

ISR -   Integrated stress response

ISRIB -   Integrated stress response inhibitor

Jak1 -   Junus kinase 1

KC –   Keratinocyte chemoattractant

kgp -   Lysine specific gingipain

LAMP2 –   Lysosome-associated membrane protein 2

LC3 -   Microtubule-associated protein 1A/1B-light chain 3

LLPS –   Liquid-liquid phase separation

LPS –   Lipopolysaccharide

MEF –   Mouse embryonic fibroblast

MEM –   Minimum essential medium

MFC –   Multifactorial complex

MOI -   Multiplicity of infection

mRNA –   Messenger ribonucleic acid

mRNP –   Messenger ribonucleoprotein particles

mTOR -   Mammalian target of rapamycin

mTORC1 –   Mammalian target of rapamycin complex 1

MYD88 -   Myeloid differentiation primary response 88

NFT –   Neurofibrillary tangle

NF-κB –   Nuclear factor kappa-light chain-enhancer of activated B cells

NOD1 –   Nucleotide-binding oligomerisation domain-containing protein 1

OMV -   Outer membrane vesicle

PBS -   Phosphate buffered saline

PBST -   PBS supplemented with tween

Pi –   Phosphate molecule

PI -   Propidium iodide

PIC –   Preinitiation complex

PI3K -   Phosphatidylinositol 3-kinase

PABP –   Poly-A-binding protein

PBS –   Phosphate buffered saline

PBST –   Phosphate buffered saline supplemented with Tween 20

PDK1 -   Phosphoinositide-dependent kinase 1

PERK –   Protein kinase R-like endoplasmic reticulum kinase

PKA -   Protein kinase A

PKCδ -   Protein kinase C delta

PKR –   Protein kinase double-stranded RNA-dependent

poly-A-mRNA – poly-adenylated-messenger ribonucleic acid

PP1 –   Protein phosphatase 1

rgpA -   Arginine-specific gingipain A

rgpB -   Arginine-specific gingipain B

ROS –   Reactive oxygen species

SA -   Sodium arsenite

Stat3 -   Signal transducer and activator of transcription 3

STEC –   Shiga toxin-producing Escherichia coli

SubAB –   Subtilase cytotoxin

SUnSET –   Surface sensing of translation

S6K1 –   p70-S6 kinase 1

TC –   Ternary complex

TLCK -   Na-tosyl-lysine chloromethyl ketone

TLR -   Toll like receptor

TNF-α -   Tumour necrosis factor alpha

TBS –   Tris buffered saline

TBST –   Tris buffered saline supplemented with Tween 20

T333 –   Type III secretion system

UBAP2L –   Ubiquitin associated protein 2 like

UPR -   Unfolded protein response

UTR –   Untranslated region

UV –   Ultraviolet

XBP1 –   X-box binding protein 1

4E-BP1 -   4E binding protein 1

43S PIC –   43S preinitiation complex

1 Chapter 1: Introduction

1.1 The Oral Microbiome and Periodontal Disease

1.1.1 The Oral Microbiome

Microorganisms were first discovered in 1683 when, using primitive light microscopy, Antony van Leeuwenhoek first observed 'little animals' whilst sampling dental plaques obtained from his mouth (Clifford, 1932). Given that the initial discovery was found in a human sample it is no surprise that in the years since, microorganisms, and subsequently their interactions with hosts have been the subject of intense research interest. It is now well established that microorganisms are a tacit component of a healthy human body, with the average human of 70kg harbouring 10-100 trillion microbes, whose combined weight is accounts for around 200 grams (Turnbaugh *et al.,* 2007; Bianconi *et al.,* 2013). These microorganisms, collectively termed the microbiome, not only form a structurally ordered and coordinated community with inter- and intra-species communication across domains, but also function in a critical, symbiotic, reactive, and reciprocal relationship with the host (Turnbaugh *et al.,* 2007; Daniel *et al.,* 2021; Duddy & Bassler, 2021).

Subsequent research has identified the oral cavity as one of the most microbiologically diverse environments of the human body, currently estimated to harbour over 700 prevalent taxa (Dewhirst *et al.,* 2010), with between 100-500 species present at any given time within healthy individuals (Paster *et al.,* 2006; Zaura *et al.,* 2009). The stability of the relationship between oral microbiome and host is exemplified by findings that the core bacterial genera are common to hominid oral biofilms since before Catarrhine-Platyrrhine split circa 40 million years ago (Fellows Yates *et al.,* 2021). This diversity may in part be explained by the variety of distinct microenvironments that the oral cavity offers, ranging from the hard enamel of the teeth to the epithelial and mucosal membranes of the gingiva (Xu *et al.,* 2015). These environments are defined by the near constant presence of fluids, with supragingival surfaces being exposed to saliva and the subgingival surfaces exposed to gingival crevicular fluid (Xu *et al.,* 2015)

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(Figure 1-1). The resulting biological nuances have led to tissue-specific tropisms in the composition of the microbial communities which exist in each niche (Lamont, Koo, & Hajishengallis, 2018). Whilst the oral environment harbours a vast array of microbes, in healthy individuals the oral microbiome is comprised mainly of the genera *Corynebacterium*, *Capnocytophaga*, *Fusobacterium*, *Leptotrichia*, *Actinomyces*, *Streptococcus*, *Neisseria*, *Haemophilus/Aggregatibacteria*, *Porphyromonas*, *Rothia*, *Lautropia*, *Veillonella* and *Prevotella*, with these accounting for 85% and 80% of the microbes found in the supra- and sub-gingival surfaces, respectively (Mark Welch *et al.,* 2016). Of these, the supragingival community harbours a larger proportion of aerobic Gram-positive genera, whereas the lowered oxygen content of the subgingival environment favours Gram-negative anaerobes such as *Bacteriodes* and spirochaetes (Xie *et al.,* 2000; Mark Welch *et al.,* 2016). The oral microbiome is however not limited to prokaryotes alone, with fungal eukaryotes such as *Candida* and *Malassezia,* being prominent commensals (Ghannoum *et al.,* 2010; Dupuy *et al.,* 2014), however bacteriophages and archaea have also been discovered within the oral cavity (Lepp *et al.,* 2004; Belmok *et al.,* 2020; Dame-Teixeira *et al.,* 2020).
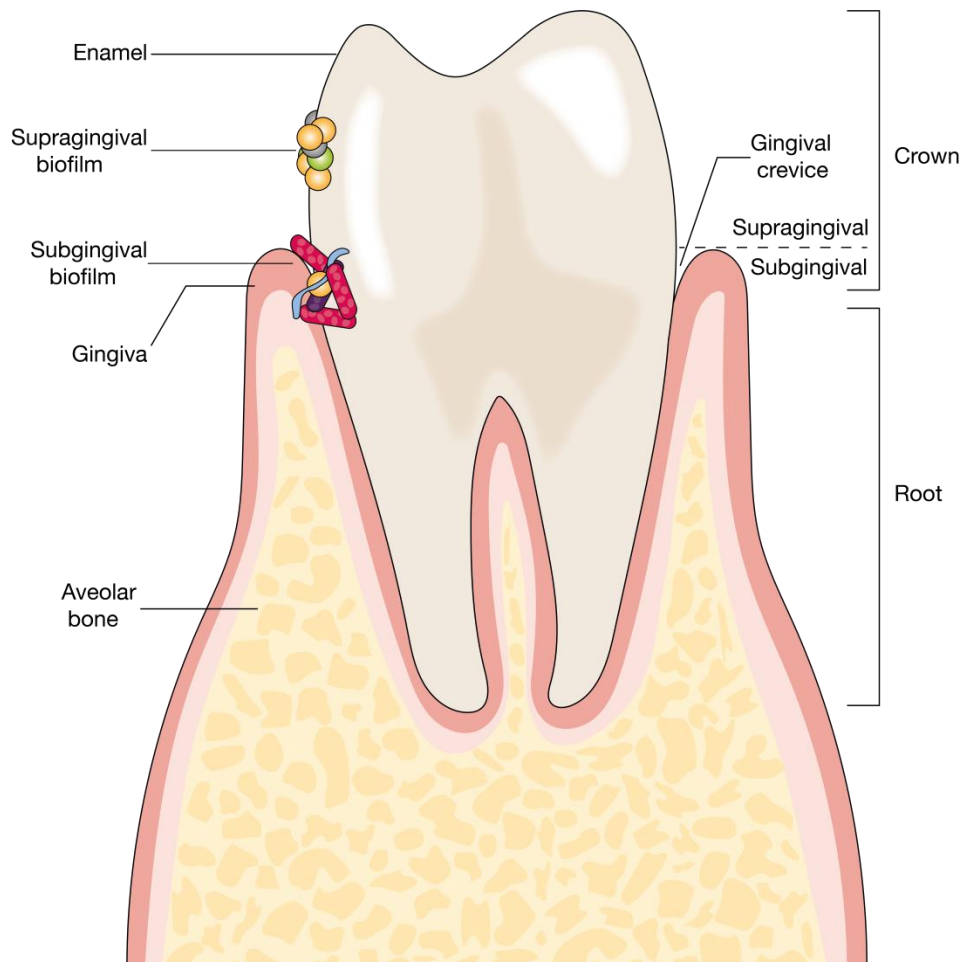
Figure 1-1 Schematic demonstrating a healthy periodontium. A structural representation of a tooth, the gingiva and alveolar bone, with a health associated supra- and subgingival biofilm.

1.1.1.1 Oral Biofilms

The microbes within the oral environment exist as structurally and functionally ordered communities, termed biofilms, which can be observed as dental plaque (Bowen *et al.,* 2018). Existing as a biofilm allows microbial communities to interact with and respond to various signalling molecules, thereby coordinating responses, such as gene expression and nutrient sharing, across the whole community (Miller & Bassler, 2001; Kolenbrander *et al.,* 2002; Janus *et al.,* 2017; Duddy & Bassler, 2021). This organised response allows the resident microbiome to greatly increase its resistance to exogenous factors such as antibiotics, to which minimal inhibitory concentrations have been shown to increase up to 100-fold when bacteria are cultured in biofilms, compared to planktonic culture (Brown, *et al.,* 1988). A schematic of oral biofilm formation can be found in Figure 1-2.
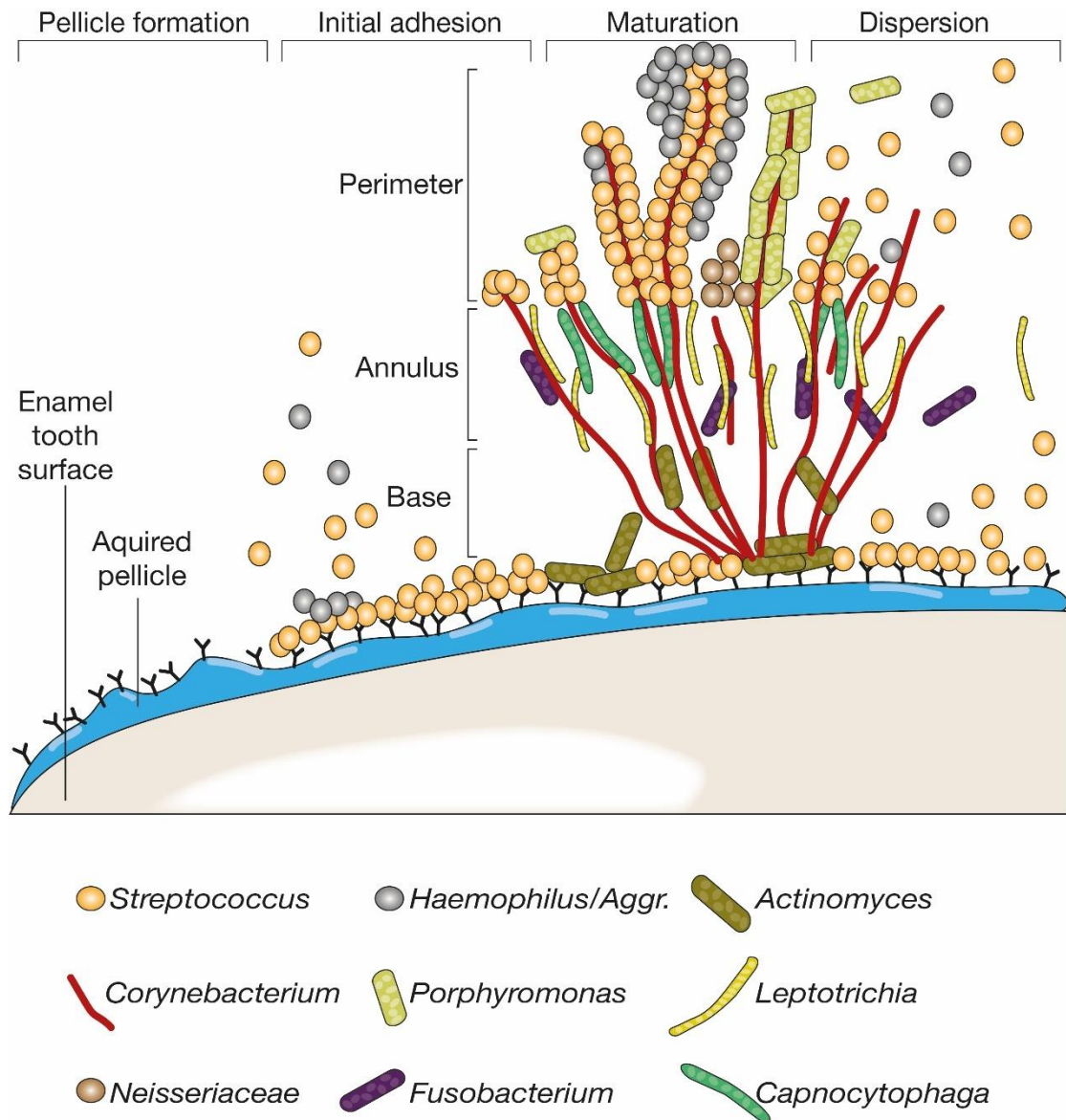
Figure 1-2 Oral biofilm formation. Biofilm formation begins with the adherence of host salivary glycoproteins to the enamel surface of the tooth forming a structure called the pellicle to which initial coloniser species such as *Streptococcus* and *Haemophilus/Agg.* bind. As the biofilm matures *Corynebacterium* binds to the initial colonisers and forms filaments radiating outwards around which other species aggregate. The perimeter has a relatively high oxygen content and harbours mainly aero-tolerant species whereas the annulus is anoxic and harbours anaerobes. Biofilms can also disperse for reasons such as adverse conditions or shear force. Adapted from Mark Welch *et al.,* (2016).

Formation of the oral biofilm is unique as it requires host salivary glycoproteins that attach to the clean enamel surface of teeth, resulting in a light film coating known as the pellicle, which facilitates biofilm attachment (Hannig, 1997; Huang, *et al.,* 2011). Following pellicle formation, pioneer coloniser species of bacteria can recognise and reversibly bind receptors such as α-amylase, statherin, proline-rich proteins, sialylated mucins and salivary agglutinin within the pellicle through interaction with bacterial surface attachment structures such as fimbriae and adhesins (Filoche *et al.,* 2010; Huang *et al.,* 2011). Of the pioneer species *Streptococcci* are particularly adept, recognising most receptors within the pellicle, however, *Actinomyces* spp, *Haemophilus* spp, *Capnactyphaga* spp, *Veillonella* spp, and *Neisseria* are also characteristic genera of pioneer species attaching to the pellicle (Ritz, 1967; Foster & Koldenbrander, 2004; Dige *et al.,* 2009). Upon initial colonisation an extracellular matrix is formed from the secretion of extracellular polymeric substances by pioneer species, which allows the aggregation of bacteria which are unable to bind to the pellicle only (Hasan & Palmer, 2014; Jakubovics *et al.,* 2021). The resulting aggregations allow for subsequent attachments of bacteria allowing for the development of a mature biofilm within 48 hours (Nyvad & Fejerskov, 1987).

Biofilm maturation is mediated by attachment of members of the *Corynebacterium* genus to early colonisers bound to the pellicle (Dige *et al.,* 2009; Mark Welch *et al.,* 2016). The *Corynobacteria* form filaments radiating away from the tooth surface providing a central structure to which other genera can attach in a hedgehog like organisation (Mark Welch *et al.,* 2016). The perimeter of the biofilm is mainly dominated by clusters of *Streptococcus, Porphyromonas* and *Neisseriaceae* with *Haemophilus/Aggregatibacter* attaching to the *Streptococcus* clusters, which resemble the outside of a cauliflower (Mark Welch *et al.,* 2016). The dominance of aero tolerant species within the perimeter highlights the aerobic nature of this environment. However, within a range of 10-20µM from the biofilm surface the environment becomes anoxic (Wessel *et al.,* 2014). *Streptococci* contribute to the maintenance of the anaerobic

environment by producing CO_2, along with hydrogen peroxide and lactate as by-products of metabolism (Ramsey *et al.,* 2011; Zhu & Kreth, 2012). While lactate and hydrogen peroxide are inhibitory to many organisms, hydrogen peroxide is detoxified by both *Corynebacterium* and *Aggregatibacter*, and lactate is a preferential substrate for *Aggregatibacter* (Brown & Whiteley, 2007), which aids the selection of these bacteria within the perimeter. The anoxic annulus, just in from the biofilm perimeter, is dominated by anaerobic and microaerophilic bacteria from the genera *Fusobacterium*, *Leptotrichia* and *Capnocytophaga* (Bernard *et al.,* 1991; Diaz *et al.,* 2000; Diaz *et al.,* 2002; Woo *et al.,* 2010; Mark Welch *et al.,* 2016). *Capnocytophaga* require CO_2 and hence reside towards the edge of the annulus close to the CO_2 producing *Streptococcus* (Bernard *et al.,* 1991; Mark Welch *et al.,* 2016). The base of the biofilm is occupied mainly by *Corynebacterium* and to a lesser extent *Actinomyces* (Mark Welch *et al.,* 2016). It is predicted that a major part of *Corynebacterium* metabolism occurs within the base region of the bacterial community. However, as the *Corynebacterium* filaments extend through the entire biofilm these will experience a variety of different conditions, likely having varied local physiology (Mark Welch *et al.,* 2016).

The mature biofilm structure allows for water channels and porous layers that facilitate the permeation of nutrients and signalling molecules throughout the biofilm (Hasan & Palmer, 2014; Mark Welch *et al.,* 2016). This structure is active and transient, with bacteria able to leave the biofilm either as single cells or clusters via erosion, sloughing or seeding due to the shear fluid force generated by the constant movement of saliva within the mouth. However, they may also disperse actively to relocate during adverse conditions, such as lack of nutrients (Huang *et al.,* 2011).

1.1.2   The polymicrobial synergy and dysbiosis model of periodontal disease

Whilst the oral microbiome exists symbiotically with the host during health, if host-community interactions break down and become dysbiotic, then site

specific diseases of the periodontium follow (Baker *et al.,* 2016; Hajishengallis & Lamont, 2014; Lamont & Hajishengallis, 2014). The impact of gingival disease is so widespread that it is estimated that gingival disease affects up to 90% of the world's population (Marsh, 2003; Pihlstrom *et al.,* 2005).

Periodontal disease is caused by a compositional shift of the oral biofilm from a symbiotic, generalist population to a dysbiotic, specialist disease associated community with increased pathogenic potential (Dabdoub *et al.,* 2016). The disease associated community dysregulates and drives a destructive host immune response in a mechanism termed polymicrobial synergy and dysbiosis (Hajishengallis & Lamont, 2012). Onset of disease is initiated by both environmental factors, such as inflammation, pH, redox potential and nutrient availability, and by certain members of the bacterial community named 'key-stone' pathogens. These factors enrich the microbiome for pathogenic associated community members (Hajishengallis & Lamont, 2012; Cugini *et al.,* 2013; Hajishengallis & Lamont, 2016). This change in microbiome composition perturbs host homeostasis leading to upregulation of immune inflammatory responses, ultimately causing periodontal disease, which in turn creates an environment favouring a pathogenic microbiota, effectively forming a positive feedback loop selecting further towards dysbiosis and disease (Hajishengallis & Lamont, 2012; Hajishengallis & Lamont, 2014). Hence it is not a singular pathogen or factor which initiates periodontal disease but a breakdown of host-microbiome crosstalk, which induces a compositional shift resulting in an oral microbiome with increased pathogenic potential (Hajishengallis & Lamont, 2012).

As postulated by the polymicrobial synergy and dysbiosis hypothesis, in animal models of disease multispecies infections have shown greater pathogenic potential than monospecies infections (Lamont & Hajishengallis, 2014; Chukkapalli *et al.,* 2017; Ebersole *et al.,* 2017). This is probably due to interspecies interactions within the oral microbial community, where

organisms can provide substrates to other species facilitating attachment, cross-feeding, or allowing cooperative metabolism (Lamont & Hajishengallis, 2014; Murray *et al.,* 2014; Short *et al.,* 2014; Hajishengallis & Lamont, 2016; Michie *et al.,* 2016). Physical interactions and signalling molecules are also capable of changing virulence gene expression through the community which further increases the pathogenic potential of the oral microbiome (Hajishengallis & Lamont, 2016; Stacy *et al.,* 2016). Hence given the role of the community within periodontal disease the idea of opposing commensal and pathogenic groupings of bacterial communities has now been replaced by a concept of a fluid continuum (Lin & Koskella, 2015; Hajishengallis & Lamont, 2016; Casadevall, 2017; Nelson & May, 2017; Chen *et al.,* 2018).

1.1.3   Periodontitis

Broadly speaking periodontal disease encompasses two sequential inflammatory conditions, gingivitis, and periodontitis. Gingivitis, the acute form of oral inflammation, is characterised by mild inflammation and is easily reversible through routine oral care (Albandar & Rams, 2002). However, if left untreated gingivitis may progress to periodontitis, the chronic form of periodontal disease (Albandar & Rams, 2002; Pihlstrom *et al.,* 2005). Periodontitis is characterised by severe inflammation of the gingiva, deepening of the gingival crevice and, in its severe form, tooth loss owing to loss of the tissues, ligaments and alveolar bone of the periodontium (Pihlstrom *et al.,* 2005). Clinically, the British Society of Periodontology define the first stage of diagnosis of periodontitis as having a periodontal pocket probing depth of >3.5mm with evidence of interdental recession. Periodontitis is further broken down into four stages. Stage I: early to mild periodontitis with <15% interproximal bone loss. Stage II: moderate periodontitis with bone loss in the coronal third of the root. Stage III: severe periodontitis with bone loss in the mid third of the roof. Stage IV: very severe periodontitis with bone loss in the apical third of the root (Deitrich *et al.,* 2019). Severe periodontitis affects around 743 million people globally, costing the US economy alone $54 billion a year in healthcare and lost

productivity; hence it is 6th on the World Health Organisation list for Global Burden of Disease (Borgnakke, Ylostalo *et al.,* 2013; Tonetti *et al.,* 2017). A schematic of the progression of periodontitis is shown in Figure 1-3.
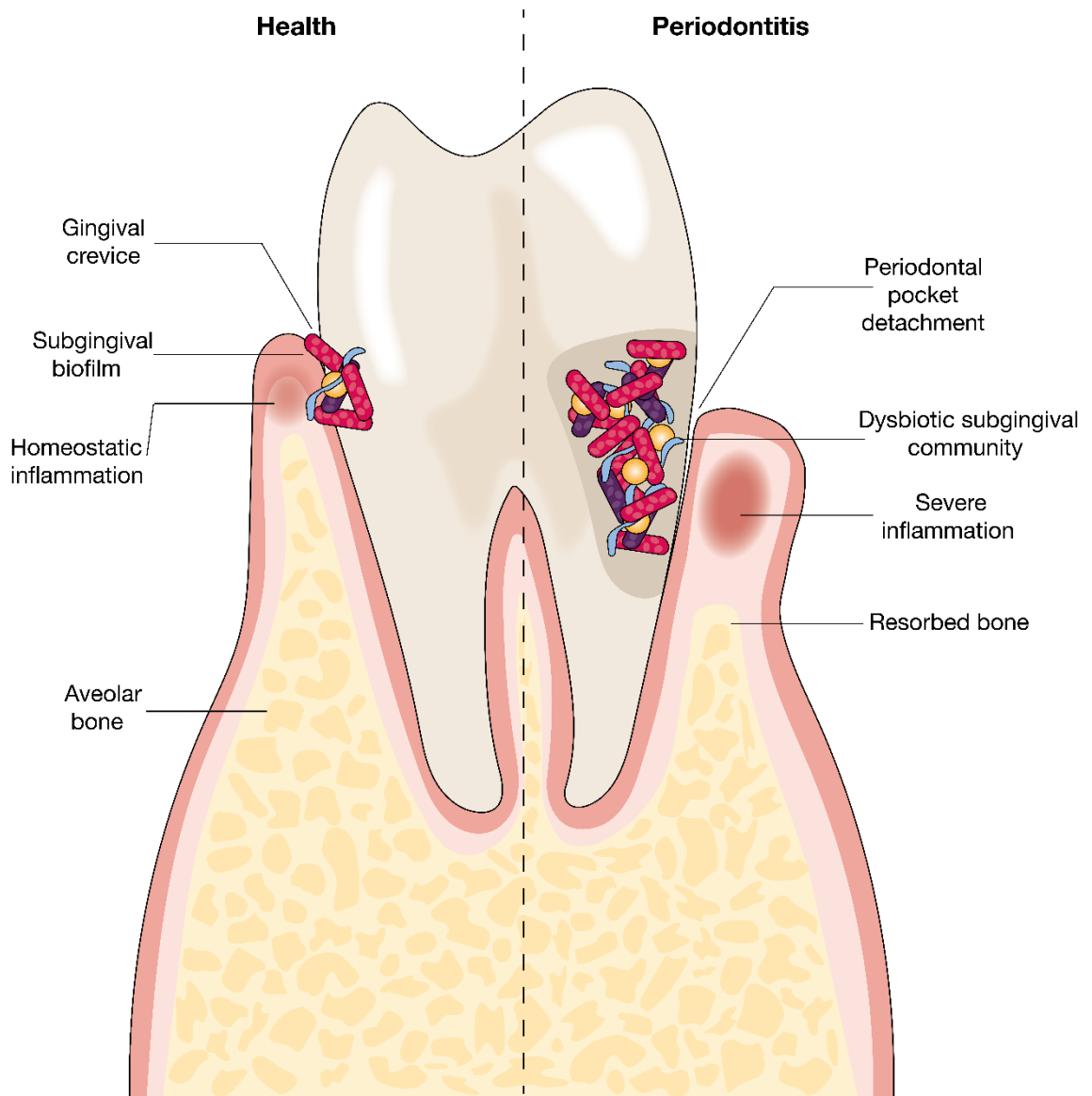
Figure 1-3 The progression of periodontitis. In health mouths there is a symbiotic subgingival biofilm, the tissues around the gingival crevice are well attached to the tooth root, intact bone and low level of homeostatic inflammation. However, during periodontitis there is a dysbiotic subgingival community, severe inflammation, periodontal pocket detachment and tooth and bone resorption.

Early studies of the oral microbiome in periodontitis grouped bacteria into consortia based on their disease status, with commensals (non-disease associated), accessory pathogens (health associated, however partially pathogenic during disease) and pathogens (Socransky *et al.,* 1998). These consortia were named using a traffic light system with three identified Gram-negative pathogens, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, termed the 'red-complex' (Socransky *et al.,* 1998; Holt & Ebersole, 2005; Socransky & Haffajee, 2005). However, in more recent years studies have now identified a plethora of other pathogenic oral bacteria, including Gram-positive species such as *Filifactor alocis* and *Peptoanaerobacter somatis*, Gram-negative species such as *Megasphaera spp.* and *Selenomonas spp.*, and members of the genera *Prevotella, Syngeristes* and *Desulfobulbus* (Dewhirst *et al.,* 2010; Griffen *et al.,* 2012; Abusleme *et al.,* 2013; Dewhirst, 2016; Diaz *et al.,* 2016).

Whilst generally present at low levels in the subgingival region during health, an ecological succession leading to increased load of these pathogenic bacteria beyond a certain threshold can instigate the inflammatory condition periodontitis (Marsh, 2003). Periodontitis patients have been found to have a higher subgingival bacterial load, accompanied by heightened gingival inflammation (Abusleme *et al.,* 2013). This is supported by findings that in animal models anti-inflammatory treatments not only lower the severity of periodontitis but also decrease the bacterial load and effectively reverse dysbiosis (Hasturk *et al.,* 2007; Hajishengallis *et al.,* 2011; Abe *et al.,* 2012; Eskan *et al.,* 2012; Moutsopoulos *et al.,* 2014; Lee *et al.,* 2016), further highlighting the symbiotic link between inflammatory responses and bacteria in periodontitis

As postulated by the polymicrobial synergy and dysbiosis model of disease, the host upregulation of inflammatory signalling in response to dysbiosis during periodontitis further selects for a microbiome with heightened pathogenic potential. The resulting inflammatory responses induce tissue damage, releasing nutrients such as degraded collagen, heme, amino acids

or iron sources into the gingival crevice (Hajishengallis, 2014; Diaz *et al.,* 2016). These nutrients are then released into the gingival crevicular fluid; a serum-like exudate which bathes the periodontitis associated bacterial community (Marsh, 2003). The transcriptome of the periodontitis associated microbiota displays a preference for acquisition of these nutrients, with upregulation of genes involved with proteolysis, iron acquisition, peptide transport and lipopolysaccharide synthesis, the latter of which could act to further heighten inflammation (Duran-Pinedo *et al.,* 2014).Therefore the link between periodontitis and inflammation is reciprocative, in that inflammation fosters a pro-periodontitis dysbiotic microbiome, but this dysbiosis itself fuels inflammation, creating a 'self-feeding' positive feedback loop that if left unchecked constantly pushes the progression of periodontitis (Duran-Pinedo *et al.,* 2014; Hajishengallis, 2014; Diaz *et al.,* 2016; Yost *et al.,* 2017).

To allow this inflammation-dysbiosis feedback loop to run unchecked, host immune-antibacterial responses must somehow be subverted. This requirement for immune subversion is exemplified by differential susceptibility to periodontitis, with certain individuals displaying a high load of dysbiotic flora within their dental plaque whilst still retaining periodontal health; likely due to the high genetic variability of human innate immunity (Socransky & Haffajee, 1994; Kinane & Hart, 2003; Kinane, *et al.,* 2007; Laine, *et al.,* 2012). Given that inflammatory responses are a prerequisite for the progression of periodontitis, the strategy of generalised immune suppression employed by many bacterial communities would be counterproductive for the periodontitis-associated oral microbiome, as it requires upregulation of host pro-inflammatory responses for nutrient acquisition (Finlay & McFadden, 2006; Lamont, Koo, & Hajishengallis, 2018). Hence to mitigate this, during periodontitis the microbiome actively uncouples host inflammatory responses from their bactericidal activity, which is best characterised by the actions of the key-stone pathogen *P. gingivalis* (Hajishengallis, Darveau, & Curtis, 2012)*,* discussed further in section 1.1.4.

1.1.4   Porphyromonas gingivalis

P. gingivalis is a Gram-negative, rod-shaped, anaerobic bacterium belonging to the *Bacteroidetes* genus (Mayrand & Holt, 1988). Owing to its requirement of hemin from host cells, in the laboratory setting *P. gingivalis* is grown on blood containing agar plates and its pigmentation changes from beige to black over 2-4 days growth (Holt *et al.,* 1999). *P. gingivalis* can be found at low abundance in the microbiome of healthy individuals, accounting for less than five percent of the microbial community (Forng *et al.,* 2000), and is only found in 25% of patients displaying oral health or mild periodontitis (Griffen *et al.,* 1998). However, increased levels of *P. gingivalis* are phenotypic of periodontitis, with its ability to induce periodontitis first being shown in 1988 in nonhuman primates (Holt *et al.,* 1988). These early studies pointed towards *P. gingivalis* being the sole cause of periodontitis (Baker *et al.,* 1999; Holt *et al.,* 1988), and fittingly *P. gingivalis* has been identified in 79% of patients with periodontal disease (Griffen *et al.,* 1998). However, more recent studies have shown that *P. gingivalis* cannot induce periodontitis in germ free mice, and subsequently it has become clear that *P. gingivalis* induces the onset of periodontitis by facilitating a compositional shift of the oral microbiome from symbiotic to dysbiotic, in fitting with the polymicrobial symbiosis model of disease (Hajishengallis *et al.,* 2011). Hence, the ability of *P. gingivalis* to induce community wide change, which results in dysbiosis, even at low abundance within the oral microflora, has led to its characterisation as a 'key-stone' pathogen (Darveau *et al.,* 2012; Hajishengallis, Darveau, & Curtis, 2012).

To elicit its pathogenic potential *P. gingivalis* has the ability to both invade cells and utilise various virulence factors including lipopolysaccharide (LPS), fimbriae, capsular polysaccharide (CPS), hemagglutinins and gingipains to foster conditions which drive periodontitis (Xu *et al.,* 2020). A selection of the virulence factors employed by *P. gingivalis* and key mechanisms by which *P. gingivalis* evades and subverts host immune responses are detailed in the following sections.

1.1.4.1   Adherence and Invasion

A key factor contributing to the pathogenicity of *P. gingivalis* is its ability to invade host cells, which was first discovered in the 1990s using antibiotic protection assays to demonstrate its invasive capacity (Lamont *et al.,* 1992). Further studies have since shown that *P. gingivalis* can invade not only oral tissues, such as multi-layered pocket epithelium (Papapanou *et al.,* 1994), but also other areas such as heart and aortic tissues (Deshpande *et al.,* 1998) and even brain tissues (Poole *et al.,* 2015).

There are five main stages of *Porphyromonas gingivalis* invasion, adherence, entry, trafficking, persistence and exit or transmission (Reyes *et al.,* 2013b). Initial adhesion is mediated by various adhesins, such as fimbriae or hemagglutinins (discussed further below), on the surface of *P. gingivalis* adhering to host cell surface ligands (Weinberg *et al.,* 1997; Deshpande *et al.,* 1998). Once adhered *P. gingivalis* can enter host cells via a mechanism involving lipid rafts, facilitating movement into the host membrane (Yamatake *et al.,* 2007), cytoskeletal rearrangement (Lamont *et al.,* 1995; Yilmaz *et al.,* 2003), and protein synthesis and actin polymerisation from metabolically active host cells (Deshpande *et al.,* 1999; Dorn *et al.,* 1999). Invasion takes around eight minutes and once internalised *P. gingivalis* can persist for up to eight days within the host cell (Belton *et al.,* 1999). The freshly internalised *P. gingivalis* is then trafficked via the host autophagic pathway (Dorn *et al.,* 2001), which it pre-activates before entry (Reyes *et al.,* 2013a). To survive the autophagic pathways, *P. gingivalis* hijacks their function by creating a novel intracellular vesicle for persistence and replication by localising to autophagosomes. *P. gingivalis* then inhibits the fusion of these autophagosomes with lysosomes sparing itself from the formation of the degradatory autolysosome (Dorn *et al.,* 2001). Functionally, trafficking localises *P. gingivalis* to the perinuclear region, where it can persist and replicate (Lamont *et al.,* 1995; Belton *et al.,* 1999), although the ability of to replicate intracellular has not been highly observed by all studies (Wang *et al.,* 2007). *P. gingivalis* can exit cells via the endocytic recycling

pathways in a manner requiring actin polymerisation and microtubule assembly (Takeuchi, Furuta, & Amano, 2011; Takeuchi *et al.,* 2011). However, *P. gingivalis* can also spread cell-to-cell, utilising cytochalasin D to polymerise filamentous protrusions of actin, which pass *P. gingivalis* to neighbouring cells (Yilmaz *et al.,* 2002). Interestingly, it has been found that *P. gingivalis* is more invasive after it has been internalised once into cells, which may implicate *P. gingivalis* reinvasion in the progression of periodontal disease (Suwannakul *et al.,* 2010).

1.1.4.2   Lipopolysaccharide

As with all Gram-negative bacteria *P. gingivalis* is sheathed by LPS, which is an outer membrane component that functions to provide structural integrity and protection, whilst also eliciting a strong host immune response (Caroff & Karibian, 2003). The *P. gingivalis* LPS is also a major activator of host pro-inflammatory immune responses, stimulating host cells to increase protein synthesis of pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1, IL-6, IL-8, IL-18 and tumour necrosis factor alpha (TNF-α), which causes tissue damage (Kadono *et al.,* 1999; Zhou *et al.,* 2005; Bostanci *et al.,* 2007a; Bostanci *et al.,* 2007b;), and eventually leads to bone resorption (Chiang *et al.,* 1999; Kato *et al.,* 2013; Nishida *et al.,* 2001). *P. gingivalis* LPS varies from the LPS of other Gram-negative bacteria, in that it is not only a weaker cytokine stimulator than other Gram-negative bacterial LPS (Liu *et al.,* 2008), but can also antagonize the cytokine-stimulating ability of other bacterial pathogens (Bostanci *et al.,* 2007). Seemingly conversely, in human umbilical cord vein cells, *P. gingivalis* LPS has also been shown to decrease expression of nuclear factor kappa B (NF-κB), a transcription factor whose activation triggers cytokine production (Wang *et al.,* 2020). LPS has also been found to causes 'chemokine paralysis' in gingival epithelial cells by preventing the production of the neutrophil activating chemokine IL-8 (Darveau *et al.,* 1998). *P. gingivalis* LPS can decrease the migration of neutrophils to infection sites by lowering expression of the cell adhesion molecule E-selectin (Darveau *et al.,* 1995). *P. gingivalis* is capable of

secreting LPS in vesicles termed outer membrane vesicles (OMVs) (Grenier & Mayrand, 1987); these are known to penetrate periodontal tissues allowing for increased control of host immune responses (Moore *et al.,* 1986; Mccoy *et al.,* 1987).

Structurally LPS consists of three subunits, an oligosaccharide core, an O-antigen and a lipid A component (Ogawa & Yagi, 2010). However, *P. gingivalis*-derived LPS is structurally unique in that variation in the O-antigen structure can confer antigenic differences between *P. gingivalis* strains (Paramonov *et al.,* 2001; Paramonov *et al.,* 2009). The lipid A component displays the opposite receptor-activating capacity of most Gram-negative bacteria as it activates toll like receptor 2 (TLR2), whilst antagonising TLR4 (Darveau *et al.,* 2004), which may attenuate immune responses (discussed further in section 1.1.4.7) (Hajishengallis, Wang, & Liang, 2009a). Furthermore, in response to changes within the surrounding microenvironment *P. gingivalis* also has the ability to modify the acetylation pattern of lipid A. For example, during low hemin levels the *P. gingivalis* LPS acetylation pattern favours activation of TLR4, whereas in conditions of high hemin availability (indicative of inflammation) the acetylation pattern favours TLR4 antagonism (Darveau *et al.,* 2004; Nemoto *et al.,* 2006; Al-Qutub *et al.,* 2006), allowing for regulation of host immune signalling in response to environmental changes.

1.1.4.3   Capsular Polysaccharide

Capsular polysaccharide (CPS) is not a feature of all *P. gingivalis* strains and of those in which it, is the composition varies, giving rise to at least six different serotypes (Laine *et al.,* 1997; Sims *et al.,* 2001). The *P. gingivalis* CPS increases resistance to polymorphonuclear leukocytes (Sundqvist *et al.,* 1991), and influences the ability of *P. gingivalis* to adhere to epithelial cells (Dierickx *et al.,* 2003). Indeed, in mouse models of periodontitis, encapsulated *P. gingivalis* strains have been found to be more invasive and to induce increased severity of periodontitis, compared to their non-

capsulated counterparts (Laine *et al.,* 1997). However, CPS is not only involved in *P. gingivalis* host interactions as studies have found CPS to aid the coaggregation of *P. gingivalis* to the oral bacterium *Fusobacterium nucleatum* (Rosen & Sela, 2006). Furthermore, different serotypes of *P. gingivalis* CPS have been shown to elicit different levels of immune activation (d'Empaire *et al.,* 2006; Vernal *et al.,* 2009). Interestingly, it has been found that *P. gingivalis* short fimbriae can be covered by CPS; effectively hiding *P. gingivalis* from host immune surveillance (Brunner *et al.,* 2010; Singh *et al.,* 2011).

1.1.4.4   Fimbriae

P. gingivalis fimbriae are thin proteinaceous, adhesive, filamentous appendages that protrude from the bacterial cell surface, and were first characterised by Yoshimura and colleagues in 1984 (Yoshimura *et al.,* 1984). *P. gingivalis* can express two forms of fimbriae, a long form and a minor form (Hasegawa *et al.,* 2009). Each mature fimbria is composed of 5 protein polymer subunits, with the long form encoded by the *fimA* gene cluster and the minor form encoded by the *mfa* gene cluster, however they are mainly comprised of protein encoded by the *fimA1* (long fimbrial protein A chain 1) and *mfa1* (minor fimbrial protein 1 chain A) genes respectively (Nishiyama *et al.,* 2007; Hasegawa *et al.,* 2009; Nagano *et al.,* 2010; Hasegawa *et al.,* 2013; Ikai *et al.,* 2015; Hasegawa *et al.,* 2016). There is a great amount of variance within the amino acid terminals and DNA sequences of the long fimbriae and as such they can be classified into six distinct types; types 1-V and type 1b (Nakagawa *et al.,* 2000; Nakagawa *et al.,* 2002). Different strains of *P. gingivalis* express different types of the long fimbriae giving rise to a range of presentations. For example, *P. gingivalis* strains ACTCC 381 and NCTC 11834 are densely fimbriated with type I fimbriae, whereas strains W83 and W50 are sparsely fimbriated with type IV fimbriae (Sojar *et al.,* 1997; Amano *et al.,* 1999).

The fimbriae of *P. gingivalis* are heavily implicated in adhesion to bacteria, host cells and substrates (Yoshimura *et al.,* 2009; Enersen *et al.,* 2013). The long fimbriae are not only involved in adhesion but also in invasion and host downstream signalling events, as such *P. gingivalis* lacking the long fimbriae have a lower invasive capacity (Njoroge *et al.,* 1997; Weinberg *et al.,* 1997).

P. gingivalis fimbriae evoke host immune responses, resulting in stimulation of cytokine production by macrophages and monocytes (Ogawa *et al.,* 1994; Amano *et al.,* 2004). Long fimbriae, encoded by the *FimA* gene cluster, trigger immune responses via interactions with TLR2 (Harokopakis *et al.,* 2006; Hajishengallis, Wang, & Liang, 2009; Coats *et al.,* 2019), CXC chemomkine receptor 4 (CXCR4) (Hajishengallis, Wang, Liang, Triantafilou, & Triantafilou, 2008; Hajishengallis, G. *et al.,* 2013), culster of differentiation 14 (CD14) (Hajishengallis, Ratti, & Harokopakis, 2005; Harokopakis & Hajishengallis, 2005) and complement receptor-3 (CR3) (Takeshita *et al.,* 1998; Hajishengallis *et al.,* 2005; Harokopakis *et al.,* 2006; Hajishengallis *et al.,* 2007; Hajishengallis *et al.,* 2008). Somewhat contrastingly, minor fimbriae encoded, by the *mfa* gene cluster, have been shown to bind to the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor of human dendritic cells. Binding facilitates *P. gingivalis* invasion and persistence by inhibiting host autophagy and intracellular bacterial killing, whilst inhibiting dendritic maturation and cytokine production (Zeituni *et al.,* 2009; Zeituni *et al.,* 2010; El-Awady *et al.,* 2015; El-Awady *et al.,* 2019; Meghil *et al.,* 2019). However, purified Mfa1 fimbriae have been shown to interact with TLR2 and CD14 in monocytes and macrophages, stimulating the production of proinflammatory cytokines such as IL-1α, IL-β, IL-6, IL-8 and TNF-α, and induce osteoclast differentiation, which together are highly implicated in bone resorption (Hamada *et al.,* 2002; Hiramine *et al.,* 2003; Amano *et al.,* 2004).

Both the major and minor fimbriae contribute towards the stability of oral communities within the subgingival regions. The minor fimbriae facilitate autoaggregation and co-aggregation with molecules of other bacteria

species, such as *Streptoccal* ssp proteins (Lamont *et al.,* 2002; Lin *et al.,* 2006), whilst the major fimbriae are also involved in binding interactions with other bacterial molecules such as *Streppococal* glyceraldehyde-3-phosphate dehydrogenase (GADPH) (Maeda *et al.,* 2004). These aggregation events help the bacterial communities resist the shear forces of saliva and gingival crevicular fluid, promoting persistence within the subgingival environment.

1.1.4.5   Gingipains

Gingipains, a group of key *P. gingivalis* virulence factors, are secreted and cell surface anchored cysteine proteases that contribute to bacterial function by cleaving host cell surface bound receptors and by inhibition of the host complement signalling, which aids nutrient acquisition and bacterial survival (Andrian *et al.,* 2006; Shoji *et al.,* 2004). A detailed discussion of gingipains can be found in section 5.1.

1.1.4.6   Hemagglutinins

Hemagglutinins are a class of virulence factors employed by *P. gingivalis* that are implicated in bacterial adhesion and nutrient acquisition (Han *et al.,* 1996; Shi *et al.,* 1999; Song *et al.,* 2005; Connolly *et al.,* 2017). These virulence factors are expressed on the bacterial cell surface either in association with fimbriae or with non-fimbrial components (Han *et al.,* 1996). *P. gingivalis* expresses at least eight hemagglutinins, however only a subset have been characterised within the remit of host-cell interactions, which are encoded by a group of *hag* genes, *hagA-hagE* (Connolly *et al.,* 2017). Both the RgpA and Kgp gingipains of *P. gingivalis* are multi-domain proteins that contain hemagglutinin domains such as hagA (Potempa *et al.,* 2003).

The role of hemagglutinins within iron scavenging is well documented. As *P. gingivalis* does not have a siderophore scavenging system, hemagglutinins mediate absorption of heme from erythrocytes by facilitating binding to

these cells, as well as gingipain activity and agglutination (Lepine *et al.,* 1996; Lewis *et al.,* 2006; Olczak *et al.,* 2007). However, hemagglutinins have been implicated in adherence. HagB has been documented to aid adherence of *P. gingivalis* to endothelial cells, however it was not found to be involved in invasion (Song *et al.,* 2005). More recent work has identified that both HagB and HagC are implicated in adherence to oral epithelial cells, in this case HagB was also found to be involved in invasion (Connolly *et al.,* 2017). Hemagglutinins are also involved in interbacterial adhesion, where HagA plays a crucial role in facilitating the coaggregation of *P. gingivalis* and *T. denticola* (Guo *et al.,* 2010; Ito *et al.,* 2010), previously discussed in section 1.1.4.5.

1.1.4.7 Immune subversion

P. gingivalis employs a wide variety of immune subversive techniques to avoid host immune surveillance whilst leaving inflammatory responses heightened, including inducing chemokine paralysis (Darveau *et al.,* 1998), and inhibition or degradation of NF-κB (Calkins *et al.,* 1998; Takeuchi *et al.,* 2013). By far the most well documented mechanism of *P. gingivalis* subverting host immune responses is through interactions with TLRs, their co-receptors and the complement system discussed further in this section. *P. gingivalis* fimbriae are known to interact with the CD14-TLR2 complex, inducing TLR2 mediated NF-κB activation, ultimately stimulating the production of pro-inflammatory cytokines in gingival epithelial cells (Asai *et al.,* 2001; Hajishengallis Ratti, & Harokopakis, 2005; Hajishengallis *et al.,* 2006), selecting towards a proinflammatory environment. Functionally, to uncouple the host bactericidal from proinflammatory responses *P. gingivalis* hijacks TLR and complement crosstalk, discussed below.

1.1.4.7.1 Dysregulation of macrophage and monocyte activity

Monocytes and macrophages are key innate immune components whose actions are heavily implicated in inflammatory and bactericidal activities (Ma

et al., 2019), and as such their function is a valuable target for *P. gingivalis* to foster the pro-inflammatory environment, which is a key driver of dysbiosis. A schematic of *P. gingivalis'* dysregulation of immune signalling in monocytes and macrophages can be found in Figure 1-4.

P. gingivalis FimA interacts with the CD14-TLR2 complex, resulting in an inside out signalling pathway, where RAC1, Phosphatidylinositol-3-kinase (P13K) and cytohesin 1 (CYT1) transactivate complement receptor 3 (CR3) (Harokopakis & Hajishengallis, 2005; Harokopakis *et al.,* 2006; Hajishengallis, Wang, & Liang, 2009), increasing adhesion and transmigration of the pro-inflammatory monocytes to the infection foci (Harokopakis *et al.,* 2006). *P. gingivalis* utilises the activation of this pathway to gain safe passage into macrophages, where once internalised, this pathway contributes to increased survival (Hajishengallis *et al.,* 2006). Activated CR3 can directly bind *P. gingivalis* fimbriae, activating extracellular signal-regulated protein kinase (ERK) 1 and ERK2, thereby inhibiting TLR mediated IL-12 production, causing impaired immune clearance of *P. gingivalis* and increased bone loss (Hajishengallis *et al.,* 2006; Wang *et al.,* 2007).

The arginine gingipain B, RgpB, of *P. gingivalis* cleaves the C5 complement component to generate increased concentrations of biologically active C5a (Liang *et al.,* 2011; Wingrove *et al.,* 1992), without the requirement for host complement activation (Hajishengallis *et al.,* 2017). This activates the C5a-receptor (C5aR), mobilising intracellular calcium, thereby inducing the activation of cAMP (Wang *et al.,* 2007). C5a alone does not strongly activate cAMP, however FimA also induces crosstalk between CXCR4 and TLR2, further activating cAMP (Hajishengallis, Ratti, & Harokopakis, 2005; Pierce *et al.,* 2009). Heightened cAMP activity results in the activation of the cAMP-dependent protein kinase A (PKA), which inhibits inducible nitric oxide synthase (iNOS)-mediated bactericidal activity in human macrophages and mouse monocytes (Hajishengallis, Wang, Liang, Triantafilou & Triantafilou, 2008).
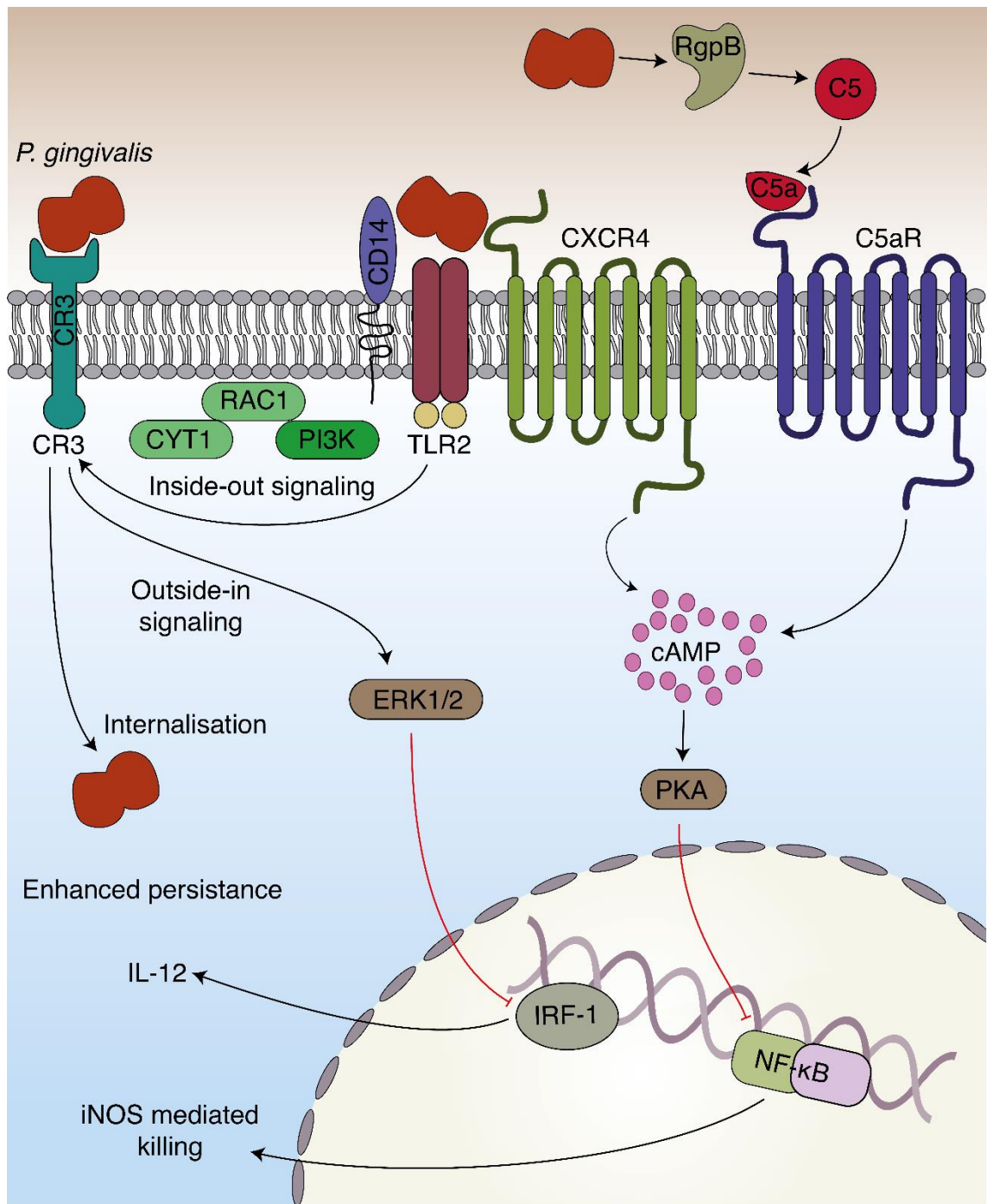
Figure 1-4. *P. gingivalis* mediated dysregulation of innate immune responses in monocytes and macrophages. *P. gingivalis* FimA binds to the CD14-TLR2 complex and initiates inside-out signalling, involving RAC1, PI3K and CYT1 to activate CR3. Activated CR3 facilitates *P. gingivalis* internalisation and inhibits the production of IL-12 via the ERK1/2 pathway. The RgpB of *P. gingivalis* cleaves C5, which activates C5aR, which concurrently with CXCR4-TLR2 crosstalk, activated by *P. gingivalis* fimbriae, activates cAMP. Increased cAMP activity induces PKA stimulation, which negatively regulates NF- κB and thereby inhibits iNOS mediated bacterial killing. Figure adapted from Hajishengallis (2009).

1.1.4.7.2 Uncoupling of inflammation from bacterial clearance in neutrophils

Neutrophils make up to 95% of the total leukocytes within the gingival crevice and hence are in contact with *P. gingivalis* (Delima & Van Dyke, 2003; Nussbaum & Shapira, 2011). However, during periodontitis neutrophils do not successfully control the dysbiotic microbiome, even though they mostly remain viable (Newman, 1980; Chapple & Matthews, 2007; Ryder, 2010; Vitkov *et al.,* 2010). This manipulation of neutrophil function during periodontitis is largely mediated by the actions of *P. gingivalis* hijacking signalling pathways linking complement and TLRs. A schematic of *P. gingivalis'* interactions with neutrophil function can be found in Figure 1-5.

As described above, *P. gingivalis* can generate high concentrations of active C5a (Wingrove *et al.,* 1992; Liang *et al.,* 2011). This coupled with the ability of *P. gingivalis* to activate TLR2 (Burns *et al.,* 2006), leads to interactions between the C5aR and TLR2 (Maekawa *et al.,* 2014). The resulting crosstalk leads to ubiquitylation of the TLR2 adaptor myeloid differentiation primary response 88 (MYD88) by the E3 ubiquitin ligase SMURF1, thereby targeting MYD88 for proteasomal degradation, which inhibits the bactericidal responses of neutrophils (Maekawa *et al.,* 2014). Concurrently, C5aR-TLR2 signalling activates PI3K, via another TLR2 adaptor MAL, which inhibits the RhoA GTPase and actin polymerisation to inhibit phagocytic uptake of *P. gingivalis,* and other community members. However, this also upregulates pro-inflammatory responses, effectively uncoupling the antimicrobial from the proinflammatory responses in neutrophils (Maekawa *et al.,* 2014), which serves to increase nutrient availability, bacterial fitness and further foster a dysbiotic microbiota.
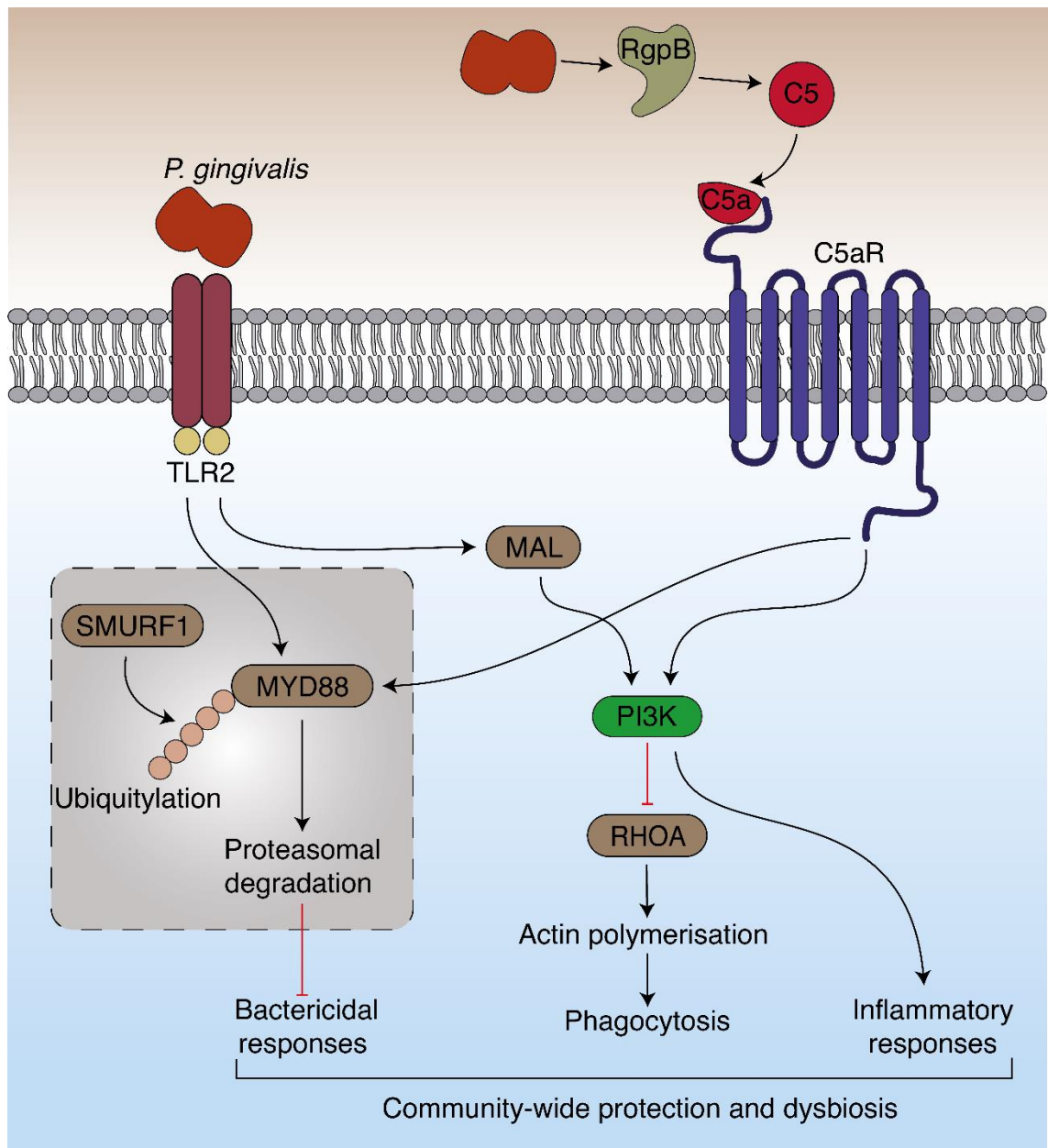
Figure 1-5 *P. gingivalis* uncouples bactericidal from pro-inflammatory responses in neutrophils. *P. gingivalis* RgpB generates high concentrations of biologically active C5a, which activates C5aR signalling. This induces proteasomal degradation of the TLR2 adaptor MYD88 owing to ubiquitylation by SMURF1, which inhibits host bactericidal responses. Concurrently, crosstalk between TLR2 and C5aR activates MAL, which induces PI3K activity, inhibiting RhoA and consequently actin polymerisation and phagocytic uptake of *P. gingivalis* and other community members. TLR2-C5aR crosstalk also induces inflammatory responses. Therefore, as such *P. gingivalis* uncouples antimicrobial responses from inflammatory responses. Figure adapted from Hajishengallis (2015).

1.1.4.8 Links to other diseases

Given the prevalence of tissue destruction during periodontal infection (Marsh, 2003), coupled with the highly invasive capacity of *P. gingivalis* (Lamont *et al.,* 1992)*,* during chronic infections *P. gingivalis* gains access to the blood stream and translocates to other areas of the host. In these circumstances *P. gingivalis* has been implicated in a variety of diseases (Xiaojing *et al.,* 2000), of which a selection is briefly outlined in the following sections.

1.1.4.8.1 Cardiovascular disease

The link between periodontal infection and heart disease has been postulated since the 1960s (Mackenzie & De Millard, 1963). However, it was not until 1989 that two studies simultaneously showed that oral infections have an etiological role in cardiovascular disease (Mattila *et al.,* 1989; Syrjanen *et al.,* 1989). Indeed, more recent studies have found the periodontium to be a key source of bacteria and pro-inflammatory mediators within the bloodstream, which lead to cardiovascular pathologies (Xiaojing *et al.,* 2000). Given that immune-subversion by *P. gingivalis* during periodontitis inherently fosters a pro-inflammatory environment (discussed in section 1.1.4.7), it is no surprise that the inflammatory burden of periodontitis has been implicated to have a significant effect on atherosclerosis, an inflammatory cardiovascular disease in which internal plaque build-up hardens arteries (Schenkein & Loos, 2013). Indeed, *P. gingivalis* has been detected in atherosclerotic plaques (Kozarov *et al.,* 2005), is known to invade cardiovascular and endothelial cells (Deshpande *et al.,* 1999), and has been extracted in a cultivable state from atheromatous tissues (Rafferty *et al.,* 2011). Whilst these previous results are observational, a study using a mouse model of atherosclerosis has shown that persistent periodontal infection induced by *P. gingivalis* increases the formation of atherosclerotic plaques (Velsko *et al.,* 2014), further implicating *P. gingivalis* in atherosclerotic disease progression.

1.1.4.8.2 Oral cancer

Cancers of the head and neck regions present mainly as squamous cell carcinoma (Chi *et al.,* 2015). The exact role of *P. gingivalis* within the context of oral cancers is yet to be fully characterised, however *P. gingivalis* is positively associated with oral squamous carcinoma. In oesophageal squamous cell carcinoma increased levels of salivary *P. gingivalis* are linked to heightened disease progression (Zhang *et al.,* 2019) and fittingly *P. gingivalis* has been found to be overabundant in oesophageal cancer tissues (Gao *et al.,* 2016). Furthermore, detection of *P. gingivalis* in oral squamous carcinoma or oesophageal squamous carcinoma lesions has been linked to worsen clinicopathological outcomes (Gao *et al.,* 2016; Chen *et al.,* 2020; Gao *et al.,* 2021).

By the very nature of tumourigenesis, increased or uncontrollable proliferation is a tacit requirement of tumour progression. To this end, in primary gingival epithelial cells *P. gingivalis* infection has been shown to upregulate host expression of genes involved in proliferation (Handfield *et al.,* 2005; Mans *et al.,* 2006; Geng *et al.,* 2017; Zhang *et al.,* 2019). Furthermore, infection has also been shown to reduce levels of the p53 tumour suppressor, which accelerates the progression through S-phase of the cell cycle (Kuboniwa *et al.,* 2008). Increased proliferation was also shown to be dependent upon FimA expression (Kuboniwa *et al.,* 2008); however, gingipains may also contribute to this response through activation of notch signalling (Al-Attar *et al.,* 2018). Studies investigating *P. gingivalis* infection in tumour cells derived from the alveolus have identified activation of alpha-defensins post infection, which has also been shown to increase cell proliferation (Hoppe *et al.,* 2016). However, during *P. gingivalis* infection there is also disruption of host immune responses, a potential driving factor of cancer progression (Whitmore & Lamont, 2014). This coupled with the ability of *P. gingivalis* to suppress pro-apoptotic pathways such as janus kinase 1 (Jak1)/Akt/signal transducer and activator of transcription 3 (Stat3)

(Mao *et al.,* 2007) and increase the host proliferative potential, shows that *P. gingivalis* has potential to drive cancer progression.

1.1.4.8.3 Diabetes

Heightened risk of developing periodontitis among diabetes patients was first described in 1993 (Loe, 1993). Indeed, subsequent studies have found that patients with type I and II diabetes have a 3 to four-fold increased risk of developing periodontitis (Kocher *et al.,* 2018; Wu *et al.,* 2020; Genco & Borgnakke, 2020; Romano *et al.,* 2021; Zheng *et al.,* 2021). However, the link between diabetes and periodontitis appears to be bi-directional (Grossi & Genco, 1998; Taylor, 2001), as in non-diabetic subject's periodontitis increases the risk of high blood glucose and subsequently the prevalence of pre-diabetes and type II diabetes (Borgnakke *et al.,* 2013; Ziukaite *et al.,* 2018; Graziani *et al.,* 2018; Wu *et al.,* 2020; Chang *et al.,* 2020). A recent systematic review has confirmed the bi-directional nature of this relationship, finding that patients suffering from type II diabetes displayed a 34% increased risk of periodontitis, and that severe periodontitis increased the risk of type II diabetes by 53% (Wu *et al.,* 2020).

P. gingivalis has also been shown to aggravate the disease phenotype in mouse models of diabetes (Tian *et al.,* 2020). Functionally *P. gingivalis* may mediate this progression in a number of ways, including increasing blood glucose and decreasing insulin production. In human hepatocyte cells *P. gingivalis* can enhance glucose output by inhibiting translocation of forkhead box protein O1 (Takamura *et al.,* 2016). Both *P. gingivalis* and its OMVs have the ability to translocate to the liver of mice with experimentally induced periodontitis, where they decrease hepatic glycogen synthesis via inhibition of the Akt-glycogen synthase kinase- 3 beta (GSK3β) pathway (Ishikawa *et al.,* 2013; Seyama *et al.,* 2020). *P. gingivalis* can also translocate to the pancreas and impact upon insulin by changing the structure of insulin secreting islets (Ilievski *et al.,* 2017). Furthermore, in both human and animal pancreatic samples *P. gingivalis* has been shown to induce epigenetic

changes that dedifferentiate insulin producing cells within the pancreas (Diomede *et al.,* 2017; Ilievski *et al.,* 2020).

1.1.4.8.4 Alzheimer's disease

The presence of extraneuronal amyloid beta (Aβ) plaques and intraneurofibrillary tangles in the brain, coinciding with clinically defined symptoms of cognitive decline form the basis of Alzheimer's disease (AD) diagnosis at autopsy (Hyman *et al.,* 2012; Dugger & Dickson, 2017). Although periodontal diseases and neurodegeneration are seemingly distinct, the periodontium and the brainstem are linked by the trigeminal ganglion (Cook *et al.,* 2013). Indeed, there is likely communication between these two regions, as tooth pain is registered in the brain and several trigeminal nerves are distributed throughout the periodontium (Goto *et al.,* 2020). Interestingly it has been shown that in AD transgenic mice, extraction of molar teeth releases Aβ and triggers neurodegeneration via the trigeminal nerve pathway (Goto *et al.,* 2020). Hence, this potential communicative relationship may provide a link between periodontal trauma and adverse neurological outcomes.

There are several further lines of evidence that in certain circumstances, *P. gingivalis* may interact with and potentially progress the pathophysiology of AD. Firstly, both *P. gingivalis* and its gingipains were recently detected in brains of patients with AD (Dominy *et al.,* 2019) and further studies have also identified *P. gingivalis* LPS within the brains of human AD patients and in the brains of mouse models of AD (Memedovski *et al.,* 2020; Poole *et al.,* 2013). Secondly, neuroinflammation is thought to be a key driver of cognitive decline (Holmes *et al.,* 2003). *P. gingivalis* LPS is known to promote neuroinflammation via TLR4 and NF-κB signalling in rats (Zhang *et al.,* 2018) and *P. gingivalis* infection-induced neuroinflammation has been postulated to heighten cognitive impairment in C57BL/6 mice (Hu *et al.,* 2020). These findings are supported by studies demonstrating that *P. gingivalis* induces proinflammatory cytokine release in brain tissues of infected mice (Ding *et*

al., 2018) and *P. gingivalis* LPS activates rat microglia (macrophage like cells of the central nervous system), which consequently induces cytokine and chemokine release (Memedovski *et al.,* 2020).

P. gingivalis infection also increases Aβ accumulation in periodontal tissues of mouse models, human gingival tissues and in human serum (Gil-Montoya *et al.,* 2017; Leira *et al.,* 2019; Nie *et al.,* 2019). Furthermore, gingipains have been shown to have similar proteolytic activity to cathepsin B (Dominy *et al.,* 2019), the enzyme that cleaves ABPP to produce Aβ deposition in sporadic AD (Hook *et al.,* 2008). Hence given that both *P. gingivalis* and its gingipains have been detected in the brains of AD patients and Aβ deposition was observed post colonisation of mouse brain by *P. gingivalis* (Dominy *et al.,* 2019), it is plausible that *P. gingivalis* is capable of progressing AD pathophysiology by increasing Aβ plaque formation.

Another classic hallmark of AD is the formation of neurofibrillary tangles (NFTs), which are formed due to hyperphosphorylation of tau, a microtubule stabilising protein. In AD hyperphosphorylated tau dissociates from microtubules, which results in the collapse of the microtubule network, consequently resulting in the aggregation of non-membranous masses along with tau, termed NFTs (Grundke-Iqbal *et al.,* 1986; Koepke *et al.,* 1993). This loss of microtubule stability owing to NFT formation ultimately disrupts protein trafficking and cellular function, which is a driving factor of AD pathology (Kinney *et al.,* 2018). There are several lines of evidence that *P. gingivalis* may interact with NFTs. Firstly, *P. gingivalis* has been identified within the NFT lesions in the brains of AD patients at autopsy (Dominy *et al.,* 2019; Ryder, 2020). The relevance of *P. gingivalis* within these NFTs is highlighted by studies showing that when *P. gingivalis* is injected into the brains of rats tau is inhibited from stabilising microtubules, which leads to the self-assembly of tau into structures similar to NFTs, and neuroinflammatory signalling (Tang *et al.,* 2021). Furthermore, phosphorylation of tau in response to *P. gingivalis* infection has also been

observed in mouse models (Ilievski *et al.,* 2018) and cell cultures (Haditsch *et al.,* 2020).

However, *P. gingivalis* gingipains may also be implicated in AD pathophysiology, as they have also been found to co-localise with microtubules with the NFTs in the brains of AD patients (Dominy *et al.,* 2019). This study also found that gingipains can degrade tau into multiple fragments, of which six contain microtubule binding domains with VQIINK and VQIVYK signatures (Dominy *et al.,* 2019). If this proteolytic activity occurs in infection sites within the brain it may be a driving factor behind increased NFT formation, as constituent components of NFTs also display these VQIINK and VQIVYK signatures (Barbier *et al.,* 2019). Hence, given that *P. gingivalis* and its gingipains have been positively identified within NFTs of AD patient brains and gingipains can directly cleave tau into fragments, with similar capacity to bind collapsed microtubules to other NFT components, it is entirely plausible that *P. gingivalis* may actively progress NFT formation during AD (Barbier *et al.,* 2019; Dominy *et al.,* 2019).

1.2 The Integrated Stress Response, ISR

Within eukaryotic cells, the Integrated Stress Response is a mechanism that, in response to changes in either intracellular or extracellular conditions, has the capability of switching between cellular survival or inducing cell death (reviewed by Pakos-Zebrucka *et al.,* 2016). Stimuli can include both physiological and pathological changes and once triggered results in the reduction of global protein synthesis, allowing the cell to focus energy into overcoming stress (Brostrom & Brostrom, 1997) mediated via the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) (Siekierka *et al.,* 1982; Donnelly *et al.,* 2013; Figure 1-6 A–C). However, during ISR activation, there is also increased translation of a selection of stress response mRNAs via translation of upstream open reading frames (Ryoo & Vasudevan, 2017). This includes mRNAs coding for transcription factors, such as activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and growth arrest and DNA damage-inducible protein (GADD34), which act as effectors of the ISR (Lee *et al.,* 2009; Palam *et al.,* 2011; Hinnebusch & Lorsch, 2012) specifically upregulating the expression of genes involved in cellular reprogramming under stress conditions (Karpinski *et al.,* 1992; Harding *et al.,* 2003; B'Chir *et al.,* 2013; Figure 1-6 D,E).

Of the ISR effectors, ATF4, a basic leucine zipper transcription factor, is the best studied (Karpinski *et al.,* 1992; Vallejo *et al.,* 1993; Ameri & Harris, 2008). Once activated, ATF4 regulates the expression of genes involved in stress responses, amino acid (AA) synthesis, metastasis, angiogenesis and differentiation, allowing for a stress-specific cellular response (Ameri & Harris, 2008). During hypoxia, endoplasmic reticulum (ER) stress, and AA starvation, ATF4 also upregulates transcripts involved in autophagy (Rzymski *et al.,* 2010; B'Chir *et al.,* 2013; Deegan *et al.,* 2015). One mechanism by which this is achieved is the inhibition of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) via translational upregulation of regulated in development and DNA-damage response 1, which functions to

activate autophagic responses (Whitney *et al.,* 2009; Kroemer *et al.,* 2010; Dennis *et al.,* 2013; Figure 1-6 D,F).

Through the action of ATF4, the ISR can induce cell death via upregulation of downstream targets including the transcription factors CHOP and ATF3 (Puthalakath *et al.,* 2007). One mechanism for this function is via CHOP increasing the expression of additional pro-apoptotic factors from the Bcl-2 homology 3-only group of the Bcl-2 family (Puthalakath *et al.,* 2007; Galehdar *et al.,* 2010). It has also been suggested that ATF4 and CHOP may interact directly to form heterodimers to heighten the expression of pro-apoptotic genes, such as Bim (Teske *et al.,* 2013).

In contrast, the ISR can also induce cellular survival and overcome the stress. In this case, upon cessation of stress, GADD34 activates protein phosphatase 1 (PP1), which dephosphorylates eIF2α (Connor *et al.,* 2001; Novoa *et al.,* 2001), thus terminating the ISR and returning the cell to homeostatic translation (Novoa *et al.,* 2001; Novoa *et al.,* 2003; Figure 1-6 G). As such, the ISR can induce the directly opposing outcomes of cellular survival or death.
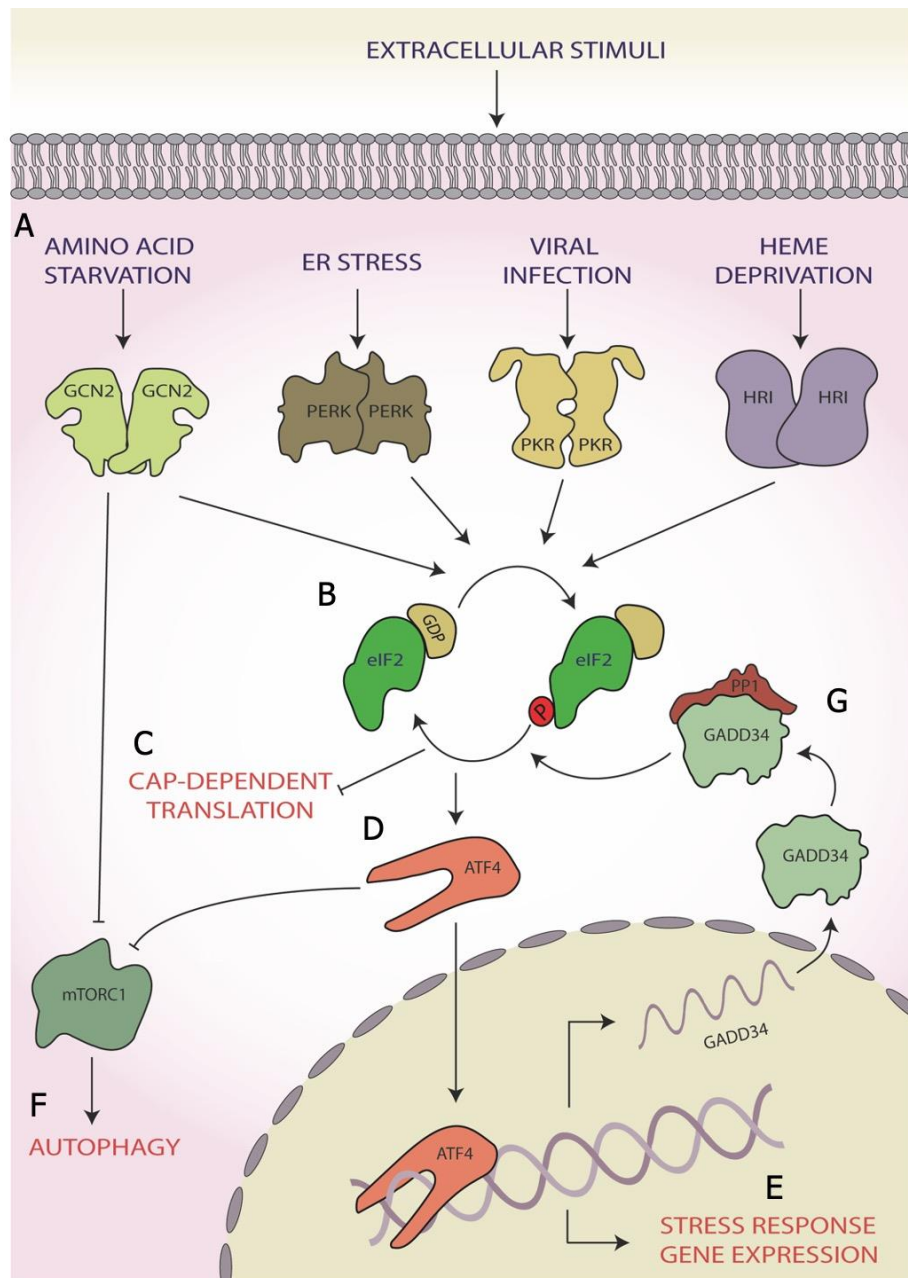
Figure 1-6 The integrated stress response. (A) A range of cellular stress stimuli activate one of four stress response kinases, GCN2, PERK, PKR, and HRI kinases, which (B) phosphorylate eIF2α. (C) This results in abrogation of canonical translation initiation, (D) which selectively upregulates the translation of ISR effector mRNAs, such as ATF4. (E) These effectors bind to and target genes involved in cellular reprogramming for expression. (F) GCN2 and ATF4 also both induce autophagy via inhibition of mTORC1. (G) If stress is overcome, the stress-inducible phosphatase GADD34 dephosphorylates eIF2α, returning homeostatic translation initiation and terminating the ISR. Figure from Knowles *et al.,* (2021), used under creative commons licence.

1.2.1 Translation Initiation

The core process targeted by the ISR is translation initiation (Harding *et al.,* 2003; Pakos-Zebrucka *et al.,* 2016), which is a key step in the regulation of protein synthesis, resulting in the formation of an elongation competent 80S ribosome, aided by the action of multiple eukaryotic initiation factors (Hinnebusch & Lorsch, 2012). The mechanism of translation initiation is outlined in Figure 1-7.

1.2.1.1 Binding of initiator methionyl tRNA to the 40S ribosomal subunit

The initial stage of the process sees binding of a ternary complex (TC), consisting of the initiator methionyl tRNA and GTP bound eIF2, to the 40S ribosomal subunit to form a 43S preinitiation complex (43S PIC) (Hinnebusch & Lorsch, 2012). eIF2 is a heterotrimer consisting of 3 subunits α, β and γ. The GTP binding occurs on the γ subunit with α and β stabilising the interaction (Nika *et al.,* 2001; Yatime *et al.,* 2004; Naveau *et al.,* 2010). The TC to 40S binding event is aided via the actions of eIFs 1, 1A, which cause a conformational change to the 40S ribosomal subunit (Passmore *et al.,* 2007) and eIFs 3 and 5, which promote binding of the TC to the 40S ribosomal subunit (Asano *et al.,* 2000; Majumdar *et al.,* 2003). In addition to the TC eIF1, 2, 3 and 5 have also been shown to have the ability to interact together forming a multifactorial complex (MFC) (Sokabe *et al.,* 2012). However, in mammalian cells the rate at which ᵢMet-tRNA is delivered to the ribosome is not affected by whether it is complexed with only eIF2-GTP or in combination with the MFC, indicating that the MFC may not have a significant role in translation initiation (Sokabe *et al.,* 2012).

1.2.1.2 mRNA recruitment to the PIC and scanning

The 43S PIC is then joined to the 5' mRNA cap near to the 5' untranslated region (UTR) readying it for ribosome binding through unwinding of the 5' cap region by the eIF4F complex, eIF3 and poly-A-binding protein (PABP)

(reviewed in Hinnebusch, 2011). eIF4F is a complex comprised of eIF4E, the DEADBOX helicase eIF4A and eIF4G, which acts as a scaffold binding protein, eIFs 4E (Mader *et al.,* 1995; Yanagiya *et al.,* 2009), 4A (Imataka & Sonenberg, 1997), which together act alongside 3 (Lefebvre *et al.,* 2006) and PABP (Imataka *et al.,* 1998; Park *et al.,* 2011). eIF4E facilitates mRNA binding via association with the mRNA 5' cap between two tryptophan residues on the surface of eIF4E, this binding is further enhanced by interaction between a region of eIF4G and the N-terminal region of eIF4E (Niedzwiecka *et al.,* 2002). eIF4E subsequently recruits eIF4A to the 5' cap, which then unwinds the mRNA with eIF4H and eIF4B synergistically enhancing its helicase activity (Marintchev *et al.,* 2009; Nielsen *et al.,* 2011; Özeş *et al.,* 2011). eIF4G forms a further point of control for eIF4A helicase activity via regulation of its conformation (Oberer *et al.,* 2005; Schütz *et al.,* 2008; Hilbert *et al.,* 2011; Nielsen *et al.,* 2011; Özeş *et al.,* 2011). eIF4G has also been shown to aid recruitment of the 43S PIC to the 5' mRNA cap through interactions with eIF3 and eIF5 (Asano *et al.,* 2001) while eIF4E is complexed with the 5' cap PABP binds the 3' mRNA poly-A-tail (Kahvejian *et al.,* 2005). This simultaneous binding of eIF4E to the cap and PABP to the poly-A-tail, and both to their separate binding sites on eIF4G forces the mRNA into a circular structure, known as the 'closed loop' (Kahvejian *et al.,* 2001; Hinnebusch & Lorsch, 2012).

Once bound the 43S PIC scans the mRNA in a 5' to 3' direction in search of a start codon, which is detected via the complementary sequence of the Met-tRNA$_i$ anticodon. eIF1 and eIF1A hold the 40S ribosome in a scanning-competent conformation (Passmore *et al.,* 2007). The ATP-dependent helicase eIF4A then breaks any weak secondary structures of the mRNAs 5' UTR, in a manner where the requirement for ATP and eIF4A is directly proportional to the degree of secondary structure (Jackson, 1991; Svitkin *et al.,* 2001). The helicase activity of eIF4A alone is not powerful enough to completely break the secondary mRNA structures. In the case of weak secondary structures the assistance of eIF4As co-factor eIF4B is enough (Dmitriev *et al.,* 2003; Rozovsky *et al.,* 2008; Özeş *et al.,* 2011). However, efficient scanning of mRNA with highly ordered secondary structures

requires the helicase DexH-Box protein DHx29 to bind directly to the 40S subunit, melting the hindering structures (Pisareva *et al.,* 2008).
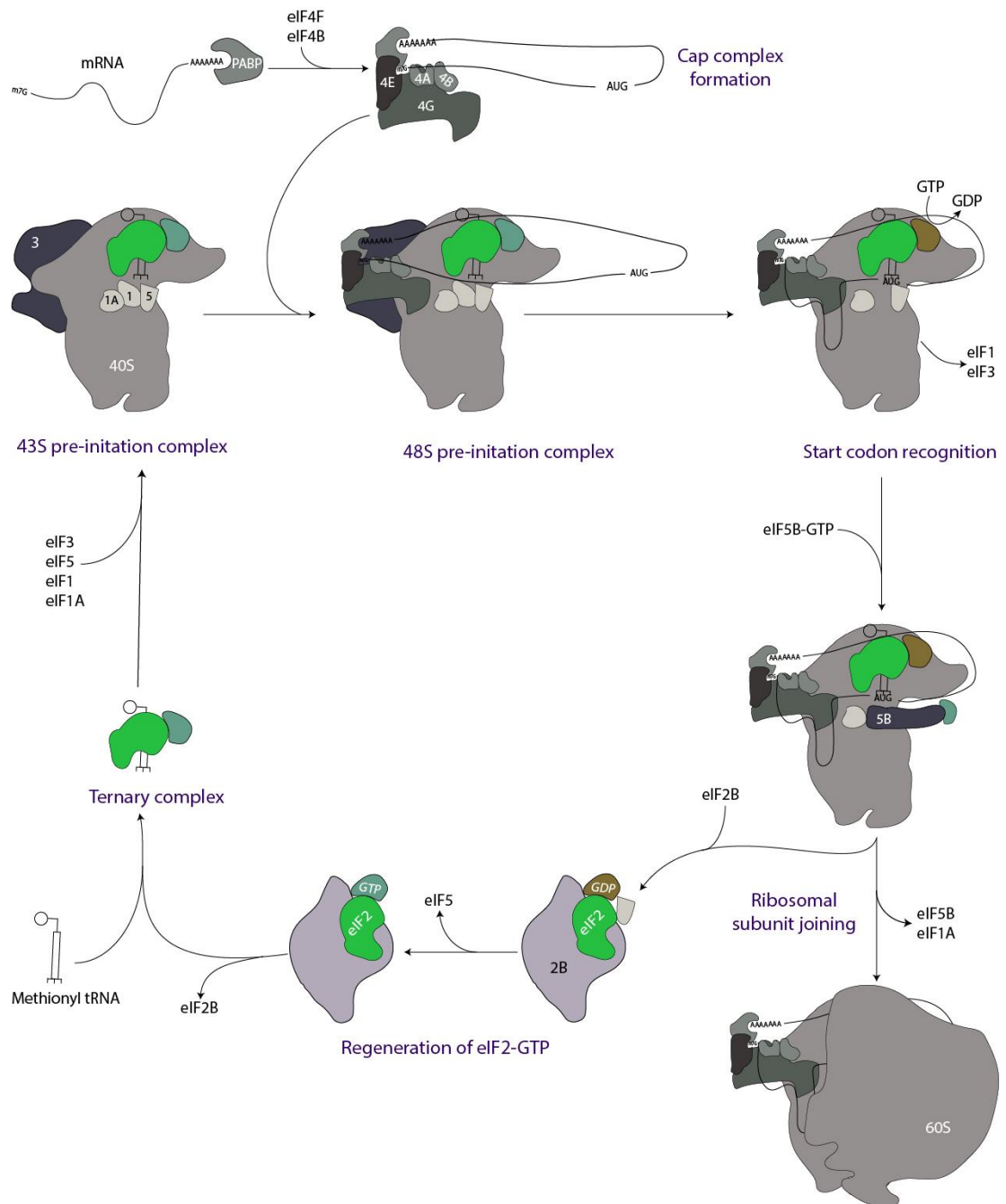
Figure 1-7 Eukaryotic Translation Initiation. Translation begins with formation of a ternary complex (TC). The TC then joins to the 40S ribosomal subunit to form a 43S preinitiation complex (43S PIC), which associates with the cap complex then associates to form a 48S PIC. The ribosome then scans the mRNA till a start codon is found at which point eIF2-GTP is hydrolysed to eIF2-GDP, catalysing association with 60S ribosome and releasing eIF2-GDP. eIF2-GTP is then regenerated by eIF2B allowing for subsequent rounds of translation.

1.2.1.3   Commitment to the start codon and ribosome subunit joining

The 43S PIC scans the mRNA in a 5' to 3' direction until a correctly positioned start codon is located. This is usually the first AUG triplet with a purine (A/G) in the -3 and a 'G' in the +4 position, relative to the 'A' of AUG (Kozak, 1991). During start codon recognition eIF2-GTP is hydrolysed to GDP, aided by eIF5 acting as a GTPase activator for eIF2γ (Huang *et al.,* 1997; Paulin *et al.,* 2001). The resulting inorganic phosphate molecule (Pi) is however not immediately released due to position of the eIF2 43S PIC during the scanning-competent formation. Rapid dissociation of Pi only occurs upon the GTPase activator eIF5 displacing eIF1 from its binding site, releasing it from the 43S PIC (Algire *et al.,* 2005; Nanda *et al.,* 2009). The following GTP hydrolysis lowers eIF2's binding affinity for ᵢMet-tRNA (Erickson & Hannig, 1996; Pisarev *et al.,* 2006; Kashiwagi *et al.,* 2016), which is further lowered by the association with eIF3 and eIF5 (Sokabe *et al.,* 2012), leading to its partial dissociation from the 43S PIC in complex with eIF5 (Algire *et al.,* 2005; Singh *et al.,* 2006).

Start codon recognition causes scanning arrest, and removal of the extreme C-terminal domain of eIF1A from the ribosome, enabling the recruitment of the GTPase eIF5B to the 43S PIC via interactions mediated by its C-terminal domain (Marintchev *et al.,* 2003; Olsen *et al.,* 2003). The presence of eIF5B-GTP accelerates the binding of the 60S ribosomal subunit with the 40S subunit, leading to the ejection of eIF3, eIF1 and any residual eIF2-GDP (Unbehaun *et al.,* 2004). This event results in the formation of the 80S ribosome (Acker *et al.,* 2006), leading to hydrolysis of eIF5B bound GTP. GDP bound eIF5B has a lower affinity for 80S IC, ultimately resulting in its release (Lee *et al.,* 2002; Shin *et al.,* 2002; Shin *et al.,* 2007). Finally, and only once eIF5B has been released, eIF1A dissociates from the 80S IC (Acker *et al.,* 2006), leaving it ready to begin elongation.

1.2.1.4 Recycling of eIF2-GDP

During the course of translation initiation eIF2-GTP is hydrolysed to eIF2-GDP and released from the 43S PIC in complex with eIF5 (Hinnebusch & Lorsch, 2012; Jennings *et al.,* 2013). In order for subsequent rounds of translation to occur eIF2-GDP must be released from eIF5, which acts as a GDP dissociation inhibitor (GDI) (Jennings *et al.,* 2013), and the eIF2-GTP must be restored (Hinnebusch & Lorsch, 2012). eIF2 has a higher affinity for GDP than GTP (Erickson & Hannig, 1996) and hence there is a requirement for the guanine nucleotide exchange reaction to regenerate eIF2-GTP to be catalysed, which is performed by the multisubunit protein complex in eIF2B (Hinnebusch & Lorsch, 2012). In its native form eIF2B exists as a decamer, consisting of two copies of each of its five subunits (Gordiyenko *et al.,* 2014; Wortham *et al.,* 2014). The γ and ε subunits confer the catalysis of nucleotide exchange, whilst the α, β and δ subunits act have a regulatory function (Pavitt *et al.,* 1997; Kimball *et al.,* 1998; Pavitt *et al.,* 1998; Williams *et al.,* 2001). Firstly, eIF2B acts as a GDI displacement factor, recruiting eIF2 from the eIF2-GDP/eIF5 complex (Jennings *et al.,* 2013). Secondly, eIF2B functions as a guanine nucleotide exchange factor (GEF) (Price & Proud, 1994; Jennings *et al.,* 2013).

The recognition of eIF2 and the formation of the eIF2-eIF2B complex occurs in a bipartite manner (Kenner *et al.,* 2019). The N-terminal region of eIF2α binds to the cleft between eIF2β and eIF2Bδ, with positively charged eIF2α S-loop facilitating resolvable interactions with negatively charged residues of the eIF2Bβ subunit (Kenner *et al.,* 2019). The second component of the bipartite binding occurs between eIF2γ and eIF2Bε (Kenner *et al.,* 2019). eIF2γ contains classic GTP-binding motifs including the phosphate binding P-loop and switch helicases 1 and 2 (Kenner *et al.,* 2019). The HEAT domain and core of eIF2Bε interact with eIF2γ favouring an open loop formation (Kenner *et al.,* 2019). These binding interactions position eIF2γ in a position where eIF2Bε and eIF2Bγ can exert its GEF activity (Gomez & Pavitt, 2000; Gomez *et al.,*

2002; Alone & Dever, 2006; Kenner *et al.,* 2019), thereby restoring eIF2-GTP ready to form renewed TCs.

1.2.1.5   eIF2α phosphorylation dependent translational arrest

The point of convergence for the ISR pathways hinges upon inhibition of translation initiation via the phosphorylation of eIF2α (Siekierka *et al.,* 1982; Donnelly *et al.,* 2013). In its phosphorylated state, eIF2α becomes a competitive inhibitor of eIF2B GEF activity (Dever *et al.,* 1995; Rowlands *et al.,* 1988). Under normal conditions two molecules of eIF2-GDP bind to eIF2B with the eIF2γ subunits positioned for interaction with the catalytic eIF2Bε subunits, facilitating GTP regeneration via nucleotide exchange (Kenner *et al.,* 2019). However, phosphorylation of eIF2α, at serine 51, results in previously unfavourable electrostatic interactions between serine 51 and arginine 53 and 63, causing a refolding of the eIF2α S-loop exposing previously hidden hydrophobic residues (Kenner *et al.,* 2019). These can interact with hydrophobic residues on eIF2Bα and eIF2Bδ, favouring a stress induced conformation of the eIF2-eIF2B complex where phosphorylated eIF2α binds on the opposite side of eIF2B with eIF2Bα and eIF2Bδ, leaving the catalytic domains of eIF2Bε dormant (Kenner *et al.,* 2019). This blocks the GEF activity of eIF2B causing a deficit in cellular eIF2-GTP (Kenner *et al.,* 2019), which ultimately leads to a cellular translational shutdown.

eIF2α phosphorylation is mediated by a family of four serine/threonine stress response kinases (Wek *et al.,* 2006). Whilst all four kinases share significant sequence similarity in their kinase domain (Donnelly *et al.,* 2013), each contains a unique regulatory domain, allowing for differential regulation via distinct stressors (Meurs *et al.,* 1990; Chen *et al.,* 1991; Berlanga *et al.,* 1998; Shi *et al.,* 1998; Dong, J. *et al.,* 2000; Harding *et al.,* 2000; Rafie-Kolpin *et al.,* 2000). Protein kinase double-stranded RNA-dependent (PKR) (gene EIF2AK2) classically responds to double-stranded RNA generated during viral infections (Clemens & Elia, 1997). PKR has also been found to respond to oxidative and ER stress as well as cytokine signalling and reactive oxygen

species (ROS) (Cheshire *et al.,* 1999; Ito *et al.,* 1999; Ruvolo *et al.,* 2001; Onuki *et al.,* 2004; Nakamura *et al.,* 2010; Anda *et al.,* 2017). The protein kinase R-like ER kinase (PERK, EIF2AK3) forms one arm of a larger three-armed response to misfolded proteins in the ER, collectively termed the unfolded protein response (UPR) (Walter & Ron, 2011). It is typically activated by ER stress, brought on by the accumulation of misfolded proteins in the ER lumen (Harding *et al.,* 2000; Walter & Ron, 2011) and by changes to ATP and Ca^{2+} in the ER independently of misfolded proteins (Sanderson *et al.,* 2010). PERK can also be activated by oxidative stress and hypoxia (Koumenis *et al.,* 2002; Harding *et al.,* 2003). General control non-depressible 2 (GCN2, EIF2AK4), the most highly conserved eIF2α kinase (Yang *et al.,* 2000; Donnelly *et al.,* 2013), is activated primarily by AA starvation (Wek *et al.,* 1995) but can also been activated by reactive oxygen species (ROS), viral infection and ultraviolet (UV) radiation (Berlanga *et al.,* 1998; Grallert & Boye, 2007; Pyo *et al.,* 2008). Heme-regulated inhibitor (HRI; EIF2AK1), a kinase mainly associated with protection against toxic globin aggregates in erythroid cells, is involved in protection against ROS induced by sodium arsenite as well as proteasome inhibition (Chen *et al.,* 1991; Han *et al.,* 2001; Lu *et al.,* 2001; McEwen *et al.,* 2005; Yerlikaya *et al.,* 2008).

1.2.1.6 Cap-dependent translational control

Although not an element of the ISR, regulation of cellular translational inhibition is not limited to eIF2α phosphorylation; a further point of control is cap-complex formation (Sonenberg & Hinnebusch, 2009). Due to low availability, the cap binding protein eIF4E is a rate limiting factor in translation (Rau *et al.,* 1996). The cap-binding activity of eIF4E is negatively regulated by an inhibitory protein, known as 4E binding protein 1 (4EBP1) (Pause *et al.,* 1994). 4EBP1 contains an amino acid sequence (YxxxxLφ), which is highly conserved between all proteins which bind eIF4E and is known as an eIF4E binding motif (Mader *et al.,* 1995). When 4EBP1 is active this motif facilitates its binding to eIF4E, preventing the association of eIF4E with eIF4G, thereby inhibiting translation initiation (Pause *et al.,* 1994; Mader *et al.,* 1995).

The activity of 4EBP1 is regulated primarily via mTORC1 (Qin *et al.,* 2016). mTORC1 is a highly conserved serine/threonine kinase, which exhibits control of cell growth and metabolism (Betz & Hall, 2013). Upon its activation the mTORC1 is positioned in close proximity to the 5' mRNA cap complex, where it phosphorylates 4EBP1, catalysing its dissociation from eIF4E, thereby allowing the recruitment of eIF4G and translation initiation to commence (Gingras *et al.,* 1999). Multiple phosphorylation sites have been identified in 4E-BP1: T37, T46, S65, T70, S83, S101 and S112 (Gingras *et al.,* 2001; Wang *et al.,* 2003). However, there is a body of conflicting data as to which phosphorylation sites are most implicated in the reduction of binding affinity between 4EBP1 and eIF4G. T37 and T46 have been shown to have minimal effect upon 4EBP1 binding to eIF4E (Gingras *et al.,* 1999; Heesom & Denton, 1999), but both have also been observed to cause a major reduction in eIF4E binding affinity (Burnett *et al.,* 1998). Interestingly, although T37 seems to be vital to the reduction of binding affinity *in vitro* its effectiveness is less marked *in vivo* (Burnett *et al.,* 1998). Moreover, it is unclear whether 4EBP1 is directly controlled by mTORC1, as inhibition of mTORC1 by rapamycin does not completely inhibit 4EBP1 phosphorylation (Wang *et al.,* 2005). More recently glycogen synthase kinase-3 (GSK3β) was identified as capable of phosphorylating 4EBP1 independently of mTORC1 (Shin *et al.,* 2014). These findings indicate a further level of complexity to 4EBP1 regulation whereby mTORC1 may act alone or in conjunction with other kinases to control 4EBP1 activity. Of particular interest is that mTORC1 is partially controlled via the actions of the eIF2α kinase GCN2, which is thought to partially mediate the inhibition of mTORC1; the exact mechanism remains to be fully elucidated (Averous *et al.,* 2016). mTOR inhibition results in a reduction of available eIF4E, stalling translation at the cap-binding phase, adding a secondary level of complexity to stress induced translational stalling.

A further point of translation control by mTORC1 activity lies within the phosphorylation of the S6 kinase 1, which leads to its dissociation from eIF3 allowing for formation of the 43S preinitiation complex (Holz *et al.,* 2005). Furthermore, active S6 Kinase 1 phosphorylates eIF4B, leading to its

recruitment to the eIF4A helicase during cap complex formation (Shahbazian *et al.,* 2006). A schematic of translational control by mTORC1 and eIF2α can be found in Figure 1-8.
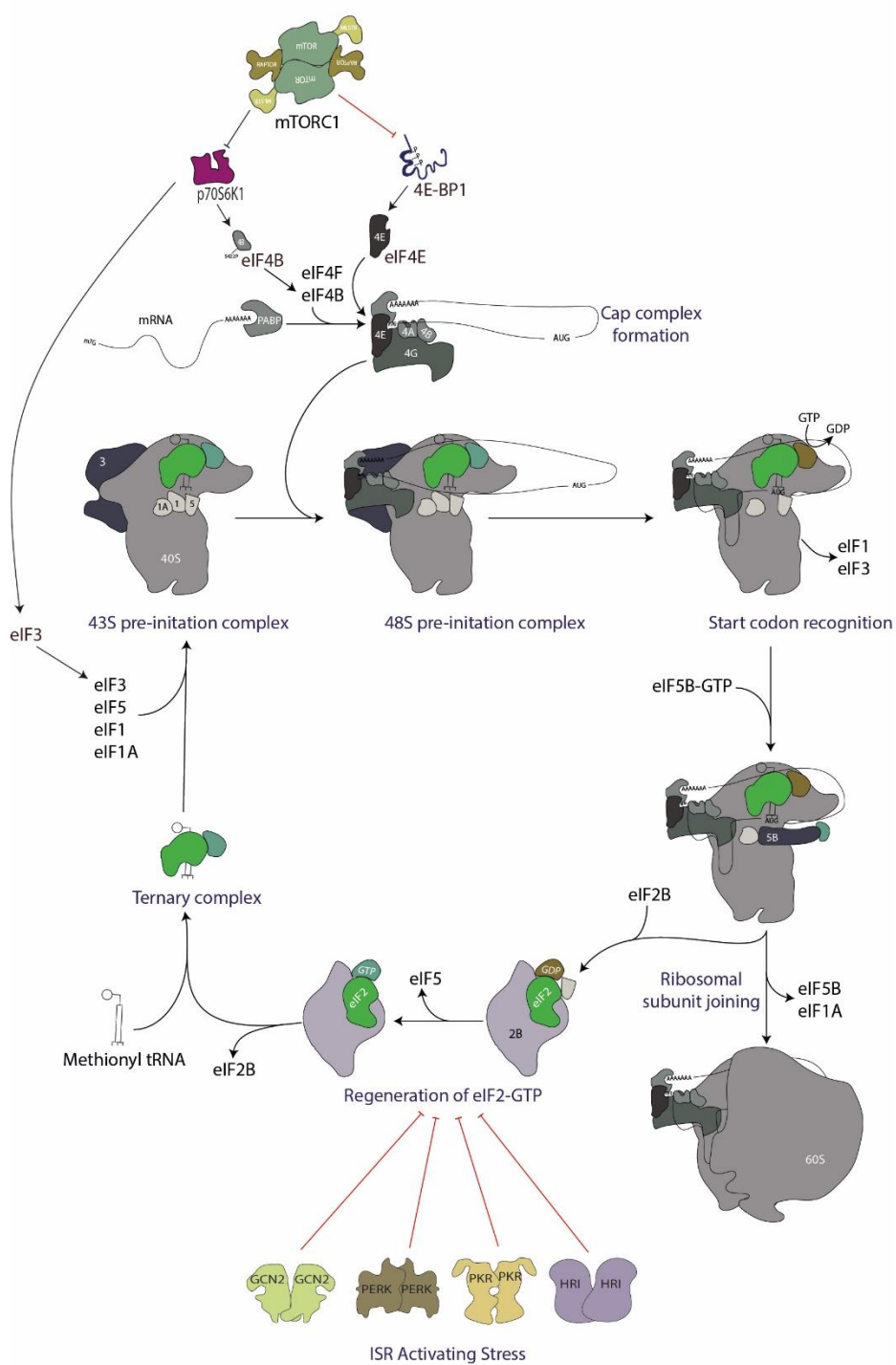
Figure 1-8 mTORC1 and eIF2 mediated translational control. ISR activating stresses activate stress response kinases, phosphorylating eIF2, which inhibits eIF2B GEF activity and results in a deficit of eIF2-GTP, thereby stalling translation. Active mTORC1, phosphorylates S6 Kinase 1, which has a 2-fold impact, firstly releasing eIF3 from S6 Kinase 1, allowing for 43S pre-initiation complex formation, and secondly phosphorylating eIF4B stimulating its recruitment to eIF4A. mTORC1 also phosphorylates 4E-BP1, releasing eIF4E and allowing for cap-complex formation.

1.2.1.7 Stress granule formation

Activation of the ISR results in translational stalling, polysome disassembly and subsequent release of stalled messenger ribonucleic acid (mRNA) transcripts into the cytoplasm (Costa-Mattioli & Walter, 2020). To allow for regulation of these stalled transcripts, cells aggregate these mRNAs along with translation initiation factors and RNA binding proteins into membraneless bioconcendensates termed stress granules (Protter & Parker, 2016). The initial trigger of stress granule assembly is increased cytoplasmic mRNA concentrations owing to polysome disassembly, and subsequent mRNA release, which if inhibited by cycloheximide treatment abrogate stress granule formation (Kedersha *et al.,* 2000). Alongside poly-adenylated-mRNA (poly-A-mRNA) stress granules typically contain 40S ribosomal subunits, eIF2, eIF3, PABP, components of the 5' cap complex (eIF4E, eIF4G, and eIF4A) and binding proteins such as G3BP1 and TIA-1 (Kedersha *et al.,* 1999; Kedersha *et al.,* 2002; Kimball *et al.,* 2003; Tourriere *et al.,* 2003; Mazroui *et al.,* 2007). Stress granules are also known to contain a vast and diverse proteome. Analysis of a stable stress granule substructure, known as the core (Jain *et al.,* 2016), revealed high concentrations of RNA binding proteins and components of many signalling pathways (Jain *et al.,* 2016), implicating stress granule assembly and dynamics in the stress dependent regulation of many cellular systems.

Internally stress granules exhibit a phase dense stable 'core' substructure containing high concentrations of proteins and mRNAs, which is surrounded by a less dense 'liquid' shell (Souquere *et al.,* 2009; Jain *et al.,* 2016). Stress granules are dynamic structures and as such undergo constant alterations, mediated by ATP driven remodelling complexes (Jain *et al.,* 2016), this ongoing reorganisation means many of their components have a residence time of only around 30 seconds (Mollet *et al.,* 2008; Jain *et al.,* 2016). However, it has been shown that a certain subset of proteins resides for longer, pointing towards an ordered system whereby the outer shell exhibits rapid component exchange whilst the core is less dynamic and harbours its

components in a more stable manner (Jain *et al.,* 2016). Interestingly, there is recent evidence implying the existence of distinct cores, occupied by either the nucleating protein G3BP1 or UV-induced ubiquitin associated protein 2 like (UBAP2L; Cirillo *et al.,* 2020), potentially pointing towards further regulation of components within stress granules.

Stress granule assembly occurs in a stepwise manner (Wheeler *et al.,* 2016; Figure 1-9). Assembly is driven by liquid-liquid phase separation (LLPS), whereby increased local concentrations of free or protein bound RNAs induces binding events, ultimately resulting in these aggregating into a condensed phase separate to the cytoplasmic phase surround them (Brangwynne *et al.,* 2009). Initially, release of mRNAs from polysomes during translational inhibition increases cytoplasmic mRNA concentrations, which induces a conformational change in G3BP1, which reveals its intrinsically disordered regions (IDRs; Guillén-Boixet *et al.,* 2020). These IDRs contain repetitive amino acid sequences that confer multivalency, enabling increased binding events and a crosslinking network, essential for LLPS (Banani *et al.,* 2017; Boeynaems *et al.,* 2018). The IDR presenting form of G3BP1 rapidly binds RNA, which in turn results in recruitment of other stress granule proteins, increasing local concentrations of protein-protein, protein-RNA and RNA-RNA binding events (Guillén-Boixet *et al.,* 2020). Once the concentration of these events surpasses the so-called 'percolation-threshold' there is LLPS which drives the condensation of small stress granules (Guillén-Boixet *et al.,* 2020; Sanders *et al.,* 2020; Yang *et al.,* 2020). These undergo fusion to form mature biphasic stress granules, in an ATP driven, microtubule dependent manner (Loschi *et al.,* 2009; Jain *et al.,* 2016; Wheeler *et al.,* 2016). Disruption of the tubulin network impairs stress granule formation, supporting the role of microtubules in stress granule assembly (Ivanov *et al.,* 2003). Furthermore, stressful conditions result in α-tubulin acetyltransferase 1 (ATAT1) mediated hyper-acetylation of the cellular tubulin network at lysine 40 of α-tubulin (Li *et al.,* 2019). Hyper-acetylation of α-tubulin stimulates increased binding and activity of microtubule motor proteins dynein and kinesin, which are highly implicated in

the movement of stress granules (Reed *et al.,* 2006; Cai *et al.,* 2009; Hammond *et al.,* 2010), which may likely aid in facilitating rapid, regulated stress granule assembly and disassembly as a protective response to stress.

Upon recovery from stress, stress granules dissociate in a manner that mostly correlates with the resumption of protein synthesis (Mazroui *et al.,* 2007). The system of clearance is roughly the opposite of assembly. Firstly, mRNAs titrate out of the granules as they resume translation, causing dissipation of the shell structure (Wheeler *et al.,* 2016), followed by disassembly of the large biphasic stress granules into smaller core structures, which are then cleared by autophagy (Buchan *et al.,* 2013; Wheeler *et al.,* 2016).
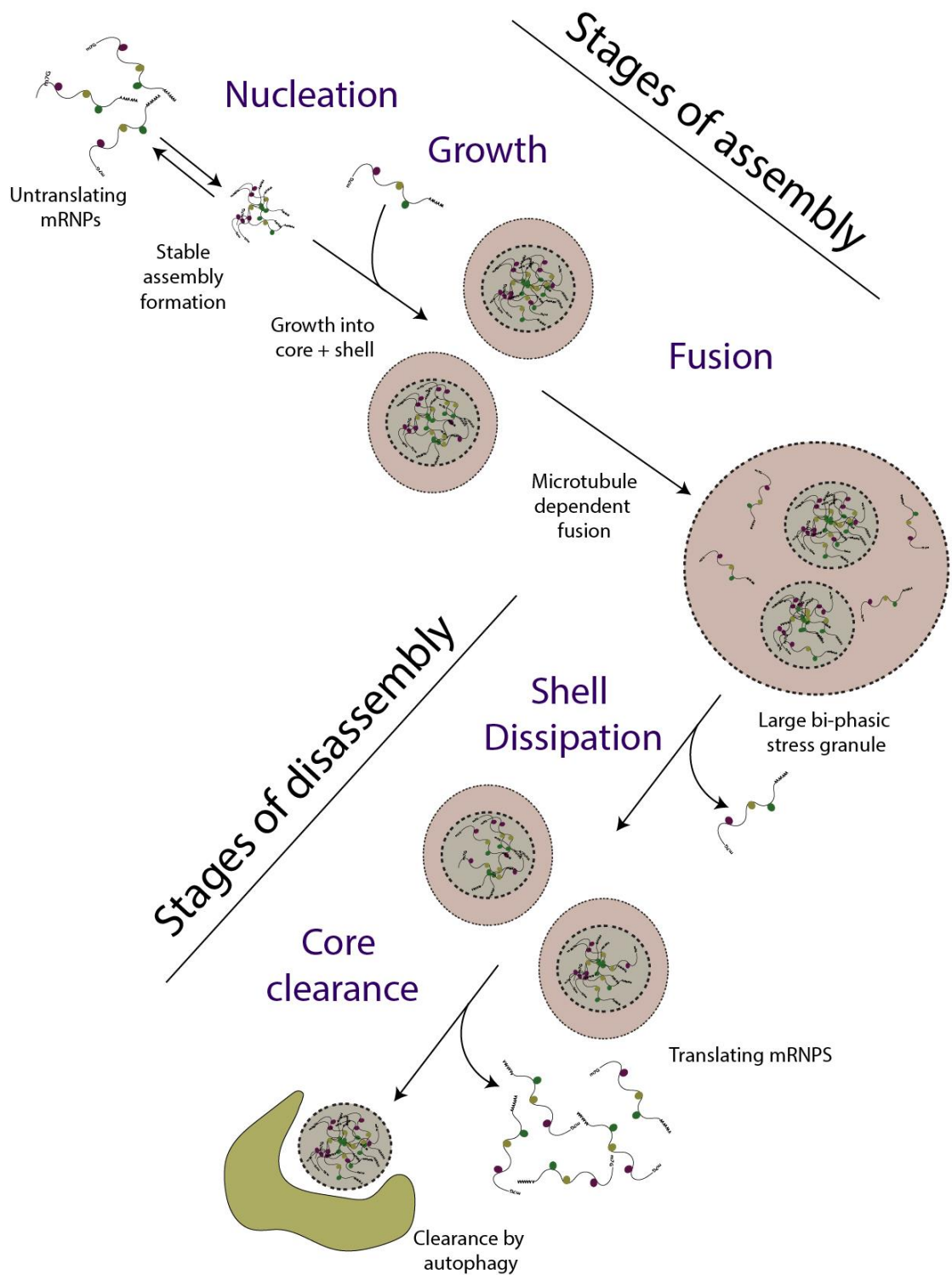
Figure 1-9 The stages of stress granule assembly and disassembly.
granule assembly begins with the aggregation of untranslating mRNPs into a
stable core. The stable cores drive liquid-liquid phase separation creating a
liquid phase shell around the core. These smaller stress granules then fuse
into larger biphasic-stress granules. Disassembly occurs in roughly the
reverse manner to assembly with mRNPs leaching out as they return to
translation and the core structure being cleared by autophagy. Adapted
from Wheeler *et al.,* (2016).

The composition of stress granules is known to vary dependent on stress stimuli (Aulas *et al.,* 2017). More recent studies found the composition of stress granules to be non-homogenous and it has thus been proposed that stress granules can be classified into three main types (Hofmann *et al.,* 2021). Type I stress granules are formed during eIF2α mediated translational arrest, whereas type II are formed during eIF2α independent translational arrest, finally type III stress granules are formed under chronic stress and lack many eIFs (Reineke & Neilson, 2019). Whilst Both type I and II stress granules are associated with cellular survival, type III are generally associated with death (Reineke & Neilson, 2019).

1.2.2 Bacterial interactions with the Integrated Stress Response

In recent years, it has become apparent that the ISR forms an integral part of the host innate immune response to pathogens (reviewed by Rodrigues *et al.,* 2018). This is supported by studies showing that pathogens can induce eIF2α phosphorylation via PERK, GCN2 and HRI (Tattoli *et al.,* 2012; Tsutsuki *et al.,* 2016; Abdel-Nour *et al.,* 2019). Given that the ISR plays a crucial role in controlling cellular fate during stress (Costa-Mattioli & Walter, 2020), pathogens with means to dampen or hijack the ISR pathway are likely able to influence cellular signalling and ultimately benefit from long-term survival and promote persistence of infection. Indeed, it is well-documented that viruses manipulate specific elements of the ISR during infection. Hepatitis C virus, Japanese encephalitis virus and human cytomegalovirus directly inhibit the viral specific eIF2α kinase PKR (Toroney *et al.,* 2010; Tu *et al.,* 2012; Ziehr *et al.,* 2016), and Kaposis sarcoma-associated virus indirectly inhibits PKR via inhibition of its activator PACT (Sharma *et al.,* 2017). Another point of ISR modulation displayed by viruses is to dampen eIF2α phosphorylation. Junin virus directly inhibits eIF2α (Linero *et al.,* 2011), and hepatitis C virus activates GADD34 to dephosphorylate eIF2α during stress (Ruggieri *et al.,* 2012).

However, recent evidence suggests that some bacterial species might also be manipulating the host ISR, inducing a variety of cellular outcomes. The following sections will focus on how five bacterial model organisms, Shiga toxin-producing *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Porphyromonas gingivalis* can manipulate specific components of the ISR to gain control over cellular fate and immune signalling, creating an environment favouring bacterial viability, replication, and infection.

1.2.2.1 Shiga Toxin-Producing *Escherichia coli*

Shiga toxin-producing *E. coli* O157:H7 (STEC) is a widespread pathogen presenting severe risk to human health, causing haemorrhagic colitis and haemolytic uremic syndrome (Riley *et al.,* 1983; Ko *et al.,* 2016). Annually, the prevalence of acute STEC infection is thought to be ~2.8 million cases worldwide, with infection progressing to HUS in 3,890 cases and resulting in death in 230 cases (Majowicz *et al.,* 2014).

Virulent strains of STEC have been shown to target the ISR to induce host cell death mediated via a secreted virulence factor termed subtilase cytotoxin (SubAB; Tsutsuki *et al.,* 2016;Figure 1-10 A). SubAB is a secreted toxin consisting of two subunits; the B subunit binds the host extracellular toxin receptor and facilitates toxin internalisation, whereas the A subunit is a serine protease, which, in conjunction with the B subunit, facilitates the intracellular virulent effects of the pathogen (Morinaga *et al.,* 2007). The main target of SubAB is the cleavage of the PERK chaperone binding immunoglobulin protein (BiP; Figure 1-10 B), resulting in the dimerisation and activation of PERK, inducing eIF2α phosphorylation (Tsutsuki *et al.,* 2016; Figure 1-10 C). ISR activation triggered in this manner causes stress granule formation, which is dependent upon death-associated protein 1 activation (Tsutsuki *et al.,* 2016). Inhibition of protein kinase C δ (PKCδ) and phosphoinositide-dependent kinase 1 (PDK1) are implicated in the formation of these stress granules, as chemical inhibition of both also heightens stress

granule formation in response to SubAB (Tsutsuki *et al.,* 2016). Furthermore, death-associated protein 1 knockdown increased basal levels of phospho-PDK1(S196), thereby inhibiting stress granule formation, further implicating PDK1 inhibition in formation of stress granules in response to SubAB (Tsutsuki *et al.,* 2016; Figure 1-10 D). Interestingly, in rat intestinal epithelioid cells, PDK1 has been shown to inhibit cell death in response to H_2O_2 (Song *et al.,* 2009). Therefore, the inhibition of PDK1 coupled with prolonged PERK activation (Lin *et al.,* 2009), which is known to promote apoptosis, may push the host cell towards death (Lin *et al.,* 2009; Tsutsuki *et al.,* 2016; Figure 1-10 E). In contrast, in human lung cancer cells, PKCδ activation induces cell death via the CHOP-ATF3 arm of the ISR (Xu *et al.,* 2012). Hence, the exact role PKCδ inhibition by SubAB in cell death requires further attention. Although driving the host cell towards death may seem counterproductive for bacterial survival, it is thought that Shiga-toxic *E. coli* displays altruism (Loś *et al.,* 2012); in this context, ISR-mediated destruction of host cell and internalised bacteria could provide nutrients to the wider STEC community.
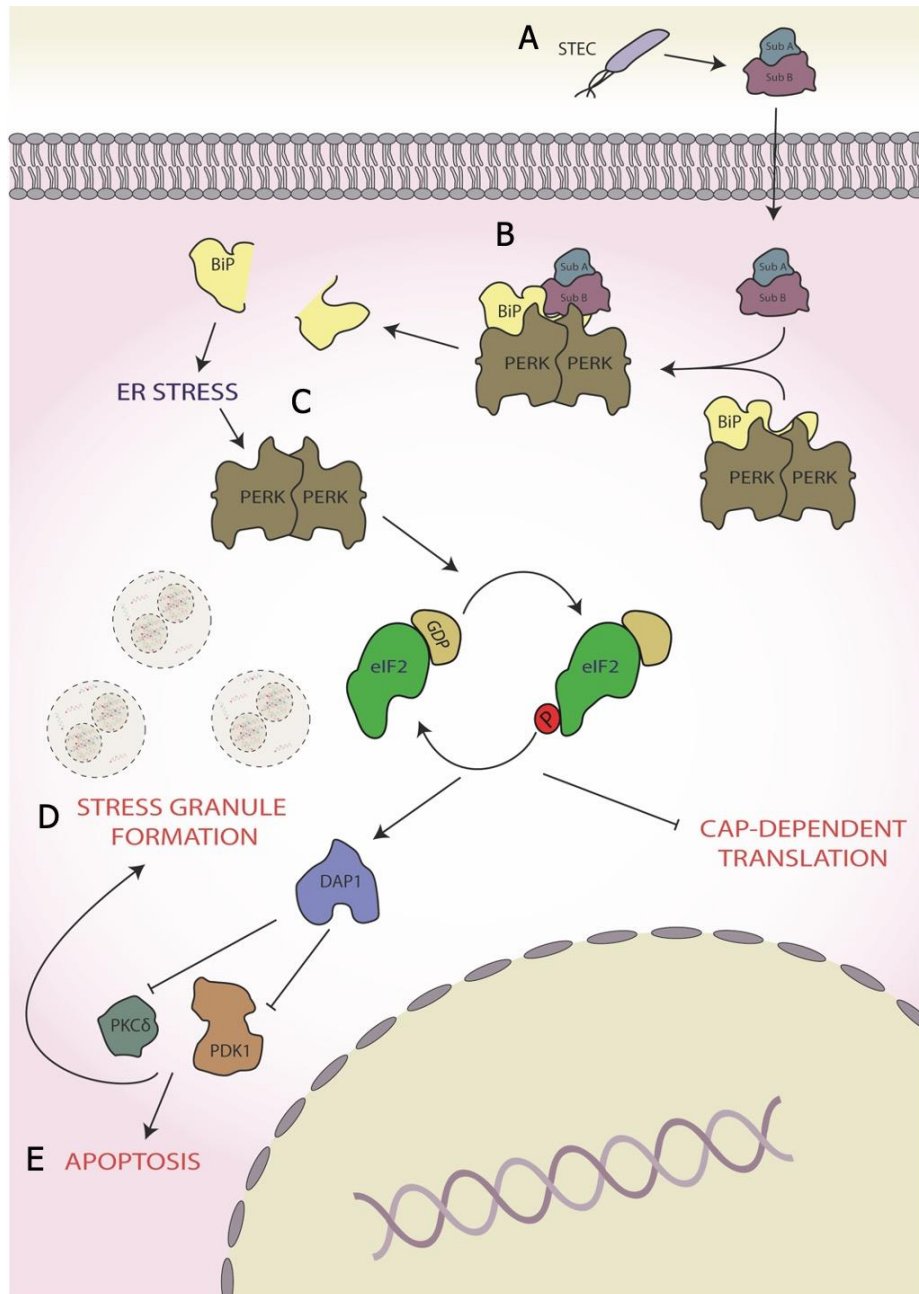
Figure 1-10 Shiga toxin-producing _Escherichia coli._ (A) During infection, STEC secretes subAB, (B) which cleaves PERKs chaperone BiP, and (C) leading to the activation of PERK and subsequent phosphorylation of eIF2α. (D) This results in the formation of stress granules in a manner dependent on DAP1 inhibition of PDK1 and PKCδ, (E) pushing the cell towards death. Figure from Knowles _et al.,_ (2021), used under creative commons licence.

1.2.2.2   Shigella flexneri

Shigella is a genus of Gram-negative, facultative anaerobic bacteria that primarily infect the gastrointestinal tract, causing acute shigellosis (Fernandez & Sansonetti, 2003). Whilst closely related to *E. coli*, *Shigella* possesses unique methods of pathogenicity (Ud-Din & Wahid, 2014). Diarrhoea is an early symptom of infection as the bacteria moves through the small intestine, but the primary target of *Shigella* is the invasion of colonic epithelial cells from the basolateral surface (Phalipon & Sansonetti, 2007). Once internalised, the bacteria replicate and spread from cell to cell. The infection also causes inflammatory colitis via secreted toxins (Eashida *et al.,* 2015). The mechanism of *Shigella* invasion has been reviewed elsewhere (Carayol & Tran Van Nhieu, 2013; Liu *et al.,* 2019).

Infection with Group B serogroup *S. flexneri* has been shown to robustly induce the ISR, resulting in the activation of two eIF2α kinases, GCN2, and HRI (Tattoli *et al.,* 2012; Abdel-Nour *et al.,* 2019; Figure 1-11). During the initial stage of infection, *S. flexneri* induces AA starvation through membrane damage, which results in the activation of GCN2 (Tattoli *et al.,* 2012) (Figure 1-11 A). In its active form, GCN2 inhibits mTORC1 (Figure 1-11 F), as demonstrated via its dispersal from lysosome-associated membrane protein 2 (LAMP2), and increases the transcription of the AA stress-related gene asparagine synthetase, a response that increases for up to 4 h post-infection (Tattoli *et al.,* 2012; Abdel-Nour *et al.,* 2019). However, *S. flexneri* is able to activate mTORC1 via direct delivery of its OspB effector into host's cellular cytoplasm using the *S. flexneri* Type III secretion system (T3SS), which interacts with the IQ motif of the GTPase-activating protein 1 (Lu *et al.,* 2015), an upstream regulator of mTORC1 (Tekletsadik *et al.,* 2012) (Figure 1-11 I), ultimately resulting in increased host cell proliferation around the infection foci during the later stages of early infection. This increased cellular proliferation reduces *S. flexneri* spread but is thought to provide a preferential intracellular niche acting as a protective measure (Lu *et al.,* 2015).
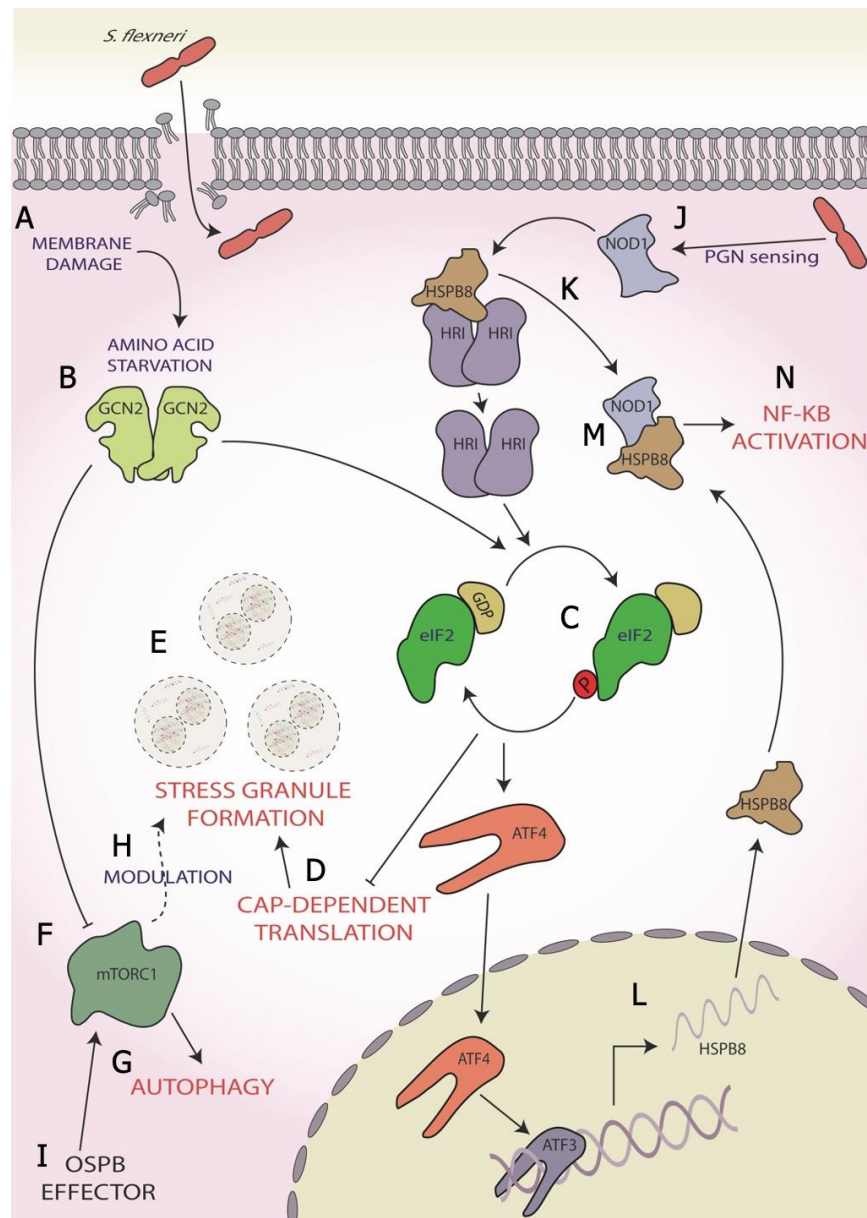
Figure 1-11 Shigella flexneri. (A) Membrane damage caused during *S. flexneri* internalisation induces AA starvation, (B) activating GCN2, and (C) subsequently phosphorylation of eIF2α. (D) This results in the inhibition of cap-dependent translation initiation (E) and consequently the formation of stress granules. (F) GCN2 also inhibits mTORC1; (G), inducing autophagy and (H) modulating the frequency and composition of stress granules during exogenous stress induction. (I) mTORC1 activity is reactivated during later-stage infection via *S. flexneri's* OspB effector. (J) Concurrently, *S. flexneri's* peptidoglycan is detected by NOD1, (K) which induces dissociation of the chaperone HSPB8 from HRI, causing its activation and subsequent eIF2α phosphorylation. (L) This results in the activation of ATF4, which along with ATF3, upregulated the expression and translation of HSPB8. (M) This nascent HSPB8 associates with NOD1 (N) leading to the activation of pro-inflammatory responses by NF-κB activation. Figure from Knowles *et al.,* (2021), used under creative commons licence.

In addition, like STEC infection, *Shigella* infections also result in the aggregation of stalled messenger ribonucleoproteins (mRNPs) into stress granules (Tattoli *et al.,* 2012; Vonaesch *et al.,* 2016; Abdel-Nour *et al.,* 2019; Figure 1-11 E). The activation of the ISR leads to the upregulation of ATF3, ATF4, and GADD34 (Abdel-Nour *et al.,* 2019) and consequently the robust upregulation of the transcription and expression of ISR and inflammatory-related genes (Tattoli *et al.,* 2012; Abdel-Nour *et al.,* 2019). Interestingly, in the presence of ISR-inducing exogenous stresses such as mitochondrial, oxidative and heat shock stress, an increase in frequency and a decrease in an area of stress granules formed are observed in *S. flexneri*-infected cells at 2–3.5 h post-infection (Vonaesch *et al.,* 2016). The composition of the stress granules is also altered with the selective exclusion of eIF3B, eIF4G, and eIF4B via a mechanism downstream of eIF2α (Vonaesch *et al.,* 2016). Since the phenotype observed is similar to the hindered assembly of stress granules seen following chemical disruption of the tubulin network with nocodazole (Fujimura *et al.,* 2009; Kolobova *et al.,* 2009; Vonaesch *et al.,* 2016) and the movement of eIF3B and eIF4B is controlled by microtubule assembly (Shanina *et al.,* 2001; Harris *et al.,* 2006; Figure 1-11 E), this differential stress granule composition may be dependent upon microtubule dysregulation. Interestingly, stresses such as selenite and hydrogen peroxide, which bypass eIF2α phosphorylation instead inhibiting mTORC1 function, also result in the formation of atypical stress granules lacking components such as eIF3 (Emara *et al.,* 2012; Fujimura *et al.,* 2012), thereby also implicating the *S. flexneri* infection-induced inhibition of mTORC1 in the differential stress granule formation (Tattoli *et al.,* 2012; Vonaesch *et al.,* 2016). As the formation of stress granules during *S. flexneri* infection has only been investigated up to 5 h (Abdel-Nour *et al.,* 2019), whether the formation of stress granules is altered similarly during later-stage infection when mTORC1 is reactivated remains unknown. If this does not occur later when mTORC1 is reactivated, this would aid the hypothesis that modulation is at least partially dependent on mTORC1 inhibition. Furthermore, whether this modulation to stress granule formation during infection may provide any

evolutionary benefit to *S. flexneri* or is simply a downstream effect of ISR modulation remains unknown.

In addition to ISR activation via membrane damage, intracellular sensing of bacterial peptidoglycan by the host PRR, nucleotide-binding oligomerisation domain-containing protein 1 (NOD1), also induces ISR activation and expression of the pro-inflammatory cytokine nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) in an HRI-dependent manner (Abdel-Nour *et al.,* 2019; Figure 1-11 J). NOD1 activation results in dissociation of the HRI chaperone, heat shock protein beta-r 8 (HSPB8), from HRI, consequently activating HRI (Abdel-Nour *et al.,* 2019; Figure 1-11 K). Activated HRI induces robust eIF2α phosphorylation (Figure 1-11 C), resulting in heightened HSPB8 transcription in a manner dependent on ATF4 and ATF3 signalling (Abdel-Nour *et al.,* 2019; Figure 1-11 L). This nascent HSPB8 can interact with the previously dissociated HSPB8 and NOD1 to form a signalosome (Figure 1-11 M) and during *S. flexneri* infection causes the upregulation of host immune inflammatory responses and macrophage activation through the NF-κB pathway (Abdel-Nour *et al.,* 2019; Figure 1-11 N). As this pathway is also triggered by misfolded proteins within the cytosol and is comparable with the UPR in the ER, it was coined the cytosolic UPR (cUPR; Abdel-Nour *et al.,* 2019).

Shigella flexneri infection results in the induction of the ISR, which can be viewed as a protective response activating pro-inflammatory responses via the cUPR (Abdel-Nour *et al.,* 2019) and autophagy via mTORC1 inhibition (Tattoli *et al.,* 2012), during early infection. However, (Abdel-Nour *et al.,* 2019) found that the eIF2α S51A mutant, which cannot be phosphorylated, resulted in a significantly increased frequency of intracellular *S. flexneri* compared to cells with phosphorylated eIF2α (Abdel-Nour *et al.,* 2019). Taken together, these data indicate that intracellular *S. flexneri* replication is heightened during ISR activation, and it is plausible that the phosphorylation state of eIF2α may at least partially control bacterial spread and viability.

However, during later-stage infection, *S. flexneri*-mediated reactivation of mTORC1 not only increases host cell viability but also decreases bacterial spread around the infection foci (Lu *et al.,* 2015). Whilst the impact of mTORC1 reactivation on bacterial infection has not been investigated, focussing on the ISR may provide further insights into this mechanism. Furthermore, infection results in GADD34 expression; however, whether this occurs during later-stage infection, when mTORC1 is reactivated, is as of yet unknown (Abdel-Nour *et al.,* 2019). As GADD34 induces the dephosphorylation of eIF2α via the activation of PP1 (Connor *et al.,* 2001; Novoa *et al.,* 2001), the potential of sustained GADD34 expression during later stage of *S. flexneri* infection (Abdel-Nour *et al.,* 2019) may lead to termination of the ISR, potentially aiding the cell in returning to homeostatic conditions.

There is also evidence that inhibition of mTORC1 leads to eIF2α phosphorylation in cancer cell lines (Harvey *et al.,* 2019). If this also occurs during *S. flexneri* infection, the reactivation of mTORC1 may further push the cellular equilibrium of eIF2α towards the non-phosphorylated form. Therefore, if dephosphorylation of mTORC1 was coupled with the GADD34 expression, this could act as a two-part shift to favour a state with minimal eIF2α phosphorylation. Though counter-intuitive, whilst defective eIF2α signalling favours *S. flexneri* invasion (Abdel-Nour *et al.,* 2019), it may lead to increased host viability, which has been suggested to benefit *S. flexneri*, the latter remaining in infected cells for much of its life cycle (Killackey *et al.,* 2016). Thus, whether mTORC1 reactivation or persistent GADD34 expression leads to eIF2α dephosphorylation during infection, and the consequential effects upon host cell viability and *S. flexneri* persistence and replication, is an area requiring further attention. Given that increased host cellular replication during OspB-mediated mTORC1 reactivation is thought to provide a preferential niche for *S. flexneri* survival (Lu *et al.,* 2015), it is entirely plausible that this potential ISR termination may feed into this, helping to create an even further immune-privileged environment for *S.*

flexneri. Furthermore, the effect of OspB only occurs later during the infection (Lu *et al.,* 2015), whereas the initial phenotype of GCN2 activation and mTOR inhibition require internalisation and the resulting membrane damage (Tattoli *et al.,* 2012; Abdel-Nour *et al.,* 2019), which is intriguing as internalisation of OspB only requires extracellular contact between the bacteria and host cell (Hueck, 1998). Therefore, elucidation of the interaction between *S. flexneri* and these pathways may provide valuable insights into the pathogenic mechanisms of S. flexneri in chronic infections.

1.2.2.3 *Salmonella enterica*

Salmonella enterica serovar Typhimurium is an enteric pathogen primarily associated with food-borne gastrointestinal disease (Fabrega & Vila, 2013) and is thought to affect 1.3 billion people annually, leading to approximately 3 million deaths globally (Pui *et al.,* 2011). During infection, *Salmonella typhimurium* adheres to the host's intestinal epithelium, resulting in extensive cytoskeletal rearrangements (Finlay *et al.,* 1991). These modifications cause membrane ruffles, which eventually engulf the bacteria in large vesicles known as *Salmonella*-containing vesicles, creating an intracellular compartment in which *Salmonella* can survive and replicate (Steele-Mortimer, 2008). *Salmonella* infection is detected by host TLRs and NOD proteins, which initiates the NF-κB signalling cascade and results in cytokine and chemokine production, leading to an inflammatory state (Souvannavong *et al.,* 2007; Spiller *et al.,* 2008; Winter *et al.,* 2009; Marijke Keestra *et al.,* 2011).

As with *S. flexneri*, membrane damage induced by *Salmonella* invasion causes intracellular AA starvation with the activation of ISR through the eIF2α kinases GCN2 (Tattoli *et al.,* 2012; Figure 1- A,B). This AA starvation-induced activation of GCN2 also initially inhibits the activity of mTORC1, via the dispersal from LAMP2 and results in the activation of autophagy (Tattoli *et al.,* 2012; Figure 1- C,D). Interestingly, within 4 h of infection, the raptor/rag/regulator pathways can reactivate mTORC1, effectively saving

Salmonella from autophagy; however, the mechanism by which this occurs has yet to be fully determined (Tattoli *et al.,* 2012; Figure 1- E).
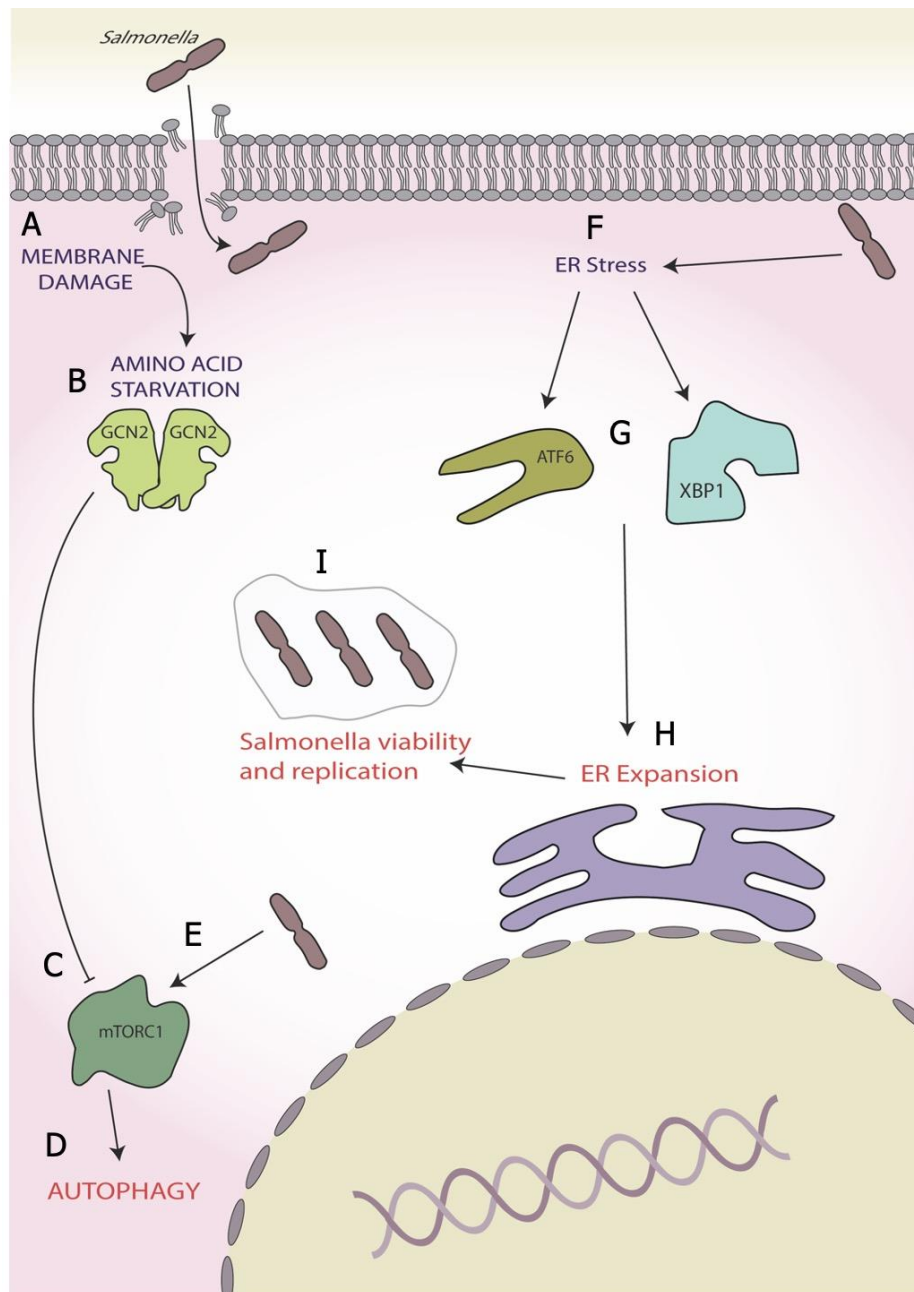
Figure 1-12 *Salmonella*. (A) Membrane damage caused during *Salmonella* internalisation induces AA starvation, (B) leading to GCN2 activation, and (C) inhibition of mTORC1, and (D) ultimately inducing autophagy. (E) During later-stage infection, *Salmonella* reactivates mTORC1, thereby inhibiting autophagy. (F) Salmonella also induces ER stress, (G) activating the UPRs ATF6 and XBP1 arms, (H) leading to expansion of the ER, and (I) which increases intracellular *Salmonella* viability and replication. Figure from Knowles *et al.,* (2021), used under creative commons licence.

In later stages of infection, e.g., 12–24 h post-infection, *Salmonella* has also been shown to induce ER stress (Figure 1- F), robustly activating the UPR and leading to the activation of X-box binding protein 1 (XBP1) and ATF6 (Antoniou *et al.,* 2018; Figure 1- G), which is known to increase lipid biogenesis and increase ER expansion (Sriburi *et al.,* 2004; Sriburi *et al.,* 2007; Figure 1- H). During *Salmonella*-induced ER stress, human leukocyte antigen (HLA)-B27 becomes misfolded, causing SCV to move away from host Golgi apparatus (Antoniou *et al.,* 2018). This, coupled with ER expansion, is thought to allow for increased space for the SCV and is supported by observations that ER stress induction by thapsigargin and misfolded HLA-B27 increase intracellular *Salmonella* viability and replication (Antoniou *et al.,* 2018; Figure 1- I). Thus, *Salmonella* effectively utilises the ISR and UPR in two opposing ways, firstly by reversing the autophagic responses brought on by the ISR during early-stage infection via mTORC1 reactivation and then inducing ER stress to allow for preferential replication conditions in later stages of infection.

1.2.2.4   *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a Gram-negative, rod-shaped, mono-flagellated bacterium, is one of the most frequent causative agents for hospital-acquired infections resulting in loss of life (Buhl *et al.,* 2015), with immunocompromised patient's survival rates being disproportionately lowered (Migiyama *et al.,* 2013). Chronic lung infections caused by *P. aeruginosa* are a common cause of death in patients with cystic fibrosis and chronic obstructive pulmonary disease, with those affected often experiencing recurrent infections (Murphy *et al.,* 2008; Yum *et al.,* 2014).

During infection, *P. aeruginosa* secretes a wide variety of proteins including the extracellular adhesin CdrA (Borlee *et al.,* 2010), the diffusible quorum-sensing molecule N-(3-oxododecanoyl)-homoserine lactone (HSL) (Smith *et al.,* 2002), and virulence factors [e.g., alkaline protease A (ArpA; Vasil & Ochsner, 1999) and HasAp (Létoffé *et al.,* 1996), all of which are known to

induce ER stress (Grabiner *et al.,* 2014; van 't Wout *et al.,* 2015; Figure 1-9 A). In mouse embryonic fibroblasts (MEFs), HSL, which has a key role in *P. aeruginosa* cell-to-cell communication within the structurally ordered biofilm (Smith *et al.,* 2002), induces ER stress via the release of Ca^{2+} from ER stores (Figure 1-9 B). This causes an imbalance in ER homeostasis and activates PERK (Figure 1-9 C), which phosphorylates eIF2α and results in a global shutdown of protein synthesis (Smith *et al.,* 2002; Grabiner *et al.,* 2014; Figure 1-9 D,E). Interestingly, this translational stalling reduces the expression and secretion of the pro-inflammatory chemokine keratinocyte chemoattractant (KC), the mouse equivalent of IL-8 (Grabiner *et al.,* 2014; Figure 1-9 F). Thus, HSL may lead to the suppression of KC secretion through eliciting the host ISR, aiding *P. aeruginosa* to evade host inflammatory and antibacterial responses during the early stages of infection (Grabiner *et al.,* 2014). This observation contrasts with the robust expression of IL-8 seen in *S. flexneri* infection (Abdel-Nour *et al.,* 2019) and suggests a species-specific response (Abdel-Nour *et al.,* 2019). However, further studies are required to probe this and ascertain whether this response is species specific or cell dependent due to the differential approaches used with one study using mouse-derived MEFs (Grabiner *et al.,* 2014) and the other using the human cervical epithelial cell line HeLa (Abdel-Nour *et al.,* 2019).

Figure 1-9. *Pseudomonas aeruginosa*. (A) The quorum-sensing molecule HSL secreted by *P. aeruginosa* (B) induces ER stress, (C) activating PERK, and (D) which leads to eIF2α phosphorylation. (E) The consequential translational stalling (F) results in decreased expression of the pro-inflammatory cytokine IL-8 production. (G) A protease, ArpA, secreted by *P. aeruginosa* (H) activates HRI, (I) leading to the specific upregulation of GADD34, and (J) which increases host cell viability. Figure from Knowles *et al.,* (2021), used under creative commons licence.

Another example of *P. aeruginosa*-mediated ISR manipulation is through the secretion of ArpA (Figure 1-9 G), a protease involved in, amongst other pathways, hosts siderophore-mediated iron scavenging (Vasil & Ochsner, 1999; Kim *et al.,* 2006; van 't Wout *et al.,* 2015). Whereas HSL induces ER stress through activation of the p53 MAPK pathway, ArpA specifically activates HRI (Figure 1-9 H), which induces the expression of GADD34 (van 't Wout *et al.,* 2015; Figure 1-9 I) and is protective against *P. aeruginosa* cytotoxicity, allowing for prolonged host cell survival (van 't Wout *et al.,* 2015; Figure 1-9 J). The mechanism by which HRI activation and GADD34 expression increase host viability is currently unknown, but increased GADD34 expression could lead to increased PP1 activity and consequently dephosphorylation of eIF2α (Connor *et al.,* 2001; Novoa *et al.,* 2001). This deactivation of the ISR could prove to be a promising system for *P. aeruginosa* to push the cell towards survival, thereby increasing viability. However, given the recent findings of (Abdel-Nour *et al.,* 2019), it is entirely plausible that activation of HRI by ArpA may activate the cUPR, which has been shown to be protective against *S. flexneri* infection. Whether this increased viability is due to eIF2α dephosphorylation or cUPR activation, or a combination of both, remains to be elucidated and requires further attention.

Pseudomonas aeruginosa hereby displays a two-part manipulation of the host ISR, both dampening inflammatory responses and increasing host cell viability. These reduced inflammatory and immune responses may act as the critical tipping point, leading to the decreased survival of immunocompromised patients (who already have impaired immune responses), as it could result in unregulated and therefore heightened *P. aeruginosa* growth.

1.2.2.5   *Porphyromonas gingivalis*

Porphyromonas gingivalis is a Gram-negative, anaerobic bacterium and the "keystone pathogen" of the chronic oral inflammatory gum disease, periodontitis (Socransky *et al.,* 1998). Infection triggers host immune

responses resulting in inflammation of the gingival tissues, which in some cases progresses to periodontitis, resulting in alveolar bone resorption and ultimately tooth loss (Pihlstrom *et al.,* 2005). *P. gingivalis* is known to modulate several host cell responses including the inhibition of antimicrobial responses whilst leaving pro-inflammatory signalling active, thereby providing nutrients from inflammatory spoils (Hajishengallis & Lambris, 2011). To achieve this, *P. gingivalis* employs a range of virulence factors including LPSs, fimbriae and lysine- and arginine-specific cysteine proteases, termed gingipains (Jia *et al.,* 2019). Gingipains are cell surface-anchored proteins (Andrian *et al.,* 2006), which can also be excreted in membrane-bound vesicles (Grenier *et al.,* 1989) and therefore can account for up to 85% of proteolytic activity around the *P. gingivalis* infection site (de Diego *et al.,* 2013).

A recent study using human umbilical vein cells as host cells suggested that the virulence of *P. gingivalis* (strain 381) may involve the UPR and ISR (Hirasawa & Kurita-Ochiai, 2018; Figure 1-14). In this study, the authors showed that whilst infection ultimately resulted in apoptosis after 21-h infection, early-stage infection (~8 h) resulted in ER stress characterised by increased expression of CHOP and BiP at both the transcriptional and translational levels coupled with increased caspase-12 activity (Hirasawa & Kurita-Ochiai, 2018; Figure 1-14 A–C). In addition, enhanced autophagy, characterised by the increased expression of autophagy markers Beclin-1, microtubule-associated protein 1A/1B-light chain 3 (LC3), and acidic vesicular organelles (Figure 1-14 D), was also observed. This response was inhibited by pretreatment with an ER stress inducer salubrinal, an inhibitor of PP1c, that results in blockage of eIF2α dephosphorylation (Boyce *et al.,* 2005). Furthermore, siRNA knockdown of LC3 resulted in increased apoptosis, thereby implicating ER stress-induced autophagy as a protective response against *P. gingivalis*-induced apoptosis (Hirasawa & Kurita-Ochiai, 2018). These results are corroborated by studies in mice where administration of *P. gingivalis* induced ER stress with increased expression of both CHOP and BiP (Yamada *et al.,* 2015).
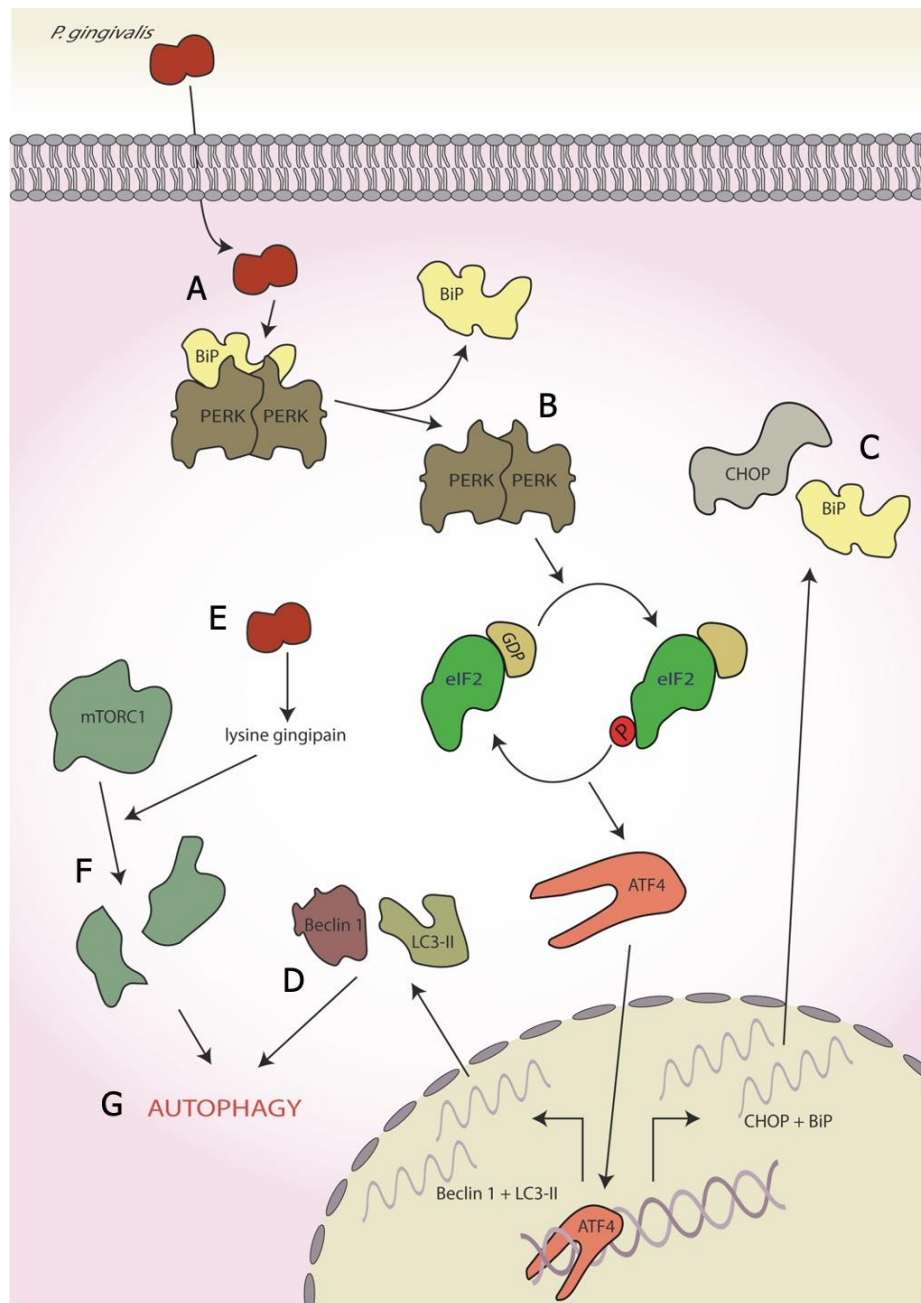
Figure 1-14 *Porphyromonas gingivalis*. (A) *P. gingivalis* infection induces ER stress, (B) leading to PERK activation, and (C) the expression of CHOP and BiP. (D) Concurrently, it also induces to autophagy as determined by the markers Beclin-1 and LC3-II. (E) *P. gingivalis* secretes a lysine-specific cysteine protease, termed the lysine gingipain, (F) which degrades mTORC1, and (G) leading to the induction of autophagy. Figure from Knowles *et al.,* (2021), used under creative commons licence.

Interestingly, the lysine-specific gingipain of *P. gingivalis* has been shown to degrade mTORC1 and modulate levels of mTORC1-associated proteins in oral epithelial cells after 4 h of infection (Stafford *et al.,* 2013; Figure 1-14 E,F). However, this mTOR degradation requires *P. gingivalis* internalisation, indicating that these effects are probably not mediated by the secretory fraction of gingipains produced by extracellular *P. gingivalis* (Stafford *et al.,* 2013). Inactivation of mTOR is known to induce autophagy (Jung *et al.,* 2010), fitting with the early stage autophagy seen by (Hirasawa & Kurita-Ochiai, 2018; Figure 1-14 G). Furthermore, mTOR inhibition by rapamycin suppresses tunicamycin-induced ER stress, resulting in autophagy (Dong *et al.,* 2015). Therefore, gingipain-mediated degradation of mTOR may help dampen ER stress induced by *P. gingivalis* infection, aiding host cell survival in the early stages of infection by delaying the onset of apoptosis.

1.2.2.6 Future Perspectives towards bacterial interactions with the ISR

Infection by pathogens triggers concerted whole organism immune responses by the host, which are often initiated at the cellular level. In fact, individual host cells can respond independently to adverse conditions via a variety of intracellular signalling systems, with the ISR being a key mediator of these responses and determining cellular fate (Costa-Mattioli & Walter, 2020). In recent years, it has become apparent that the ISR may have a wider role in host immune responses (Cláudio *et al.,* 2013; Pulendran, 2015). Here, we discuss recent advances in understanding host–microbe interactions, which demonstrate that bacterial pathogens can interact with the host ISR during infection, directly manipulating cellular fate and immune signalling. This demonstrates that a comprehensive understanding of pathogenic interactions with the ISR is crucial for the elucidation of microbial disease progression.

Particularly striking is that both *S. flexneri* and *P. aeruginosa* infection result in the activation of HRI, with *S. flexneri* inducing HRI activation leading to initiation of immune signalling as demonstrated by NF-κB activation (Abdel-

Nour *et al.,* 2019) and *P. aeruginosa* inducing HRI activation leading to increased host cell viability (van't Wout *et al.,* 2015). Interestingly, during monospecies bacterial infection (employed by most studies discussed here), HRI activation is required for *Y. pseudotuberculosis* and *Listeria monocytogenes* to achieve their virulence associated intracellular activities, where a lack of HRI interferes with the pathogens T3SS virulence factors (Shrestha *et al.,* 2013). In contrast, at an organismal level, HRI-deficient mice have been shown to be more susceptible to *L. monocytogenes* infection and less able to mount a system-level cytokine response (Bahnan *et al.,* 2018). This suggests potentially different outcomes depending on whether infection is monospecies or polymicrobial in nature, and hence, the exact role of HRI needs further attention. To date, HRI-specific inhibitors have been identified (Rosen *et al.,* 2009), and PERK inhibitors have already shown promise in combatting neurodegenerative diseases that impact upon the ISR (Moreno *et al.,* 2013). Greater understanding of the exact role of HRI in bacterial infection is therefore an area that may allow for the targeting of HRI as a novel antimicrobial therapy using inhibitors or activators in an infection-specific manner. Findings that internalisation efficiency of *Chlamydia trachomatis* was independent of HRI activity and that it was increased by loss of PKR (Shrestha *et al.,* 2013) support PKR as another potential therapeutic target.

Given the paradoxical role of the ISR, any change to a particular signal can have vastly different outcomes dependent upon the circumstance. For example, in neuronal cells, inhibition of PERK is protective during stress induction (Moreno, *et al.,* 2013); conversely in pancreatic cells, it induces type I interferon activation, proving to be fatal (Yu *et al.,* 2015). These opposing outcomes of ISR dysregulation are also apparent during bacterial infection. Where PERK activation by STEC and *Porphorymonas gingivalis* infection ultimately leads to cell death (Tsutsuki *et al.,* 2016; Hirasawa & Kurita-Ochiai, 2018), PERK activation by *P. aeruginosa* reduced the secretion of the pro-inflammatory cytokine KC, the mouse equivalent of IL-8, potentially aiding immune evasion (Grabiner *et al.,* 2014). Conversely, *S.*

flexneri infection and activation of both GCN2 and HRI showed the opposite of this phenotype, leading to robust upregulation of IL-8 (Abdel-Nour *et al.,* 2019). Although it remains to be elucidated whether these changes are cell type or infection specific, these conflicting phenotypes demonstrate the range of outcomes that bacterial manipulation of the ISR can have on immune signalling and cellular fate; however, further work is required to evidence this.

Furthermore, (Abdel-Nour *et al.,* 2019) found that inhibition of eIF2 signalling via the knock-in eIF2α S51A mutant induced a significant increase in intracellular *S. flexneri*, thereby implicating eIF2α signalling in the control of bacterial internalisation. The potential of eIF2α to control bacterial internalisation is corroborate, although inversely, by the findings of (Shrestha *et al.,* 2012), who reported that invasion of the Far East scarlet-like fever causing pathogen *Yersinia pseudotuberculosis* resulted in a 25-fold increase in MEFs containing the eIF2α S51A knock-in compared with wild type. The authors also identified functional eIF2α signalling as a prerequisite for cytokine expression and demonstrated that *Y. pseudotuberculosis* specifically dampens eIF2 phosphorylation during a range of cellular stresses through the action of a virulence factor YopJ, which is inserted directly into host cells via a T3SS. Ultimately, this resulted in decreased pro-inflammatory cytokine expression (Shrestha *et al.,* 2012). These findings further demonstrate the potential for ISR manipulation as an immuno-evasive mechanism of bacterial pathogens.

During prolonged ER stress, LPS is known to trigger a TLR-dependent reprogramming of the ISR (Woo *et al.,* 2012). LPS is detected by TLRs, which triggers a signalling cascade mediated by the action of its downstream adaptor TRIF and results in decreased serine phosphorylation of eIF2Bε, thereby increasing eIF2B GEF activity in a manner independent of eIF2α phosphorylation (Woo *et al.,* 2012). This increased eIF2B GEF activity results in suppression of CHOP, increasing cell survival, and increased

translation of the pro-inflammatory cytokine TNF-α (Woo *et al.,* 2012). Both *P. aeruginosa* and *P. gingivalis* induce the production of host TNF-α dependent on their LPS (Nativel *et al.,* 2017; Raoust *et al.,* 2009). Therefore, given that both *P. aeruginosa* and *P. gingivalis* infections are long term and chronic and can induce ER stress and PERK activation, it is entirely plausible that the TNF-α expression may be at least partially dependent on the TLR/TRIF eIF2B pathway (Grabiner *et al.,* 2014; van 't Wout *et al.,* 2015; Yamada *et al.,* 2015; Hirasawa & Kurita-Ochiai, 2018). Indeed, *P. gingivalis* survival is known to hinge upon increased inflammatory signalling, whilst dampening host antimicrobial responses, all in a TLR-dependent manner (Hajishengallis & Lambris, 2012). Here, investigation into this potential reprogramming of the ISR during infection may yield crucial information into *P. gingivalis* virulence and may point to the potential of therapeutic targeting of eIF2B activity during chronic infection. Given that Woo *et al.,* (2012) only investigated the TLR-dependent ISR reprogramming under ER stress, it is entirely possible that this signalling cascade may also occur during other stresses; therefore, all of the bacteria discussed above may induce this response. The limiting factors would be host cell survival time and cytotoxicity of infection, as this cascade occurs primarily during long-term stress (Woo *et al.,* 2012).

It should be noted that most of the studies to date have investigated the role of a single species upon a single cell type, whereas most bacterial infections are polymicrobial (Brogden *et al.,* 2005). As with host immune responses, pathogenic bacteria are known to interact with other microbes; indeed, the virulence and disease severity of both *P. gingivalis* and *Salmonella* infection are thought to be reliant upon their ability to manipulate the wider bacterial community (reviewed in Hajishengallis *et al.,* 2012). *P. aeruginosa* is also known to secrete products, which have a community wide effect in cystic fibrosis patients, ultimately shaping microbial community dynamics within the lung (Reviewed in O'Brien & Fothergill, 2017). Whilst, pyocyanin, a quorum-sensing molecule secreted by *P. aeruginosa* in response to Gram-negative cell wall fragments, is thought to reduce microbial community

diversity to select for a more pathogenic community (Norman *et al.,* 2004; Korgaonkar & Whiteley, 2011; Korgaonkar *et al.,* 2013), pyocyanin also functions to generate ROS (Xu *et al.,* 2013), a known inducer of the ISR. Given that secretion of pyocyanin is governed by inter-bacterial communication, which is inherently non-linear (Dietrich *et al.,* 2006), alterations in pyocyanin concentrations could induce differential ROS production over time. This could plausibly result in oscillation of host ISR activation, adding another layer of complexity to the ISR dampening interaction seen during monospecies *P. aeruginosa* infection. Furthermore, *Bifidobacterium* spp. protects mice from STEC toxicity via the production of acetate, which inhibits the subAB toxin produced by STEC (Fukuda *et al.,* 2011; Fukuda *et al.,* 2012). This lowering of toxicity may well be due dampening of STEC-mediated ISR activation in host tissues around the infection sites, especially as subAB self-internalises (Morinaga *et al.,* 2007), which is likely be in contact with the extracellular acetate before the internalisation event. Therefore, given the role of the wider bacterial community upon virulence, studying the interactions between polymicrobial communities and the host ISR may lead to advances in the understanding of host–pathogen interactions, reflect physiological conditions and act as a platform for possible therapies.

1.3   Project Overview

In recent years, the regulation of translation initiation and its shutdown during stress have become increasingly implicated within cellular responses to microbial infections. However, the dynamics of host cellular translational control during stress and *Porphyromonas gingivalis* infection have not been previously investigated. *P. gingivalis* is known to interact with or degrade multiple cellular regulators of translation initiation, and furthermore the process of infection generates potentially stressful conditions. This project therefore aims to elucidate the molecular mechanisms of interactions between *P. gingivalis* and host cellular translational control, with or without a chemically recapitulated oxidative stress environment.

The hypothesis is that *P. gingivalis* infection will induce ISR activating stress within its host and heighten the tightly controlled down-regulation of translation during cellular stress.

This will be met by addressing the following aims:

- Determining whether *P. gingivalis* infection induces the host ISR activation
- Investigating the effects of *P. gingivalis* infection during a chemically recapitulated ISR activating environment
- Investigate the effect of *P. gingivalis* infection on stress granule formation
- Probing the molecular mechanisms of the interaction between *P. gingivalis* and translational regulation during ISR activation

2 Chapter 2: Materials and Methods

2.1 Materials

All chemical reagents utilised in this study unless otherwise stated were purchased from Sigma-Aldrich or ThermoFisher Scientific. All manufacturers and suppliers' addresses are shown in Table 2-1.

Table 2-1 Manufacturers and suppliers

Supplier	Location
Abcam	Cambridgeshire, UK
Bio-Rad	Watford, UK
Carl Zeiss	Cambridge, UK
Cell Signalling Technologies	Leiden, NL
Difco Laboratories	East Molesey, Surrey, UK
Don Whitley Scientific	Bingley, West Yorkshire, UK
Gibco	Loughborough, UK
Hawksley	Lancing, Sussex, UK
Invitrogen	Paisley, Renfrewshire, UK
LAB-M	Bury, UK
Leica	Sheffield, UK
Li-Cor	Cambridge, UK
Merck.	Feltham, UK
Pro-Lab Diagnostics	Birkenhead, UK
Roche	Poole, UK
Sigma-Aldrich	Poole, UK
Statsdirect Ltd	Birkenhead, UK
TCS Biosciences	Buckingham, UK
ThermoFisher Scientific	Loughborough, UK
UK Health Security Agency	Porton Down, Salisbury, UK

2.2 Antibodies

The *P. gingivalis* mAb 1B5 antibody was a kind gift from Professor Mike Curtis (Centre for Host Microbiome Interactions, Kings College London, London, UK) and the *P. gingivalis* whole cell antibodies were a kind gift from Professor Graham Stafford (School of Clinical Dentistry, University of Sheffield, UK). Details of the *P. gingivalis* antibodies used as detailed in Table 2-2.

Table 2-2 Primary bacterial specific antibodies used in this study

Antibody Name	Species	Raised Against
mAb 1B5	Mouse	*Porphyromonas gingivalis* LPS
PG Whole Cell 1	Rabbit	*Porphyromonas gingivalis* NCTC11834
PG Whole Cell 2		*Porphyromonas gingivalis* NCTC11834

Table 2-3 Primary mammalian specific antibodies used in this study

Antibody	Species	Catalogue Number	Clonality	Company
α-Tubulin	Mouse	2144	Polyclonal	Cell Signalling
Acetyl-α-Tubulin	Rabbit	1252	Polyclonal	Cell Signalling
eIF2α	Mouse	ab181467	3A7B11	Abcam
p-eIF2α	Rabbit	44-728G	Polyclonal	ThermoFisher
eIF3b	Rabbit	ab133601	ERP5804	Abcam
GAPDH	Mouse	G9545	Polyclonal	Sigma
GAPDH	Rabbit	PLA0125	Polyclonal	Sigma
G3BP	Mouse	ab56574	2F3	Abcam
HDAC6	Mouse	7558	D2E5	Cell Signalling
Puromycin	Mouse	MABE343	12D10	Merck
p-p70-S6Kinase (T389)	Rabbit	9234	108D2	Cell Signalling
Tubulin	Rat	ab6161	YOL1/34	Abcam
4E-BP1	Rabbit	9452	Polyclonal	Cell Signalling
p-4E-BP1 (Thr 37/46)	Rabbit	2855	236B4	Cell Signalling

Table 2-4 Secondary antibodies used for immunofluorescence in this study

Antibody	Species	Catalogue Number	Clonality	Company
Alexafluor 488 anti Mouse	Goat	ab150113	Polyclonal	Abcam
Alexafluor 488 anti Rabbit	Goat	ab150077	Polyclonal	Abcam
Alexafluor 488 anti Rat	Goat	ab150157	Polyclonal	Abcam
Alexafluor 568 anti Mouse	Goat	ab175473	Polyclonal	Abcam
Alexafluor 633 anti Mouse	Goat	A-21050	Polyclonal	ThermoFisher
Alexafluor 633 anti Rabbit	Goat	A-21070	Polyclonal	ThermoFisher

Table 2-5 Secondaries antibodies used for western blotting in this study

Antibody	Species	Catalogue Number	Clonality	Company
680RD anti Mouse	Goat	926-68070	Polyclonal	Li-Cor
680RD anti Rabbit	Goat	926-68071	Polyclonal	Li-Cor
800CW anti Mouse	Goat	926-32210	Polyclonal	Li-Cor
800CW anti Rabbit	Goat	926-32213	Polyclonal	Li-Cor

2.3 Bacterial Culture

2.3.1 Bacterial Strains

All bacterial strains used were a kind gift from Professor Graham Stafford (School of Clinical Dentistry, University of Sheffield, UK).

Table 2-6 Bacterial Strains and mutants used within this study.

Species	Genotype	Mutation	Relevant characteristics
Porphyromonas gingivalis	NCTC11834	Wild type	NCTC Strain
	ATCC381	Wild type	ATCC Strain
	ATCC53978 (W50)	Wild type	ATCC Strain
	W50 K1A	Δ *kgp::Em^R*	*kgp* deletion mutant of ATCC53978 (Aduse-Opoku *et al.,* 2000)
	W50 E8	Δ *rgpA::Em^R* Δ *rgpB::Tet^R*	*rpg*A, *rgp*B double deletion mutant of ATCC53978 (Aduse-Opoku *et al.,* 2000)
	W50 EK18	Δ *rgpA::Em^R* Δ *rpgB::Tet^R* Δ *kgp::Chlor^R*	*rpg*A, *rgp*B, *kgp* triple deletion mutant of ATCC53978 (Stafford, *et al.,* 2013)

Em^R – erythromycin resistance, *Tet^R* – tetracycline resistance, *Chlor^R* – chloramphenicol resistance.

2.3.2 Bacterial growth

2.3.2.1 On solid agar

P. gingivalis were grown and maintained on fastidious anaerobe agar (Lab M) containing oxalated horse blood (5 % (v/v); TCS Biosciences) and supplemented with antibiotics (outlined in section 2.3.1) as required under anaerobic conditions (10 % CO_2, 10 % H_2 and 80 % N_2) at 37 °C in a Don Whitley Scientific A25 anaerobic workstation. Bacteria were subcultured every 3-4 days for maintenance. Throughout this study, bacteria used to infect cells were no older than 3-4 days old post-subculturing.

2.3.2.2 As liquid culture

P. gingivalis were grown as liquid cultures in brain heart infusion broth (BHI, Difco laboratories) supplemented with 0.5 % (w/v) yeast extract, hemin (5 µg/ml), vitamin K (0.5 µg/ml) and cysteine (0.1% (w/v). Purity of liquid cultures was confirmed by Gram staining (section 2.3.4) before use.

2.3.3 Bacterial counting

Bacterial cells used for infection of mammalian cells (section 2.5) were counted using a Helber Bacterial Counting Chamber (Hawksley) visualised on a Leica DMi1 (Leica) light microscope at 400x magnification.

2.3.4 Gram staining

Gram staining was carried out using the Pro-Lab Diagnostics Gram Staining Kit (Pro-Lab Diagnostics) according to manufacturer's instructions. In brief, samples were heat fixed to slides, stained with crystal violet (1 min), washed with water, stained with Grams iodine (1 min), washed with water, and decolourised with differentiator (10 sec) Finally samples were washed, stained with counter stain (1 min), washed and air dried. Gram staining was visualised using a Leica DMi1 (Leica) light microscope at 400x magnification.

2.3.5   Long term bacterial storage

For long term storage, bacteria were stored at -80°C. Bacteria were grown on plates as detailed in section 2.3.2.1. One loop of bacteria was mixed into 1 mL of BHI broth (supplemented as outlined in 2.3.2.2) containing 50 % (v/v) glycerol. Once mixed the resulting mixture was immediately frozen in a -80 °C freezer.

2.3.6   Crude isolation of *P. gingivalis* outer membrane vesicles

Crude *P. gingivalis* (strain NCTC11834) OMVs were extracted as previously described (Dong *et al.,* 2018). *P. gingivalis* were grown to late exponential phase overnight in liquid culture as outlined above. The next day, cultures were adjusted by back dilution to an OD_{600} of 1.0 and then centrifuged at 8,000 x g for 5 min at 4 °C. The resulting supernatant was filtered-sterilised (0.22 µM) and further centrifuged (100,000 x g, 2h, 4 °C), after which the supernatant was discarded and the pellet resuspended in 1 x phosphate buffered saline (PBS). Protein content of the OMVs was determined as outlined in section 2.10 and used to challenge H357 cells.

2.4   Mammalian Cell Culture

The oral squamous carcinoma derived cell line (H357) (UK Health Security Agency) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, ThermoFisher Scientific) supplemented with 10 % (v/v) foetal bovine serum (FBS) and 1 % (w/v) L-glutamine (Glu).

The neuroblastoma cell line (SHSY-5Y) was maintained in F-12 Ham (Gibco) supplemented with 10 % (v/v) FBS, 1 % L-glutamine (w/v) (Glu) and 1 % (w/v) sodium pyruvate.

Primary astrocytes (a kind gift from the Campbell Lab Group, Sheffield Hallam University, UK) were maintained in minimum essential medium (MEM; Gibco) supplemented with 10 % (v/v) FBS, 1 % (w/v) L-glutamine, 1 % (w/v) sodium

pyruvate and 1 % (w/v) nonessential amino acids. All cells were cultured in a humidified environment (5 % CO_2, 37°C).

2.4.1   Cell passaging

Cells were passaged when 75 % confluent by trypsinization for 5-10 minutes until cells were fully detached, following which trypsin was neutralised using complete media. Cells were pelleted by centrifugation (200 x g, 5 mins, RT), the supernatant discarded, and the cell pellet was resuspended in fresh complete media and incubated as detailed above.

2.4.2   Cell counting

Mammalian cells were counted automatically using a Thermo Invitrogen Cell countess II FL (ThermoFisher), according to manufacturer specification. During counts cell viability was assessed using the trypan blue exclusion method and dead cells were automatically discounted from cell counts.

2.4.3   Cryopreservation of Cells

For cryopreservation, cells were trypsinised and counted as detailed above. Following this, cells were re-suspended in freezing medium (10% dimethyl sulphoxide (DMSO), 90 % FBS (V/V)) at a density of 3×10^6 cells per mL. One millilitre of the resulting solution was added to each cryovial, which were subsequently frozen at rate of 1 °C per minute using a Mr Frosty™ (ThermoFisher) freezing container for 24 hours at -80 °C. Frozen cells were transferred to liquid nitrogen storage within 1 week of initial freezing.

2.5   Infection of mammalian cells with *P. gingivalis*

Cells were seeded at a density of 6×10^4 cells/cm² on coverslips or at 3.6×10^4 per cm² in tissue culture flasks in DMEM/Glu/FBS, following which cells were incubated (5 % CO_2, 37 °C) and allowed to adhere overnight. After replacement of overnight media with fresh media, cells were challenged with

P. gingivalis at a multiplicity of infection (MOI) of 100 at the time points as detailed below. Oxidative stress was induced using sodium arsenite (SA, 250 µM) which was added for the final 30 min of infection. Cells were also treated with or without integrated stress response inhibitor (ISRIB) (200 nM, 30 min), Nocodazole (200 nM, 30 min), Rapamycin (400 nM, t=1h) or *P. gingivalis* (NCTC11834) derived LPS (at 1, 5 or 10 µg/mL, t=2 hours). Untreated cells were included as control. Following treatment, cells were either fixed with ice-cold methanol (-20 °C, 10 mins) or protein extracted as detailed in section 2.10.

2.6 Challenge of cells with conditioned media

To generate *P. gingivalis* infection conditioned media, H357 cells were infected (MOI of 100) as described above. Following infection, conditioned media was recovered and filtered (0.22 µm) to remove bacteria and other particulate matter. Untreated H357 cells were then challenged with the filtered conditioned media for 2h. For gingipain inhibition studies, H357 cells were challenged with conditioned media supplemented with either leupeptin (0.2 mM) or Na-Tosyl-Lysine Chloromethyl Ketone (TLCK, 0.5 mM) after which total protein was extracted as detailed in section 2.10 and levels of proteins of interest were probed by Western blotting as detailed in section 2.11.

2.7 Challenge of cells with high concentration conditioned media

High concentration mammalian cell culture media conditioned by *P. gingivalis* was generated as described previously (Nakayama *et al.,* 2015). Briefly, *P. gingivalis* were grown in liquid culture at concentration of 1×10^8/mL in DMEM for 24 hours (10 % CO_2, 10 % H_2 and 80 % N_2) at 37 °C. The next day the OD_{600} adjusted by back dilution to 1.0 and the purity of the *P. gingivalis* culture was confirmed by Gram staining as outlined in 2.3.4. Next, cultures were subjected to centrifugation (8,000 x g, 5 min, 4 °C). The resulting supernatant was passed through a 0.2 µm filter to remove any remaining whole bacteria. The pure conditioned media was used to challenge H357 cells for the times

indicated after which total protein was extracted as detailed in section 2.10 and levels of proteins of interest were probed by Western blotting as detailed in section 2.11.

2.8 Puromycin incorporation (SUnSET) assay

The relative rates of protein synthesis, after cells were treated as detailed above, were determined using the non-radioactive fluorescence activated surface sensing of translation (SUnSET) assay as described previously (Hodgson *et al.,* 2019). Briefly, post treatment cells were incubated in culture media containing puromycin (91 µM) and emetine (208 µM) for 5 minutes (5 % CO_2, 37 °C). Cells were then washed twice with 1 x PBS containing cycloheximide (355 µm) and total protein was extracted as detailed in section 2.10, following which puromycin uptake was probed by western blotting section 2.11.

2.9 Immunofluorescence confocal microscopy

Methanol-fixed cells were washed with PBS supplemented with Tween 20 (0.5 % v/v; PBST), following which the cells were blocked in PBS supplemented with bovine serum albumin (BSA) (1 % w/v) for a minimum of 1 hour at room temperature before incubation with primary antibodies, detailed in Table 2-2 and

Table 2-3, overnight at 4 °C. After washing with PBST (3 x 5 min), membranes were incubated with corresponding fluorescent Alexa fluor™ conjugated secondary antibodies, detailed in Table 2-4, for 1 hour at room temperature. Cells were washed with PBST and mounted using ProLong Gold™ antifade mountant containing DAPI (ThermoFisher). Protein localisation was visualised using a Zeiss LSM800 microscope (Carl Zeiss). Images were captured using ZenBlue software, either a 40x or 63x plan-apochromat oil objective and a laser with maximum output of 10mW at 0.2 % laser transmission. Stress granule frequencies, area and co-localization were quantified using the analysis module of Zeiss ZenBlue software (Carl Zeiss).

2.10 Protein extraction

After treatment cells were washed with phosphate buffered saline (PBS), before the addition of lysis buffer (PBS supplemented with 10 %(v/v) PhosStop (Roche), 10 % (v/v) complete EDTA-free protease inhibitors and 0.1 %(v/v) SDS). Total proteins were extracted using a cell scraper and cell lysates were stored at -80 °C for a minimum of 1 hour, or overnight after which proteins were recovered by centrifugation (17 200 x g, 14 min, 4 °C) and stored at -80 °C until required. Total protein extracts were quantified using the Qubit™ protein assay (ThermoFisher) according to manufacturer instructions and expression levels of proteins of interest probed by Western blotting.

2.11 Western blotting

For western blotting, total protein extracts were subjected to SDS page electrophoresis using 4-20 % polyacrylamide gradient gels (Bio-Rad) and transferred to nitrocellulose membranes using a Trans-blot Turbo transfer system (Bio-Rad). Membranes were blocked in either (5 % w/v) BSA or powdered milk prepared in Tris Buffered Saline (TBS; 37 mM NaCl, 20 mM Tris, pH 7.6) supplemented with 0.1 % (v/v) Tween 20 (TBST) for 1 hour at room temperature before incubation with primary antibodies, detailed in

Table 2-3, overnight at 4 °C. After washing with TBST (3 x 5 min), membranes were incubated with the corresponding fluorescent conjugated secondary antibodies, detailed in Table 2-5, for 1 h at room temperatures. Proteins were visualised using a Li-Cor Odyssey infrared imager (Li-Cor) and quantified using Image Studio Lite software (Li-Cor).

2.12 Statistical analysis

Significance between groups was analysed using the StatsDirect software package (Statsdirect Ltd). Data was first subjected to a Shapiro-Wilks test where data was considered parametric if $p < 0.05$. All data was found to be non-parametric. Significance between unpaired groups was determined using

a Kruskal-Wallis test, which if significant was followed by a Conover-Inman *post-hoc* test. Significance was set at p≤ 0.05; **** $P \leq 0.001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

3 Chapter 3: Crosstalk between *P. gingivalis* infection, exogenous stress and the host translational control

3.1 Introduction

Periodontitis is caused by a variety of pathogenic bacteria, with the most prominent being the keystone pathogen *Porphyromonas gingivalis* (Hajishengallis *et al.,* 2012). Progression of periodontitis leads to an increasingly cytotoxic environment within the periodontal pocket with increasing levels of bacterial metabolites and oxidative stress due to neutrophil activation (Peake & Suzuki, 2004). Under such stress conditions, host cells activate a number of signalling cascades, one of which is the concerted cellular reprogramming system, the ISR, which functions to determine cellular fate via targeting translational control (Costa-Mattioli & Walter, 2020).

Functionally, the ISR initially causes a global downregulation of protein synthesis, which sets out to conserve energy and allow the activation of a stress response gene expression program, thereby aiding the cell to either overcome the stress or initiate programmed cell death (Costa-Mattioli & Walter, 2020). A variety of stresses, including bacterial infection, activate one or more of four stress response kinases, PKR, PERK, GCN2 and HRI (discussed in section 1.2.1.5). Once activated, these stress response kinases converge upon the phosphorylation of eIF2α at serine 51 (Siekierka *et al.,* 1982; Donnelly *et al.,* 2013). Stress-induced eIF2α phosphorylation blocks the ability of eIF2B to regenerate eIF2-GTP resulting in the abrogation of global translation by inhibiting the formation of active ternary complex (Rowlands *et al.,* 1988; Dever *et al.,* 1995; Kenner *et al.,* 2019) (discussed further in section 1.2.1.5). The activation of translational shutoff pathways results in stalled mRNPs, which are aggregated into cytoplasmic condensates known as stress granules. These function to aid sorting of mRNPs into those which will be degraded, or re-initiation if stress is overcome and translation resumes (Nover *et al.,* 1989; Anderson & Kedersha, 2002).

Several bacterial pathogens are known to activate and modulate the host ISR during their infection cycles (discussed in section 1.2.2). *P. gingivalis* has previously been shown to induce activation of the Unfolded Protein Response (UPR; Hirasawa & Kurita-Ochiai, 2018), which interlinks with the ISR (Harding *et al.,* 2003). This finding coupled with the fact that periodontal infection produces possible stress through inflammation (Peake & Suzuki, 2004; Hajishengallis & Lambris, 2012) suggests that *P. gingivalis* infection may be capable of activating and interacting with the host ISR.

3.2 Aims

The overall aim of this chapter is to investigate whether *P. gingivalis* can activate or interact with the host integrated stress response. This will be achieved by:

- Investigating the potential of *P. gingivalis* infection alone to activate the ISR
- Optimising the chemical recapitulation of an oxidative ISR activating environment
- Investigating interactions between *P. gingivalis* infection and the ISR activated by chemical oxidation

3.3 Results

3.3.1 Confocal microscopy identifies internalised *P. gingivalis*

To confirm that it was possible to differentiate between internalised, adhered or cell free *P. gingivalis,* super-resolution confocal microscopy using Z-stacking was utilised together with antibodies raised against *P. gingivalis* and Ras GTPase-activating protein-binding (G3BP), cytosolically dispersed protein that is also a canonical stress granule marker. By generating three dimensional images of slices taken throughout H357 cells which had been infected (NCTC11834, MOI of 100, t=24h). *P. gingivalis* antibody signal could clearly be detected within the slices containing the cytosolically dispersed G3BP antibody signal and be clearly differentiated from *P. gingivalis* outside of the intracellular G3BP signal (Figure 3-1). These results confirm that confocal microscopy and Z-stacking could positively and reliably identify cells infected with *P. gingivalis.*
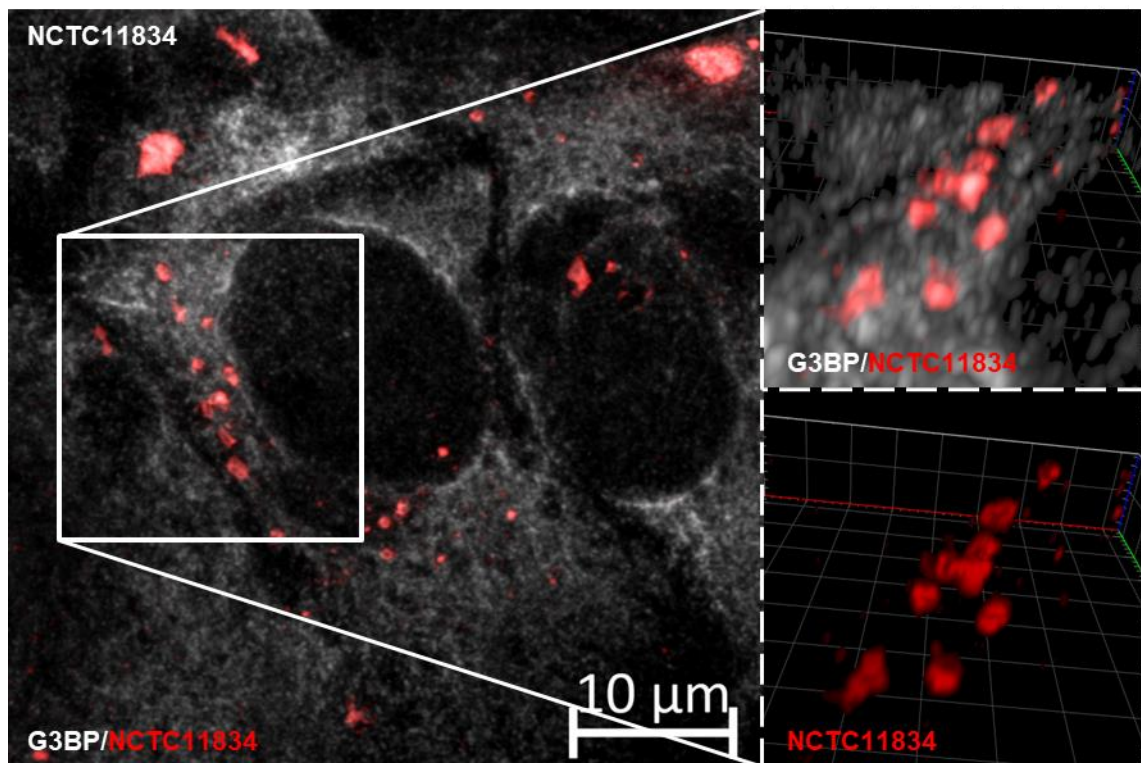
Figure 3-1 Confocal microscopy identified internalised *P. gingivalis*. H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=24h). G3BP (white) and *P. gingivalis* (red) were visualised using immunofluorescence confocal microscopy. Scale bar is 10 μm.

3.3.2 *P. gingivalis* infection alone does not induce cellular stress

Some bacterial infections can lead to an oxidative stress environment which is known to activate the host ISR (Knowles *et al.,* 2021). To determine if *P. gingivalis* can induce the host ISR during infection, the effects on global protein synthesis and eIF2α phosphorylation were monitored in infected H357 cells (t=2, 4, 6 and 24h; MOI of 100). Infection did not alter the rate of translation, as measured by puromycin incorporation (Figure 3-2 A) and eIF2α phosphorylation (Figure 3-2 B). These results indicate that *P. gingivalis* infection alone over a period of two to 24 hours does not induce the core functions of the ISR.
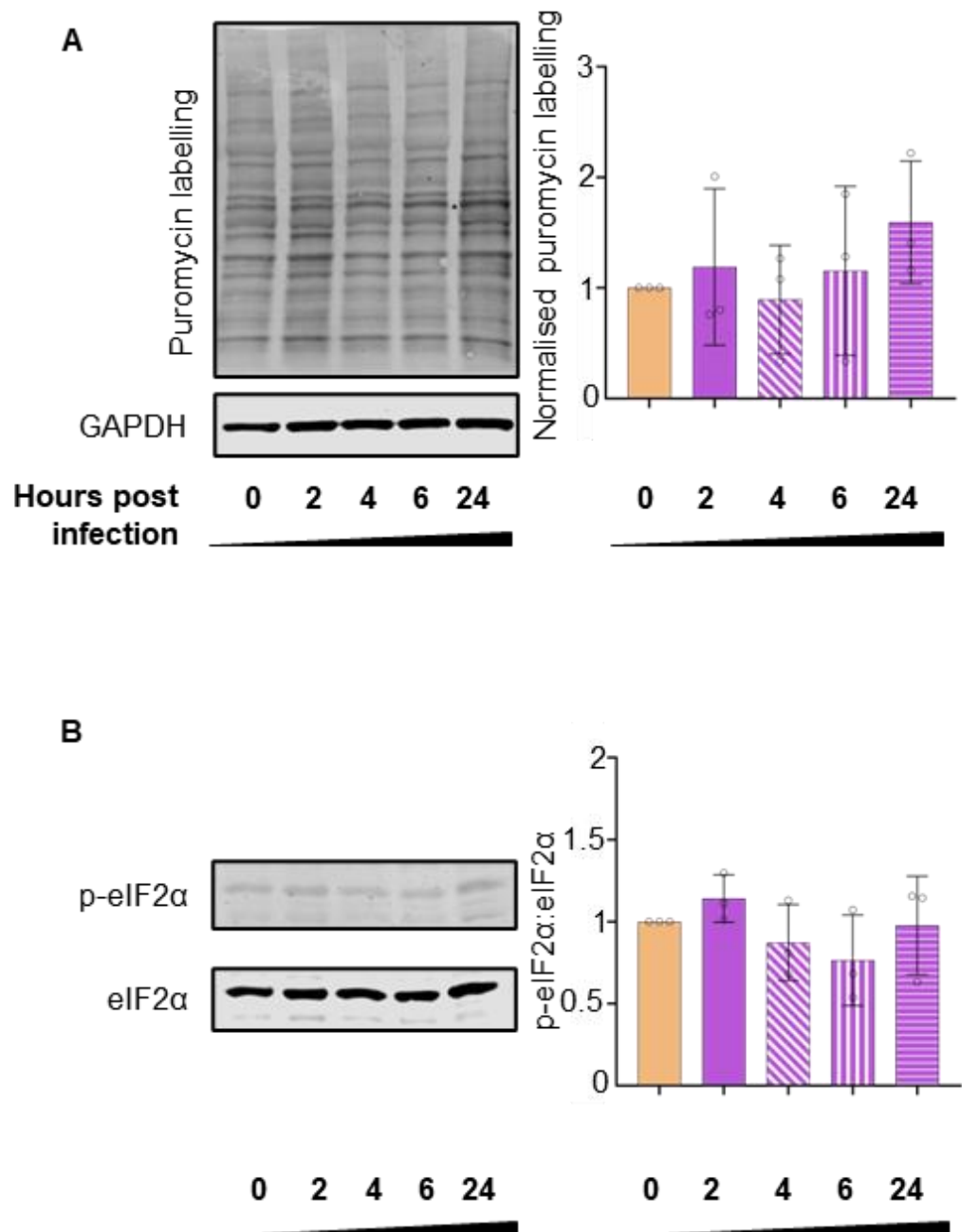
Figure 3-2 *P. gingivalis* infection does not induce ISR activation. (A) H357 cells were left untreated, infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2 to 24h). The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated. (B) Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.

3.3.3 Lack of stress induction during *P. gingivalis* infection is accompanied by lack of stress granule formation

During stress-induced translational attenuation, stalled mRNPs are aggregated into cytoplasmic foci, termed stress granules. To further assess cellular stress responses during *P. gingivalis* infection stress granule formation was assessed over a period of two, four, six and 24 hours, with oxidative stress (sodium arsenite, 250 μM) as a control. Stress granules were visualised using immunofluorescence microscopy, probing for G3BP (white) and *P. gingivalis* (red). Despite infection being observed at all time points no stress granule formation was observed over the time period investigated; further implying that *P. gingivalis* infection alone does not activate the ISR Figure 3-3).
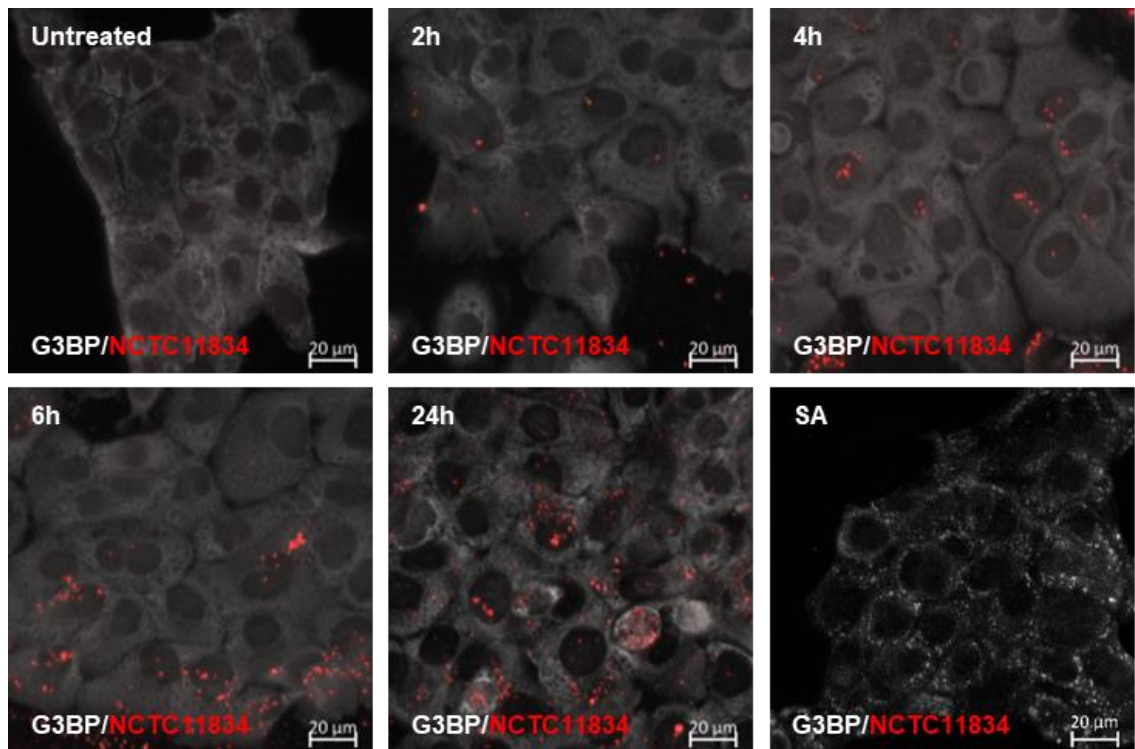
Figure 3-3 *P. gingivalis* infection does not induce stress granule formation. H357 cells were left untreated, infected with *P. gingivalis* (NCTC11834, MOI 1 in 100, t=2h to 24h) or, treated with sodium arsenite (SA; 250µM, t = 30mins). G3BP (white) and *P. gingivalis* (red) were used to visualise stress granule formation and infection, respectively, by immunofluorescence confocal microscopy. Scale bar is 20 µm.

3.3.4 Optimisation of chemical recapitulation of oxidative stress using sodium arsenite

Although *P. gingivalis* did not induce the ISR directly, it is known that host immune responses to infection such as inflammation may themselves produce a potentially stressful environment due to induction of oxidative radicals from neutrophil activation inflammation (Peake & Suzuki, 2004; Hajishengallis & Lambris, 2012). To study the impact of such an environment on infection sodium arsenite, a well-documented ISR inducing stressor (Brostrom & Brostrom, 1997; McEwen *et al.,* 2005), was used to chemically recapitulate an oxidative stress environment.

First the ability of sodium arsenite to induce the ISR and the subsequent effects on cellular viability were investigated. H357 cells were challenged with a range of concentrations of sodium arsenite (0, 125, 250 and 500 µM) for 30 mins. All concentrations induced cellular stress as indicated by decreased puromycin incorporation (Figure 3-4), increased eIF2α phosphorylation (Figure 3-5) and stress granule formation (Figure 3-6). However, 250 and 500 µM sodium arsenite showed a robust decrease in protein synthesis of 4.5-fold and 7.86-fold respectively (Figure 3-4), an increase in eIF2α phosphorylation 3.45-fold and 4.88-fold respectively (Figure 3-5), and both treatments induced G3BP stress granules in 98 % of cell (Figure 3-6). At 125 µM sodium arsenite only decreased translation 2.8-fold (Figure 3-4), increased eIF2α phosphorylation by 2.55-fold (Figure 3-5)and induced stress granules in only 73 %of cells (Figure 3-6).

In addition viability of cells was investigated and no significant change to cell death as determined by Hoechst 3342 and propidium iodide staining was observed when compared to the control (Figure 3-7). As 250 µM sodium arsenite showed a robust induction of cellular stress similar to 500 µM this concentration was chosen for future studies.
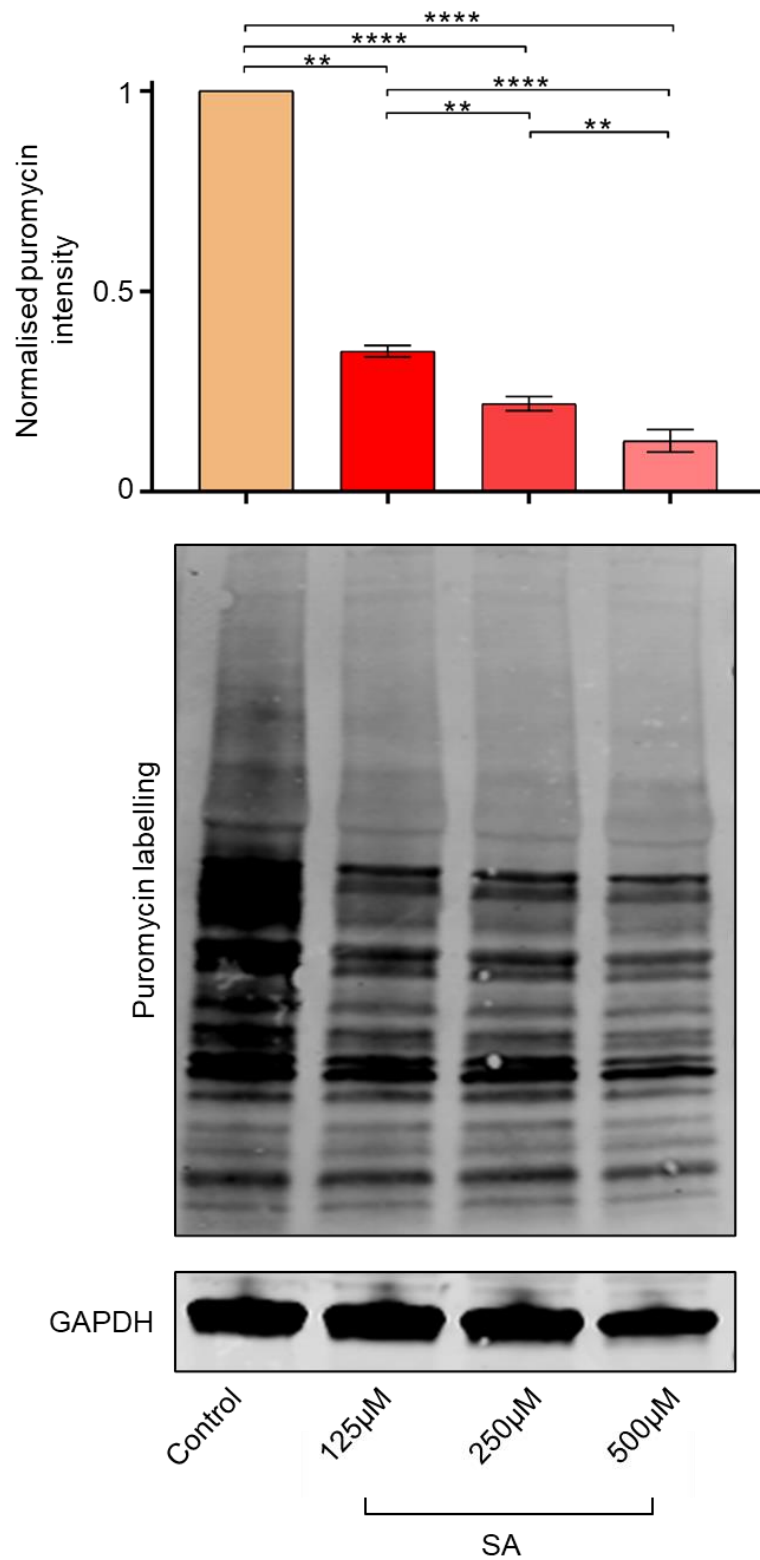
Figure 3-4 Concentration dependent inhibition of translation by sodium arsenite. H357 cells were left untreated or challenged with sodium arsenite (SA; t = 30mins). The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). **** $P \leq 0.001$; **, $P \leq 0.01$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
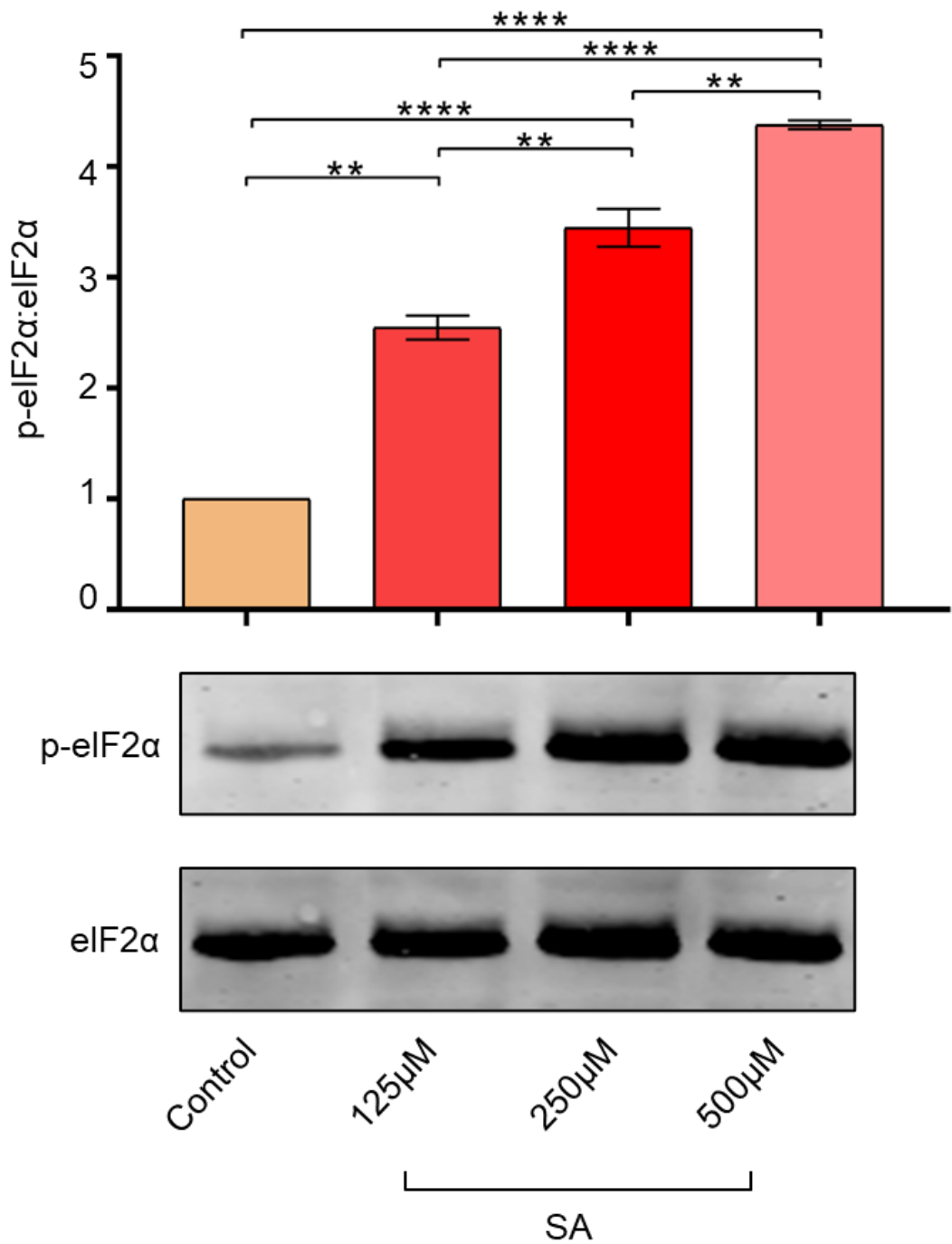
Figure 3-5 Concentration dependent eIF2α phosphorylation by sodium arsenite. H357 cells were left untreated or challenged with sodium arsenite (SA; t = 30mins). Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). **** $P \leq 0.001$; **, $P \leq 0.01$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
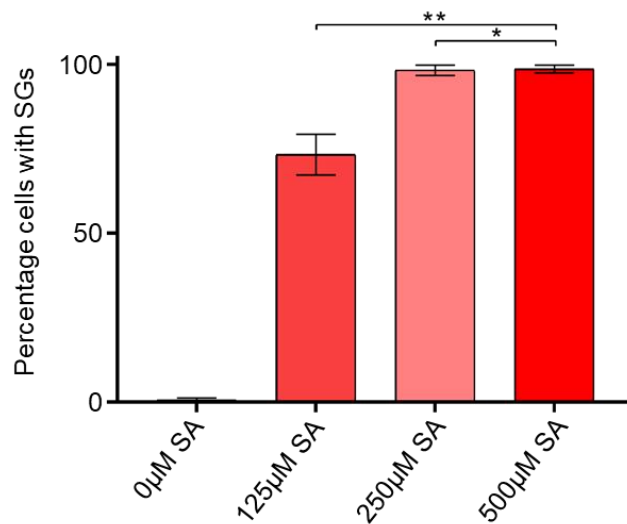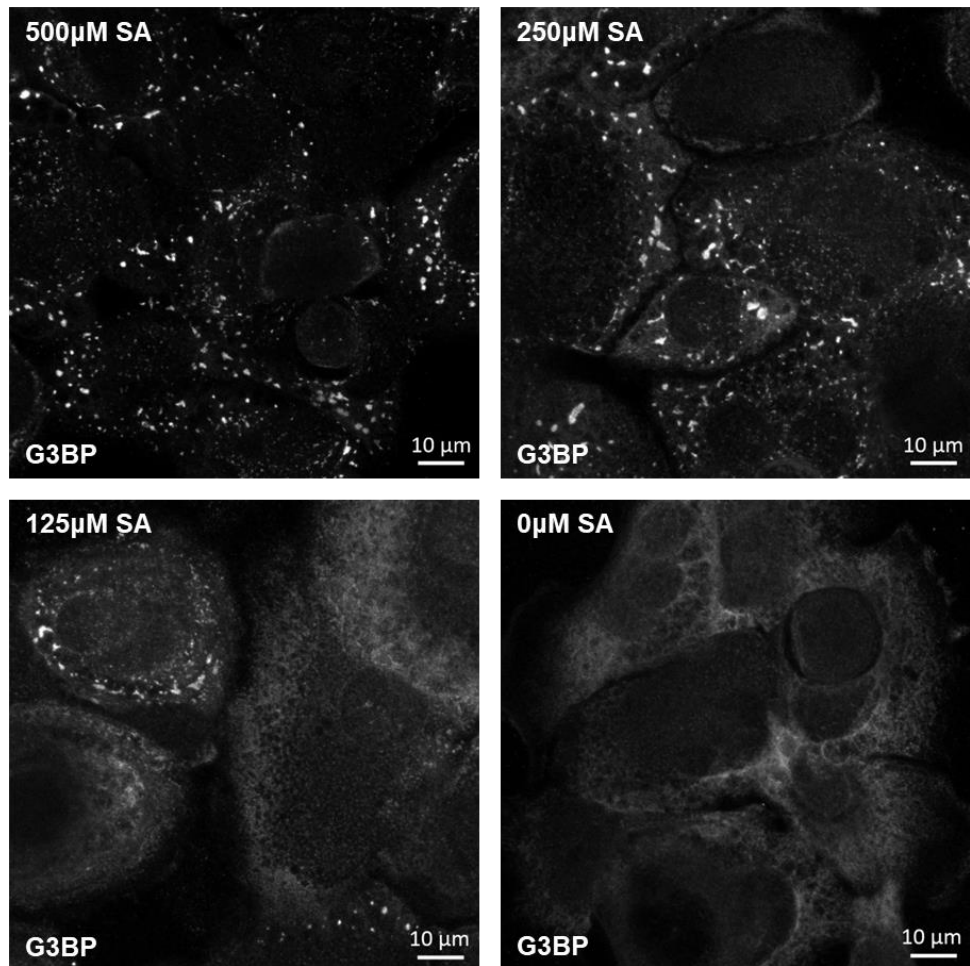
Figure 3-6 Concentration dependent stress granule formation during sodium arsenite challenge. H357 cells were left untreated or challenged with sodium arsenite (SA; t = 30mins). G3BP (white) was visualised using immunofluorescence confocal microscopy (mean ± SD, n=3, 100 cells per biological replicate). **, $P \leq 0.01$; *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test. Scale bar is 10 μm.
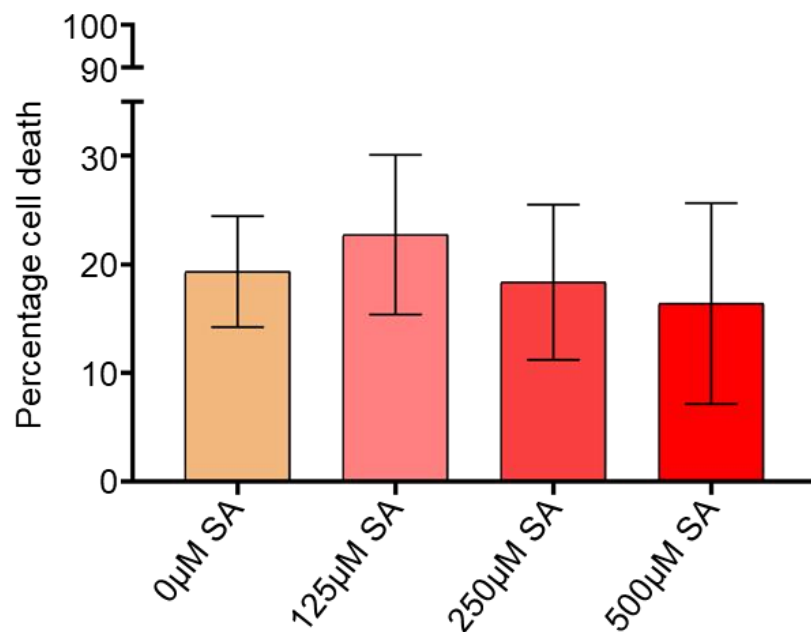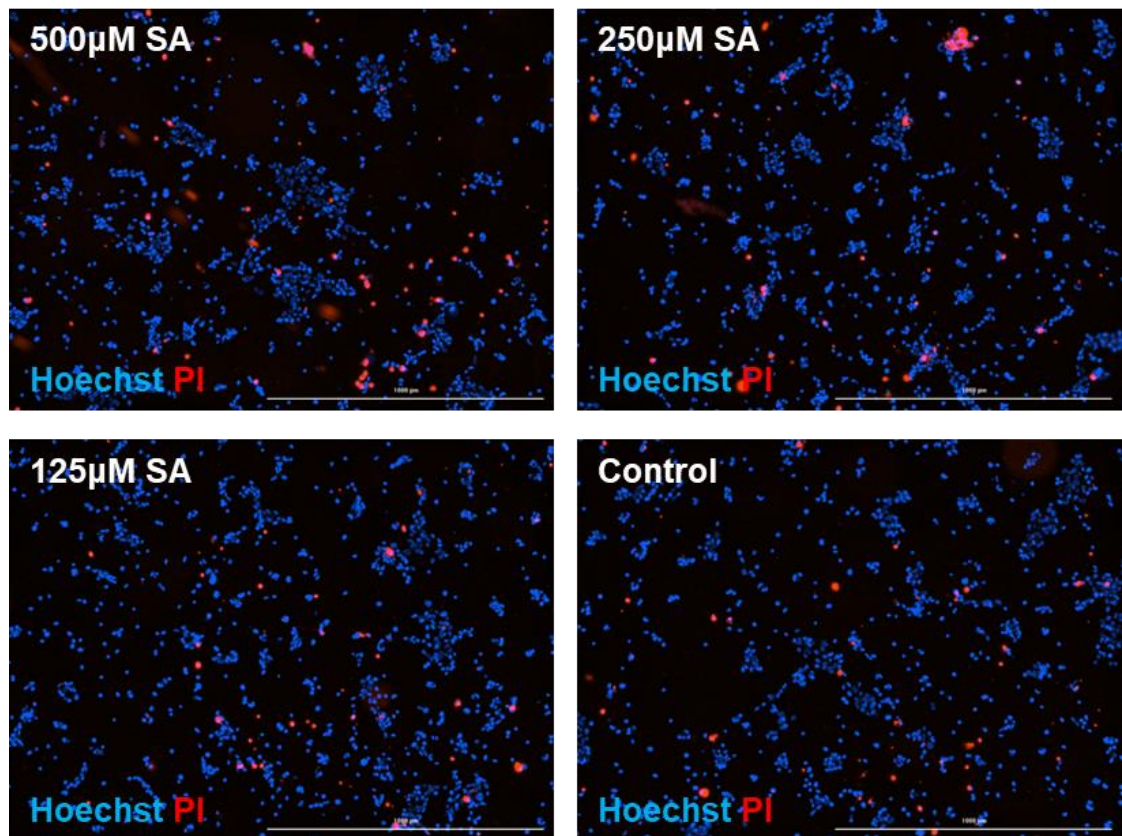
Figure 3-7 Sodium arsenite does not impact upon cell viability. H357 cells were left untreated or challenged with sodium arsenite (SA; t = 30mins), following which cell viability was assessed using Hoechst 3342 and propidium iodide staining (PI; mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test. Scale bar is 1000 μm.

3.3.5 Sodium arsenite treatment increases *P. gingivalis* invasion of oral epithelial cells without a decrease in host viability

After the selection of 250µM sodium arsenite to induce oxidative stress its effect on the rate of *P. gingivalis* invasion was determined. The percentage of cells infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) in the presence or absence of sodium arsenite was quantified by counting the number of cells with a positive *P. gingivalis* antibody signal under each condition using confocal microscopy and Z-stacking as described in section 3.3.1. In the presence of oxidative stress, an increase in *P. gingivalis* infected cells (39 ± 9 %) was observed when compared to cells infected in the absence of oxidative stress (24 ± 1.2 %) (Figure 3-8).

Sodium arsenite challenge (250 µM, t=30mins), *P. gingivalis* infection (NCTC11834, MOI of 100, t=2h) or a combination of both induced no change in H357 cell viability as assessed by Hoechst 33342 and propidium iodide staining (Figure 3-9).
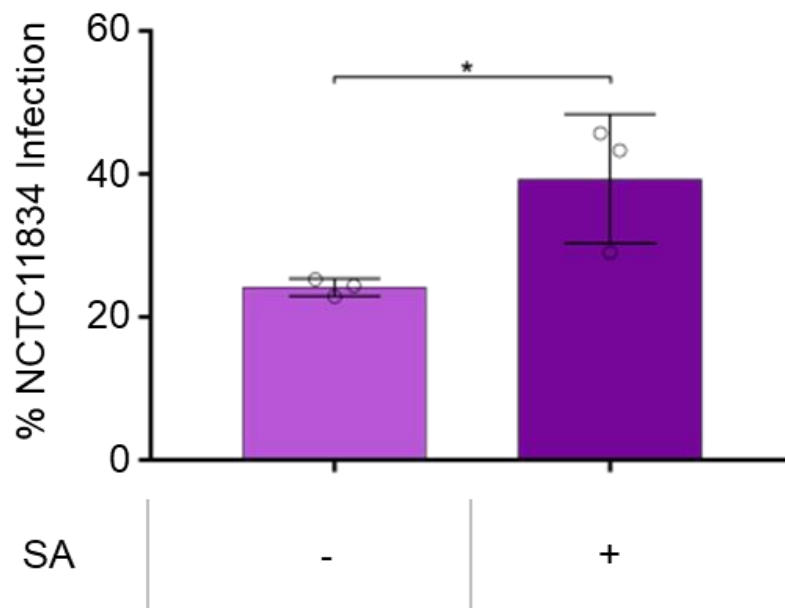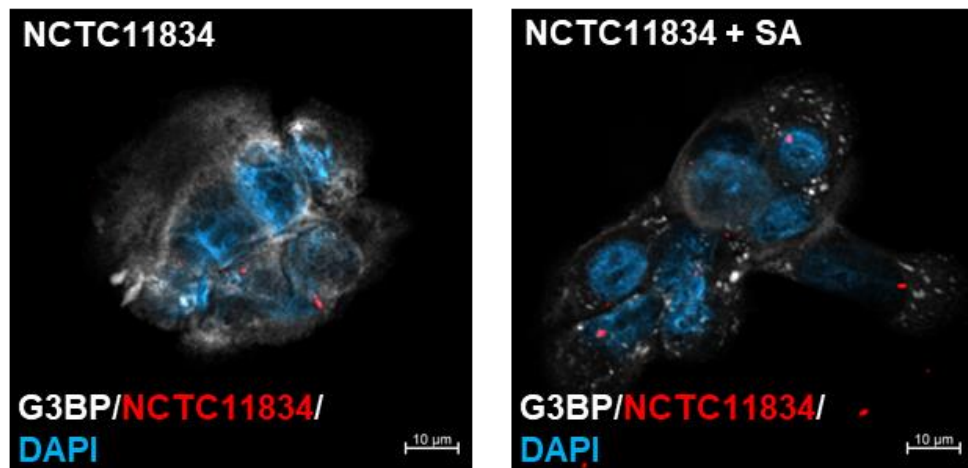
Figure 3-8 Sodium arsenite challenge increases *P. gingivalis* invasion.
H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h)
with or without sodium arsenite (SA; 250 µM) for the final 30 minutes.
Infected cells were enumerated by visualising *P. gingivalis* (red) and G3BP
(white) using immunofluorescence confocal microscopy. (mean ± SD, n=3,
100 cells per biological replicate). *, $P \leq 0.05$ according to Kruskal-Wallis
with Conover-Inman *post-hoc* test. .Scale bar is 10 µm.
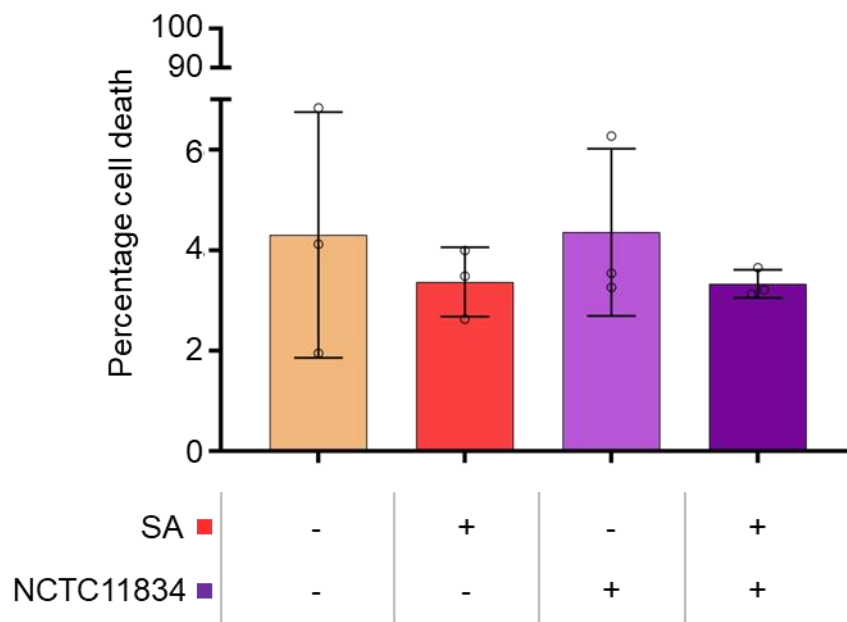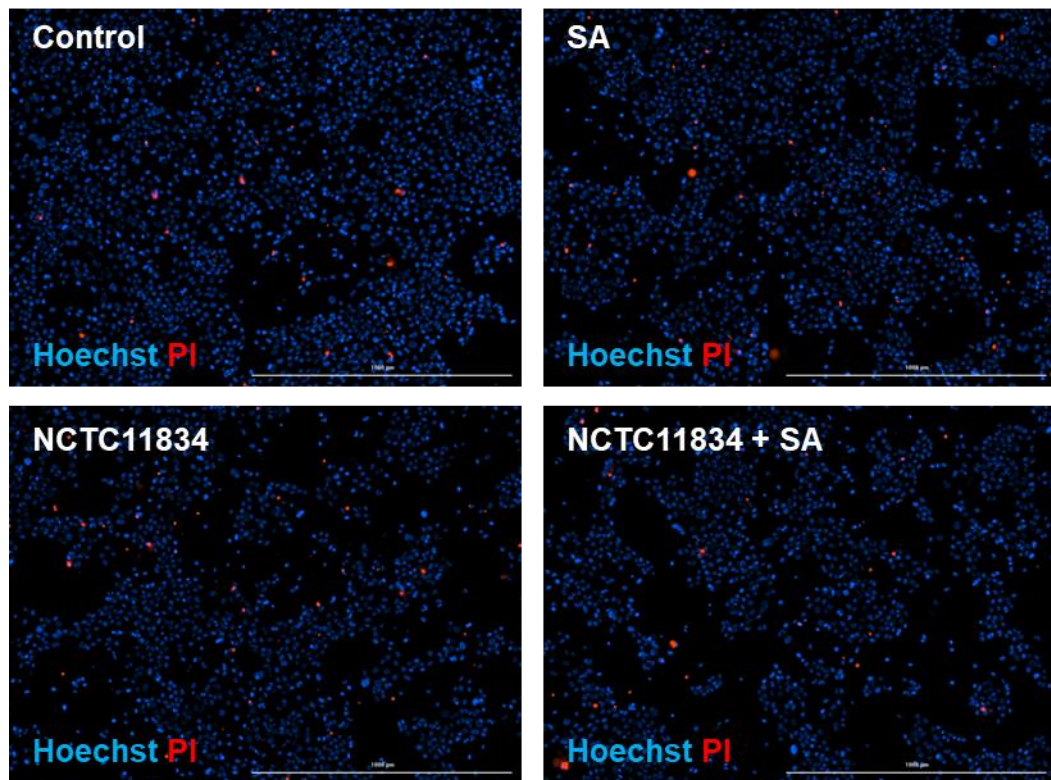
Figure 3-9 *P. gingivalis* infection and sodium arsenite do not impact upon cell viability. H357 cells were left untreated or infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without challenged with sodium arsenite (SA; 250 µM) for the final 30 mins, following which cell viability was assessed using Hoechst 3342 and propidium iodide (PI) staining (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test. Scale bar is 1000 µm.

3.3.6 *P. gingivalis* heightens translational repression during oxidative stress

Oxidative stress during *P. gingivalis* infection may occur via host immune responses (Hajishengallis & Lambris, 2012; Peake & Suzuki, 2004). Next translation initiation was monitored during *P. gingivalis* (strain NCTC11834, MOI of 100) infection of a period of two, four, six and 24 hours with and without a chemical induced oxidative stress environment (sodium arsenite, 250 µM) for the final 30 minutes. Oxidative stress resulted in a 3.72-fold decrease in translation compared to the untreated control. *P. gingivalis* infection heightened stress-induced translational inhibition 2.10-fold when cells were treated with both *P. gingivalis* and oxidative stress (Figure 3-10). As this increased inhibition of translation was observed at all infection time points, further studies were conducted after 2h infection.
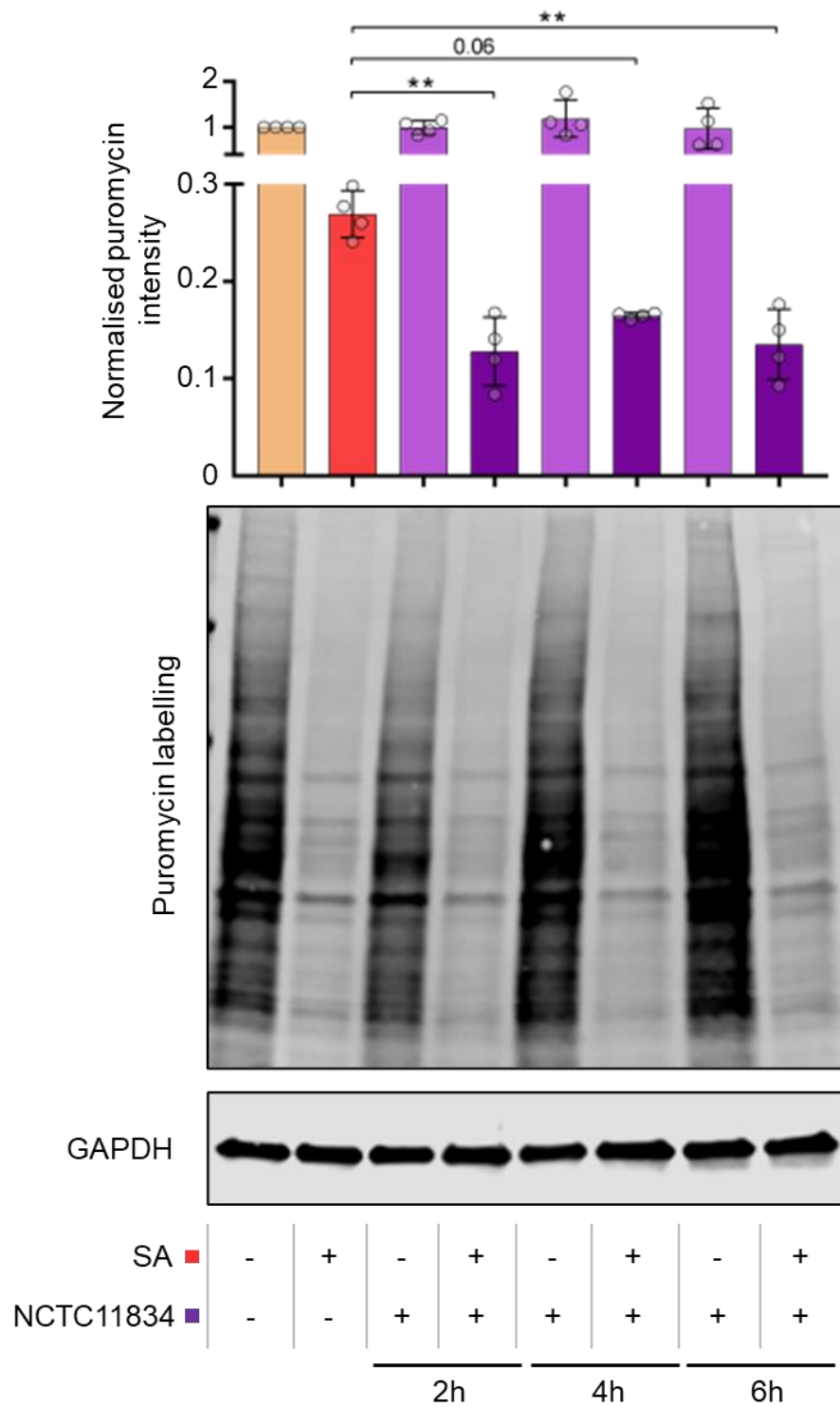
Figure 3-10 *P. gingivalis* heightens stress induced translational stalling.
H357 cells were left untreated or infected with *P. gingivalis* (NCTC11834,
MOI of 100, t=2h to 6h) with or without sodium arsenite (SA; 250 µM) for the
final 30 mins. The relative rate of protein synthesis as measured by
puromycin uptake and the concentration relative to GAPDH enumerated
(mean ± SD, n=3). **, $P \leq 0.01$ according to Kruskal-Wallis with Conover-
Inman *post-hoc* test.

3.3.7 Heightened translational repression is independent of eIF2α phosphorylation

As *P. gingivalis* heightened translational repression during oxidative stress, the phosphorylation state of eIF2α during these conditions was investigated. Similar basal level of p-eIF2α was observed in *P. gingivalis* infected (NCTC11834, MOI of 100, t=2h) cells and the untreated control. Strikingly, whilst an increase in translational repression was observed in cells co-treated with *P. gingivalis* infection and sodium arsenite, a decrease in levels of p-eIF2α was observed compared to the oxidative stress-only treatment (Figure 3-11).
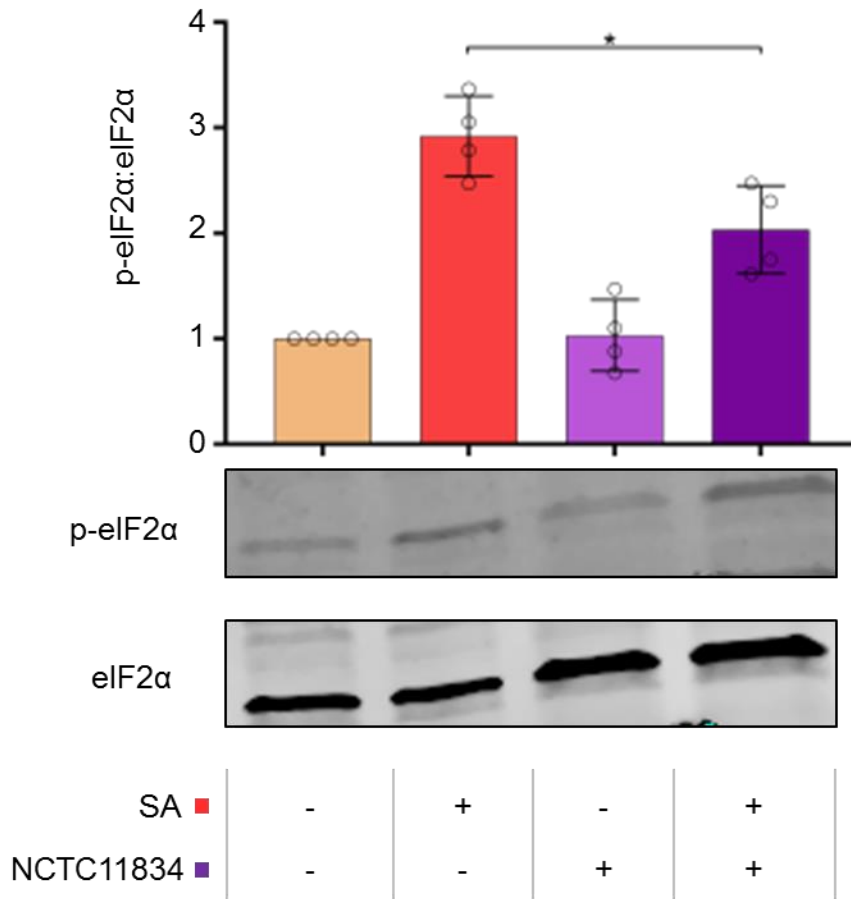
Figure 3-11 *P. gingivalis* infection lowers eIF2α phosphorylation. H357
cells were left untreated or infected with *P. gingivalis* (NCTC11834, MOI of
100, t=2h) with or without sodium arsenite (SA; 250 µM) for the final 30
mins. Levels of p-eIF2α were probed using immunoblotting and the ratio of
phosphorylated to total eIF2α were determined (mean ± SD, n=3). *, $P \leq 0.05$
according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

### 3.3.8 The integrated stress response inhibitor, ISRIB, cannot rescue translational function during *P. gingivalis* infection and oxidative stress

ISRIB is a potent ISR inhibitor, which reverses the inhibitory effect of eIF2α phosphorylation on eIF2B activity, resulting in the rescue of translational function and disassembly of stress granules (Sidrauski *et al.,* 2015). As *P. gingivalis* heightened translational repression independently of increased eIF2α phosphorylation during oxidative stress, the ability of ISRIB to rescue translational function during these conditions was next investigated. ISRIB (400 nM, t=30mins) treated H357 cells did not induce any change in translation initiation compared to the untreated control. When coupled with sodium arsenite challenge (250 µM, t=30 mins) ISRIB (400 nM, t=30mins) treated resulted in an increase in translational function (3.02-fold) when compared to the sodium arsenite only (Figure 3-12 A). When cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) and with sodium arsenite (250 µM, t=30mins), ISRIB treatment (400 nM, t=30mins) only rescued translation 1.85-fold (Figure 3-12 B). Under the same conditions it was found that ISRIB could only inhibit stress granules formation in 85% percent of cells compared to the 96% percent decrease in the sodium arsenite and ISRIB treated cells (Figure 3-13).

Taken together these data indicate that *P. gingivalis* heightens stress induced translational stalling during oxidative stress in a manner independent of eIF2α signalling as indicated by lack of heightened eIF2α phosphorylation and the inability of ISRIB to rescue translational function.
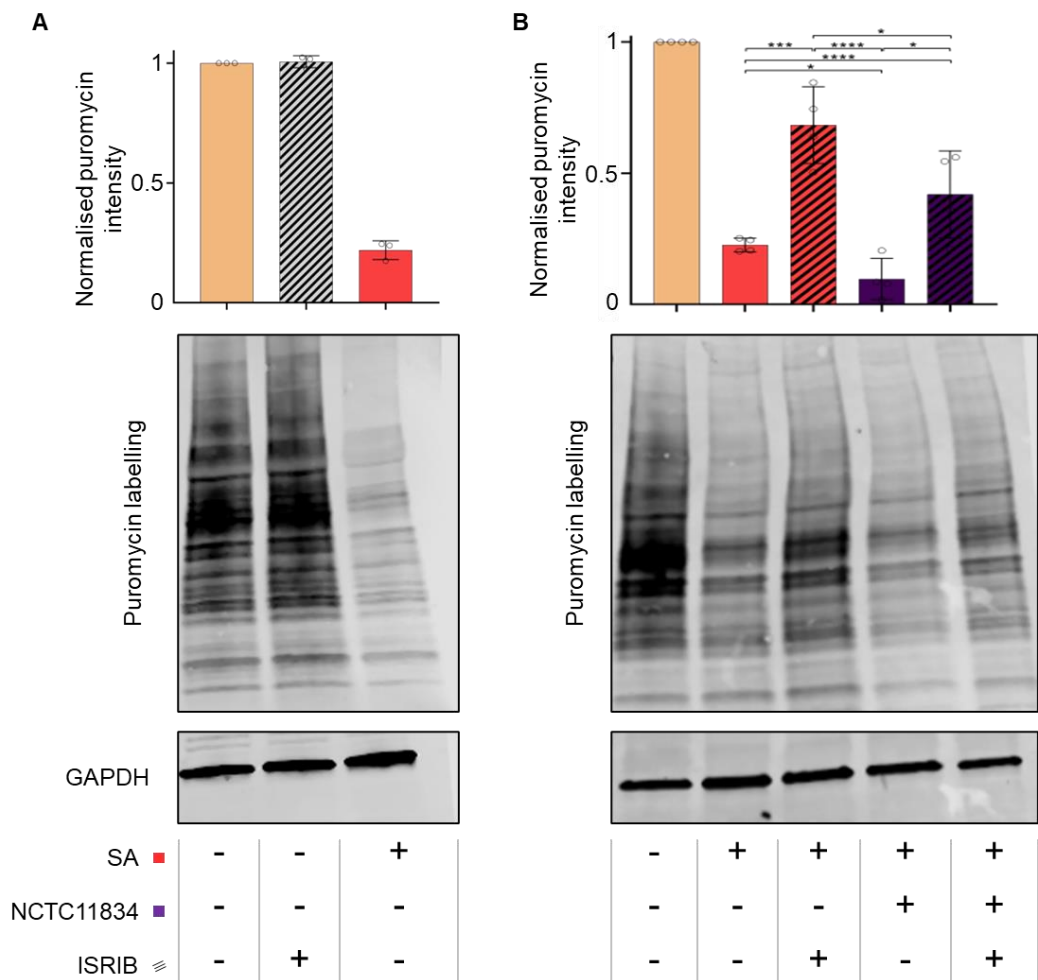
Figure 3-12 *P. gingivalis* infection inhibits the translational restorative function of ISRIB. (A) H357 cells were left untreated, treated with ISRIB (400nM, t=30mins) or challenged with sodium arsenite (SA; 250 µM, t=30mins). The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). (B) H357 cells were left untreated or infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h), with and without sodium arsenite (250 µM) and ISRIB (400 nM) for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=4). **** $P \leq 0.001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
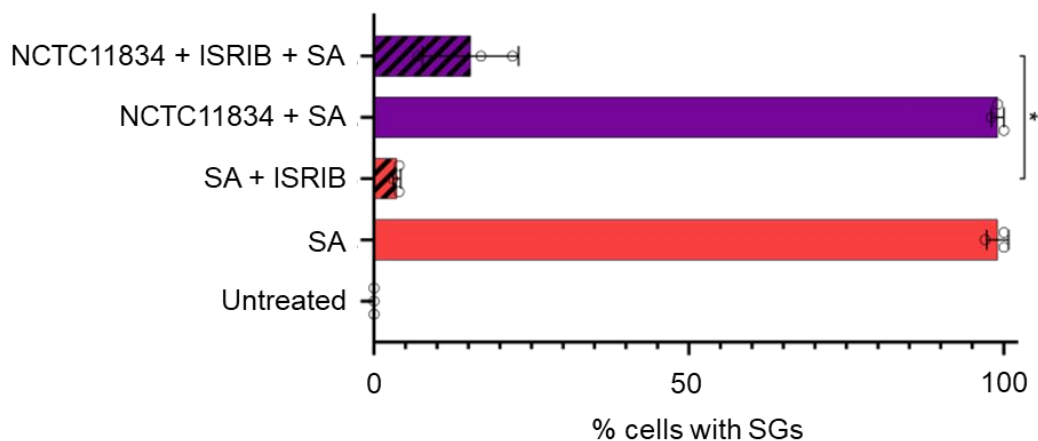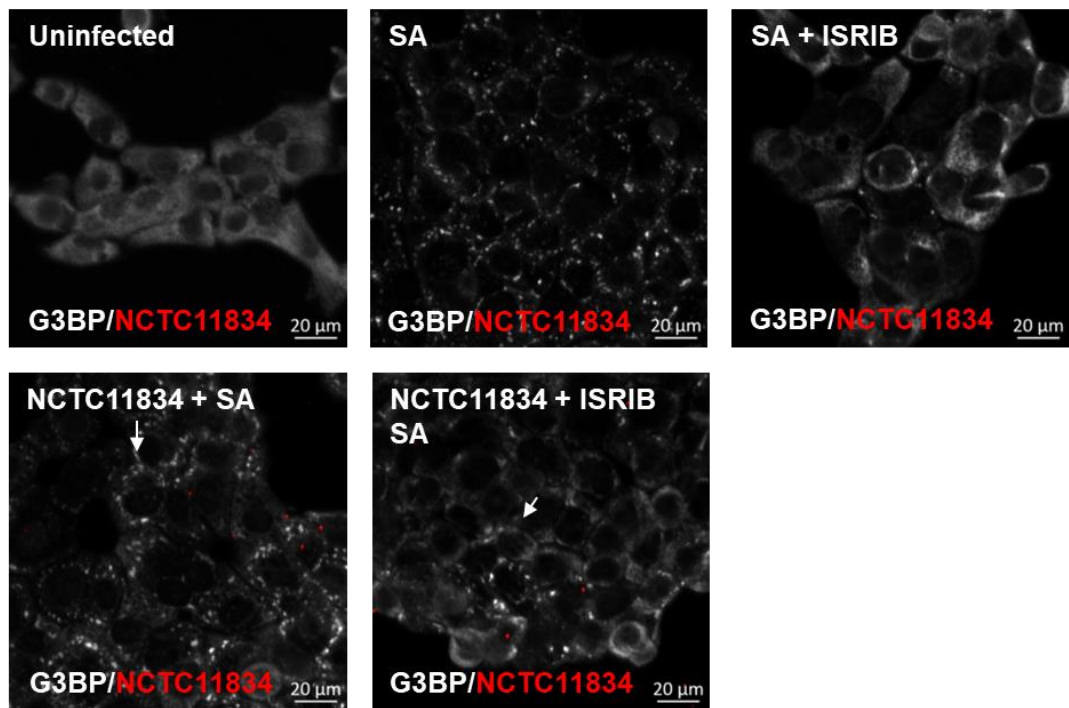
Figure 3-13 *P. gingivalis* dampens the ability of ISRIB to inhibit stress granule formation. H357 cells were left untreated or infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h), with and without sodium arsenite (SA; 250 μM) and ISRIB (400 nM) for the final 30 mins. Stress granule (SG) formation as visualised by G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks (mean ± SD, n=3, 100 cells per biological replicate). *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test. Scale bar is 20 μm.

3.4 Discussion

Recent developments within the field of ISR signalling and translational control during stress have identified these signalling cascades as a tacit component of immune responses within eukaryotes (Costa-Mattioli & Walter, 2020). However, in recent years these pathways have become increasingly implicated in host-pathogen interactions. In this context these pathways not only confer antimicrobial functions, contributing to host fitness during infection, but can also function as targets for pathogenic virulence systems to hijack, ultimately puppeteering overall host cellular functions to their own gain (reviewed in Knowles *et al.,* 2021). This study aimed to investigate the potential of *P. gingivalis* infection to interact with host translational control either on its own or during an ISR activating environment.

Earlier findings reported that mTOR was degraded by *P. gingivalis* in both oral squamous carcinoma (H357) and immortalised oral keratinocytes (OK-F6; Stafford *et al.,* 2013). As it was hypothesised that mTOR degradation may be implicated in host translational control and *P. gingivalis* infection during stress H357 cells were chosen as the model system for this study.

P. gingivalis infection has been suggested to activate the host UPR in human umbilical cord vein cells (Hirasawa & Kurita-Ochiai, 2018). As the UPR, a multifaceted signalling cascade, has arms which feed into the ISR (Harding *et al.,* 2003), we hypothesised that *P. gingivalis* infection mediated UPR activation may also stimulate ISR activity. However, over a period of two to 24 hours *P. gingivalis* did not induce ISR activation as evidenced by monitoring of levels of eIF2α phosphorylation and protein synthesis (Figure 3-2). This was further supported by a lack of stress granule formation (Figure 3-3), a downstream marker of ISR activity (Anderson & Kedersha, 2002; Pakos-Zebrucka *et al.,* 2016). It cannot be excluded that the UPR activation during *P. gingivalis* infection may activate the ISR in certain cell types as the previous study employed human umbilical cord endothelial cells

(Hirasawa & Kurita-Ochiai, 2018), whereas this study utilised oral squamous epithelial carcinoma cells. However, it is also possible that whilst no translational stalling was observed here, arms of the UPR may have been active independently of those feeding the ISR (discussed further in Pavitt & Ron, 2012)).

P. gingivalis infection alone does not activate the core functions of the ISR. However, inflammatory mediators such as those released during periodontitis (Cekici *et al.,* 2014), which result in the activation of neutrophils are well known to induce reactive oxygen species that can lead to oxidative stress (Mayadas *et al.,* 2014). Hence the impact of *P. gingivalis* infection during oxidative stress was investigated. To recapitulate an oxidative stress like environment, sodium arsenite was utilised to chemically induce oxidative stress. Sodium arsenite is one of the most characterised ISR inducing agents, which robustly and reliably induces oxidative stress and ISR activation via activation of the HRI kinase (Brostrom & Brostrom, 1997; McEwen *et al.,* 2005). The action of sodium arsenite inducing oxidative stress, which mimics the oxidation observed during the chronic inflammatory state characteristic of periodontitis (Cekici *et al.,* 2014). These findings, coupled with the reports documenting the expression of oxidative stress resistance genes expressed by *P. gingivalis* (Henry *et al.,* 2012) made sodium arsenite a relevant stress inducer.

Previous studies investigating stress granules and ISR function have induced acute stress using 500 µM sodium arsenite (Kedersha *et al.,* 1999; Rabouw *et al.,* 2020). However, here in H357 cells sodium arsenite 250 µM challenge for 30 minutes decreased protein synthesis (Figure 3-4) and increased eIF2α phosphorylation (Figure 3-5). This treatment also robustly induced stress granule formation (Figure 3-6) without increasing cell death as assessed by Hoechst 33342 and propidium iodide staining (Figure 3-7). Therefore, further studies utilised 250 µM sodium arsenite for 30 minutes to induce acute oxidative stress.

P. gingivalis is known to invade between 14 % of cells at an MOI of 100 over 90 minutes (Lamont *et al.,* 1995). A slightly higher invasion of 20 % after 2 hours infection was observed in the H357 cell line used in this study. Interestingly, in the presence of sodium arsenite induced oxidative stress *P. gingivalis* invasion increased 1.63-fold (Figure 3-8).The exact cause remains elusive, however *P. gingivalis* is known to express oxidative stress resistance genes (Henry *et al.,* 2012), whilst also activating host antioxidant glutathione responses, effectively protecting its host against oxidative stress (Choi *et al.,* 2013). Furthermore, *P. gingivalis* has a layer of hemin around its cell surface which can act as a buffer against oxidative radicals, further increasing its tolerance of oxidative stress (Johnson *et al.,* 2004; Smalley *et al.,* 2000). Thus the multitude of defence's *P. gingivalis* employs against oxidative stress coupled with sodium arsenite's ability to decrease mammalian membrane integrity (Yancy *et al.,* 2005), may underpin the increased invasive capability of *P. gingivalis* during the sodium arsenite-induced oxidative stress.

Sodium arsenite induced stress is known to result in translational repression (Mcewen *et al.,* 2005a). Here, during oxidative stress *P. gingivalis* heightened translational attenuation was seen (Figure 3-10). Inhibition of protein synthesis during ISR activation functions to conserve energy, allowing the cell to focus this energy upon a stress response gene expression program, ultimately either returning to homeostasis or initiating programmed cell death (Pakos-Zebrucka *et al.,* 2016). However, here heightened translational repression during stress and *P. gingivalis* infection did not result in increased cell death as no change was found in H357 viability(Figure 3-9).

Translational attenuation during sodium arsenite induced oxidative stress is mediated by the phosphorylation of eIF2α (Mcewen *et al.,* 2005b). The central function of eIF2α signalling on translational control and cellular signalling has made it a target for a wide range of viruses to hijack host translational function (Reviewed in Liu, Y. *et al.,* 2020). In the case of *P. gingivalis* although heightened translational repression was observed during

oxidative stress no corresponding increase in eIF2α phosphorylation was observed (Figure 3-11). The lack of requirement of eIF2α signalling to heighten translational repression during oxidative stress and *P. gingivalis* infection was corroborated by the inability of ISRIB, and ISR inhibitor, to rescue translational function or inhibit stress granule assembly (Figure 3-12) and (Figure 3-13). ISRIB allosterically competes for a binding site on eIF2B which induces a conformational change, antagonising the inhibitory effects of eIF2α phosphorylation (Schoof *et al.,* 2021; Zyryanova *et al.,* 2021). ISRIB only works within a defined window of stress (Rabouw *et al.,* 2019). However, given that the levels of eIF2α phosphorylation do not concomitantly increase, it is unlikely that *P. gingivalis* infection during exogenous stress increased stress severity out of the window of action. Hence, the data point towards a mechanism independent of eIF2α signalling as the effector of heightened translational repression during oxidative stress and *P. gingivalis* infection.

4 Chapter 4: *P. gingivalis* modulates stress granule formation during exogenous stress and is accompanied by cytoskeletal dysregulation

4.1 Introduction

Cellular stress results in global translational shutdown caused by a deficit in eIF2-GTP regeneration and the inhibition of 5' mRNA cap binding. These events ultimately result in stalled pre-initiation complexes and polysome run off, which increase the pool of non-productive translation initiation complexes termed mRNPs (Anderson & Kedersha, 2002; Kedersha *et al.,* 2002). These stalled mRNPs can instead be sequestered into non-membranous cytoplasmic condensates known as stress granules (Nover *et al.,* 1989; Anderson & Kedersha, 2002), which function to aid in sorting of mRNPs into those that will be degraded or re- initiated if the anti-translational capacity of stress is overcome (Anderson & Kedersha, 2002).

Stress granule nucleation is initially driven by protein and RNA binding capacities of the canonical stress granule component G3BP1, which function to drive LLPS. However, stress granules also contain a plethora of other components, including RNA binding proteins, such as TIA-1 and TIA-R, as well as various translation initiation factors and poly adenylated mRNA (Kedersha *et al.,* 1999; Kedersha *et al.,* 2002; Kimball *et al.*, 2003; Tourriere *et al.,* 2003; Mazroui *et al.,* 2007). Stress granules are active structures that form within minutes, dissolve and a similar pace and have component residence times from seconds to permanent (Kedersha *et al.,* 2000). Owing to their dynamic nature stress granules require ongoing retrograde transport along functioning microtubules (Loschi *et al.,* 2009; Wheeler *et al.,* 2016).

Interestingly, the bacterial pathogen *S. flexneri* has been shown to alter the composition of stress granules during exogenous stress, leading to an increase in stress granule frequency coupled with the selective exclusion of eIF3b from these granules (Vonaesch *et al.,* 2016). Given that in chapter 3 *P. gingivalis* was shown to alter stress induced translational stalling, independently of eIF2α phosphorylation, the potential of interactions with stress granule dynamics is investigated here.

4.2 Aims

In this chapter the impact of *P. gingivalis* infection on stress granule formation during an oxidative stress environment will be investigated by:

- Evaluating the frequency and composition of stress granules formed during oxidative stress and *P. gingivalis* infection
- Elucidating the role of the microtubule network in the relationship between *P. gingivalis* infection and stress granules during oxidative stress.

4.3 Results

4.3.1 *P. gingivalis* infection increases the frequency of G3BP containing stress granules

P. gingivalis infection heightened translational repression during oxidative stress without inducing or inhibiting stress granule formation (Chapter 3). Here the interactions between *P. gingivalis* and stress granules formation were further investigated.

H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with and without sodium arsenite (250 µM) for the final 30 mins, following which stress granule formation was monitored. As found previously, *P. gingivalis* alone did not induce stress granule formation and in the presence of sodium arsenite challenge did not inhibit stress granule formation (Figure 4-1).

To establish whether the stress granules formed during *P. gingivalis* infection with sodium arsenite challenge varied from those formed during sodium arsenite only treatment, the frequency and area of the stress granules formed were next quantified. Cells treated with sodium arsenite induced on average the formation of 36.2 ± 30.2 stress granules per cell with an average area of 2.25 ± 3.16 µm^2. Within the *P. gingivalis* (NCTC11834, MOI of 100, t=2h) and sodium arsenite treated population the frequency of stress granules increased on average to 59.4 ±42.71 per cells, with a similar average area (Figure 4-2).

The effects of *P. gingivalis* can be strain specific, therefore the effects of *P. gingivalis* strains ATCC381 and ATCC53978 (W50) were also investigated to establish whether these responses were conserved. Similarl to NCTC11834, both the ATCC381 and ATCCW50 strains of *P. gingivalis* increased the mean frequency of stress granules to 64.48 and 58.66 per cell respectively (Figure 4-2). While NCTC11834 and ATCC381 decreased

the mean stress granule area to 1.3 ± 1.57 μm^2 and 1.39 ± 1.51 μm^2, this trend fell short of statistical significance. However, W50 significantly decreased the average area to 0.8704 ± 1.02 μm^2 per cell (Figure 4-2).

These findings indicate that *P. gingivalis* increases stress granule frequency during sodium arsenite induced oxidative stress. In addition, the data suggests that whilst this response is conserved and may not be strain specific, decrease in stress granule area may be strain dependent.
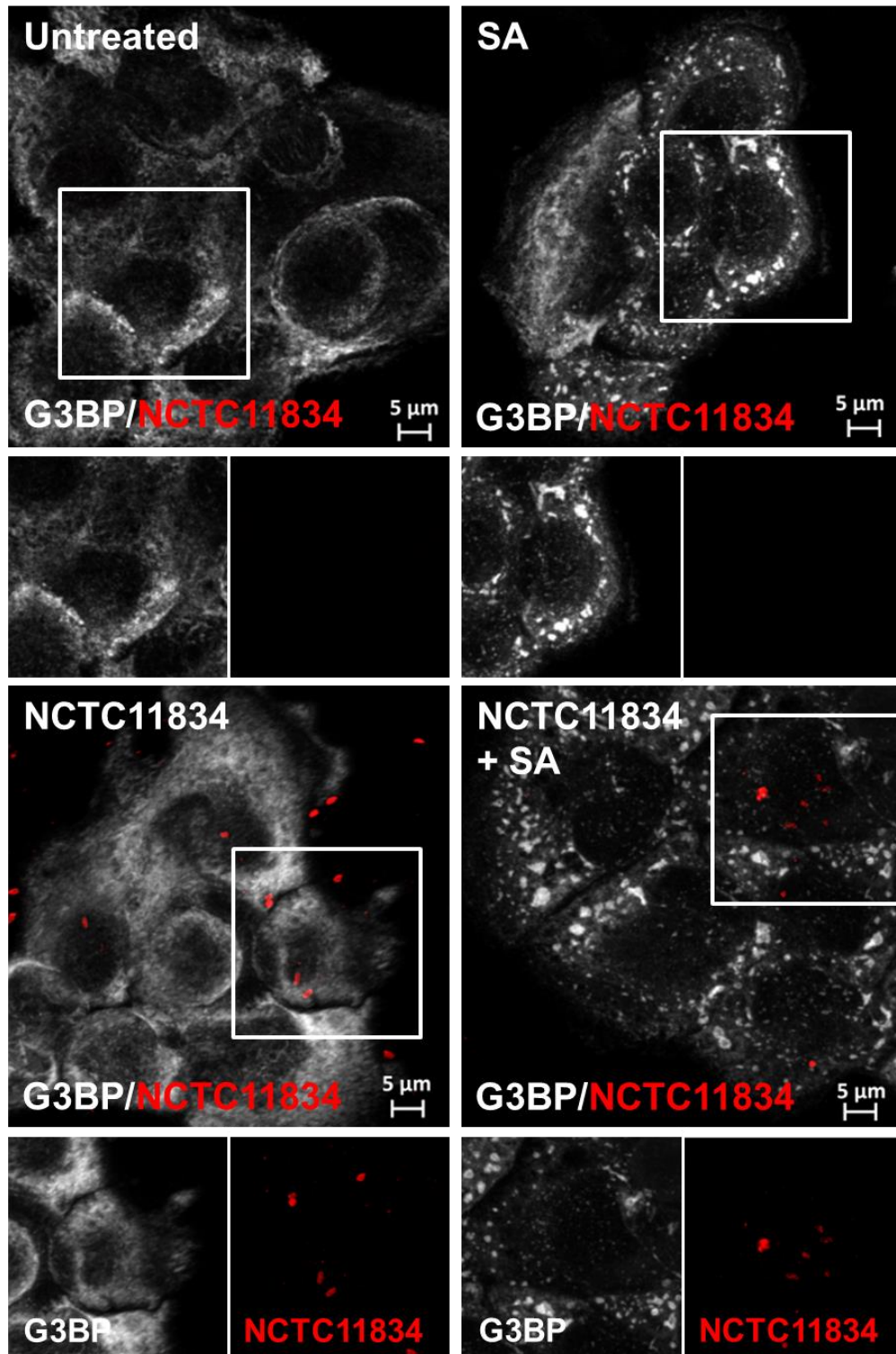
Figure 4-1 *P. gingivalis* does induce or inhibit stress granule formation during oxidative stress. H357 cells were infected with *P. gingivalis* NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA) for the final 30 mins. Stress granule formation as visualised by G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks. Scale bar is 5 μm.
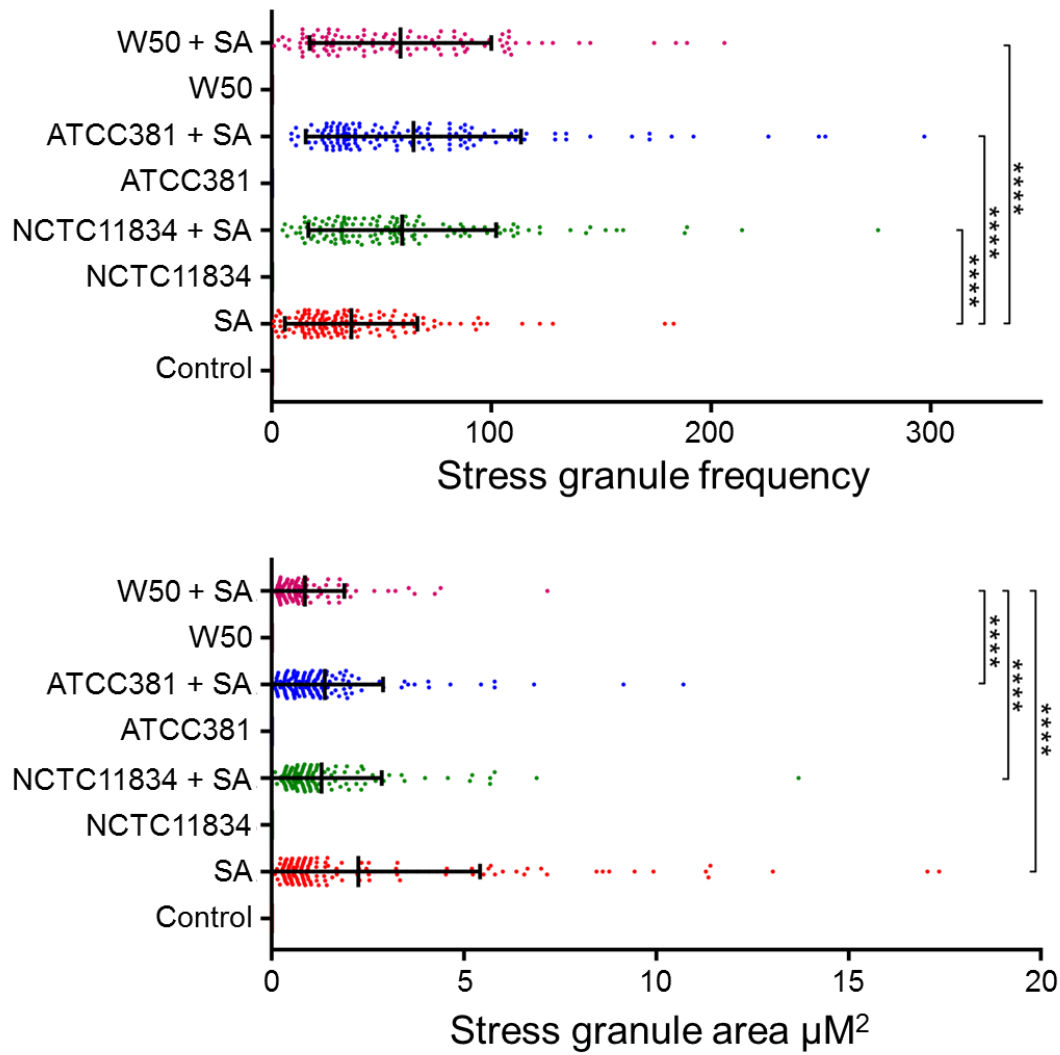
Figure 4-2 *P gingivalis* increases the frequency of stress granules during oxidative stress. H357 cells were infected with *P. gingivalis* (NCTC11834, ACTC381, ACTCW50, MOI of 100, t=2h) with or without sodium arsenite (SA) for the final 30 mins. Stress granule formation as visualised by G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks (mean ± SD, n=3, 50 cells per biological replicate [other than W50 of which one replicate only had 20 cells]). **** $P \leq 0.001$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

4.3.2 *P. gingivalis* infection induces delocalisation of eIF3b from G3BP stress granules

The composition of stress granules is known to vary in a stress dependent manner (Aulas *et al.,* 2017). As stress granule frequency increased during *P. gingivalis* infection and oxidative stress the composition of stress granules under these conditions was next investigated.

H357 cells were infected with *P. gingivalis* (NCT11834, MOI of 100, t=2h) with or without sodium arsenite (250 µM) challenge for the final 30 mins, following which the localisation of G3BP (white) and eIF3b (purple) was analysed using immunofluorescence confocal microscopy. Co-localisation of eIF3b to G3BP was visualised using colour intensity profiles along a line through a cell and the percentage co-localisation was then enumerated (Figure 4-3 A, B). During oxidative stress, eIF3b co-localised highly with G3BP positive stress granules (mean 76 ± 28 %). However, in the presence of *P. gingivalis* infection and oxidative stress, the mean percentage co-localisation decreased to 48 ± 30 % (Figure 4-3 C).
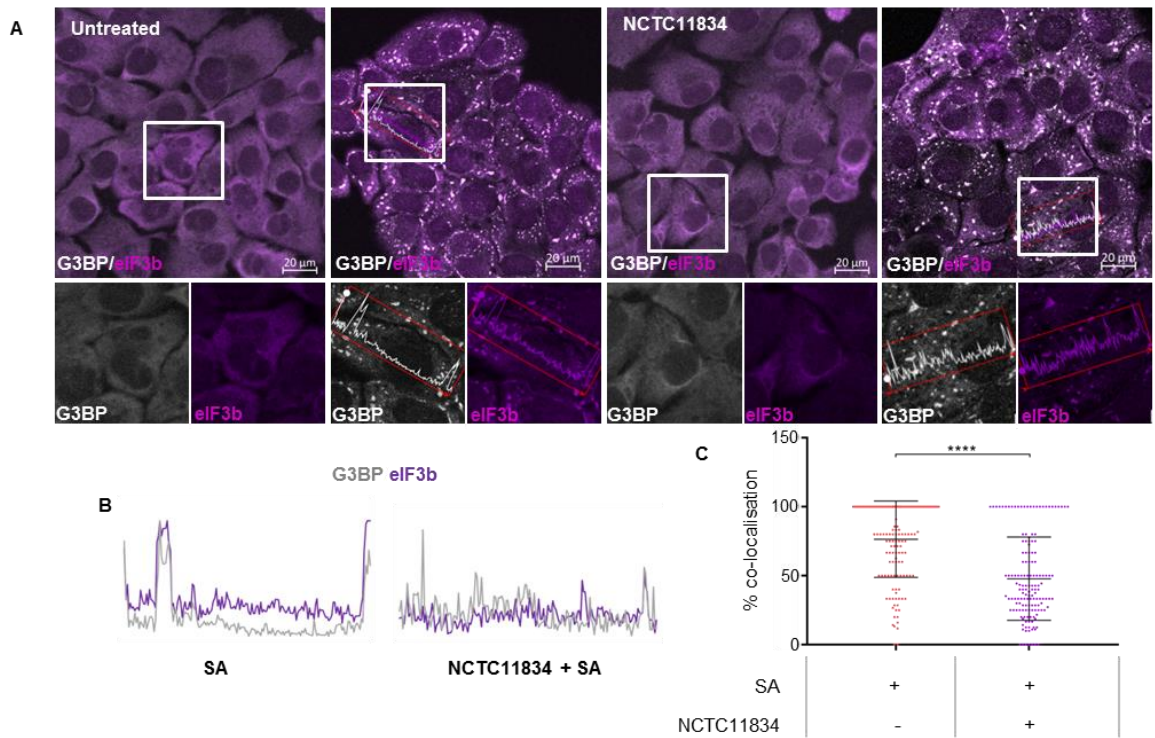
Figure 4-3 *P. gingivalis* infection induces de-localisation of eIF3b from stress granules during oxidative stress. (A) H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100; t=2h) with or without sodium arsenite (SA) for the final 30 mins. Co-localisation of G3BP1 (white) and eIF3B (purple) in stress granules was assessed by immunofluorescence. (B) Representative line segments of colour profiles taken from H357 cells challenged with sodium arsenite with or without *P. gingivalis* infection, where intensity peaks correspond to stress granules. (C) Percentage of colocalisation of eIF3b and G3BP1 (mean ± SD, n=3, 50 cells per biological replicate). **** $P \leq 0.001$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test. Scale bar is 20µm.

4.3.3 *P. gingivalis* infection does not degrade G3BP or eIF3b

During infection *P. gingivalis* is known to degrade a multitude of host proteins (Sroka *et al.,* 2001). To determine whether the modulation of stress granule formation and composition may be due to the degradation of G3BP and eIF3b the expression of these proteins was next monitored.

H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (250 μM) challenge for the final 30 mins. No degradation of G3BP and eIF3b was observed under any condition (Figure 4-4); this suggests that the ability of *P. gingivalis* to modulate stress granule frequency and composition is independent of G3BP or eIF3b degradation.
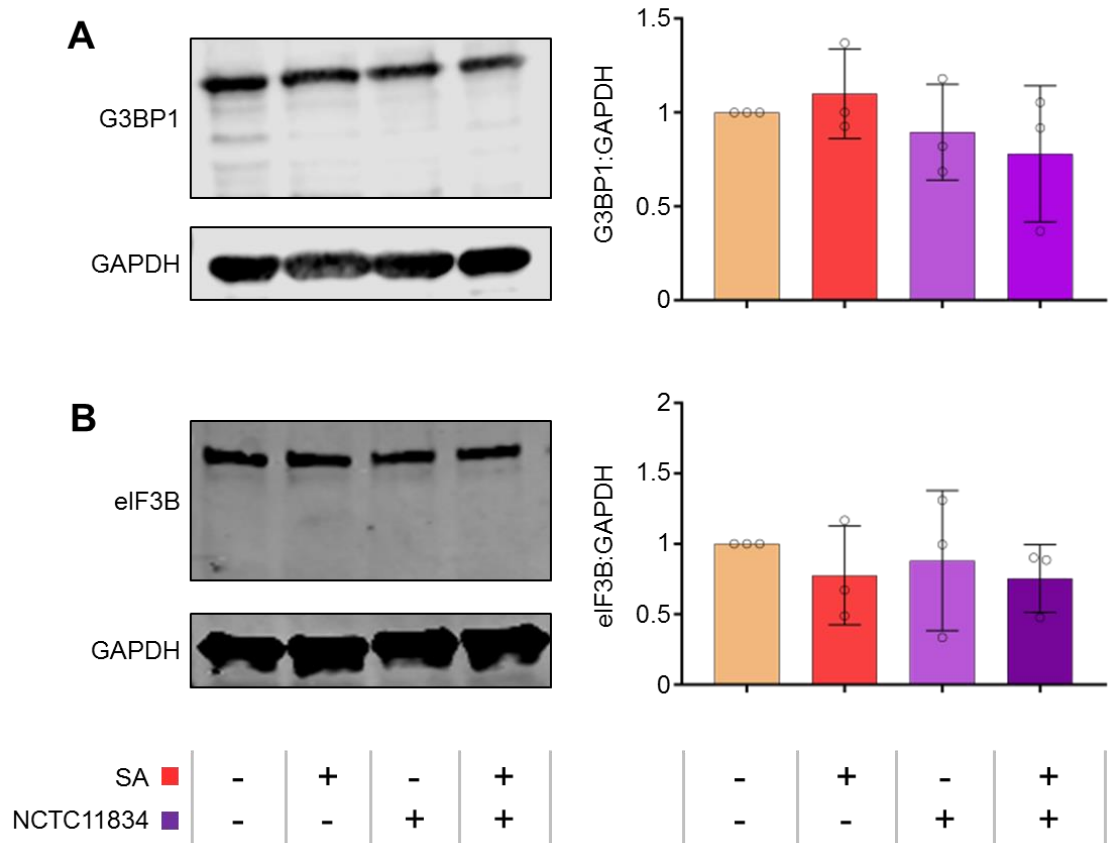
Figure 4-4 *P. gingivalis* **and exogenous stress do not alter G3BP or eIF3B expression.** H357 cells were left untreated or infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 µM) challenge for the final 30 mins. Expression levels of (A) G3BP1 and (B) eIF3B were probed using immunoblotting. Concentration relative to the loading control GAPDH was first determined before being normalised to the untreated sample (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.

4.3.4 *P. gingivalis* infection does not induce visual changes to the microtubule network

With stress granule frequency and composition varying under oxidative stress and *P. gingivalis* infection; the underlying mechanisms of stress granule assembly were investigated. The assembly of mature stress granules requires aggregation of small stress granule cores and shells into larger foci along polymerising microtubules (Nadezhdina *et al.,* 2010; Wheeler *et al.,* 2016).

The integrity of microtubule network was assessed in cells infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (250 µM) for the final 30 mins by probing for α-tubulin. No qualitative changes to the integrity of the tubulin network was observed in all test samples, compared to the total lack of microtubular structures observed with the positive control, nocodazole (200 nM, t=30mins) treatment (Figure 4-5).
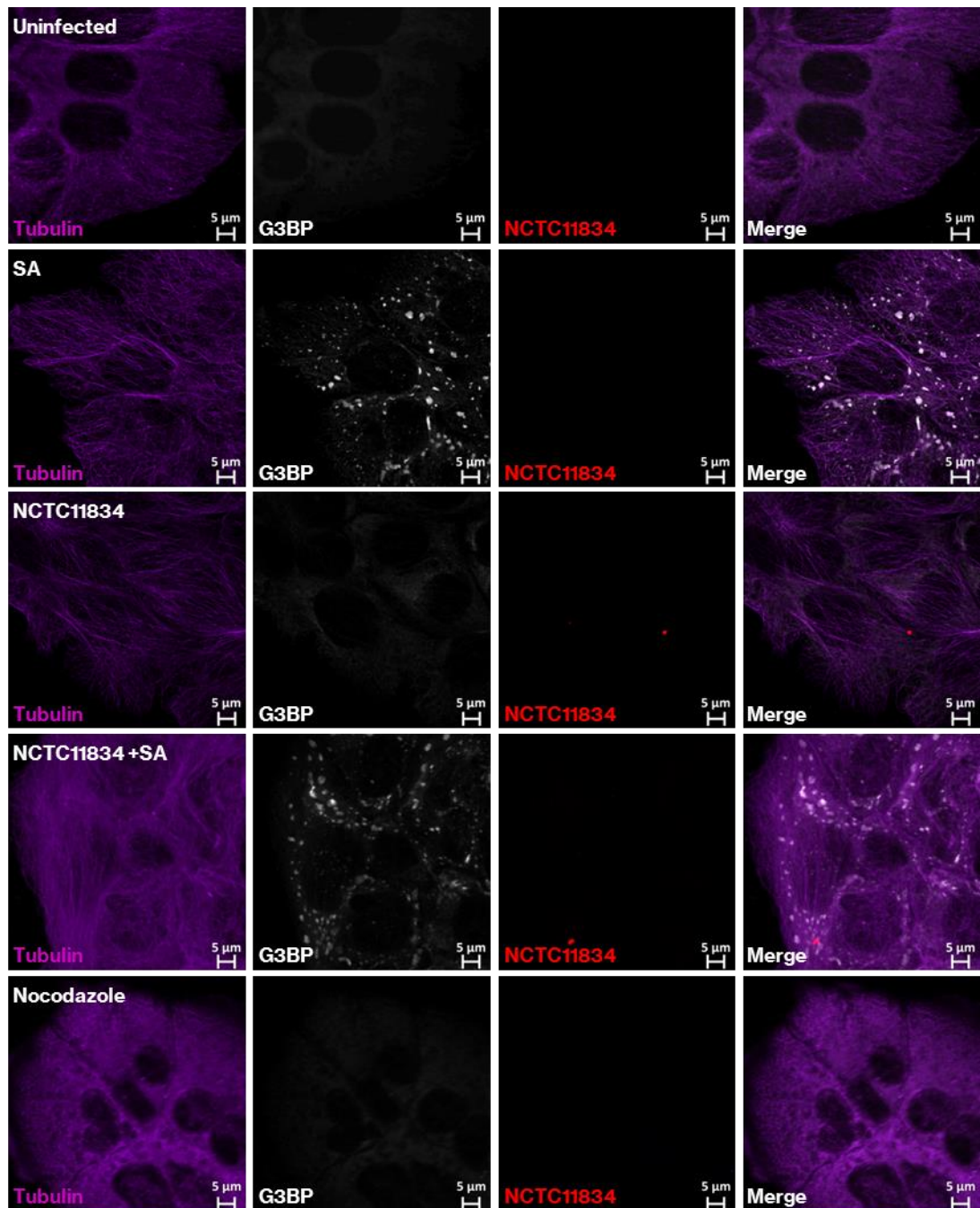
Figure 4-5 *P. gingivalis* does not disrupt microtubule formation during oxidative stress. H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of100, t=2h) with or without sodium arsenite (SA; 250 µM) challenge for the final 30 mins. Nocodazole (200 nM, t=30mins) treatment was included as a control for tubulin disruption. Stress granule, α-tubulin integrity and *P. gingivalis* were visualised using confocal microscopy. Scale bar is 5 µm.

4.3.5 *P. gingivalis* infection lowers stress induced tubulin acetylation in a manner independent of HDAC6 expression.

As the function of tubulin can be modified post-translationally (Eshun-Wilson *et al.,* 2019) the levels of acetyl-α-tubulin were monitored using immunoblotting.

Cells both infected and uninfected displayed basal levels of tubulin acetylation. Oxidative stress resulted in a 3.6-fold increase in acetylation Figure 4-6). This response was dampened 1.42-fold when cells were infected with *P. gingivalis* prior to the addition of oxidative stress Figure 4-6).

To investigate the means of tubulin deacetylation during oxidative stress, the expression of the principal tubulin deacetylation enzyme histone deacetylase 6 (HDAC6; Zhang, Y. *et al.,* 2003) was also monitored. *P. gingivalis* infection did not alter HDAC6 expression above basal levels whilst oxidative stress increased HDAC6 levels 3.64-fold. The oxidative stress-induced increase in HDAC6 expression was not observed when infection was coupled with oxidative stress (Figure 4-7), pointing towards a HDAC6 independent mechanism α-tubulin acetylation inhibition during oxidative stress and *P. gingivalis* infection.
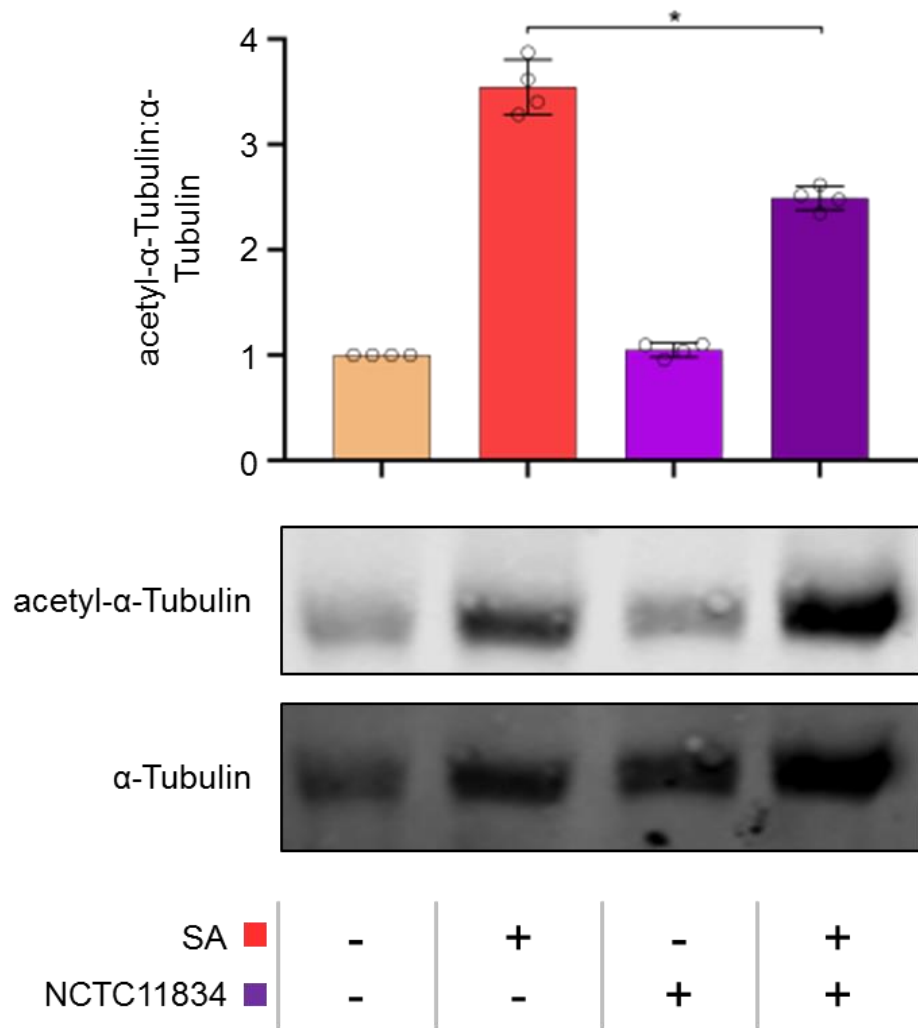
Figure 4-6 *P. gingivalis* infection lowers expression of stress induced tubulin acetylation. H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 μM) challenge for the final 30 mins. Expression levels of α-tubulin and the ratio of acetyl-α-tubulin to α-tubulin were assessed by immunoblotting (mean ± SD, n=4). *, *P* ≤ 0.05 according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
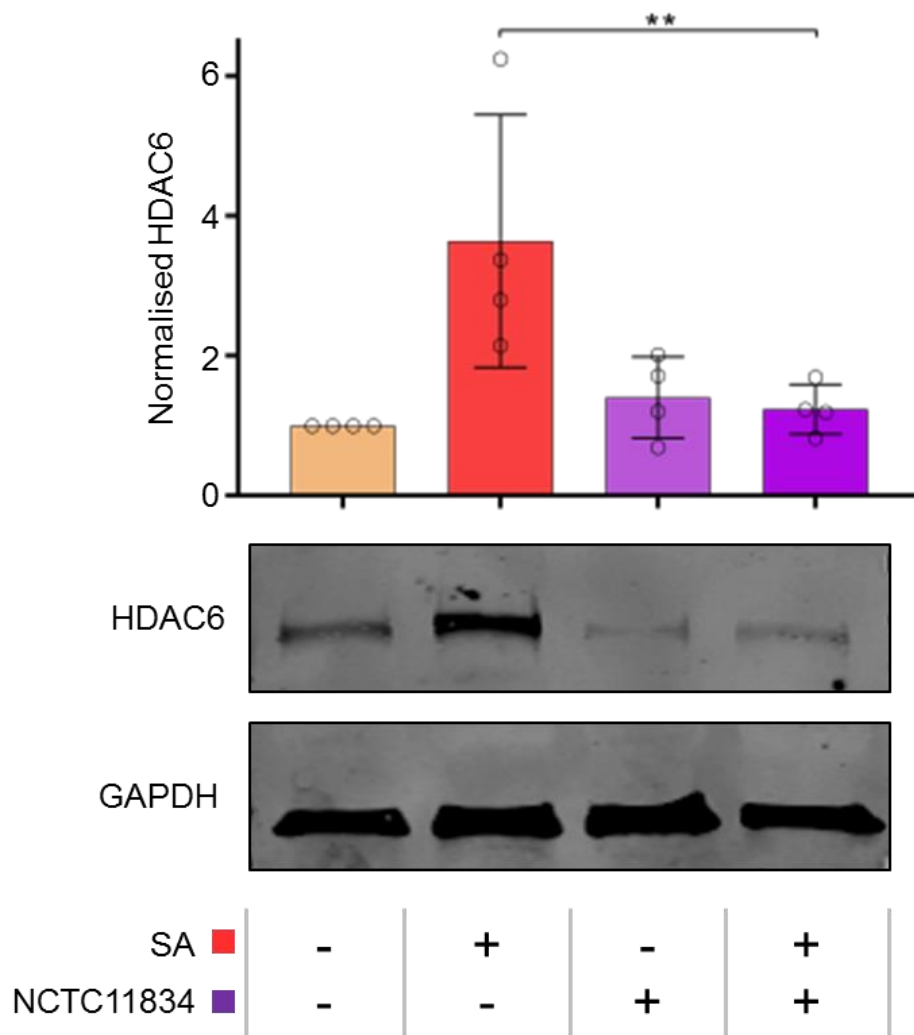
Figure 4-7 *P. gingivalis* infection inhibits increased stress induced HDAC6 expression. H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 µM) challenge for the final 30 mins. Expression of HDAC6 and its concentration relative to GAPDH were determined via immunoblotting (mean ± SD, n =4). **, $P \leq 0.01$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

4.4 Discussion

The potential for *P. gingivalis* to dysregulate host stress granule formation during oxidative stress was investigated. To date there have been limited studies conducted into the impact of bacterial infection on stress granule formation. Recent work has however begun to shine a light on bacterial infection mediated stress granule formation, with *Salmonella*, *Listeria* and *E. coli* infections all being capable of inducing stress granule formation (Abdel-Nour *et al.,* 2019; Tsutsuki *et al.,* 2016). In this study, *P. gingivalis* infection did not induce stress granule formation (Figure 3-3). However, studies in *S. flexneri* have reported modulation of stress granule formation during exogenous stress (Vonaesch *et al.,* 2016). In the presence of sodium arsenite-induced oxidative stress, infection by *P. gingivalis* (NCTC11384, ATCC381 and W50) were found to increases to the frequency of stress granules formed per cell, compared to the stress only control (Figure 4-2).

Interestingly the W50 strain produced a more marked decrease in stress granule area (Figure 4-2). Whilst this result may be due to the lower number of cells counted in the final repeat biological replicate of W50 infection, *P. gingivalis* NCTC11834 and ACTC381 are both evolutionarily highly related with similar genetic constitutions, whereas W50 has significant variations both its 16S rRNA and core protein sequences (Chen *et al.,* 2017). A key variation between W50 and the more similar NCTC11834 and ATCC381 is the long fimbriae, a key cell surface expressed virulence factor (Amano *et al.,* 1999), where the ACTCW50 *fimAIV* long fimbriae are thought to be more virulent (Enersen *et al.,* 2008). The NCTC11834 strain of *P. gingivalis* is more densely fimbriated than its W50 counterpart (Sojar *et al.,* 1997; Amano *et al.,* 1999)., and this in part is thought to play a role in its increased capacity for invasion of H357 cells (Suwannakul *et al.,* 2010). Here it is unclear whether the variation in impact upon stress granule area is dependent on differential fimbriae expression and increased invasion as there are further significant genomic differences between strains.

Stress granule composition is known to vary depending on the stress stimuli (Aulas *et al.,* 2017), indeed during *S. flexneri* infection during exogenous stress it was shown that eIF3b is partially excluded from stress granules (Vonaesch *et al.,* 2016). *P. gingivalis* infection also modulated the composition of stress granules, inducing a lower localisation of eIF3b from G3BP positive stress granules (Figure 4-3). *P. gingivalis* infection is known to result in the degradation of key host proteins including mTOR (Stafford *et al.,* 2013), however this phenotype was independent of any degradation of either G3BP or eIF3b (Figure 4-4).

Stress granule aggregation requires ongoing retrograde transport along functioning microtubules (Nadezhdina *et al.,* 2010; Wheeler *et al.,* 2016). An increased frequency of stress granules has previously been observed during chemical disruption of microtubule assembly with nocodazole (Vasquez *et al.,* 1997; Nadezhdina *et al.,* 2010). As *P. gingivalis* is known to degrade cytoskeletal protein components such as β-actin (Kinane *et al.,* 2012) the involvement of potential tubulin degradation in stress granule modulation during *P. gingivalis* infection and oxidative stress was next investigated. No visible changes were found microtubule network during oxidative stress and *P. gingivalis* infection, compared to the nocodazole control (Figure 4-5). Microtubule network activity can however be controlled post-translationally through acetylation and phosphorylation (Magiera & Janke, 2014). Interestingly, during stress the tubulin network becomes hyper-acetylated at lysine 40 of α-tubulin (Li *et al.,* 2019), which stimulates increased binding and activity of microtubule motor proteins dynein and kinesin, which are involved in the movement of stress granules (Reed *et al.,* 2006; Cai *et al.,* 2009; Hammond *et al.,* 2010). In this study *P. gingivalis* dampened the levels of sodium arsenite oxidative stress-induced α-tubulin acetylation (Figure 4-6), independently of increased HDAC6 expression, the major α-tubulin deacetylase (Figure 4-7). HDAC6 is also a critical stress granule component, which when ablated results in inhibition of stress granule formation (Kwon *et al.,* 2007). Therefore, the lowered HDAC6 expression and reduced α-tubulin

acetylation may play a crucial role in stress granule dysregulation during *P. gingivalis* infection and oxidative stress.

5 Chapter 5: Identification of the molecular mechanisms of *P. gingivalis* interactions with host translational control during stress

5.1 Introduction

To elicit control over host cellular functions *P. gingivalis* employs a range of virulence factors including LPS, gingipains, CPS, fimbriae and hemagglutinins (discussed further in section 1.1.4).

One of the most prominent virulence factors employed by *P. gingivalis* are gingipains (O'Brien-Simpson *et al.,* 2003), a group of cysteine proteases that belong to the C25 family (Eichinger *et al.,* 1999). There are two classes of these trypsin-like proteases produced by *P. gingivalis*, namely arginine specific, encoded by the *rgpA* and *rgpB* genes, and lysine specific, encoded by the *kgp* gene (Travis *et al.,* 1997). To exert their proteolytic activity the arginine gingipains cleave the Arg-X dipeptide bonds and lysine gingipain cleaves at the carboxyl (COOH) side of the lysine residue (Pike *et al.,* 1994).

Gingipains may also be secreted in OMVs, facilitating further reach of their proteolytic function (Grenier *et al.,* 1989). As such, gingipains account for 85% of the extracellular proteolytic activity of *P. gingivalis* around the infection site (Potempa *et al.,* 1997; de Diego *et al.,* 2014). This proteolytic activity is pivotal to *P. gingivalis* virulence, to the extent that a gingipain based vaccine reduced total bacterial load of *P. gingivalis* as well as protected against bone resorption in nonhuman primates (Page *et al.,* 2007). In agreement, a gingipain inhibitor has also shown potent antibacterial activity, greatly reducing inflammation in a beagle dog model of periodontal disease (Kataoka *et al.,* 2014).

Functionally, gingipains are heavily implicated in colonisation of the gingival crevice, inducing chronic inflammation, ultimately resulting in the destruction of the surrounding host tissues, which leads to the expulsion of haem and nutrients from host cells (Andrian *et al.,* 2004; Sowmya *et al.,* 2017). *P. gingivalis* colonisation is aided by gingipains facilitating attachment to the extracellular matrix via degradation of collagen, inactivation of host plasma proteinase inhibitors, activation of host matrix metalloproteinases, and

cleavage of host cell surface receptors (Potempa *et al.,* 2000; Imamura *et al.,* 2003). In addition, gingipains play a crucial role in stabilising the dysbiotic subgingival oral community, facilitating the growth of *T. forsythia* and *Aggregatibacter actinomycetemcomitans* in multispecies biofilms (Bao *et al.,* 2014; Haraguchi *et al.,* 2014), whilst facilitating coaggregation between *P. gingivalis* and other oral pathogens such as *Treponema denticola* and *S. gordonii* (Guo *et al.,* 2010; Ito *et al.,* 2010). Furthermore, gingipains are involved in maturation and processing of virulence factors such as FimA, which is secreted to the outer membrane in a precursor form that requires the proteolytic activity of the arginine specific gingipains to reach its final filamentous form (Shoji *et al.,* 2004).

P. gingivalis gingipains play a key role in hijacking of host antibacterial and immune responses, which are inhibited as gingipains degrade bactericidal proteins such as complement factors (Hajishengallis *et al.,* 2013), T-cell receptors (Yun *et al.,* 2007), and neutrophil-derived α-defensin (Carlisle *et al.,* 2009). Furthermore, gingipains have been shown to be involved in immune subversion by degradation of various pro-inflammatory cytokines such as IL-1β (Stathopoulou *et al.,* 2009), IL-6 (Banbula *et al.,* 1999), IL-8 (Uehara *et al.,* 2008) and α-tubulin acetyltransferase 1 -α (Calkins *et al.,* 1998).

Given their involvement with nutrient acquisition, particularly haem, gingipains are heavily involved in haemolysis (Smalley *et al.,* 2008; Li *et al.,* 2010) and haem uptake (Simpson *et al.,* 2004) by *P. gingivalis*. To this end gingipains also increase the availability of haem around the infection site, by increasing bleeding through increasing the expression of thrombin to increase vascular permeability (de Diego *et al.,* 2013), as well as degrading fibrinogen and host haem proteins to inhibit blood coagulation (Sroka *et al.,* 2001).

Interestingly, it has been reported that mTOR complex 1 (mTORC1), a key regulator of cellular functions such as autophagy (Rabanal-Ruiz *et al.,* 2017) and translation initiation (Holz *et al.,* 2005), is inactivated by *P. gingivalis*

gingipains in an invasion independent manner via inactivation of the PI3K/protein kinase B (AKT) signalling pathway (Nakayama *et al.,* 2015). Hence, gingipains may aid in *P. gingivalis* evading autophagy, contributing to its persistence within infected cells. However, previous studies have shown that both *P. gingivalis* (Yilmaz *et al.,* 2004) and proteolytically inactive gingipains (Ciaston *et al.,* 2022) activate the PI3K/AKT pathway to induce a pro-inflammatory environment; therefore, the effects of gingipains on the PI3K/AKT pathway may be somewhat paradoxical and are potentially situation or environment specific. A study by Stafford *et al.,* (2013) also found that once *P. gingivalis* may inhibit mTORC1 independently of PI3K/AKT. Finding that once *P. gingivalis* had internalised into its host, the lysine-specific gingipain degraded the mTOR and therefore would lead to decreased mTORC1 activity.

The potential double inhibition of mTORC1 by *P. gingivalis* gingipains (Stafford *et al.,* 2013; Nakayama *et al.,* 2015), may be linked to heightened translational repression during stress. As mTORC1 acts as a key regulator of translation initiation, facilitating cap-complex formation (Gingras *et al.,* 1999) by phosphorylating p70-S6 kinase 1 (S6K1) and 4E-BP1, which facilitate the addition of eIF4B and eIF4E to the cap-complex, respectively (Pause *et al.,* 1994; Dufner & Thomas, 1999; Gingras *et al.,* 1999; Holz *et al.,* 2005). Fittingly, when mTORC1 is inhibited this results in polysome disassembly and translational stalling (Thoreen *et al.,* 2012; Sévigny *et al.,* 2020).

5.2 Aims

This chapter aims to elucidate the molecular mechanisms by which *P. gingivalis* dysregulates oxidative stress-induced translational repression by:

- Investigating the potential of *P. gingivalis* secretory factors in heightened translational repression during oxidative stress
- Investigating the role of mTORC1 inhibition to mediate heightened translational repression during oxidative stress
- Probing the impact of *P. gingivalis* gingipains upon host translational repression during oxidative stress

5.3 Results

5.3.1 *P. gingivalis* conditioned media heightens translational repression during stress

P. gingivalis employs a range of virulence factors to manipulate host responses, some of which are secreted (discussed in detail in section 1.1.4). To investigate whether the heightened translational repression during oxidative stress was mediated via a secreted factor, cells were treated with conditioned media generated by infecting H357 cells with *P. gingivalis* (NCTC11834, MOI 1 in 100, t=2h), following which the supernatants were centrifuged and bacterial cells removed by filtration (0.2 µM). H357 cells were subsequently incubated with the resulting conditioned media for 2 hours with or without sodium arsenite challenge (250 µM) for the final 30 mins following which total protein was extracted and expression of puromycin incorporation and eIF2α phosphorylation was probed by immunoblotting.

Cells treated with conditioned media did not alter the rates of translation or eIF2α phosphorylation following the two hour incubation period (Figure 5-1 and Figure 5-2). When oxidative stress was added in the final 30 minutes of conditioned media challenge, protein synthesis and eIF2α phosphorylation also decreased in line with infected cells, compared to the oxidative stress-only control (Figure 5-1 and Figure 5-2). Taken together these data demonstrate that factors released during *P. gingivalis* infection of H357 cells are capable of heightening oxidative stress-induced translational repression in a manner independent of eIF2α signalling.
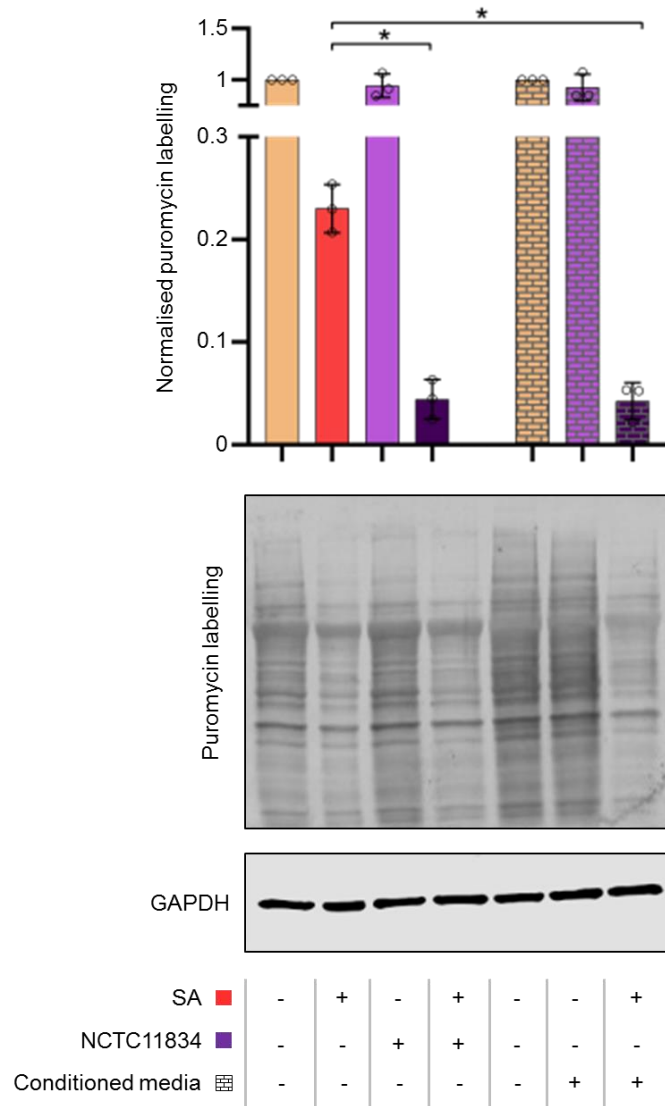
Figure 5-1 *P. gingivalis* **conditioned media heightens translational repression during exogenous stress.** Cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) or *P. gingivalis* conditioned media (as detailed in section 2.6, t=2h) with or without sodium arsenite (SA; 250 µM) challenge for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

SA 🟥	-	+	-	+	-	-	+
NCTC11834 🟪	-	-	+	+	-	-	-
Conditioned media ▦	-	-	-	-	-	+	+
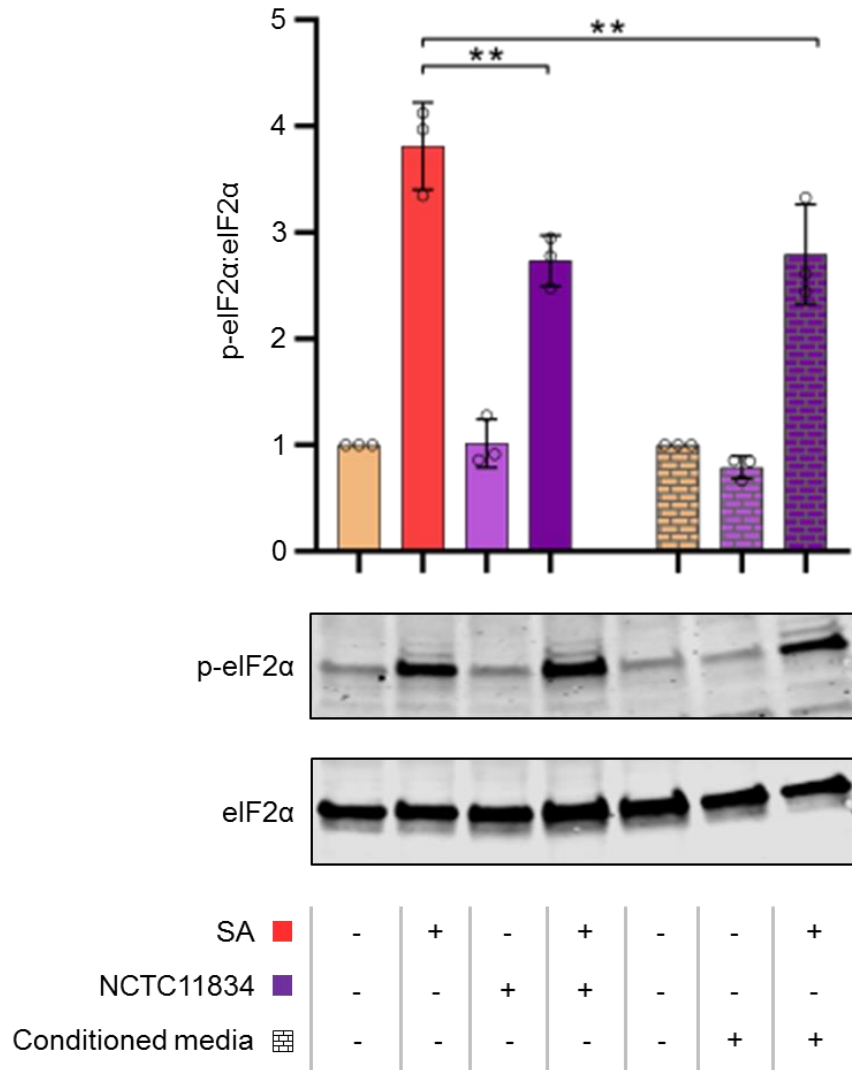
Figure 5-2 *P. gingivalis* **conditioned media dampens stress induced eIF2α phosphorylation during exogenous stress.** Cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) or *P. gingivalis* conditioned media (as detailed in section section 2.6, t=2h) with or without sodium arsenite (SA; 250 μM) challenge for the final 30 mins. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). **, $P \leq 0.01$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

5.3.2 Uninfected cells of the *P. gingivalis* infected population also displayed increased stress granule formation during oxidative stress

As *P. gingivalis* conditioned media also heightened translational repression the ability of a secretory factor to modulate stress granule formation was next investigated. During a two-hour infection period not every H357 cell is infected with *P. gingivalis*, hence stress granule formation was compared between cells positive and negative for internalised *P. gingivalis* in the infected population exposed to oxidative stress.

H357 cells were infected with *P. gingivalis* with and without sodium arsenite as previously detailed and both stress granule formation and *P. gingivalis* invasion were visualised using immunofluorescence confocal microscopy. Whilst the whole cell population was exposed to *P. gingivalis*, not all cells were infected (evidenced by *P. gingivalis* internalisation) (Figure 5-3 A). Cells infected with *P. gingivalis* increased the frequency of stress granules during oxidative stress (Figure 5-3 B). Increased stress granule frequency during oxidative stress was also observed in the uninfected cells of the *P. gingivalis* treated population. Intriguingly, an increase in stress granule area was also observed in the uninfected cells of the *P. gingivalis* treated population, although this was less pronounced (Figure 5-3 B).
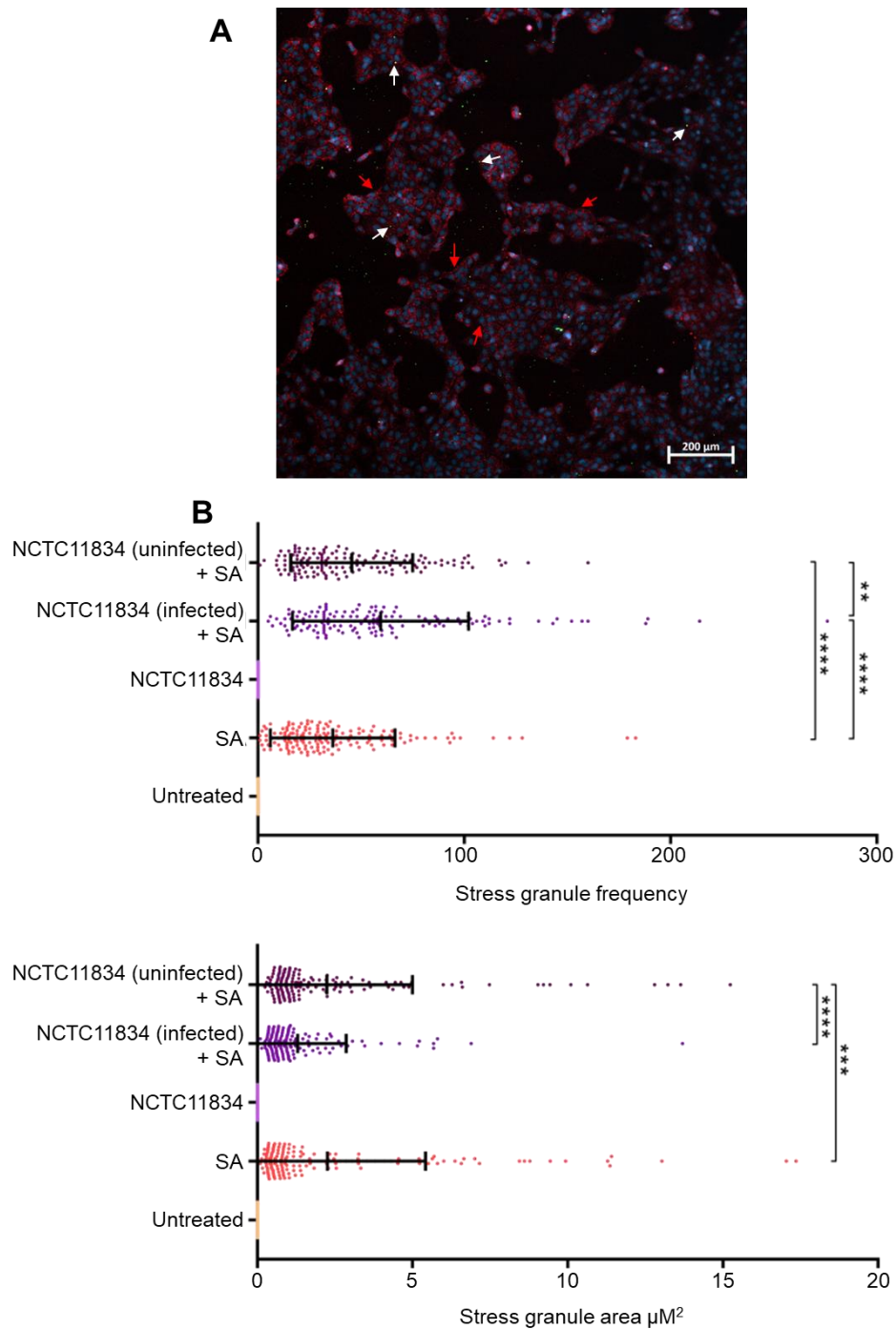
Figure 5-3 Uninfected cells of the *P. gingivalis* infected population also displayed increased stress granule frequency during oxidative stress. H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 µM) for the final 30 mins. Stress granule formation was visualised using antibodies to of G3BP1 (red) and *P. gingivalis* (green) using confocal microscopy and Z-stacks. Red arrows point to example cells without *P. gingivalis* infection and white arrows point to cells infected with *P. gingivalis* (mean± SD, n=3, 50 cells per biologic replicate). **** $P \le 0.001$; ***, $P \le 0.001$; **, $P \le 0.01$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test. Scale bar is 200µM.

5.3.3 High concentration conditioned media heightens translational repression during stress

The conditioned media previously used in section 5.3.1 was produced by bacteria infecting cells at an MOI of 100, which was likely of a low concentration of bacterial secretants and did not induce ISR activation alone. To ascertain whether the secreteome of *P. gingivalis* had the potential to induce ISR activation, cell culture media conditioned with a high concentration of *P. gingivalis* was next employed.

This high concentration media conditioned with *P. gingivalis* was generated as outlined in section 2.7. Briefly, *P. gingivalis* was cultured anaerobically overnight in cell culture medium and the conditioned media isolated from bacterial cells. The purity of *P. gingivalis* grown in liquid culture to generate the high concentration conditioned media was confirmed using Gram staining (Figure 5-4). H357 cells were challenged with this high concentration conditioned media (t=2h) with or without sodium arsenite (250 µM) for the final 30 mins. High concentration conditioned media decreased translation to a similar level as oxidative stress compared to the untreated control. When conditioned media challenge was coupled with oxidative stress for the final 30 mins, translational attenuation was heightened 1.82-fold, however this trend fell short of significance (p>0.05) (Figure 5-5). The ability of high concentration conditioned media alone to induce translational stalling was further confirmed by increased eIF2α phosphorylation (Figure 5-6).

Taken together these results indicate that at high concentrations conditioned media may be capable of inducing the integrated stress response.
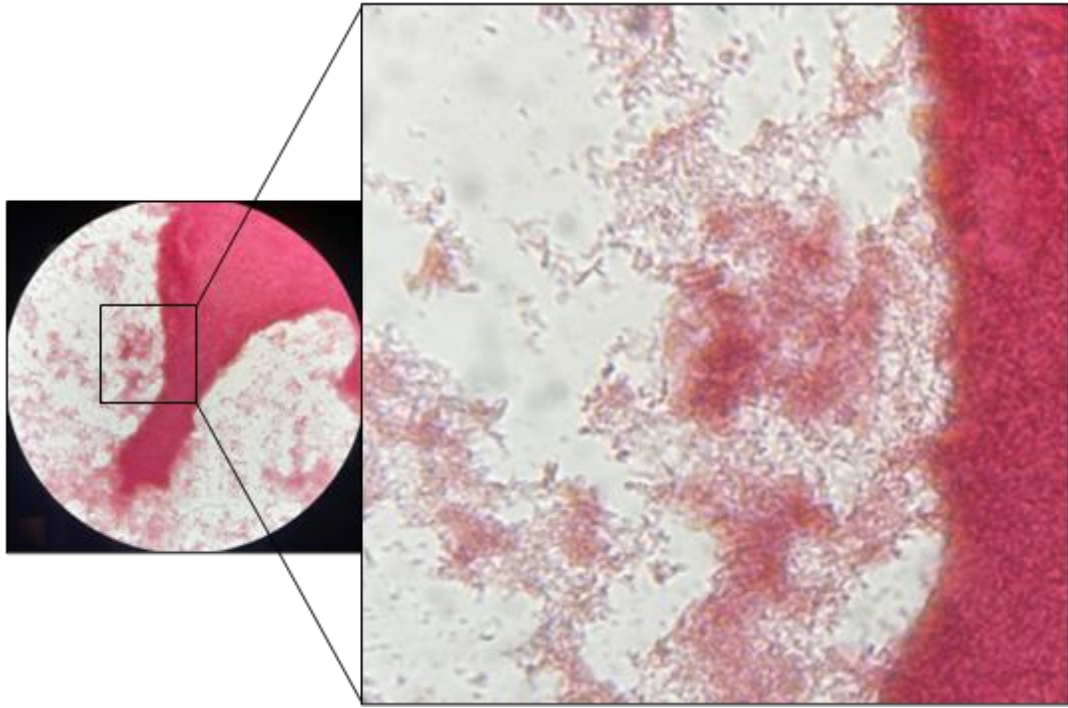
Figure 5-4 *P. gingivalis* liquid cultures used to generate high conc. conditioned media were not contaminated by Gram positive bacteria. *P. gingivalis* (NCTC11834) were grown in liquid as detailed in section 2.7 to produce high conc. conditioned media. After the centrifugation step the pellet was resuspended in PBS and subjected to Gram staining as detailed in section 2.3.4.
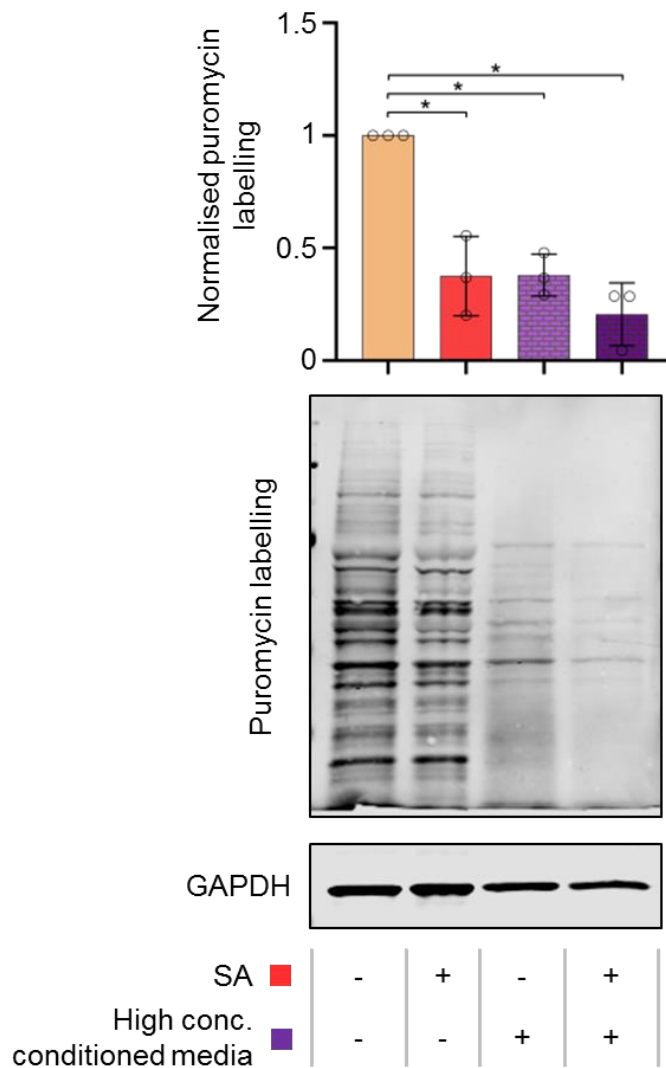
Figure 5-5 High conc. *P. gingivalis* conditioned media induces translational repression. Cells were infected with *P. gingivalis* conditioned media (as detailed in section 2.7, t=2h) with or without sodium arsenite challenge (SA; 250 µM) for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
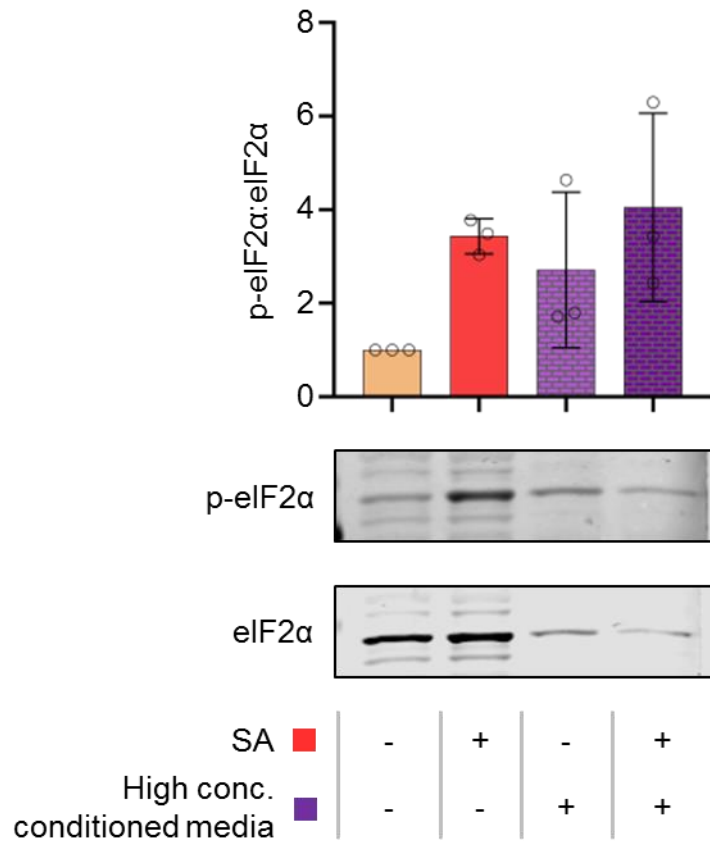
Figure 5-6 High conc. *P. gingivalis* conditioned media increases eIF2α phosphorylation. Cells were infected with *P. gingivalis* conditioned media (as detailed in section 2.7, t=2h) with or without sodium arsenite challenge (SA; 250 µM) for the final 30 mins. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). **, $P ≤ 0.01$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

5.3.4 *P. gingivalis* outer membrane vesicles heighten translational repression during stress

To facilitate the delivery of virulence factors and signalling molecules *P. gingivalis* secretes OMVs (Grenier & Mayrand, 1987). As *P. gingivalis* conditioned media had elicited heightened translational repression during oxidative stress the potential role of OMVs in this response was next investigated.

P. gingivalis (NCTC11834) OMVs were prepared as detailed in section 2.3.6. Prior to OMVs isolation the purity of *P. gingivalis* liquid cultures was determined using Gram staining (Figure 5-7). Following extraction, the potential for OMVs to induce cellular stress was determined by immunoblotting for puromycin incorporation and eIF2α phosphorylation. H357 cells were challenged with *P. gingivalis* OMVs (1, 10, 100 μg/mL, t=2h). Sodium arsenite treatment was included as a positive control. OMVs did not induce cellular stress at any concentration used, as evidenced by a lack of translational attenuation or increased eIF2α phosphorylation (Figure 5-8 and Figure 5-9).

Next the ability of OMVs to heighten translational repression was investigated. In the presence of oxidative stress OMVs heightened translational repression 2.13-fold in a manner (Figure 5-10) independent of eIF2α signalling, as eIF2α phosphorylation was not increased, but in fact decreased 1.69-fold (Figure 5-11).
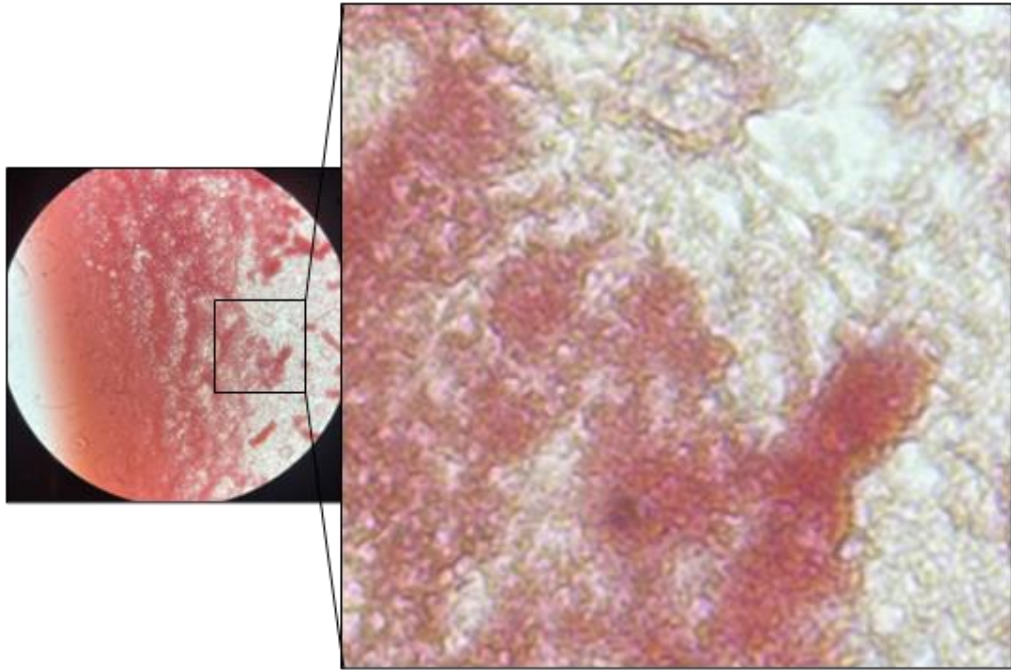
Figure 5-7 Liquid cultures used to isolate crude *P. gingivalis* OMVs were not contaminated by Gram positive bacteria. *P. gingivalis* (NCTC11834) were cultured in liquid OMVs extracted as detailed in section 2.3.6. After the centrifugation step the pellet was resuspended in PBS and subjected to Gram staining as detailed in section 2.3.4.
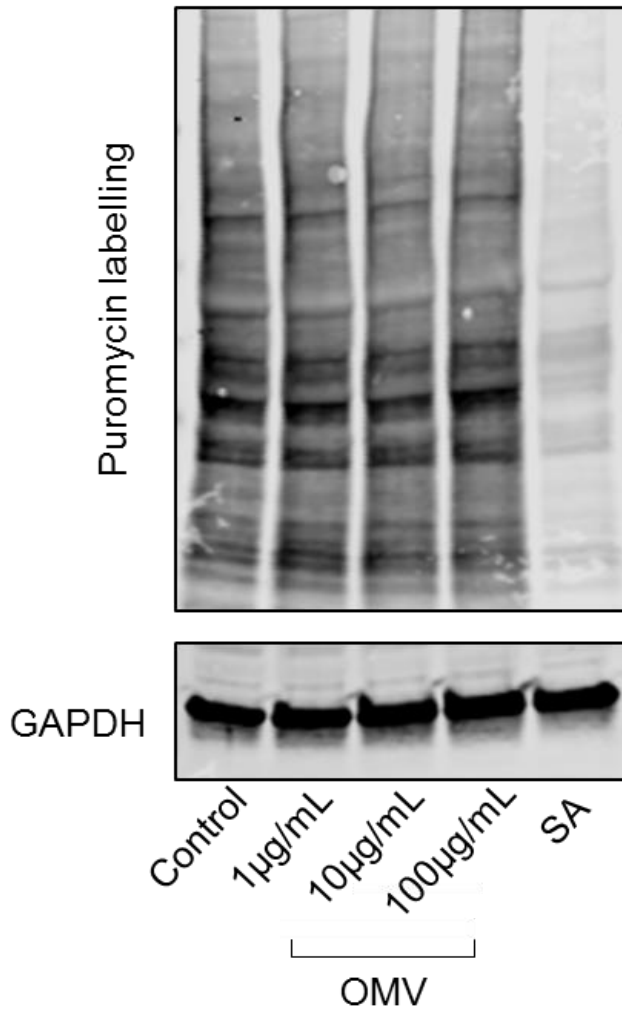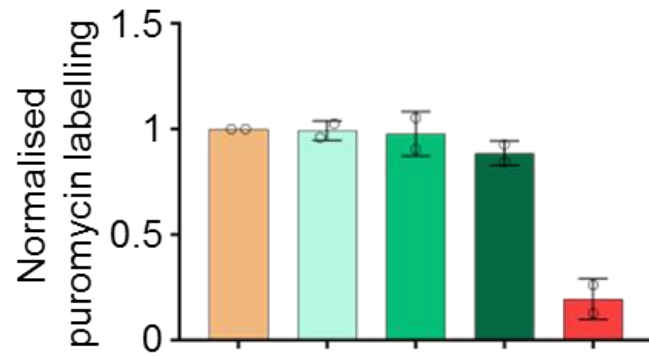
Figure 5-8 Crude *P. gingivalis* OMVs do not inhibit protein synthesis. H357 cells were challenged with crude *P. gingivalis* (NCTC11834) derived OMVs (1, 10 or 100 µg/mL, t=2h). Sodium arsenite (SA; 250 µM, t=30mins) was included as a control. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=2).
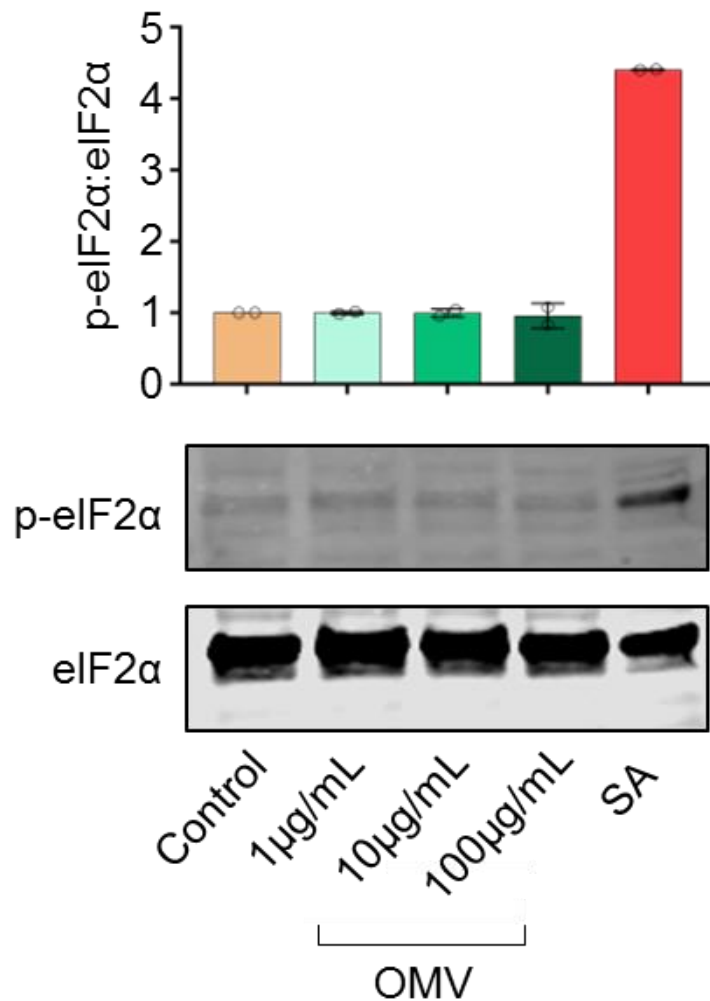
Figure 5-9 Crude *P. gingivalis* OMVs do not induce eIF2α phosphorylation. H357 cells were challenged with crude *P. gingivalis* (NCTC11834) derived OMVs (1, 10 or 100 µg/mL, t=2h). Sodium arsenite (SA; 250 µM, t=30mins) was included as a control. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=2).
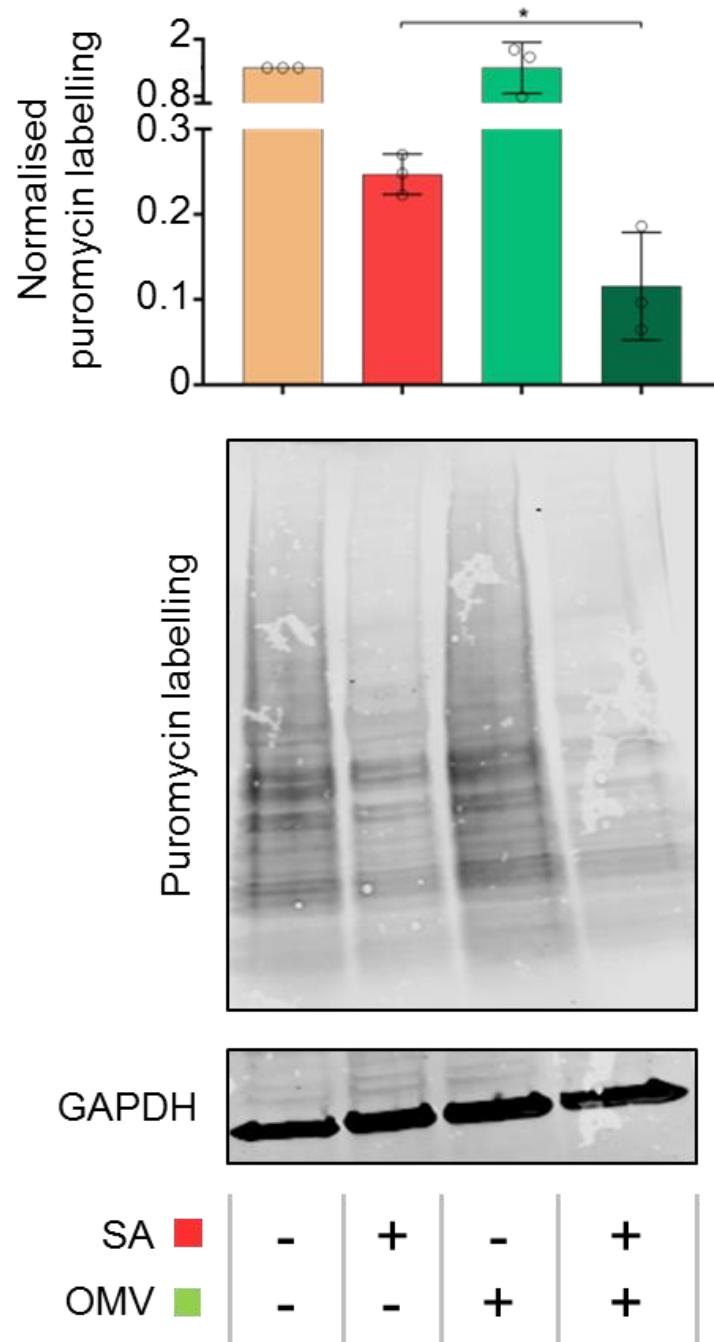
Figure 5-10 Crude *P. gingivalis* OMVs heighten translational repression during oxidative stress. H357 cells were challenged with crude *P. gingivalis* (NCTC11834) derived OMVs (100 µg/mL, t=2h), with or without sodium arsenite (SA; 250 µM) for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
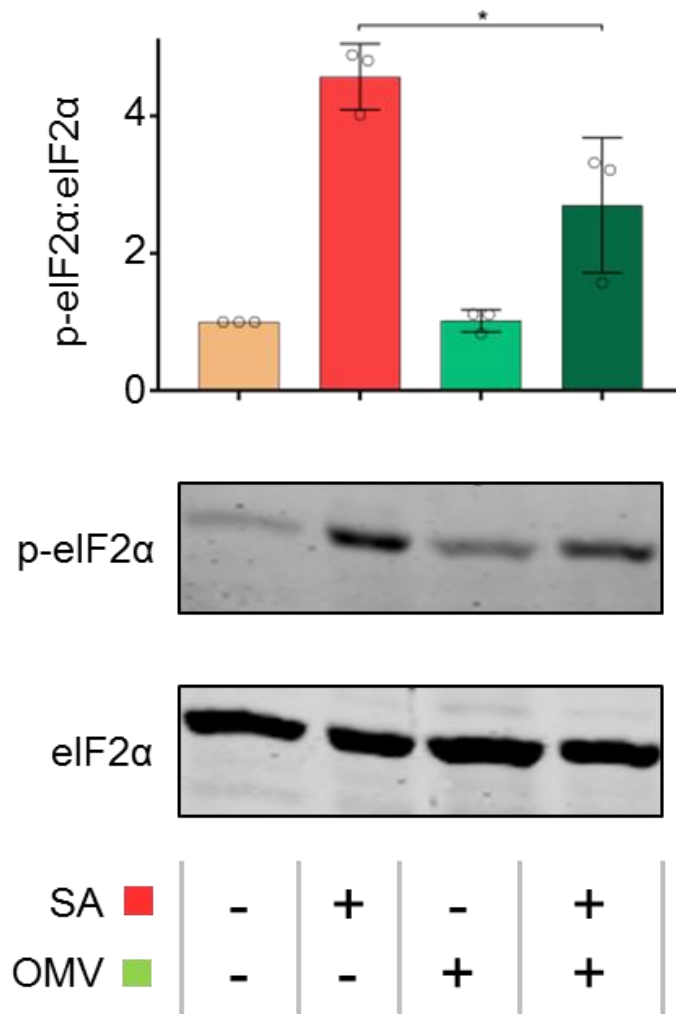
Figure 5-11 Crude *P. gingivalis* OMVs dampen eIF2α phosphorylation during oxidative stress. H357 cells were challenged with crude *P. gingivalis* (NCTC11834) derived OMVs (100 µg/mL, t=2h), with or without sodium arsenite (SA; 250 µM) for the final 30 mins. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

5.3.5 *P. gingivalis* lipopolysaccharide do not interact with host translational repression during stress

A further bacterial virulence factor employed by *P. gingivalis* is LPS. Activity of the ISR kinase HRI, and subsequent eIF2α phosphorylation has previously been reported in response to *E. coli* LPS (Liu *et al.,* 2007). Whilst our previous findings have shown that *P. gngivalis* infection the potential of any contributing role of LPS to heightened translational attenuation during stress was next investigated.

Firstly, the possibility that LPS may activate the ISR was investigated using immunoblotting. H357 cells were challenged with commercially obtained pure *P. gingivalis* LPS (Strain NCTC11834; Sigma; 1, 5, 10 µg/mL, t=2h). Sodium arsenite was included as a positive control. None of the concentrations of LPS tested induced cellular stress, evidenced by a lack of translational attenuation (Figure 5-12) or increased eIF2α phosphorylation (Figure 5-13).

Next the potential of LPS to heighten translation was assessed using immunoblotting. H357 cells were treated with LPS (10 µg/mL, t=2h) with or without sodium arsenite (250 µM) for the final 30 mins. In the presence of oxidative stress LPS did not alter translational repression (Figure 5-14) or eIF2α phosphorylation (Figure 5-15). These data indicate that heightened translational repression during *P. gingivalis* infection and oxidative stress is independent of LPS.
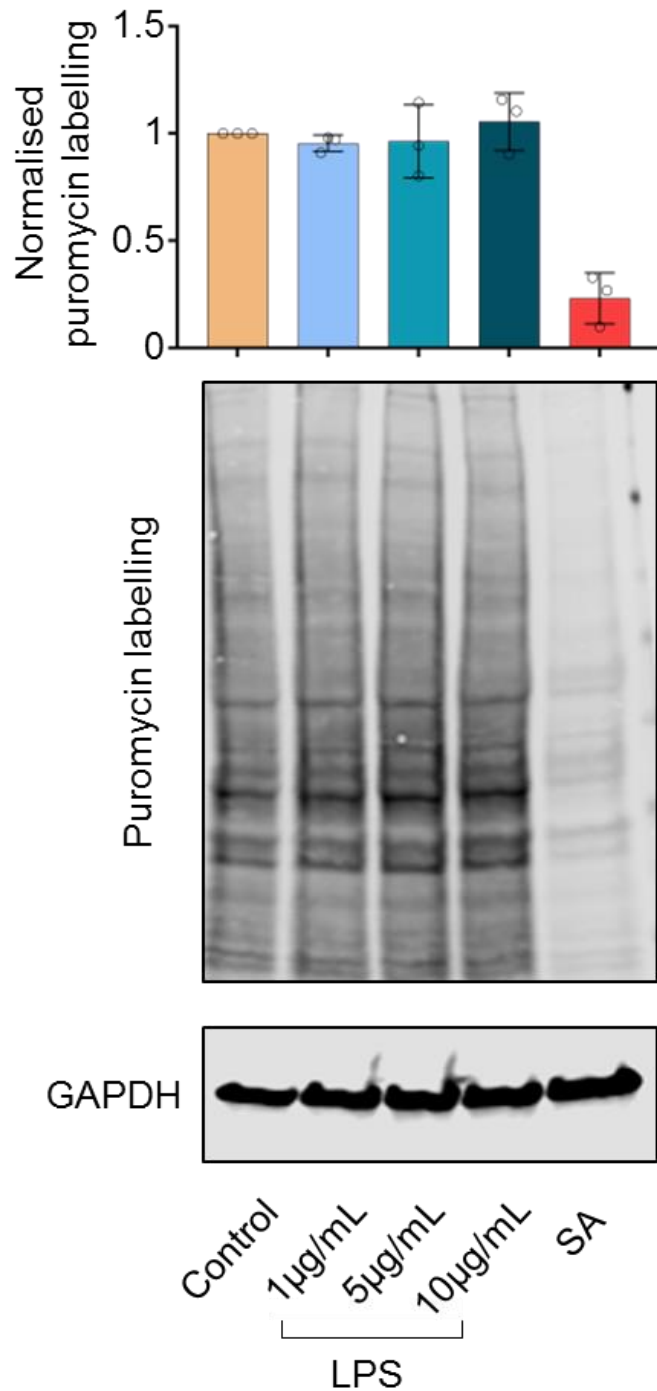
Figure 5-12 *P. gingivalis* LPS does not inhibit protein synthesis. H357 cells were challenged with *P. gingivalis* (NCTC11834)-derived LPS (1, 5 or 10 µg/mL, t=2h). Sodium arsenite (SA; 250 µM, t=30mins) was included as a control. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.
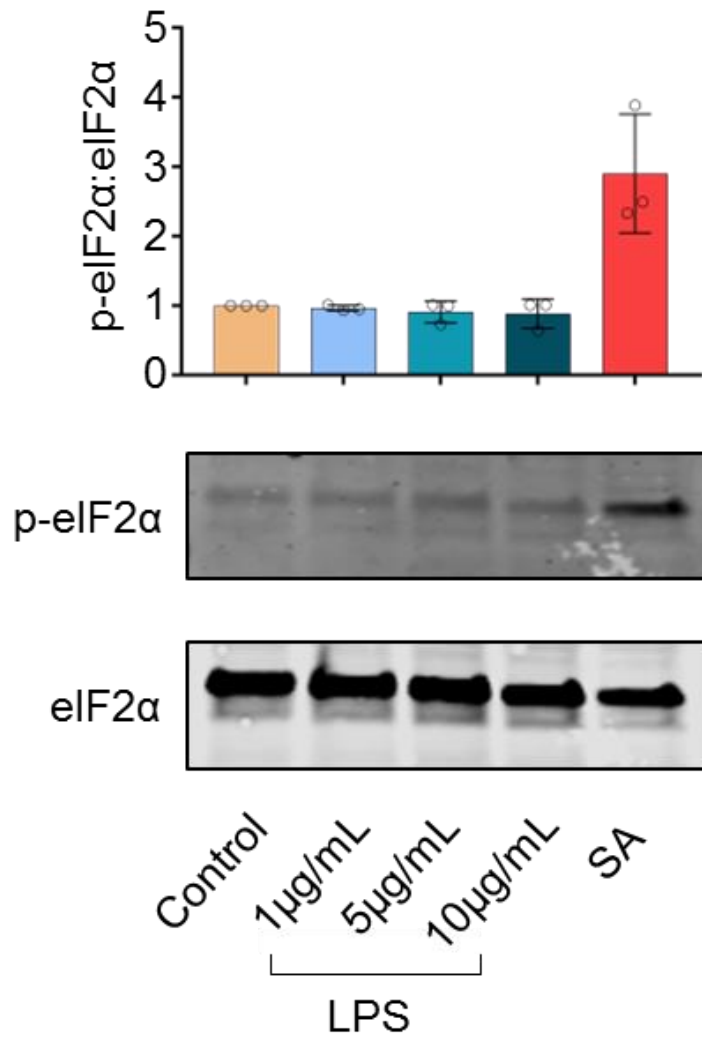
Figure 5-13 *P. gingivalis* LPS does not induce eIF2α phosphorylation.
H357 cells were challenged with *P. gingivalis* (NCTC11834)-derived LPS (1, 5 or 10 µg/mL, t=2h). Sodium arsenite (SA; 250 µM, t=30mins) was included as a control. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.
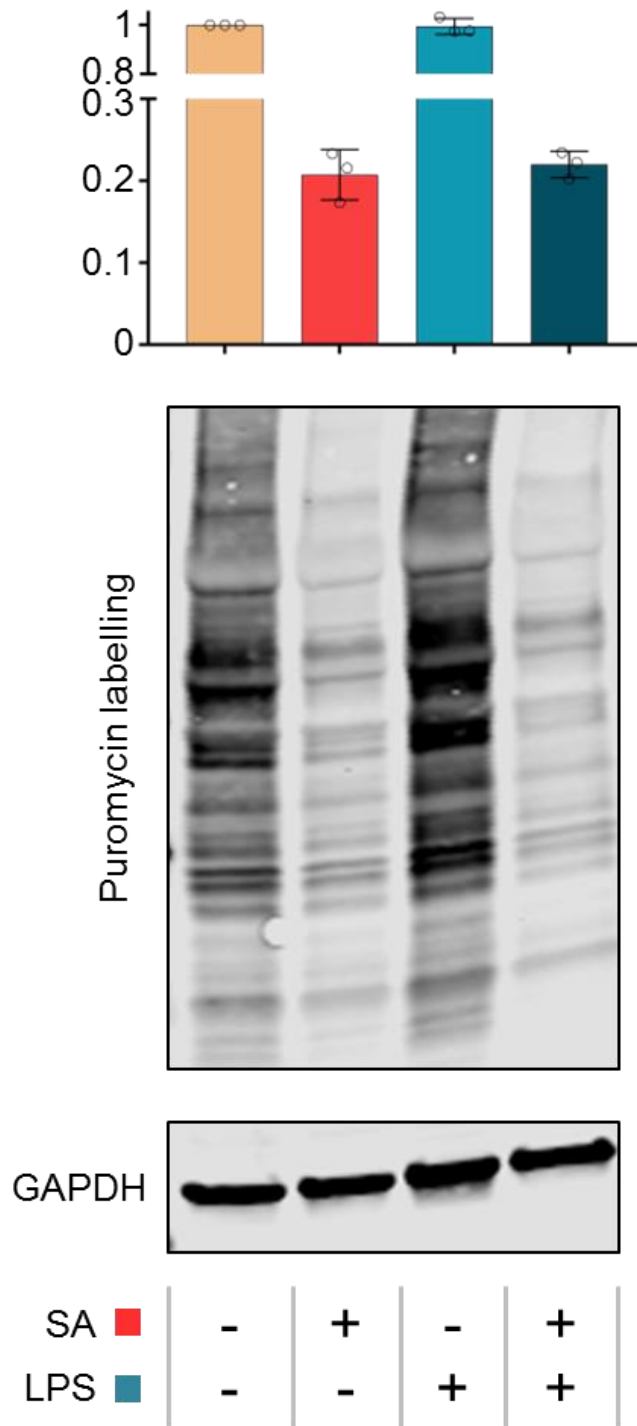
Figure 5-14 *P. gingivalis* **LPS does not change translational repression during oxidative stress.** H357 cells were challenged with *P. gingivalis* (NCTC11834)-derived LPS (10 µg/mL, t=2h), with or without sodium arsenite (SA; 250 µM) for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.
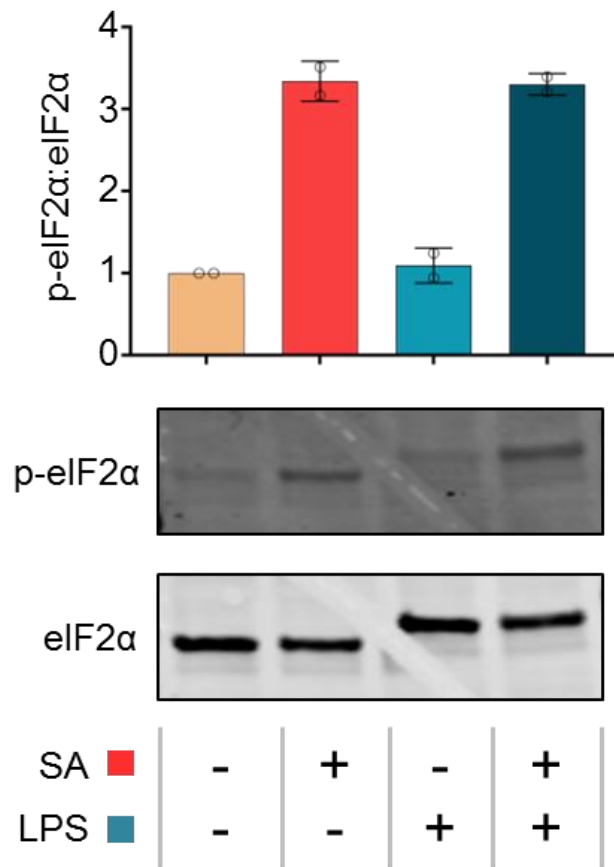
Figure 5-15 *P. gingivalis* **LPS does not change eIF2α phosphorylation during oxidative stress.** H357 cells were challenged with *P. gingivalis* (NCTC11834)-derived LPS (10 μg/mL, t=2h), with or without sodium arsenite (SA; 250 μM) for the final 30 mins. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.

5.3.6 Rapamycin treatment mirrors *P. gingivalis* mediated heightened translation repression during stress

mTORC1 has been implicated in translational control (Sévigny *et al.,* 2020). Earlier studies have indicated that *P. gingivalis* can both degrade and inhibit mTORC1 via the action of its gingipains (Stafford *et al.,* 2013; Nakayama *et al.,* 2015). As heightened translational repression during oxidative stress and *P. gingivalis* infection was independent of eIF2α signalling the potential role of mTORC1 signalling was evaluated. Firstly, the effect of the mTOR selective inhibitor, rapamycin, was investigated in relation to translation and eIF2α signalling during oxidative stress.

H357 cells were treated with rapamycin (400 nM, t=1h) with or without sodium arsenite (250 µM) for the final 30 minutes, follow by analysis of puromycin incorporation and eIF2α phosphorylation. In the presence of sodium arsenite, rapamycin treatment decreased puromycin incorporation 1.54-fold (Figure 5-16), a response which was independent of increased eIF2α phosphorylation (Figure 5-17). These results showed similarities to the heightened translational repression observed during stress and *P. gingivalis* infection.
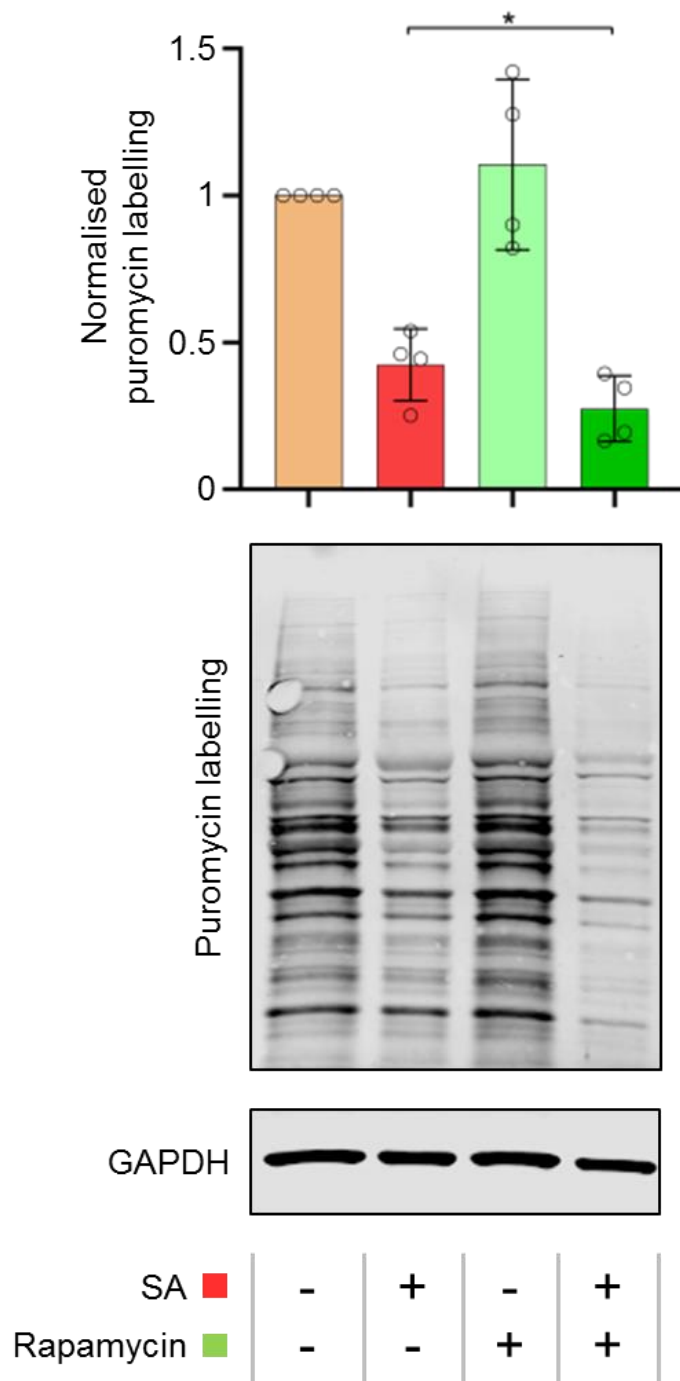
Figure 5-16 Rapamycin treatment heightens translational repression during oxidative stress. H357 cells were treated with rapamycin (400 nM, t=1h) with or without sodium arsenite (SA; 250 μM) for the final 30 minutes. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=4). *, $P \leq$ 0.05 according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
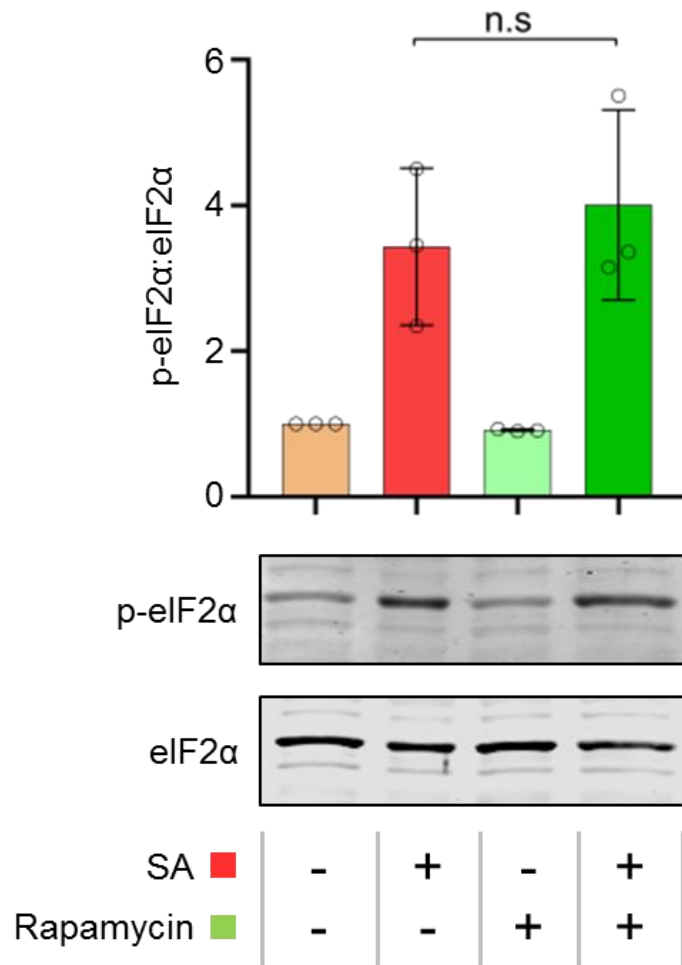
Figure 5-17 Rapamycin treatment does not alter eIF2α phosphorylation during oxidative stress. H357 cells were treated with rapamycin (400 nM, t=1h) with or without sodium arsenite (SA; 250 µM) for the final 30 minutes. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.

5.3.7 *P. gingivalis* infection decreases stress induced p70-S6-Kinase (Thr389) phosphorylation

mTORC1 activity is known to phosphorylate S6 kinase 1 (S6K1) at T389, which allows the entry of eIF4B to the cap complex (Figure 1-7), which subsequently allows formation of the 48S pre-initiation complex and start codon recognition (Pause *et al.,* 1994; Dufner & Thomas, 1999). As the data point towards mTORC1 dysregulation as the effector of heightened translational repression during stress and *P. gingivalis* infection, levels of p-70-S6K1 phosphorylation (T389) was investigated.

H357 cells were infected with *P. gingivalis* (t=2-6h) with or without sodium arsenite as described before, and levels of p-70-S6K1 phosphorylation (T389) were next determined. Rapamycin treatment (400 nM, t=1h) was included as a control for mTORC1 inhibition. Rapamycin resulted in a 2.77-fold decrease in the levels of p-p70-S6K1 (T389), whereas oxidative stress induced an increase of 2.67-fold, compared to the untreated control. In contrast, whilst *P. gingivalis* infection alone did not result in altered levels of p-p70-S6K1 (T389), infection in the presence of oxidative stress caused a 1.73-fold decrease at all timepoints investigated (Figure 5-18). These data suggest that the phosphorylation activity of mTORC1 is downregulated by *P. gingivalis* infection during stress.
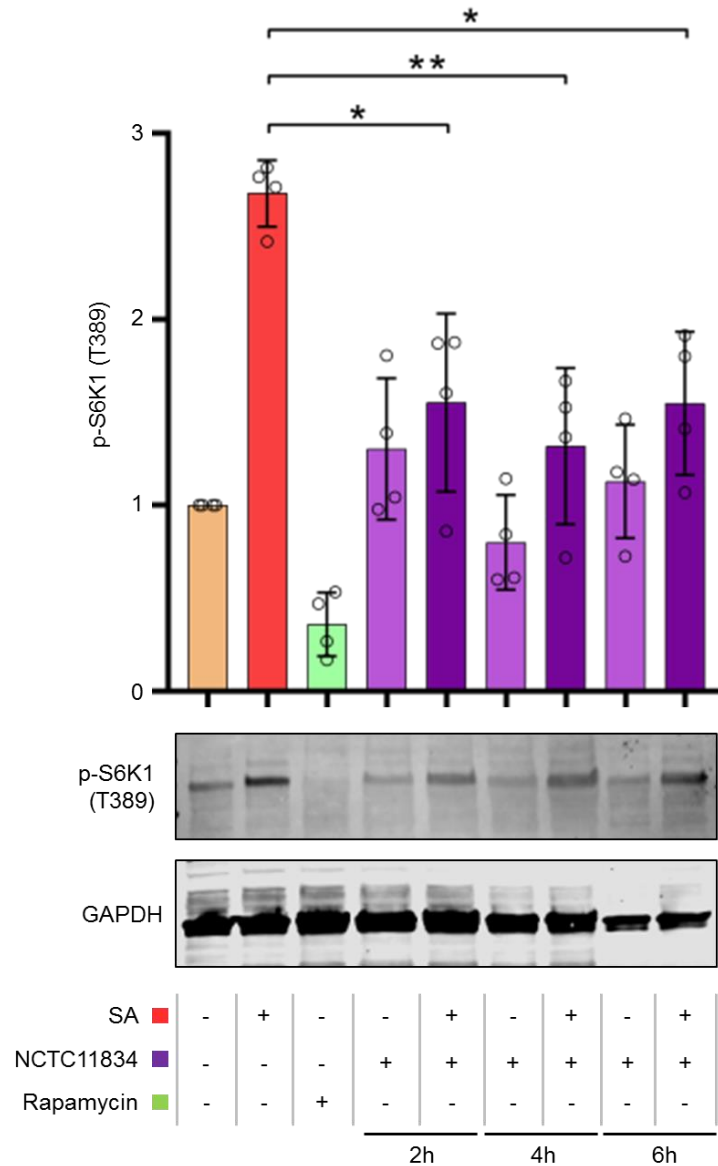
Figure 5-18 ***P. gingivalis*** **infection attenuates oxidative stress-induced p-p70-S6-Kinase (T389).** H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (250 µM) for the final 30 mins. Rapamycin treatment (400 nm, t=30mins) was included as a control. Expression of p-p70-S6-Kinase (T389) and its concentration relative to GAPDH were determined via immunoblotting (mean ± SD, n =4). **, $P \leq 0.01$; *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

5.3.8 *P. gingivalis* lowers stress induced 4E-BP1 phosphorylation

A further target of mTORC1 activity is 4E-BP1, phosphorylation of which is a tacit for cap-complex formation and subsequently functional translation initiation (Gingras *et al.,* 1999; Holz *et al.,* 2005; Sonenberg & Hinnebusch, 2009). Therefore, to further investigate the role of mTORC1 activity during stress and *P. gingivalis* infection the expression of phosphorylated 4E-BP1 was probed.

Following H357 treatment as previously described with *P. gingivalis* in the presence or absence of sodium arsenite, levels of p-4EBP1 were next quantified. Rapamycin treatment (400 nM, t=1h) was included as a control for mTORC1 inhibition. All treatments decreased p-4EBP1 at least 1.57-fold compared to the untreated control indicating that both oxidative stress and *P. gingivalis* infection during oxidative stress inhibit the activity of mTORC1 on 4E-BP1 phosphorylation, however this trend fell short of significance (p=0.0639) (Figure 5-19).
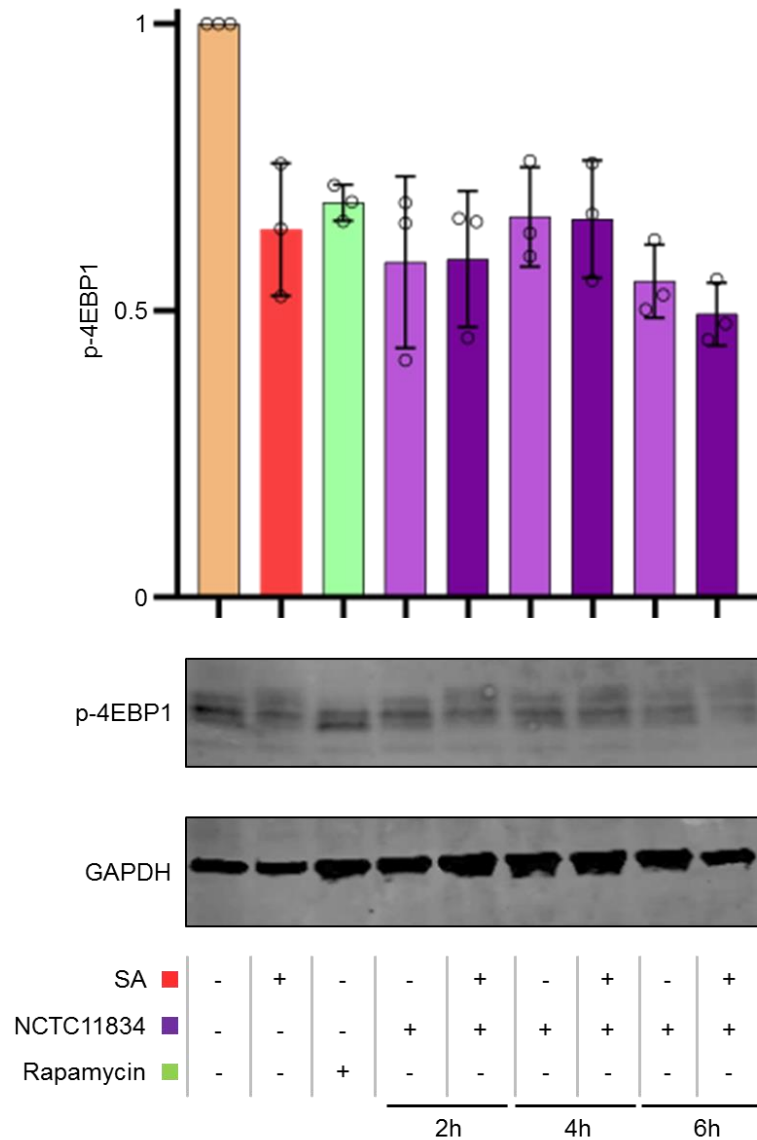
Figure 5-19 *P. gingivalis* infection lowers expression of p-4EBP1. H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 µM) for the final 30 mins. Rapamycin treatment (400 nm, t=30mins) was included as a control. Expression of p-4EBP1 and its concentration relative to GAPDH were determined via immunoblotting (mean ± SD, n =4). **, $P \leq 0.01$; *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

5.3.9 *P. gingivalis* gingipains facilitate heightened translational repression during stress

P. gingivalis gingipains have previously been shown to both inhibit and degrade mTORC1 (Stafford *et al.,* 2013; Nakayama *et al.,* 2015). As mTOR was increasingly implicated in translational dysregulation during *P. gingivalis* infection and oxidative stress the impact of gingipains on translation was probed.

H357 cells were treated with *P. gingivalis* conditioned media (generated as outlined in section 2.6, t=2h) in the presence of gingipain specific inhibitors TLCK (Lysine-specific, kgp) and Leupeptin (Arginine-specific, rgp) with or without sodium arsenite (250 µM) for the final 30 mins. Both Leupeptin and TLCK either alone or in tandem, inhibited the ability of the conditioned media to heighten translational stalling during oxidative stress (Figure 5-20).

To further confirm the role of gingipains in translational attenuation a series of isogenic gingipain null mutants in *P. gingivalis* strain W50 were studied. Neither the wild type W50 strain nor the mutant strains (K1A [-kgp], E8 [-rgp] and EK18 [-kgp-rgp]) induced a change in protein synthesis during infection (MOI of 100, t=2h) in the absence of oxidative stress (Figure 5-21). W50 infection (t=2h) in the presence of sodium arsenite (250 µM) for the final 30 mins, decreased puromycin incorporation 3.5-fold, compared to the sodium arsenite only control; however, the mutants were unable to elicit this effect (Figure 5-22).

These findings implicate gingipains in *P. gingivalis* mediated heightened translational repression during oxidative stress and hence the effect of gingipains on stress granules was investigated. In H357 cells, neither infection with the wildtype W50 strain nor gingipain mutants induced or inhibited stress granule formation in the absence or presence of sodium arsenite, respectively (Figure 5-23). In sodium arsenite treated H357 cells, infection with wild-type W50 and gingipain mutants E8 and EK18 induced an

increase in stress granule frequency, which was not observed in K1A infected cells. Neither wild-type, K1A nor EK18 changed the average stress granule area, whereas surprisingly the E8 mutant increased the area of stress granules (Figure 5-24).

Taken together these findings indicate that both lysine- and arginine-specific gingipains contribute to *P. gingivalis* mediated heightened translational repression during oxidative stress, with the lysine-specific gingipain inducing the increased stress granule frequency.
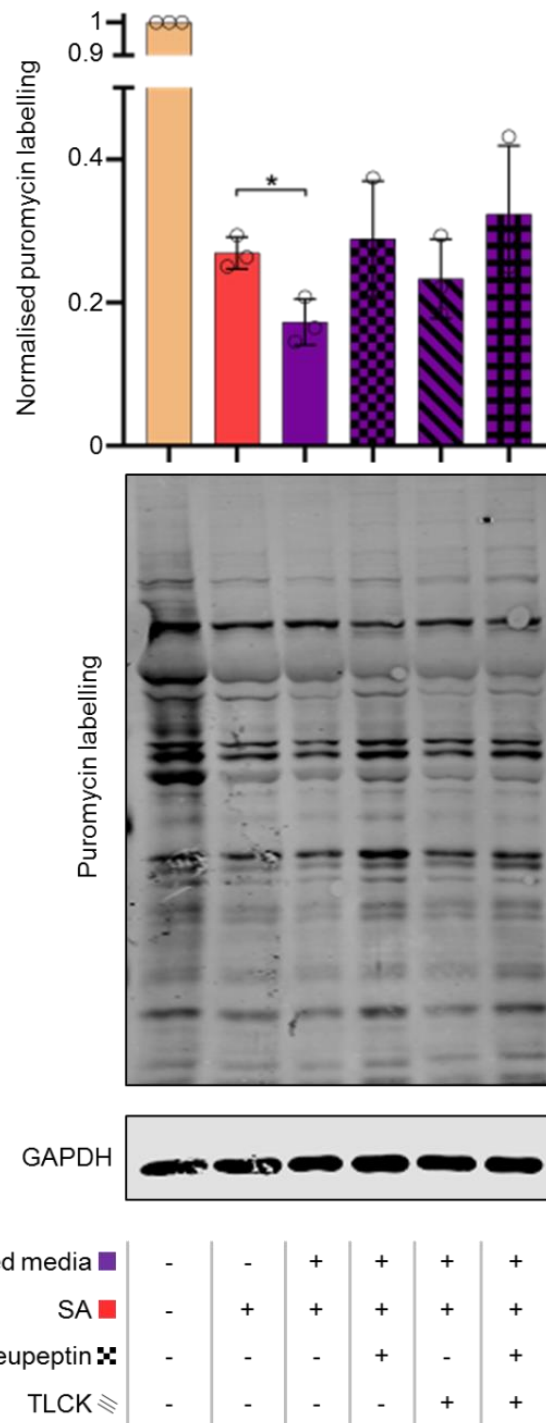
Figure 5-20 Gingipain inhibitors hinder the ability of *P. gingivalis* to heighten translational attenuation during oxidative stress. H357 cells were challenged with *P. gingivalis* conditioned media (t=2h) in the presence or absence of leupeptin or TLCK and with sodium arsenite (SA; 250 μM) for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
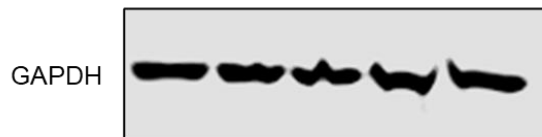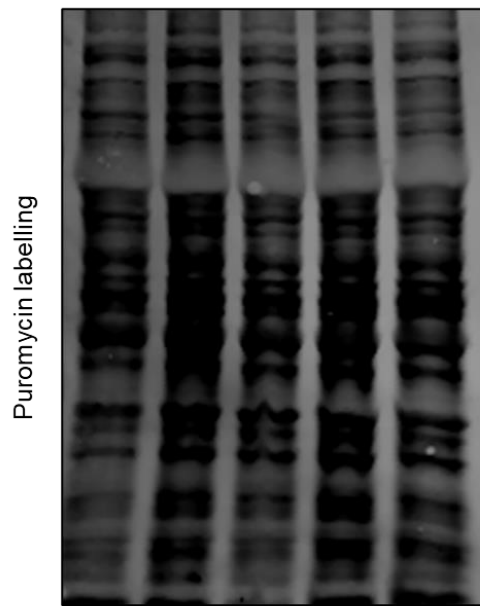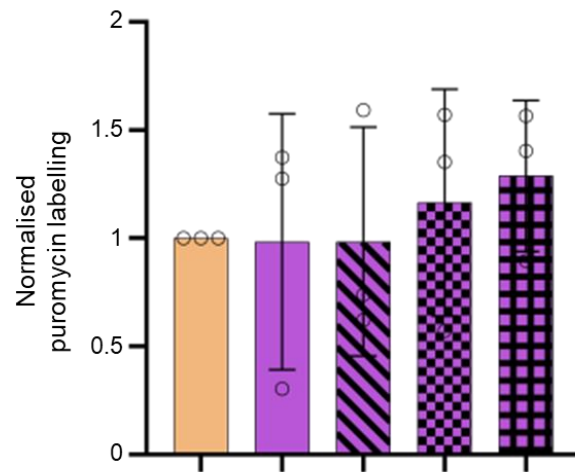
Figure 5-21 *P. gingivalis* gingipain-deficient mutants do not induce translational stalling. H357 cells were infected with *P. gingivalis* (W50, K1A (*kgp*-), E8 (*rgp*-) and EK18 (*rgp*-*kgp*-), MOI of 100; t=2h). The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=3). No significant differences in means were found with a Kruskal-Wallis test.
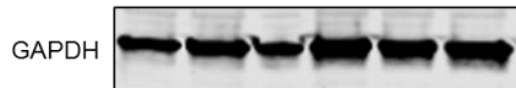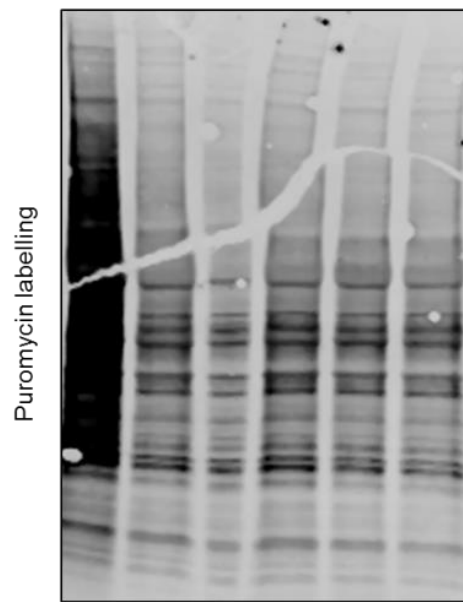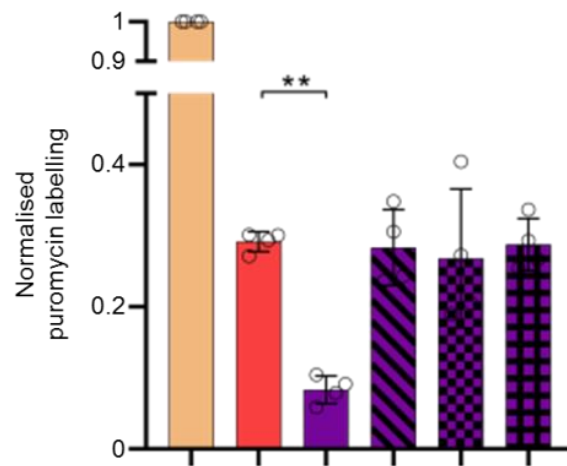
Figure 5-22 *P. gingivalis* gingipain-deficient mutants do not heighten translation attenuation during oxidative stress. H357 cells were infected with *P. gingivalis* (W50, K1A (*kgp⁻*), E8 (*rgp⁻*) and EK18 (*rgp⁻kgp⁻*), MOI of 100; t=2h), with or without sodium arsenite (SA; 250 µM) for the final 30 mins. The relative rate of protein synthesis as measured by puromycin uptake and the concentration relative to GAPDH enumerated (mean ± SD, n=4). No significant differences in means were found with a Kruskal-Wallis test. **, *P* ≤ 0.01 according to Kruskal-Wallis with Conover-Inman *post-hoc* test.
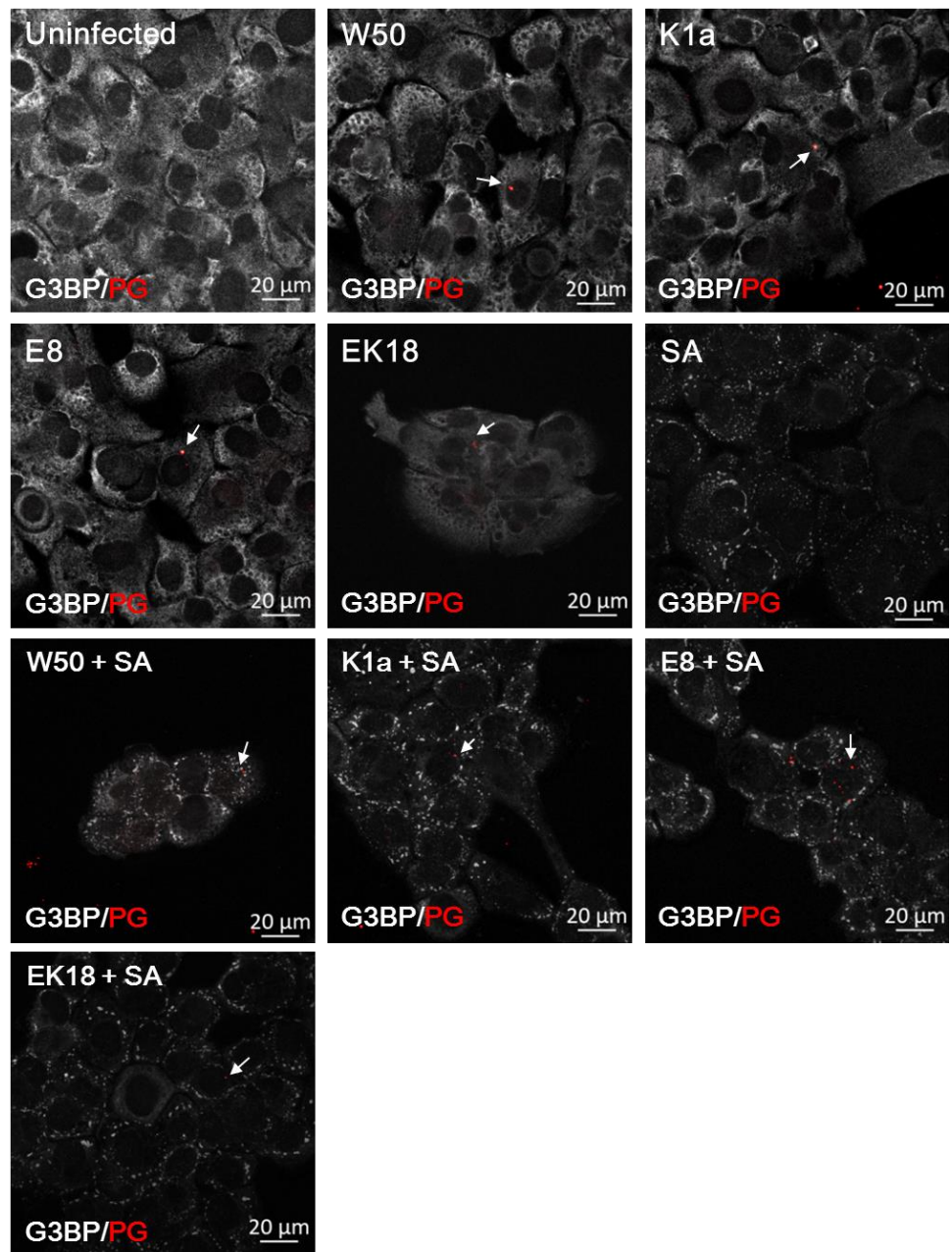
Figure 5-23 *P. gingivalis* **gingipain-deficient mutants do not induce or inhibit stress granule formation.** H357 cells were infected with *P. gingivalis* (W50, K1A (*kgp*⁻), E8 (*rgp*⁻) and EK18 (*rgp*⁻*kgp*⁻), MOI of 100; t=2h), with or without sodium arsenite (SA; 250 µM) for the final 30 mins. Stress granule formation was visualised using an antibody to G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks. Scale bar is 20 µm.
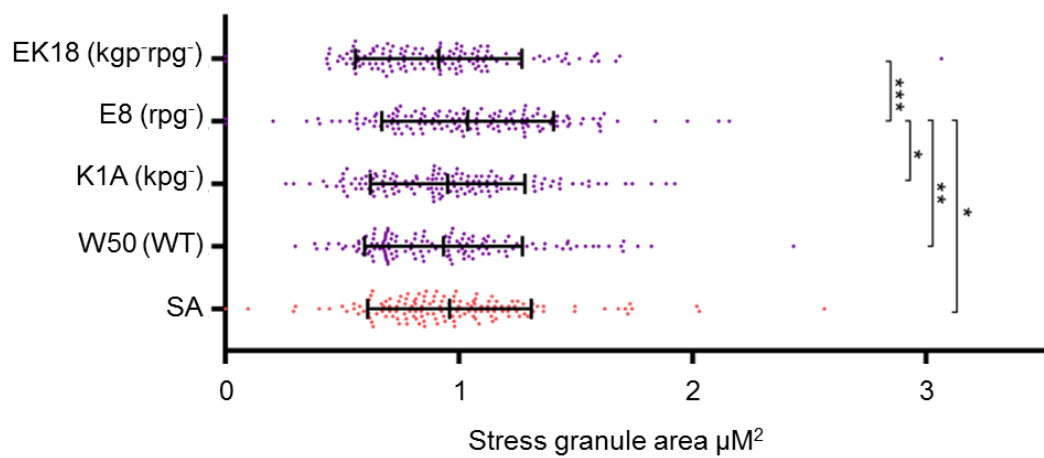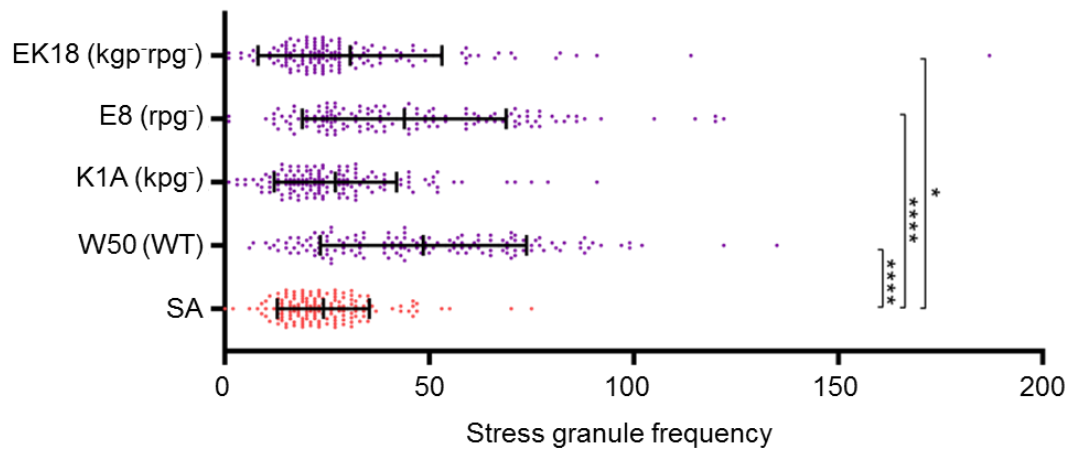
Figure 5-24 *P. gingivalis* **lysine-specific gingipain deficient mutant does not increase stress granule frequency.** H357 cells were infected with *P. gingivalis* (W50, K1A (*kgp*⁻), E8 (*rgp*⁻) and EK18 (*rgp*⁻*kgp*⁻), MOI of 100; t=2h), with or without sodium arsenite (SA; 250 μM) for the final 30 mins. Stress granule formation as visualised by G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks (mean ± SD, n=3, 50 cells per biological replicate). **** $P \leq 0.001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$ according to Kruskal-Wallis with Conover-Inman *post-hoc* test.

5.4 Discussion

P. gingivalis employs a variety of virulence factors to aid infection, including gingipains, LPS, fimbriae and capsule (Jia *et al.,* 2019). Hence the molecular mechanism by which *P. gingivalis* dysregulates translational control and stress granule formation during exogenous stress was next investigated.

One mechanism of *P. gingivalis* delivery of LPS and gingipains is extracellular expression and secretion (Grenier & Mayrand, 1987; Grenier *et al.,* 1989; Grenier *et al.,* 1995; Mantri *et al.,* 2015). Here *P. gingivalis* conditioned media heightened translational repression during oxidative stress independently of eIF2α signalling (Figure 5-1 and Figure 5-2). This response was corroborated by increased stress granule frequency uninfected (*P. gingivalis* negative) cells within the infected population (Figure 5-3). These data indicate that a factor secreted or shed by *P. gingivalis* contributes to the translational dysregulation during oxidative stress and *P. gingivalis* infection. Interestingly, at high concentration *P. gingivalis* conditioned media decreased protein synthesis and increased eIF2α phosphorylation without the requirement for additional oxidative stress (Figure 5-5 and Figure 5-6). Therefore, a constituent of *P. gingivalis* conditioned media may be capable of eliciting ISR activation at a higher concentration; similarly to many ISR activating stressors, which also have a threshold for ISR activation (Harding *et al.,* 2003; Pakos-Zebrucka *et al.,* 2016). However, given the number of *P. gingivalis* required to generate the conditioned media it is highly unlikely that the threshold would ever be reached in a physiological setting.

As *P. gingivalis* gingipains are known to both inhibit and degrade mTORC1 (Stafford *et al.,* 2013; Nakayama *et al.,* 2015) immunoblotting was carried out to monitor the expression of two downstream mTORC1 targets, p-p70-S6K1 (T389) and p-4EBP1. Sodium arsenite induced oxidative stress is known to induce high levels of S6K1 (T389) phosphorylation (Wu *et al.,* 2011), which were decreased by *P. gingivalis* infection (Figure 5-18). Furthermore, though

less conclusive, lower levels of p-4E-BP were also observed under the same conditions (Figure 5-19). This further points toward the ability of *P. gingivalis* to either degrade or inhibit mTORC1 (Stafford *et al.,* 2013; Nakayama *et al.,* 2015), which during oxidative stress conditions may be the cause of heightened translational stalling.

The role of the mTORC1 pathway was further investigated using rapamycin, an mTORC1 inhibitor. Similarly to *P. gingivalis* infection, during oxidative stress rapamycin heightened translational repression independent of eIF2α phosphorylation (Figure 5-16 and Figure 5-17), further implicating mTORC1 inhibition in mechanism of translational dysregulation employed by *P. gingivalis*.

Mechanistically *P. gingivalis* inhibits and degrades mTORC1 via the action of gingipains (Stafford *et al.,* 2013; Nakayama *et al.,* 2015), which are proteins either cell surface anchored, or within the secreteome of *P. gingivalis* where they are found both freely and packaged within OMVs (Grenier & Mayrand, 1987; Potempa *et al.,* 1995; Potempa & Travis, 1996). Both inhibition of gingipains by specific inhibitors, as well as infection with gingipain null mutant strains inhibited the heightened translational repression observed during oxidative stress, compared to gingipain active controls (Figure 5-20 and Figure 5-22). These findings implicate both the arginine- and lysine-specific gingipains both extra- and intracellularly in heightened translational repression during oxidative stress, as gingipains can also enter cells in a clathrin dependent manner (Boisvert & Duncan, 2008). This may possibly act through the PI3K pathway of gingipain-dependent mTOR inhibition, where bacterial internalisation is not prerequisite (Nakayama *et al.,* 2015).

However, the lysine-specific gingipain null *P. gingivalis* mutant failed to increase stress granule frequency (Figure 5-24), potentially implicating mTORC1 degradation, which is dependent on both *P. gingivalis* internalisation and lysine-specific gingipain expression (Stafford *et al.,* 2013). OMVs are also capable of invading cells, however the level of invasion is

only around 8% (Mantri *et al.,* 2015), in comparison to 40% invasion by *P. gingivalis* found here during oxidative stress (Figure 3-8). As gingipains are also secreted whilst *P. gingivalis* resides internally within the host cells (Xia *et al.,* 2007; Mantri *et al.,* 2015), this would imply there is an increased number of cells subjected to higher internal concentrations of active lysine gingipain in infected cells as opposed to OMV or conditioned media challenge. Therefore, the dependency of the lysine-specific gingipain and *P. gingivalis* internalisation, may explain the dampened increase in stress granule frequency in *P. gingivalis* negative compared to *P. gingivalis* positive cells, within the infected population (Figure 5-3).

These findings along with the inability of LPS to heighten translational attenuation during stress (Figure 5-14 and Figure 5-15) indicate that translational dysregulation by *P. gingivalis* during oxidative stress is dependent on gingipain activity. However, whilst both gingipain subtypes heighten translational repression, the lysine-specific gingipain is likely the main effector of stress granule modulation, requiring internalisation for optimum efficiency.

6 Chapter 6: *P. gingivalis* infection-induced dysregulation of translational control in a neurological setting

6.1 Introduction

Neurodegenerative diseases primarily present with loss of neuronal function, due to neural network disruption, synaptic dysfunction, and aberrant protein deposition within the brain (Hoover *et al.,* 2010; Scott *et al.,* 2010; Kovacs, 2019). Some of the most common neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's Disease, amyotrophic lateral sclerosis (ALS), Huntington's disease and prion diseases (Lamptey *et al.,* 2022). Collectively, neuronal pathologies are the leading cause of disability-adjusted life years (the sum of years of life lost and years lived with disability), affecting 276 million globally in 2016 (Bannick *et al.,* 2019), which presents a significant burden on global health systems and quality of life (Jin *et al.,* 2015). Currently, while there are therapeutic support options available, there is no cure for neurodegenerative disease (EbioMedicine, 2020), and hence understanding the molecular mechanisms of these diseases is vital to guide treatment options.

The ISR has become increasingly implicated as a mechanism in many neurodegenerative diseases (Bond *et al.,* 2020). ISR activity has a negative impact upon memory, with pharmacological activation of the ISR known to impair long-term memory formation in mouse models (Costa-Mattioli *et al.,* 2007), probably owing to the necessity of *de-novo* protein synthesis for long-term memory formation (Barondes & Cohen, 1966; Davis & Squire, 1984; Costa-Mattioli *et al.,* 2007). Neurodegenerative diseases such as AD, Parkinson's disease, ALS, Huntington disease and prion diseases induce chronic neuroinflammation, oxidative stress, disturbed proteostasis and aberrant protein mis-localisation (Dasuri *et al.,* 2013; Hetz & Mollereau, 2014; Scheper & Hoozemans, 2015; Chen *et al.,* 2016; Kempuraj *et al.,* 2016), all known activators of the ISR (Pakos-Zebrucka *et al.,* 2016). Fittingly, sustained elevation and dysregulation of eIF2α phosphorylation is now known to be a hallmark of many neurodegenerative pathologies (Chang *et al.,* 2002; Moreno *et al.,* 2012; Costa-Mattioli & Walter, 2020). This association between the ISR and neurodegeneration is further evidenced by

detection of phosphorylated eIF2α and ISR kinases within post-mortem brains samples of patients suffering from and animal models of AD, ALS, Parkinson's disease, Huntington disease and Down's syndrome

Many studies into the function of the ISR utilise short bursts of high dose, acute stress (Harding *et al.,* 2003). However, ER and oxidative stress phenotypic of neurodegenerative disease tend to be chronic, that is of lower intensity, but prolonged (Dasuri *et al.,* 2013; Pickering *et al.,* 2013; Niedzielska *et al.,* 2015; Cabral-Miranda & Hetz, 2017; Lanzillotta *et al.,* 2021). Under such prolonged stress GADD34 is upregulated, dephosphorylating eIF2α to allow for resumption of global protein synthesis (Novoa *et al.,* 2001). The expression of GADD34 mRNA remains at a high steady-state for a considerable time post induction by stress, which functions as a memory adaptation to limit the activation of the ISR if there is another episode or continuation of stress (Fay *et al.,* 2021; Klein *et al.,* 2022). Stress-induced adaptation to protect from further episodes of stress has also been identified in response to chronic oxidative stress in *Drosophila* and MEFs, where repeated stress induced protective gene expression with upregulation of the capacity for antioxidant and proteasomal proteolytic capabilities (Pickering *et al.,* 2013). Hence given that neurodegenerative diseases present chronic prolonged stress, and cells adapt their response to stress during such stress it is imperative to study responses with both acute and chronic stress stimuli.

The data presented in the previous chapters have demonstrated that *P. gingivalis* gingipains dysregulated host stress-induced translational control during oxidative stress. Dysregulation of translational stalling and ISR activation are highly implicated within neurodegenerative diseases (Bond *et al.,* 2020) and *P. gingivalis* is known to colonise lesions within the brains of Alzheimer's patients (Dominy *et al.,* 2019) (discussed further in section 1.1.4.8.4). Therefore, the effects of *P. gingivalis* within the neurological setting during both acute and chronic stress are investigated in the next chapter.

6.2   Aims

The aim of this chapter is to investigate the impact of *P. gingivalis* infection on ISR signalling in neuronal and glial cells. This will be achieved by addressing the following aims:

- Assessing ISR signalling during acute stress and *P. gingivalis* infection in primary human astrocytes and neuroblastoma cells (SH-SY5Y).
- Assessing ISR signalling during chronic stress and *P. gingivalis* infection in primary human astrocytes and neuroblastoma cells (SH-SY5Y).

COVID-19 Disclaimer

The data collection for this chapter was significantly hindered by the COVID-19 pandemic, which resulted in the 6-month closure of the Biomolecular Sciences Research Centre at Sheffield Hallam University. After this initial period access to the laboratory was restricted to a three-day working week for a further 6 months, which also had a significant impact upon data collection. As such, the data presented in this chapter is preliminary and significance cannot be drawn from it.

6.3 Results

6.3.1 *P. gingivalis* heightens translational repression independently of eIF2α phosphorylation in SH-S5Y and Primary Astrocytes during acute stress and infection

Earlier data presented in this study implicate *P. gingivalis* gingipains in the dysregulation of translational control during ISR activation during oxidative stress. Abnormalities in the function of the ISR play a pivotal role in many neurodegenerative disorders (Bond *et al.,* 2020) and *P. gingivalis* has been found in the brains and cerebrospinal fluid of patients suffering such conditions (Dominy *et al.,* 2019). Hence the potential role of *P. gingivalis* infection in the neurological setting was investigated using neuronal (SH-SY5Y) and primary astrocyte cells during oxidative stress.

SHSY-5Y and primary astrocyte cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (250 µM) for the final 30 minutes. In the presence of exogenous oxidative stress *P. gingivalis* lowered the rates of protein synthesis in both SH-SY5Y and primary astrocytes (Figure 6-1), compared to the stress only control. Heightened translational repression during stress and infection was independent of the increased eIF2α phosphorylation observed in both SH-SY5Y and primary astrocytes (Figure 6-2). All treatments did not alter the expression of GADD34 in either SH-SY5Y or primary astrocytes (Figure 6-3).

Taken together the data may indicate that in the neurological context *P. gingivalis* might have the ability to heighten translational repression during stress.
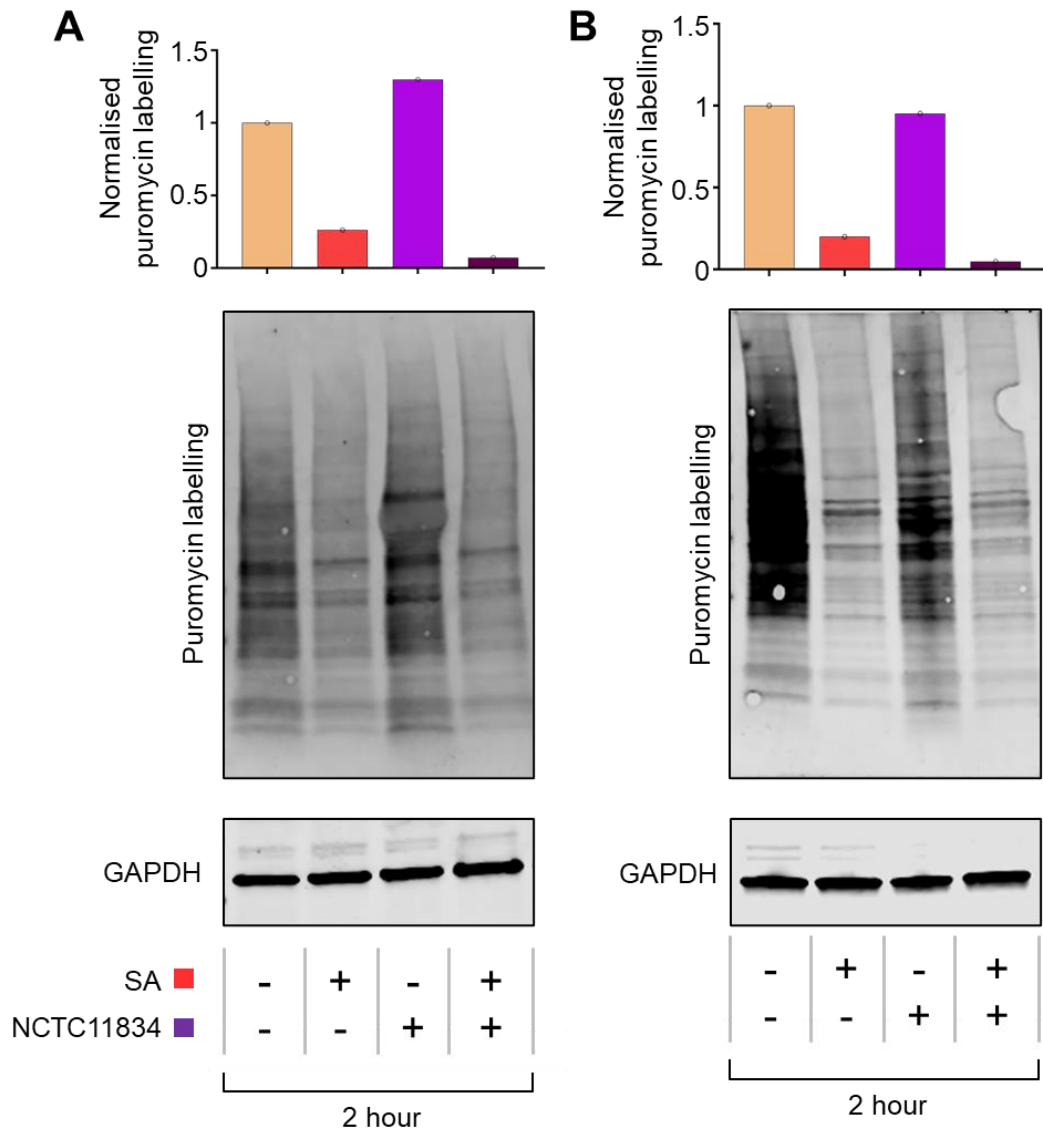
Figure 6-1 *P. gingivalis* infection heightens translational repression during acute stress in SH-SY5Y and primary astrocyte cells. (A) SH-SY5Y and (B) primary astrocyte cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 µM) for the final 30 mins. Following which, puromycin uptake and the concentration relative to GAPDH were enumerated by immunoblotting (n=1).
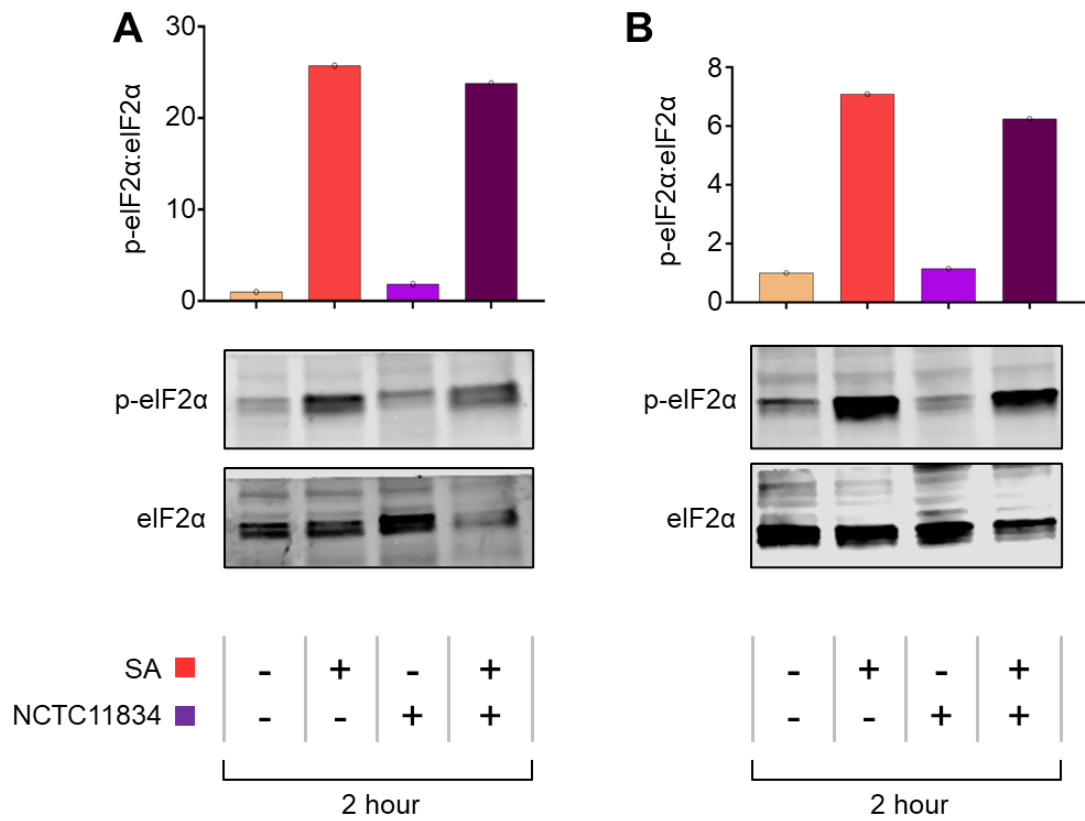
Figure 6-2 *P. gingivalis* **infection dampens acute stress induced eIF2α phosphorylation in SH-SY5Y and primary astrocyte cells.** (A) SH-SY5Y and (B) primary astrocyte cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 μM) for the final 30 mins. Levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (n=1).
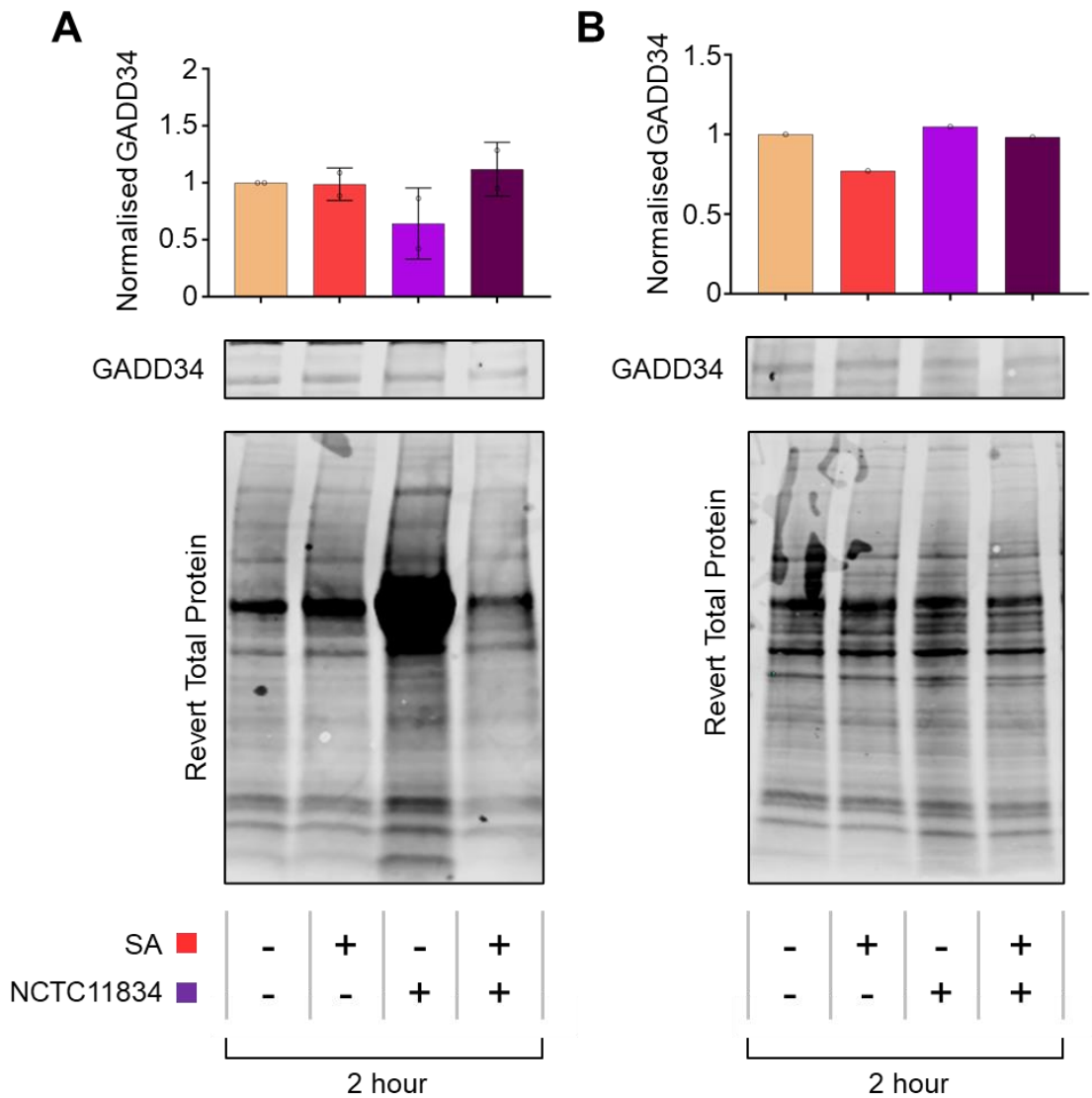
Figure 6-3 Acute stress and *P. gingivalis* do not induce GADD34 expression in SH-SY5Y and primary astrocyte cells. (A) SH-SY5Y and (B) primary astrocytes cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t=2h) with or without sodium arsenite (SA; 250 μM) for the final 30 mins. Levels of GADD34 were probed using immunoblotting and their ratio to total protein were determined (mean ± SD, (A) n=2, (B) n=1).

6.3.2 Chronic stress and *P. gingivalis* infection does not heighten translational repression in SH-S5Y and Primary Astrocytes but dampens GADD34 expression

The experimental design described in the studies thus far has utilised a short, acute dose of oxidative stress (sodium arsenite, 250 μM, 30 mins). However, it is likely that in the neurological settings stress would be long-term and chronic.

To investigate how *P. gingivalis* infection may impact upon translational function and the ISR during chronic stress SH-SY5Y and primary astrocytes were infected with *P. gingivalis* (NCTC11834, MOI of 100) with or without a low, chronic dose of sodium arsenite (15 μM) for 24 hours. Chronic oxidative stress lowered translation initiation 1.8-fold in SH-SY5Y and 4-fold primary astrocytes (Figure 6-4). However while *P. gingivalis* infection alone did not alter the rates of translation, in the presence of chronic oxidative stress a decrease in translation was not observed in SH-SY5Y (Figure 6-4A) while potentially a 1.9-fold increase in primary astrocytes was observed (Figure 6-4B). The translational attenuation during chronic oxidative stress was accompanied by a 2-fold increase in eIF2α phosphorylation in both SH-SY5Y and primary astrocytes (Figure 6-5). Whilst *P. gingivalis* infection alone did not alter eIF2α phosphorylation, in the presence of chronic oxidative stress infection resulted in a modest increase in eIF2α phosphorylation in both SH-SY5Y and primary astrocytes (Figure 6-5). Chronic oxidative stress induced heightened expression of the eIF2α dephosphorylation enzyme GADD34 a response which was damped by *P. gingivalis* infection in both SH-SY5Y and primary astrocytes (Figure 6-6).
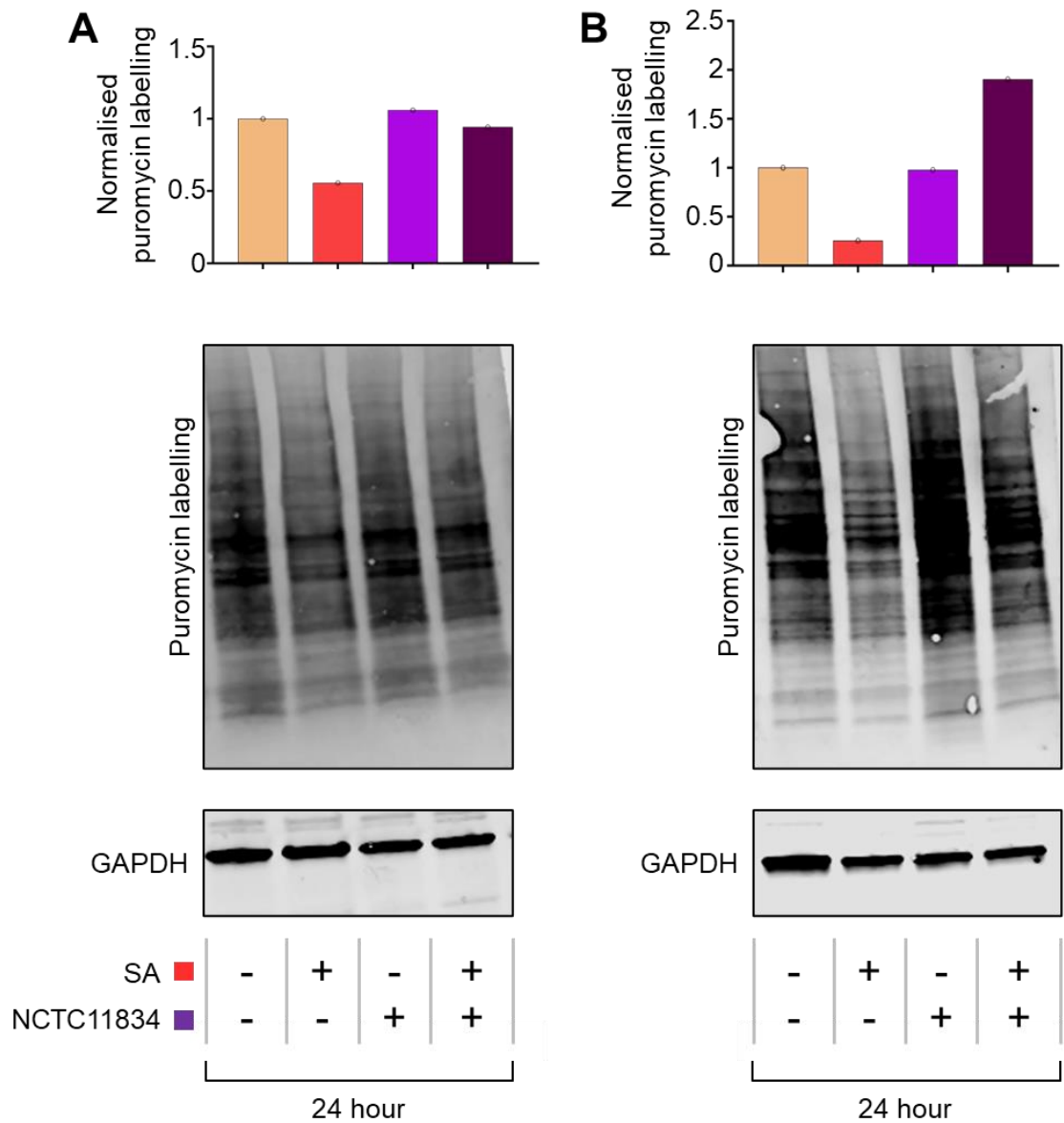
Figure 6-4 Chronic stress and *P. gingivalis* infection increase translation in SH-SY5Y and primary astrocyte cells. (A) SH-SY5Y and (B) primary astrocyte cells were infected with *P. gingivalis* (NCTC11834, MOI of 100) with or without sodium arsenite (SA; 15 µM) for 24h. Following which, puromycin uptake and the concentration relative to GAPDH were enumerated by immunoblotting (n=1).
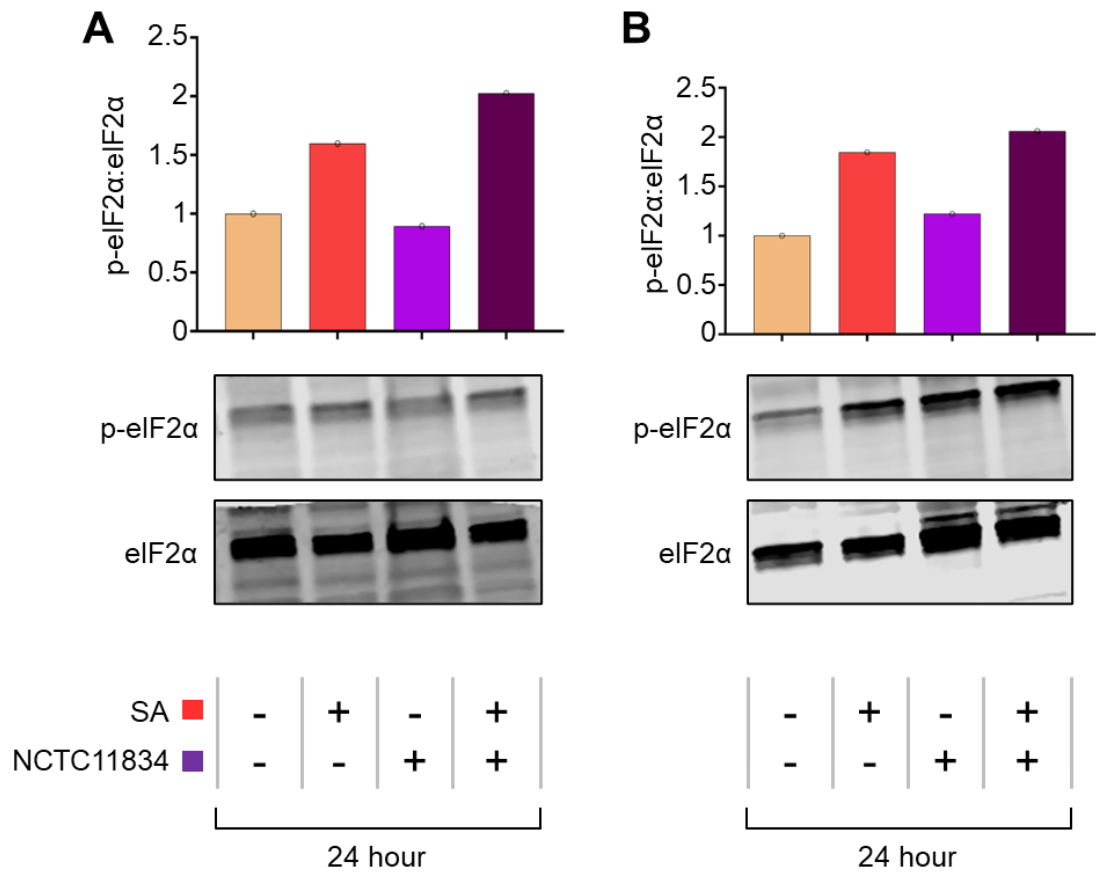
Figure 6-5 Chronic stress and *P. gingivalis* infection do not dampen eIF2α phosphorylation in SH-SY5Y and primary astrocyte cells. (A) SH-SY5Y and (B) primary astrocyte cells were infected with *P. gingivalis* (NCTC11834, MOI of 100) with or without sodium arsenite (SA; 15 µM) for 24h. Following which, levels of p-eIF2α were probed using immunoblotting and the ratio of phosphorylated to total eIF2α were determined (n=1).
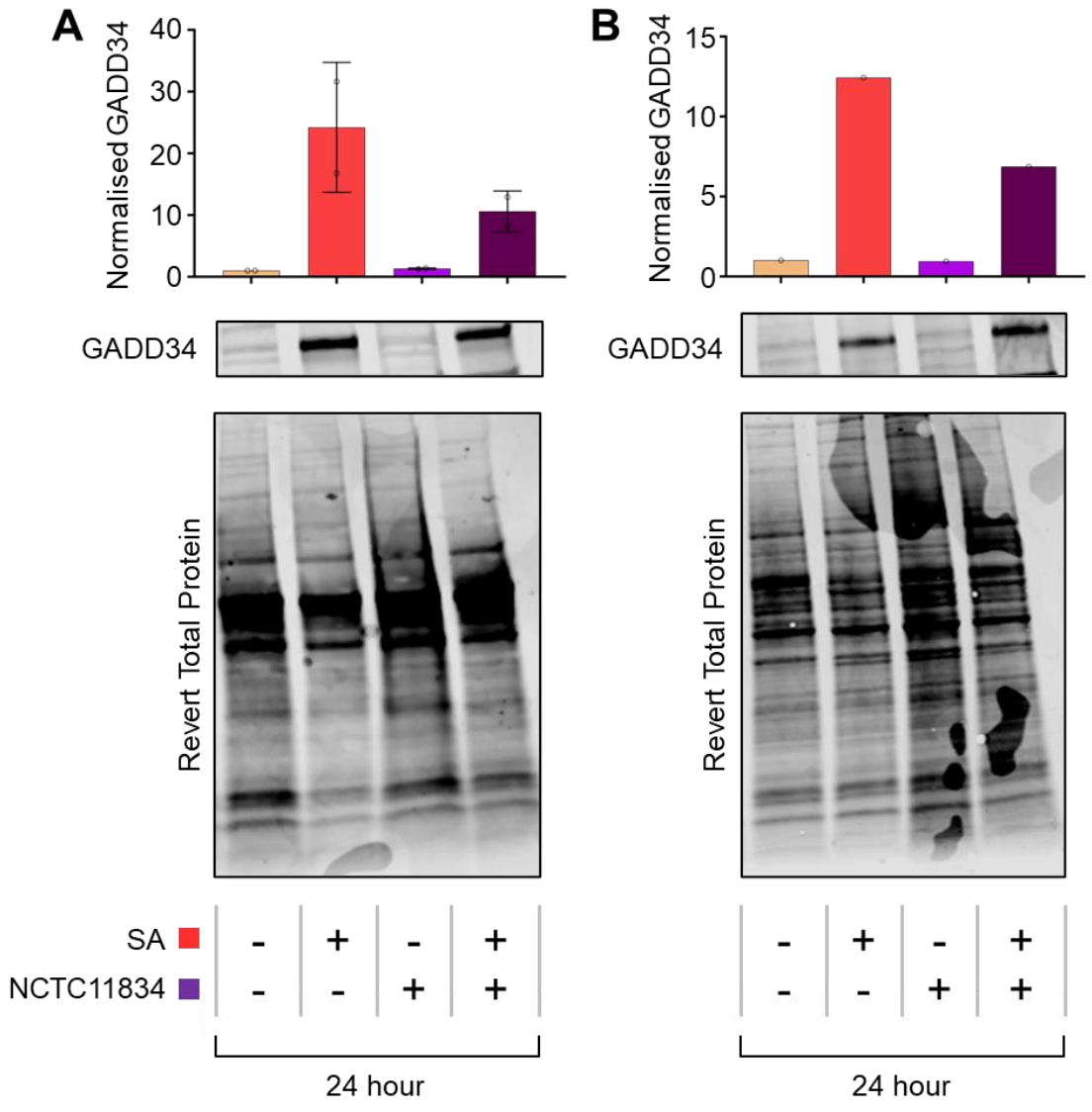
Figure 6-6 GADD34 expression is lowered during chronic stress and *P. gingivalis* infection in SH-SY5Y and primary astrocyte cells. (A) SH-SY5Y and (B) primary astrocytes cells were infected with *P. gingivalis* (NCTC11834, MOI of 100) with or without sodium arsenite (SA; 15 µM) for 24h. Following which, levels of GADD34 were probed using immunoblotting and their ratio to total protein were determined (mean ± SD, (A) n=2, (B) n=1).

6.4 Discussion

During severe periodontitis, *P. gingivalis* infection can become systemic, as evidenced by detection of *P. gingivalis* within brains and cerebrospinal fluid of Alzheimer's disease patients (Dominy *et al.,* 2019) and of the RgpA in the blood of Parkinson's disease patients (Adams *et al.,* 2019). These findings point towards the potential of *P. gingivalis*, a well-known dysregulator of host signalling pathways (discussed in section 1.1.4)*,* to interact with the pathways involved in these pathologies. Within the context of Alzheimer's disease, gingipain activity has been shown to clinically progress pathology (Dominy *et al.,* 2019), and pharmaceutical inhibition of gingipain activity has shown therapeutic promise in Alzheimer's disease mouse models (Dominy *et al.,* 2019). In fact, this has led to the successful completion of these novel gingipain inhibitors in phase I clinical trials (Dominy *et al.,* 2019; ClinicalTrials.gov NCT03331900).

Collectively, neurodegenerative pathologies such as AD, Parkinson's disease, ALS, frontotemporal dementia, Huntington disease and prion disorders are phenoccupied by a chronic inflammatory state, loss of proteostasis, aberrant protein deposition and oxidative stress within the tissues of patient brains (Bonda *et al.,* 2010; Bankston *et al.,* 2013; Dasuri *et al.,* 2013; Hetz & Mollereau, 2014; Scheper & Hoozemans, 2015; Chen *et al.,* 2016), all of which are well-documented ISR activating stressors (Pakos-Zebrucka *et al.,* 2016). Our findings indicate that *P. gingivalis* modulates protein synthesis and ISR signalling during oxidative stress (Chapters 3, 4 and 5). Hence, the role of *P. gingivalis* infection during oxidative stress was investigated within the context of the neurological environment.

Infection of both the neuroblastoma cell line SH-SY5Y and the primary astrocytes with *P. gingivalis* over a period of two hours with sodium arsenite for the final 30 mins heightened translational repression, independently of eIF2α phosphorylation. This is comparable to our observations in the oral epithelial H357 cell line (Chapter 3). This short, concentrated period of

oxidative stress is indicative of an acute stress episode, however the oxidative stress and disruption to proteostasis during neurodegenerative pathologies are long term and chronic (Dasuri *et al.,* 2013; Pickering *et al.,* 2013; Niedzielska *et al.,* 2015; Cabral-Miranda & Hetz, 2017; Lanzillotta *et al.,* 2021). Therefore, the impact of *P. gingivalis* infection during chronic sodium arsenite challenge was investigated, using a low concentration for 24 hours.

Previous studies have demonstrated chronic sodium arsenite exposure can activate not only HRI but also PRK, PERK and GCN2 in mammalian cell cultures (Dabo *et al.,* 2017), rat hippocampi (Sun *et al.,* 2017) and yeast (Zhan *et al.,* 2004), respectively. Therefore, low level chronic sodium arsenite stress may offer a closer recapitulation of physiological levels of stress in neurodegenerative pathologies, which often involve multiple stresses and kinases (Bonda *et al.,* 2010; Bankston *et al.,* 2013; Dasuri *et al.,* 2013; Hetz & Mollereau, 2014; Scheper & Hoozemans, 2015; Chen *et al.,* 2016).

In both SH-SY5Y cells and primary astrocytes when coupled with chronic sodium arsenite exposure, 24-hour *P. gingivalis* infection showed a trend towards increasing translational function, which accompanied with either no change or a modest increase in eIF2α phosphorylation. Whilst further investigations are required to evidence the significance of these trends, these outcomes seem somewhat opposing. Protein synthesis is required for the long-term formation of memories (Agranoff *et al.,* 1965; Davis & Squire, 1984; Kandel, 2001; Costa-Mattioli *et al.,* 2009) and prolonged translational attenuation causes disruption to neuronal signalling (Marciniak & Ron, 2006; Hetz & Mollereau, 2014; Scheper & Hoozemans, 2015), which taken together may imply *P. gingivalis* rescuing translational function during chronic stress may be somewhat neuroprotective. However, chronic stress and *P. gingivalis* infection showed a trend towards heightened sustained eIF2α phosphorylation, which is known to stunt long-term memory formation (Davis & Squire, 1984; Costa-Mattioli *et al.,* 2007; Costa-Mattioli *et al.,* 2009) and induce prion neurodegeneration (Moreno *et al.,* 2012). Both of these outcomes would have a negative impact upon homeostatic brain function .

Further evidence that increased eIF2α phosphorylation may play a role in AD has also been reported. Thapsigargin, a potent UPR and ISR activator, was found to phosphorylate tau (Fu *et al.,* 2010; Ho *et al.,* 2012; Lin *et al.,* 2014), a response which induces the formation of aberrant tau tangles, a key feature of AD pathology (Mamun *et al.,* 2020). Furthermore, increased expression of phosphorylated eIF2α, which localised with phosphorylated tau foci, was observed in the brains of patients with sporadic AD (Chang *et al.,* 2002), potentially implicating ISR mediated eIF2α phosphorylation in tau aggregation and consequently disease pathology. Increased phosphorylated eIF2α during *P. gingivalis* infection and chronic oxidative stress has specific relevance here, as *P. gingivalis* is known to increase tau aggregation in mouse models of AD (Dominy *et al.,* 2019). Hence, the potential for dysregulation of eIF2α phosphorylation during chronic stress and *P. gingivalis* infection and the resulting cellular and pathological outcomes in AD models is an area requiring further study.

The trend of slightly increased eIF2α phosphorylation during chronic stress and *P. gingivalis* infection was accompanied by decreased GADD34 expression. GADD34 functions to dephosphorylate eIF2α via the action of PP1 (Pakos-Zebrucka *et al.,* 2016), therefore lowered GADD34 expression may explain the slight increase in eIF2α phosphorylation observed. However, the outcome of this in the context of neurological diseases is not easily predicted. Studies investigating Guanabenz, an FDA approved anti-hypertensive compound, that inhibits the action of GADD34 have reported disparate results treating ALS (Vaccaro *et al.,* 2013; Jiang *et al.,* 2014; Vieira *et al.,* 2015). Therefore, the role of reduced GADD34 during *P. gingivalis* infection and chronic stress will require further elucidation. Of note is that (Vieira *et al.,* 2015), found paradoxical outcomes of guanabenz treatment between primary cell cultures and mouse models of ALS, indicating that cell cultures offer potentially different responses to those at the organismal level. These findings indicate that in the future, knowledge of the effects of ISR dysregulation on both different cell types and organisms will be

indispensable for understanding the interactions between *P. gingivalis,* chronic stress and neurodegenerative pathologies. This will be crucial for any successful pharmacological therapeutic targeting which may arise from such studies.

7 Chapter 7: General Discussion and Future Perspectives

The overall aim of this study was to investigate whether *P. gingivalis* interacts with host translational control during stress and elucidate the molecular mechanisms of identified interactions. Here for the first time, we show that *P. gingivalis* is capable of heightening host stress-induced translational inhibition during oxidative stress. This was accompanied by an increase in the frequency of stress granules, which had an altered composition with eIF3b delocalising from G3BP. Mechanistically, our findings point towards gingipains, and their previously reported inhibitory and degradatory impact on mTORC1 (Stafford *et al.,* 2013; Nakayama *et al.,* 2015), as the effector of these changes. A summary of the interactions between *P. gingivalis* and host stress-induced translational control identified in this study can be found in Figure 7-1 and Figure 7-2.

Somewhat strikingly, *P. gingivalis* infection alone did not induce cellular stress or ISR activation, despite previous studies finding upregulation of the UPR, a multifaceted signalling cascade which in part feeds the ISR, during *P. gingivalis* infection (Hirasawa & Kurita-Ochiai, 2018) (Discussed in detail in section 3.4). As previously discussed (Section 3.4), the chronic inflammatory state induced by *P. gingivalis* infection may be capable of producing oxidative stress capable of activating the ISR (Mayadas *et al.,* 2014). However, the dysbiotic oral microbiome in part generated by *P. gingivalis* (Darveau *et al.,* 2012), may also be capable of inducing cellular stress. To this end, in periodontitis mouse models the oral microbiota has been shown to induce NOD1 signalling (Jiao *et al.,* 2013). During *S. flexneri* infection, activation of the ISR by bacterial peptidoglycan led to increased NOD1 signalling and NF-κB activation (Abdel-Nour *et al.,* 2019). Periodontitis patients also display increased NF-κB expression (Arabaci *et al.,* 2010), therefore there is potential that the dysbiotic oral microbiota in periodontitis is inducing NF-κB expression via ISR activation and NOD1 signalling. Further study into stress responses during multispecies periodontal bacterial infections at the cellular and organismal level may offer an insightful future perspective.
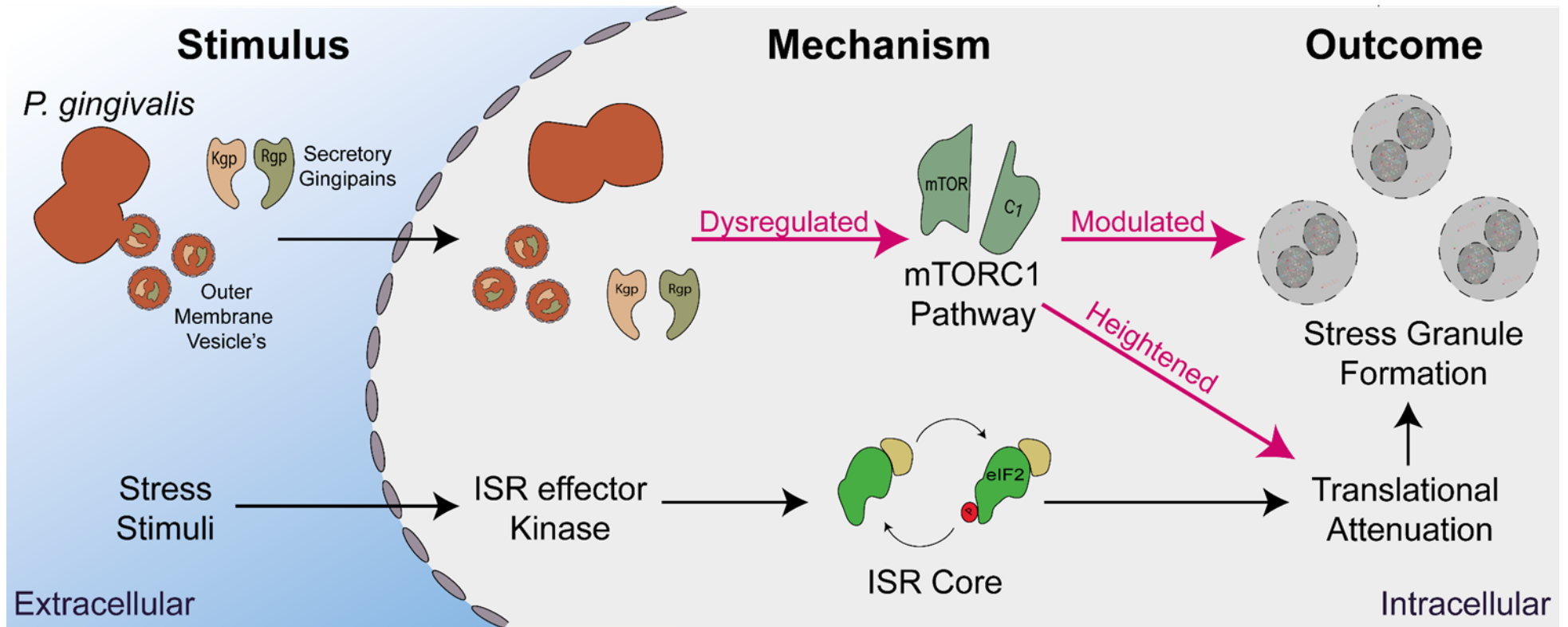
Figure 7-1 Summary of *P. gingivalis* interactions with host stress-induced translational control. Stress stimuli, such as oxidative stress, activate ISR effector kinases which phosphorylate eIF2α, resulting in translational attenuation and stress granule formation. *P. gingivalis* secretes gingipains and outer membrane vesicles in a both extra and intracellular manner, which dysregulate the mTORC1 pathway leading to heightened translational attenuation and modulated stress granule formation.
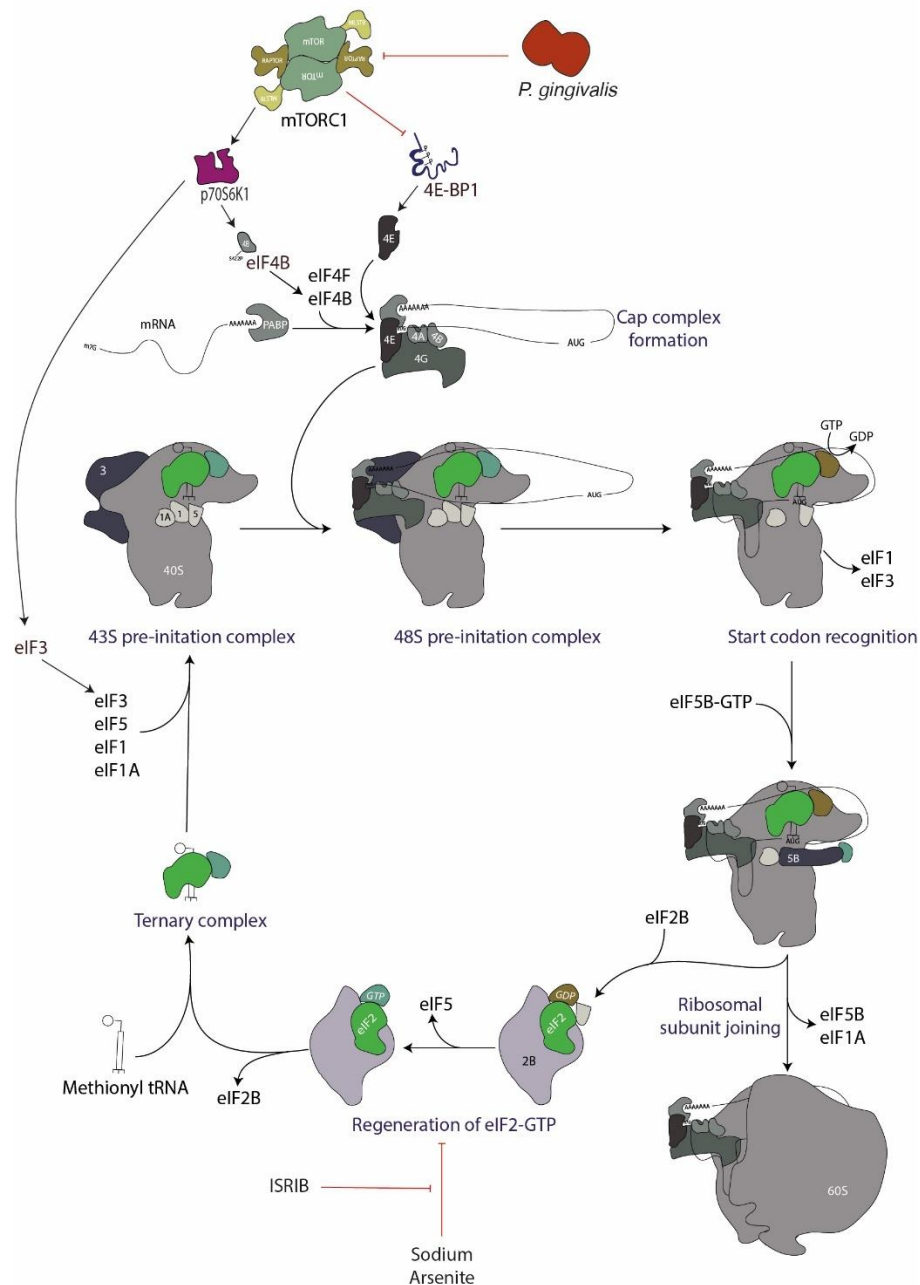
Figure 7-2 Molecular interactions between *P. gingivalis* and host translational control during sodium arsenite induced oxidative stress. Sodium arsenite induces oxidative stress, leading to the phosphorylation of eIF2, thereby inhibiting regeneration of eIF2-GTP and subsequent translational stalling. The small molecule ISRIB can rescue this function. Independently of eIF2 phosphorylation *P. gingivalis* inhibits mTORC1 activity, which may lead to decreased p70-S6 kinase and 4E-BP1 phosphorylation, subsequently inhibiting eIF4B and eIF4E, respectively, from joining the cap complex. Furthermore, decreased S6-Kinase phosphorylation may lead to decreased availability of eIF3. Through these mechanisms there may be heightened stress induced translational stalling independently of eIF2.

In the H357 oral squamous carcinoma cell line, during acute oxidative stress *P. gingivalis* gingipains heightened translational repression independently of increased eIF2α phosphorylation. The ISR inhibiting molecule ISRIB, also failed to rescue translational function during stress and *P. gingivalis* infection. A derivative of ISRIB is currently in phase I clinical trials as a treatment for ALS (ClinicalTrials,gov Identifier NCT04948645). *P. gingivalis* and gingipains have been identified in the bloodstream, cerebrospinal fluid, and brains of humans (Adams *et al.,* 2019; Dominy *et al.,* 2019). Therefore, understanding the impact of gingipains upon ISRIBs efficacy in treating ALS may offer vital understanding for the use of ISRIB to treat ALS patients, especially as patients with neurodegenerative diseases often suffer from poor oral health (Auerbacher *et al.,* 2022). Given that oral hygeine can be significantly improved by tooth brushing by non-dental professional in people with reduced ability to brush their own teeth (Barbe *et al.,* 2021), understanding whether *P. gingivalis* infections may impact the efficacy this ISRIB derivative as an ALS treatment may guide to more thorough oral hygiene care in ALS patients.

Heightened translational repression during oxidative stress and *P. gingivalis* infection was accompanied by increased stress granule frequency and the partial exclusion of eIF3b from stress granules. This is corroborated by reports that *S. flexneri* can selectively cause delocalisation of eIF3b from stress granules during exogenous stress, in a manner dependent on mTORC1 inactivation (Vonaesch *et al.,* 2016). The movement of eIF3b is regulated by mTORC1, which phosphorylates S6K1 at T389, resulting in S6K1 dissociating from eIF3b (Holz *et al.,* 2005). Oxidative stress-induced p-S6K1 (T389) (Wu *et al.,* 2011) was decreased by *P. gingivalis* infection, probably owing to inhibition or degradation of mTORC1 (Stafford *et al.,* 2013; Nakayama *et al.,* 2015). Therefore, decreased p-S6K1 (T389) could account for the lack of eIF3b in stress granules during oxidative stress and *P.*

gingivalis infection and further supports the role of mTORC1 in the exclusion of eIF3b from stress granules.

The functional outcome of eIF3b exclusion from stress granules during *P. gingivalis* infection and oxidative stress remains to be elucidated. Though many translation initiation factors are excluded from cell death associated stress granules, eIF3b remains resident (Reineke & Neilson, 2019), implying that the exclusion of eIF3b during *P. gingivalis* infection and stress has an outcome divergent from cell death. It has previously been shown that during chronic stress translation is partially restored in an eIF3 dependent manner (Guan *et al.,* 2017). Therefore, an attractive hypothesis is that, in situations where programmed cell death is not initiated during extended stress, exclusion of eIF3b from stress granules may allow for faster resumption of translation via this eIF3 dependent system. This would be supported by the preliminary findings here that in SH-SY5Y cells and primary astrocytes *P. gingivalis* infection may increase protein synthesis during chronic sodium arsenite stress, however more work is required to evidence this. Isolation and proteomic analysis of stress granules is now well established (Wheeler *et al.,* 2017), utilising these analyses for stress granules during *P. gingivalis* infection and various stress conditions would provide an interesting avenue to explore to this end.

The preliminary findings here in SH-SY5Y cells and primary astrocytes indicate that *P. gingivalis* infection may lower GADD34 expression during chronic oxidative stress and infection. Increased GADD34 expression correlates with the resumption of protein synthesis (Pakos-Zebrucka *et al.,* 2016), which makes this result surprising as protein synthesis was also increased under the same conditions. While these data require further repeats to confirm their validity, decreased GADD34 expression may benefit *P. gingivalis* as GADD34 has been shown to tune translation towards lysosomal biogenesis to enable autophagy during nutrient starvation (Gambardella *et al.,* 2020). *P. gingivalis* is well known to hijack and suppress autophagy and the formation of lysosomes to aid persistence with the host

cells (Dorn *et al.,* 2001), which inhibition of GADD34 may support. It may be interesting to investigate these responses over a longer time as *P. gingivalis* may persist in host cells for up to eight days (Belton *et al.,* 1999).

The data presented here has shown that that *P. gingivalis* is capable of dysregulating translational control during oxidative stress. In future, studies are needed to confirm that oral and systemic periodontal infections, and the subsequently upregulated immune responses, are capable of inducing ISR activating stress conditions in both the physiological oral and neurological environments. To this end, inflammation has been shown to induce stress granule formation in the atherosclerotic plaques of LDRL-/- mice (Herman *et al.,* 2019) and the brain tissues of P301L (Tau-transgenic) and FTBP-17 (AD) mice (Vanderweyde *et al.,* 2012). Therefore, as stress granules are a hallmark of ISR activation (Pakos-Zebrucka *et al.,* 2016) it is probable that such studies would identify ISR activity and stress granule formation within the tissues affected by periodontitis and tissues colonised by systemic periodontal infections. It would however be vital to evidence this not only in animal models but also in human tissues.

The identification of stress granule formation in atherosclerosis and AD mice (Vanderweyde *et al.,* 2012; Herman *et al.,* 2019) highlights the far-reaching potential of *P. gingivalis* mediated dysregulation of translation during oxidative stress, as *P. gingivalis* has been shown to persist within atherosclerotic tissues and AD brains (Kozarov *et al.,* 2005; Dominy *et al.,* 2019). The results found in this study provide a basis for further investigation of the molecular mechanisms of ISR dysregulation by *P. gingivalis* during systemic infections. Such studies may provide crucial knowledge towards both medicinal and care decisions of patients suffering from poor oral hygiene and diseases with which *P. gingivalis* has been found to interact.

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9 Ethical Approval

Cellular cross-talk during stress and periodontal infection

Ethics Review ID: ER9633388

Workflow Status: Application Approved

Type of Ethics Review Template: No human participants, human tissue or personal data

Primary Researcher / Principal Investigator

Alex Knowles
(Biomolecular Sciences Research Centre)

Converis Project Application::

Q1. Is this project: ii) Doctoral research

Director of Studies

Prachi Stafford
(Health and Wellbeing)

Supervisory Team

Susan Campbell
Neil Cross
(Biomolecular Sciences Research Centre),(Biomolecular Sciences Research Centre)

Q3b. External Investigator Details: Not Applicable

Q4. Proposed Start Date of Data Collection: 15/10/2018

Q5. Proposed End Date of Data Collection : 01/01/2022

10 Communications

Oral communications:

- Microbiology Society annual conference, 2022, Belfast, UK
- European oral microbiology workshop, 2021, Online, Tromsø, Norway
- Oral microbiology and immunology group workshop, 2021, Online
- Sheffield Hallam creating knowledge conference, 2019, Sheffield, UK

Poster presentations:

- Translation UK, 2018, 2019, 2021, 2022, various locations, UK
- Protein synthesis and translation control, 2021, Online, Heidelberg, Germany
- Translational control, 2020, Online, Cold Spring Harbour, USA

11 Published Work

Knowles, A., Campbell, S., Cross, N., Stafford, P. (2021). Bacterial Manipulation of the Integrated Stress Response: A New Perspective on Infection. *Front. Microbiol., 22*(12), 645161.

Knowles, A. A., Campbell, S. G., Cross, N. A., Stafford P. (2023). Dysregulation of Stress-Induced Translational Control by *Porphyromonas gingivalis* in Host Cells. *Microorganisms. 11*, 606.

Bacterial Manipulation of the Integrated Stress Response: A New Perspective on Infection

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Host immune activation forms a vital line of defence against bacterial pathogenicity. However, just as hosts have evolved immune responses, bacteria have developed means to escape, hijack and subvert these responses to promote survival. In recent years, a highly conserved group of signalling cascades within the host, collectively termed the integrated stress response (ISR), have become increasingly implicated in immune activation during bacterial infection. Activation of the ISR leads to a complex web of cellular reprogramming, which ultimately results in the paradoxical outcomes of either cellular homeostasis or cell death. Therefore, any pathogen with means to manipulate this pathway could induce a range of cellular outcomes and benefit from favourable conditions for long-term survival and replication. This review aims to outline what is currently known about bacterial manipulation of the ISR and present key hypotheses highlighting areas for future research.

Keywords: bacteria, ISR, eIF2alpha, infection, immunity, stress

INTRODUCTION

The relationship between microbes and hosts has shaped almost every aspect of microbial and mammalian evolution. This association is formed through an extensive series of interactions, some of which are beneficial, whilst others pose pathogenic threat. The immune system, consisting of innate and adaptive or acquired immunity, is a highly complex network enabling the human body to detect and determine the fate of foreign entities (Nicholson, 2016). During the first line of defence against pathogens, the host cell utilises the innate immune system to activate extensive signalling cascades in a concerted effort to defend against pathogenicity. These include activation of host pattern recognition receptors such as toll-like receptors (TLRs; Wright et al., 1989; Hoshino et al., 1999; Kumar et al., 2009) and Nod-like receptors (Opitz et al., 2005; Hasegawa et al., 2006), which detect structural bacterial features, termed pathogen-associated molecular patterns (Lai and Gallo, 2008; Davis et al., 2011). The resulting induction of pro-inflammatory cytokines enables host cells to initiate both intracellular and extracellular mechanisms to protect the cell and surrounding tissues (Lai and Gallo, 2008; Davis et al., 2011). These result in inflammation and the subsequent activation of macrophage- and neutrophil-mediated bacterial clearance at the infection site (Zhou et al., 2019). In some cases, autophagic responses are also triggered to remove foreign bacteria, such as *Salmonella* (Wild et al., 2011), with the host cells internalising the bacterium into double-membraned vesicles, termed autophagosomes, which are subsequently targetted for lysosomal degradation, thereby removing the foreign bacterium (Bah and Vergne, 2017).

To evade host-mediated innate immune responses, bacterial pathogens are also constantly evolving and developing mechanisms to ensure persistence within host cells and gain evolutionary success. Such mechanisms include antigenic variation (Saunders, 1990), inhibition of the humoral immune response by recruitment of complement inhibitors (Meri et al., 2013), direct interaction with complement components (Amdahl et al., 2013), evasion of autophagic responses (Ogawa et al., 2005), and residing in immune-privileged sites (Young et al., 2002). These strategies ultimately allow the bacteria to avoid detection and induce conditions favourable for bacterial survival and successful proliferation (Young et al., 2002).

In recent years, a group of highly conserved cellular pathways, collectively termed the integrated stress response (ISR), has gained increased interest in relation to host–pathogen interactions (Pakos-Zebrucka et al., 2016). The ISR, which can respond to a variety of stimuli, has been implicated in controlling the tight balance between cellular survival and death during adverse conditions, with a body of evidence implicating cross-talk between the ISR and viruses, forming a key mechanism of viral pathogenesis (Rabouw et al., 2020). The aim of this review is to explore to what extent bacteria have exploited these stress response pathways to overcome cell defences. Given that the ISR functions as a master regulator of cellular fate, understanding to what end bacteria can manipulate these pathways will allow for a better understanding of their disease pathology. Furthermore, as antibiotic resistance is on the increase, a better understanding of these host–microbe interactions may help identify novel candidate therapeutic targets.

THE INTEGRATED STRESS RESPONSE

Within eukaryotic cells, the ISR is a mechanism that, in response to changes in either intracellular or extracellular conditions, has the capability of switching between cellular survival or inducing cell death by triggering a range of signalling cascades (reviewed by Pakos-Zebrucka et al., 2016). Stimuli can include both physiological and pathological changes and once triggered results in the reduction of global protein synthesis, allowing the cell to focus energy into overcoming stress (Brostrom and Brostrom, 1997) mediated via the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α; Siekierka et al., 1982; Donnelly et al., 2013; **Figures 1A–C**). However, during ISR activation, there is also increased translation of a selection of stress response mRNAs via non-canonical translation (Ryoo and Vasudevan, 2017). This includes mRNAs coding for transcription factors, such as activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), and growth arrest and DNA damage-inducible protein (GADD34), which act as effectors of the ISR (Lee et al., 2009; Palam et al., 2011; Hinnebusch and Lorsch, 2012) specifically upregulating the expression of genes involved in cellular reprogramming under stress conditions (Karpinski et al., 1992; Harding et al., 2003; B'chir et al., 2013; **Figures 1D,E**).

Of the ISR effectors, ATF4, a basic leucine zipper transcription factor, is the best studied (Karpinski et al., 1992;

Vallejo et al., 1993; Ameri and Harris, 2008). Once activated, ATF4 regulates the expression of genes involved in stress responses, amino acid (AA) synthesis, metastasis, angiogenesis and differentiation, allowing for a stress-specific cellular response (Ameri and Harris, 2008). During hypoxia, endoplasmic reticulum (ER) stress, and AA starvation, ATF4 also upregulates transcripts involved in autophagy (Rzymski et al., 2010; B'chir et al., 2013; Deegan et al., 2015). One mechanism by which this is achieved is the inhibition of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) via translational upregulation of regulated in development and DNA-damage response 1, which functions to activate autophagic responses (Whitney et al., 2009; Kroemer et al., 2010; Dennis et al., 2013; **Figures 1D,F**).

Through the action of ATF4, the ISR can induce cell death via upregulation of downstream targets including the transcription factors CHOP and ATF3 (Puthalakath et al., 2007). One mechanism for this function is via CHOP increasing the expression of additional pro-apoptotic factors from the Bcl-2 homology 3-only group of the Bcl-2 family (Puthalakath et al., 2007; Galehdar et al., 2010). It has also been suggested that ATF4 and CHOP may interact directly to form heterodimers to heighten the expression of pro-apoptotic genes, such as Bim (Teske et al., 2013).

However, the ISR can also induce cellular survival and overcome the stress. In this case, upon cessation of stress, GADD34 activates protein phosphatase 1 (PP1), which dephosphorylates eIF2α (Connor et al., 2001; Novoa et al., 2001), thus terminating the ISR and returning the cell to homeostatic translation (**Figure 1G**; Novoa et al., 2001, 2003). As such, the ISR can induce the directly opposing outcomes of cellular survival or death.

Whilst the ISR can be initiated by multiple stimuli (e.g. AA starvation, ER stress, viral infection, and heme deprivation), the point of convergence of this response hinges upon the abrogation of canonical translation initiation via the phosphorylation of eIF2α at serine 51 (Siekierka et al., 1982; Donnelly et al., 2013; **Figure 1**). During homeostatic translation initiation, eIF2 in its active-GTP bound form associates with the initiator methionyl tRNA (tRNA$_i^{Met}$) to form a ternary complex. Upon AUG recognition, the tRNA$_i^{Met}$ is released, and eIF2-GTP is hydrolysed to eIF2-GDP (Hinnebusch and Lorsch, 2012). To enable subsequent rounds of translation, eIF2-GTP is regenerated by the guanine nucleotide exchange factor (GEF) eIF2B (Price and Proud, 1994; Jennings et al., 2013). The activation of the ISR and the subsequent phosphorylation of eIF2α at serine 51 block eIF2B GEF activity and result in a deficit of cellular eIF2-GTP and subsequently the ternary complex (Kenner et al., 2019), leading to the shutdown of most mRNA transcripts (**Figures 1B,C**). Some of these stalled complexes of translational machinery and transcripts consisting of both proteins and RNAs are sequestered into dynamic, phase dense, cytoplasmic foci termed stress granules (Nover et al., 1989; Anderson and Kedersha, 2002). Stress granules can form within minutes and dissolve at a similar pace upon stress cessation (Kedersha et al., 2000). Due to their dynamic nature, they require ongoing retrograde transport of stalled translational machinery along functioning microtubules (Loschi et al., 2009). Functionally, stress granules play a key role
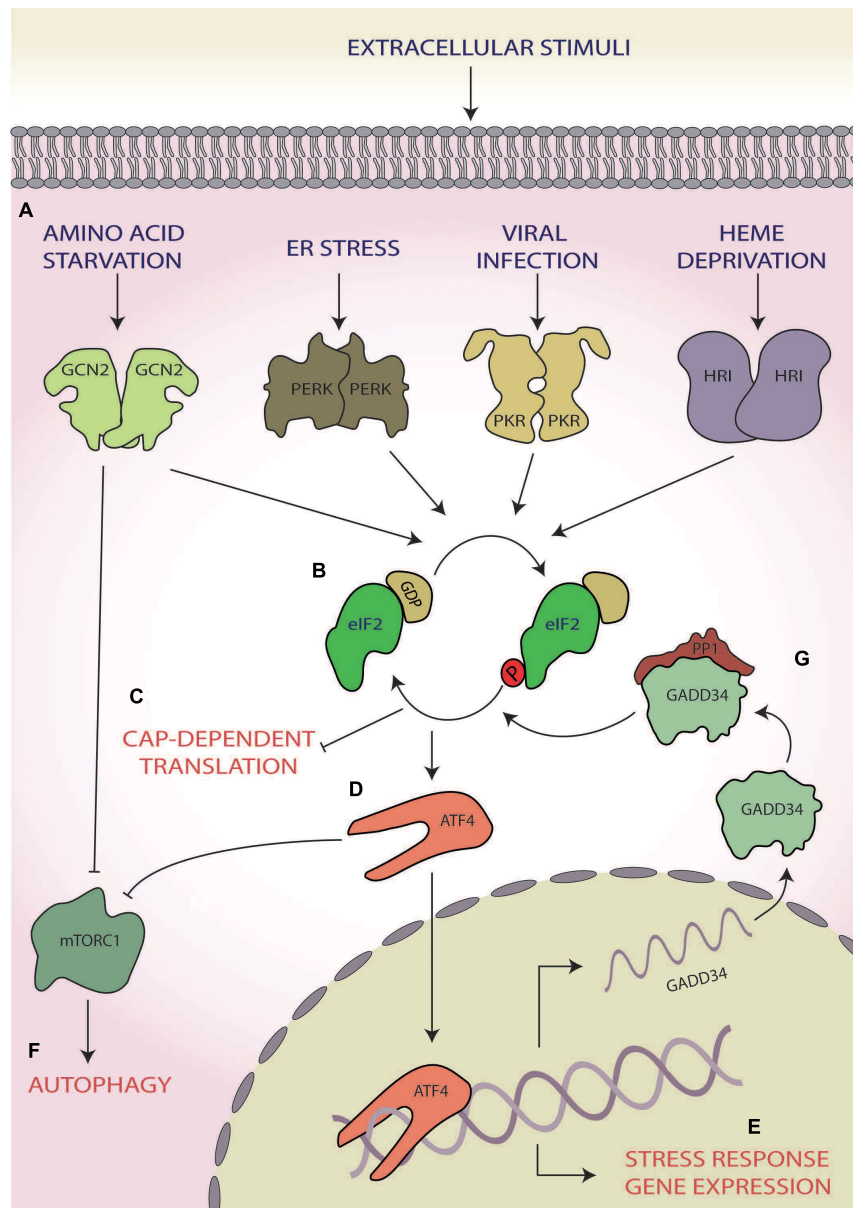
FIGURE 1 | The integrated stress response (ISR). **(A)** A range of cellular stress stimuli activate one of four stress response kinases, general control non-depressible 2 (GCN2), protein kinase R-like endoplasmic reticulum (ER) kinase (PERK), protein kinase R (PKR), and heme-regulated inhibitor HRI kinases, which **(B)** phosphorylate eukaryotic initiation factor 2 alpha (eIF2α). **(C)** This results in abrogation of canonical translation initiation, **(D)** which selectively upregulates the translation of ISR effector mRNAs, such as activating transcription factor 4 (ATF4). **(E)** These effectors bind to and target genes involved in cellular reprogramming for expression. **(F)** GCN2 and ATF4 also both induce autophagy via inhibition of mammalian target of rapamycin complex 1 (mTORC1). **(G)** If stress is overcome, the stress-inducible phosphatase growth arrest and DNA damage-inducible protein (GADD34) dephosphorylates eIF2α, returning homeostatic translation initiation and terminating the ISR.

in allowing molecules to be sorted for storage, degradation or for re-initiation of translation, thereby allowing for rapid sorting of transcripts once homeostasis is returned (Nover et al., 1989; Anderson and Kedersha, 2002).

To enact the core function of the ISR, eIF2α phosphorylation is mediated by a family of four serine/threonine stress response kinases (Wek et al., 2006). Whilst all four kinases share significant sequence similarity in their kinase domain

(Donnelly et al., 2013), each contains a unique regulatory domain, allowing for differential regulation via distinct stressors (Meurs et al., 1990; Chen et al., 1991; Berlanga et al., 1998; Shi et al., 1998; Dong et al., 2000; Harding et al., 2000; Rafie-Kolpin et al., 2000). Protein kinase double-stranded RNA-dependent (PKR) also known as EIF2AK2 classically responds to double-stranded RNA generated during viral infections (Clemens and Elia, 1997). PRK has also been found to respond to oxidative

and ER stress as well as cytokine signalling and reactive oxygen species (ROS; Cheshire et al., 1999; Ito et al., 1999; Ruvolo et al., 2001; Onuki et al., 2004; Nakamura et al., 2010; Anda et al., 2017). The protein kinase R-like ER kinase (PERK, EIF2AK3) forms one arm of a larger three-armed response to misfolded proteins in the ER, collectively termed the unfolded protein response (UPR; Walter and Ron, 2011). It is typically activated by ER stress, brought on by the accumulation of misfolded proteins in the ER lumen (Harding et al., 2000; Walter and Ron, 2011) and by changes to ATP and Ca^{2+} in the ER independently of misfolded proteins (Sanderson et al., 2010). PERK can also be activated by oxidative stress and hypoxia (Koumenis et al., 2002; Harding et al., 2003). General control non-depressible 2 (GCN2, EIF2AK4), the most highly conserved eIF2α kinase (Yang et al., 2000; Donnelly et al., 2013), is activated primarily by AA starvation (Wek et al., 1995) but can also been activated by ROS, viral infection and UV radiation (Berlanga et al., 2006; Grallert and Boye, 2007; Pyo et al., 2008). Heme-regulated inhibitor (HRI; EIF2AK1), a kinase mainly associated with protection against toxic globin aggregates in erythroid cells, is involved in protection against ROS induced by sodium arsenite as well as proteasome inhibition (Han et al., 2001; Lu et al., 2001; McEwen et al., 2005; Chen, 2007; Yerlikaya and DoKudur, 2008). Interestingly, to date, bacterial pathogens have been shown to activate all kinases with the exception of the viral specific kinase, PKR (Tattoli et al., 2012; Tsutsuki et al., 2016; Abdel-Nour et al., 2019).

BACTERIAL MANIPULATION OF THE INTEGRATED STRESS RESPONSE

In recent years, it has become apparent that the ISR forms an integral part of the host innate immune response to pathogens (reviewed by Rodrigues et al., 2018). This is supported by studies showing that pathogens can induce eIF2α phosphorylation via PERK, GCN2 and HRI (Tattoli et al., 2012; Tsutsuki et al., 2016; Abdel-Nour et al., 2019). Given that the ISR plays a crucial role in controlling cellular fate during stress (Costa-Mattioli and Walter, 2020), pathogens with means to dampen or hijack the ISR pathway are likely able to influence cellular signalling and ultimately benefit from long-term survival and promote persistence of infection. Indeed, it is well-documented that viruses manipulate specific elements of the ISR during infection. Hepatitis C virus, Japanese encephalitis virus and human cytomegalovirus directly inhibit the viral specific eIF2α kinase PKR (Toroney et al., 2010; Tu et al., 2012; Ziehr et al., 2016), and Kaposi's sarcoma-associated virus indirectly inhibits PKR via inhibition of its activator PACT (Sharma et al., 2017). Another point of ISR modulation displayed by viruses is to dampen eIF2α phosphorylation. Junin virus directly inhibits eIF2α (Linero et al., 2011), and hepatitis C virus activates GADD34 to dephosphorylate eIF2α during stress (Ruggieri et al., 2012).

However, recent evidence suggests that some bacterial species might also be manipulating the host ISR, inducing a variety of cellular outcomes. This review will focus on how five

bacterial model organisms, Shiga toxin-producing *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Porphyromonas gingivalis* can manipulate specific components of the ISR to gain control over cellular fate and immune signalling, creating an environment favouring bacterial viability, replication, and infection.

Shiga Toxin-Producing *Escherichia coli*

Shiga toxin-producing *E. coli* O157:H7 (STEC) is a widespread pathogen presenting severe risk to human health, causing haemorrhagic colitis and haemolytic uremic syndrome (Riley et al., 1983; Ko et al., 2016). Annually, the prevalence of acute STEC infection is thought to be ∼2.8 million cases worldwide, with infection progressing to HUS in 3,890 cases and resulting in death in 230 cases (Majowicz et al., 2014).

Virulent strains of STEC have been shown to target the ISR to induce host cell death mediated via a secreted virulence factor termed subtilase cytotoxin (SubAB; Tsutsuki et al., 2016; **Figure 2A**). SubAB is a secreted toxin consisting of two subunits; the B subunit binds the host extracellular toxin receptor and facilitates toxin internalisation, whereas the A subunit is a serine protease, which, in conjunction with the B subunit, facilitates the intracellular virulent effects of the pathogen (Morinaga et al., 2007). The main target of SubAB is the cleavage of the PERK chaperone binding immunoglobulin protein (BiP; **Figure 2B**), resulting in the dimerisation and activation of PERK, inducing eIF2α phosphorylation (Tsutsuki et al., 2016; **Figure 2C**). ISR activation triggered in this manner causes stress granule formation, which is dependent upon death-associated protein 1 activation (Tsutsuki et al., 2016). Inhibition of protein kinase C δ (PKCδ) and phosphoinositide-dependent kinase 1 (PDK1) are implicated in the formation of these stress granules, as chemical inhibition of both also heightens stress granule formation in response to SubAB (Tsutsuki et al., 2016). Furthermore, death-associated protein 1 knockdown increased basal levels of phospho-PDK1(S196), thereby inhibiting stress granule formation, further implicating PKD1 inhibition in formation of stress granules in response to SubAB (Tsutsuki et al., 2016; **Figure 2D**). Interestingly, in rat intestinal epithelioid cells, PDK1 has been shown to inhibit cell death in response to H_2O_2 (Song et al., 2009). Therefore, the inhibition of PDK1 coupled with prolonged PERK activation (Lin et al., 2009), which is known to promote apoptosis, may push the host cell towards death (**Figure 2E**; Lin et al., 2009; Tsutsuki et al., 2016). In contrast, in human lung cancer cells, PKCδ activation induces cell death via the CHOP-ATF3 arm of the ISR (Xu et al., 2012). Hence, the exact role PKCδ inhibition by SubAB in cell death requires further attention. Although driving the host cell towards death may seem counterproductive for bacterial survival, it is thought that Shiga-toxic *E. coli* displays altruism (Loś et al., 2012); in this context, ISR-mediated destruction of host cell and internalised bacteria could provide nutrients to the wider STEC community.

Shigella flexneri

Shigella is a genus of gram-negative, facultative anaerobic bacteria that primarily infect the gastrointestinal tract, causing
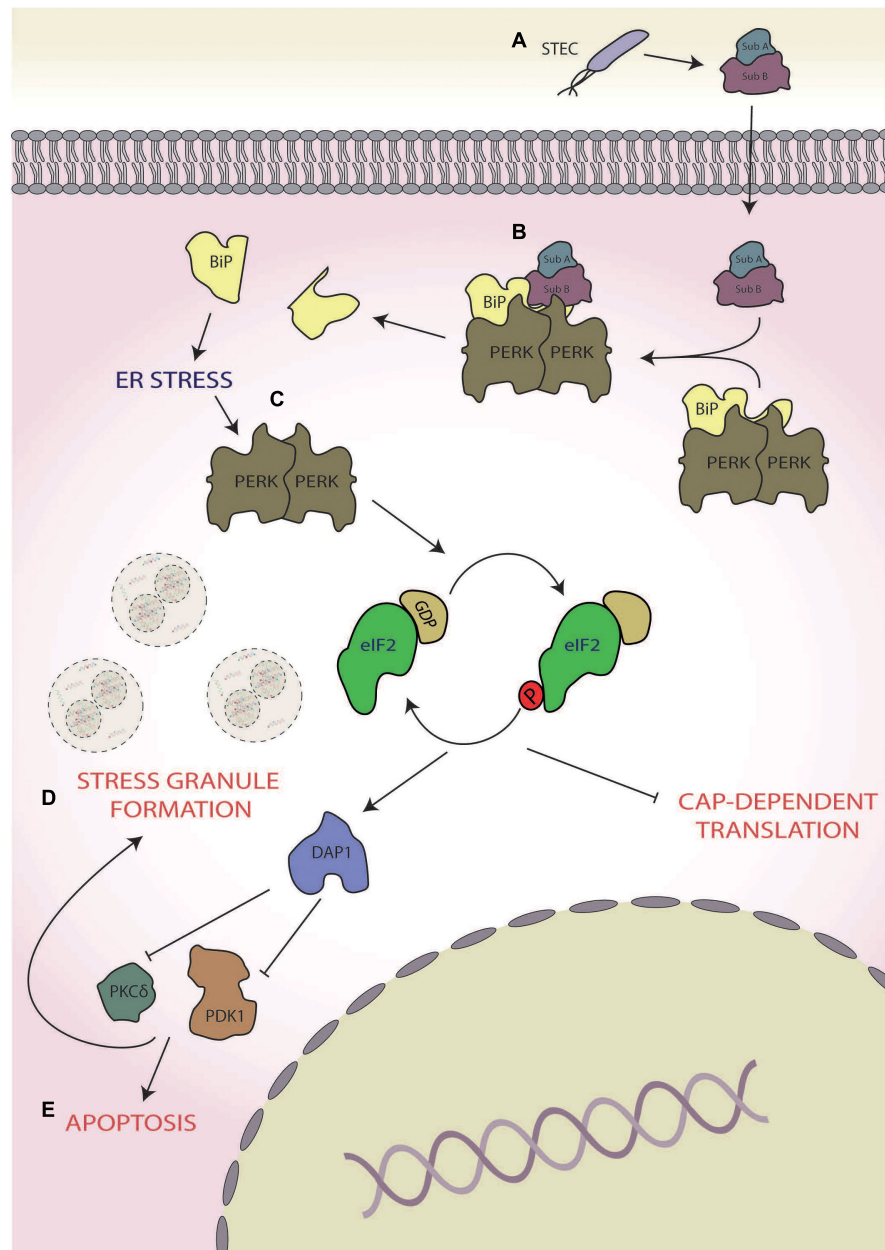
FIGURE 2 | Shiga toxin-producing *Escherichia coli* (STEC). **(A)** During infection, STEC secretes subtilase cytotoxin (subAB), **(B)** which cleaves protein kinase R-like endoplasmic reticulum (ER) kinases (PERKs) chaperone binding immunoglobulin protein (BiP), and **(C)** leading to the activation of PERK and subsequent phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α). **(D)** This results in the formation of stress granules in a manner dependent on death-associated protein 1 (DAP1) activation of phosphoinositide-dependent kinase 1 (PDK1) and protein kinase C δ (PKCδ), **(E)** pushing the cell towards death.

acute shigellosis (Fernandez and Sansonetti, 2003). Whilst closely related to *E. coli*, *Shigella* possesses unique methods of pathogenicity (Ud-Din and Wahid, 2014). Diarrhoea is an early symptom of infection as the bacteria moves through the small intestine, but the primary target of *Shigella* is the invasion of colonic epithelial cells from the basolateral surface (Phalipon and Sansonetti, 2007). Once internalised, the bacteria replicate and spread from cell to cell. The infection also causes inflammatory colitis via secreted toxins (Eashida et al., 2015). The mechanism of

Shigella invasion has been reviewed elsewhere (Carayol and Van Nhieu, 2013; Liu et al., 2019).

Infection with Group B serogroup *S. flexneri* has been shown to robustly induce the ISR, resulting in the activation of two eIF2α kinases, GCN2, and HRI (Tattoli et al., 2012; Abdel-Nour et al., 2019; **Figure 3**). During the initial stage of infection, *S. flexneri* induces AA starvation through membrane damage, which results in the activation of GCN2 (Tattoli et al., 2012; **Figure 3A**). In its active form, GCN2 inhibits mTORC1 (**Figure 3F**), as

demonstrated via its dispersal from LAMP2, and increases the transcription of the AA stress-related gene asparagine synthetase, a response that increases for up to 4 h post-infection (Tattoli et al., 2012; Abdel-Nour et al., 2019). However, *S. flexneri* is able to activate mTORC1 via direct delivery of its OspB effector into host's cellular cytoplasm using the *S. flexneri* Type III secretion system (T3SS), which interacts with the IQ motif of the GTPase-activating protein 1 (Lu et al., 2015), an upstream regulator of mTORC1 (Tekletsadik et al., 2012; **Figure 3I**), ultimately resulting in increased host cell proliferation around the infection foci during the later stages of early infection. This increased cellular proliferation reduces *S. flexneri* spread but is thought to provide a preferential intracellular niche acting as a protective measure (Lu et al., 2015).

In addition, like STEC infection, *Shigella* infections also result in the aggregation of stalled messenger ribonucleoproteins (mRNPs) into stress granules (Tattoli et al., 2012; Vonaesch et al., 2016; Abdel-Nour et al., 2019; **Figure 3E**). The activation of the ISR leads to the upregulation of ATF3, ATF4, and GADD34 (Abdel-Nour et al., 2019) and consequently the robust upregulation of the transcription and expression of ISR and inflammatory-related genes (Tattoli et al., 2012; Abdel-Nour et al., 2019). Interestingly, in the presence of ISR-inducing exogenous stresses such as mitochondrial, oxidative and heat shock stress, an increase in frequency and a decrease in an area of stress granules formed are observed in *S. flexneri*-infected cells at 2–3.5 h post-infection (Vonaesch et al., 2016). The composition of the stress granules is also altered with the selective exclusion of eIF3B, eIF4G, and eIF4B via a mechanism downstream of eIF2α (Vonaesch et al., 2016). Since the phenotype observed is similar to the hindered assembly of stress granules seen following chemical disruption of the tubulin network with nocodazole (Fujimura et al., 2009; Kolobova et al., 2009; Vonaesch et al., 2016) and the movement of eIF3B and eIF4B is controlled by microtubule assembly (Shanina et al., 2001; Harris et al., 2006; **Figure 3E**), this differential stress granule composition may be dependent upon microtubule dysregulation. Interestingly, stresses such as selenite and hydrogen peroxide, which bypass eIF2α phosphorylation instead inhibiting mTORC1 function, also result in the formation of atypical stress granules lacking components such as eIF3 (Emara et al., 2012; Fujimura et al., 2012), thereby also implicating the *S. flexneri* infection-induced inhibition of mTORC1 in the differential stress granule formation (Tattoli et al., 2012; Vonaesch et al., 2016). As the formation of stress granules during *S. flexneri* infection has only been investigated up to 5 h (Abdel-Nour et al., 2019), whether the formation of stress granules is altered similarly during later-stage infection when mTORC1 is reactivated remains unknown. If this does not occur later when mTORC1 is reactivated, this would aid the hypothesis that modulation is at least partially dependent on mTORC1 inhibition. Furthermore, whether this modulation to stress granule formation during infection may provide any evolutionary benefit to *S. flexneri* or is simply a downstream effect of ISR modulation remains unknown.

In addition to ISR activation via membrane damage, intracellular sensing of bacterial peptidoglycan by the host PRR, nucleotide-binding oligomerisation domain-containing protein

1 (NOD1), also induces ISR activation and expression of the pro-inflammatory cytokine nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) in an HRI-dependent manner (Abdel-Nour et al., 2019; **Figure 3J**). NOD1 activation results in dissociation of the HRI chaperone, heat shock protein beta-r 8 (HSPB8), from HRI, consequently activating HRI (Abdel-Nour et al., 2019; **Figure 3K**). Activated HRI induces robust eIF2α phosphorylation (**Figure 3C**), resulting in heightened HSPB8 transcription in a manner dependent on ATF4 and ATF3 signalling (Abdel-Nour et al., 2019; **Figure 3L**). This nascent HSPB8 can interact with the previously dissociated HSPB8 and NOD1 to form a signalosome (**Figure 3M**) and during *S. flexneri* infection causes the upregulation of host immune inflammatory responses and macrophage activation through the NF-κB pathway (Abdel-Nour et al., 2019; **Figure 3N**). As this pathway is also triggered by misfolded proteins within the cytosol and is comparable with the UPR in the ER, it was coined the cytosolic UPR (cUPR; Abdel-Nour et al., 2019).

Shigella *flexneri* infection results in the induction of the ISR, which can be viewed as a protective response activating pro-inflammatory responses via the cUPR (Abdel-Nour et al., 2019) and autophagy via mTORC1 inhibition (Tattoli et al., 2012), during early infection. However, Abdel-Nour et al. (2019) found that the eIF2α S51A mutant, which cannot be phosphorylated, results in a significantly increased frequency of intracellular *S. flexneri* compared with cells with phosphorylated eIF2α (Abdel-Nour et al., 2019). Taken together, these data indicate that intracellular *S. flexneri* replication is heightened during ISR activation, and it is plausible that the phosphorylation state of eIF2α may at least partially control bacterial spread and viability.

However, during later-stage infection, *S. flexneri*-mediated reactivation of mTORC1 not only increases host cell viability but also decreases bacterial spread around the infection foci (Lu et al., 2015). Whilst the impact of mTORC1 reactivation on bacterial infection has not been investigated, focussing on the ISR may provide further insights into this mechanism. Furthermore, infection results in GADD34 expression; however, whether this occurs during later-stage infection, when mTORC1 is reactivated, is as of yet unknown (Abdel-Nour et al., 2019). As GADD34 induces the dephosphorylation of eIF2α via the activation of PP1 (Connor et al., 2001; Novoa et al., 2001), the potential of sustained GADD34 expression during later stage of *S. flexneri* infection (Abdel-Nour et al., 2019) may lead to termination of the ISR, potentially aiding the cell in returning to homeostatic conditions.

There is also evidence that inhibition of mTORC1 leads to eIF2α phosphorylation in cancer cell lines (Harvey et al., 2019). If this also occurs during *S. flexneri* infection, the reactivation of mTORC1 may further push the cellular equilibrium of eIF2α towards the non-phosphorylated form. Therefore, if dephosphorylation of mTORC1 was coupled with the GADD34 expression, this could act as a two-part shift to favour a state with minimal eIF2α phosphorylation. Though counter-intuitive, whilst defective eIF2α signalling favours *S. flexneri* invasion (Abdel-Nour et al., 2019), it may lead to increased host viability, which has been suggested to benefit *S. flexneri*, the latter remaining in infected cells for much of its life cycle (Killackey et al., 2016). Thus, whether mTORC1 reactivation or
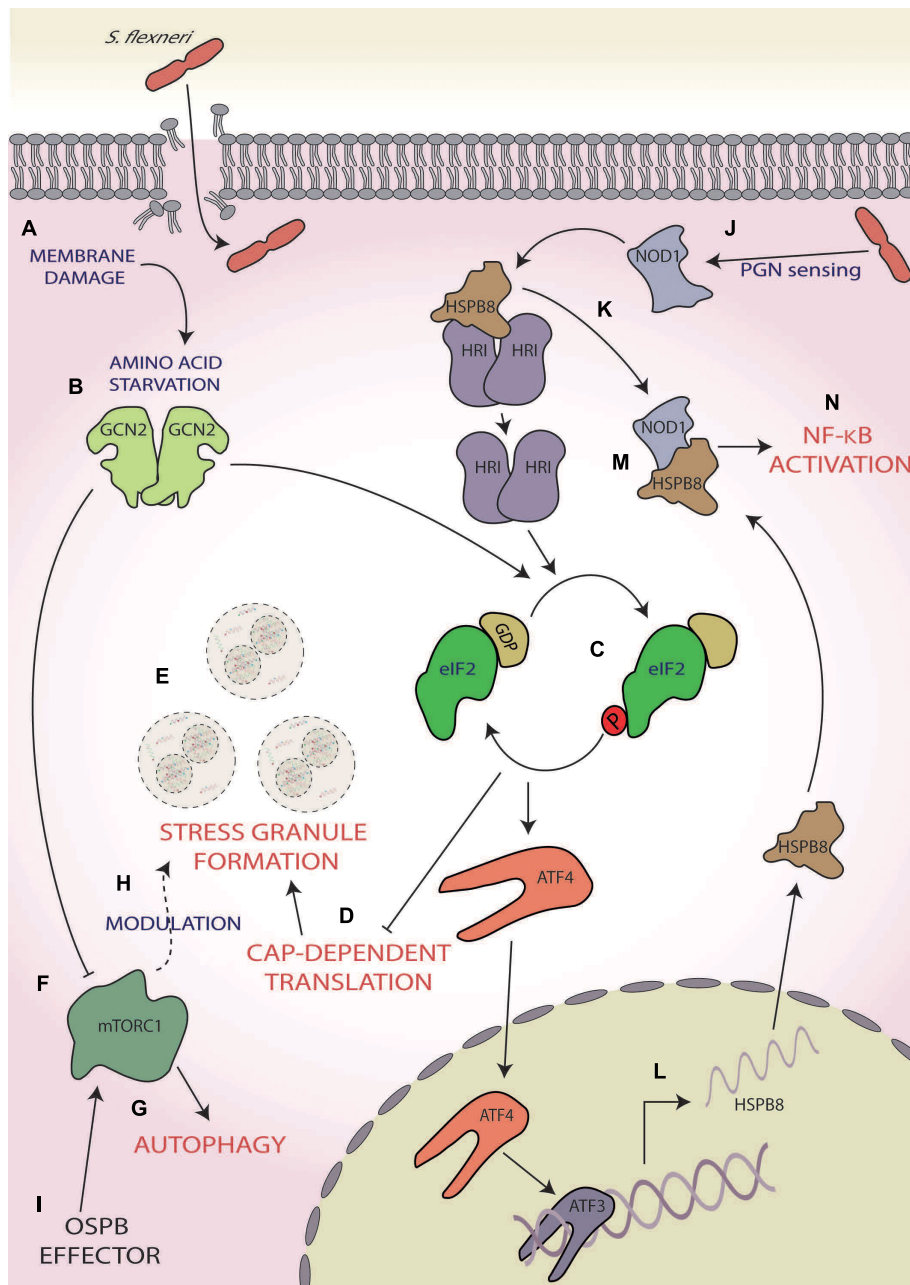
FIGURE 3 | *Shigella flexneri*. **(A)** Membrane damage caused during *S. flexneri* internalisation induces amino acid (AA) starvation, **(B)** activating general control non-depressible 2 (GCN2), and **(C)** subsequently phosphorylation of eIF2α. **(D)** This results in the inhibition of cap-dependent translation initiation **(E)** and consequently the formation of stress granules. **(F)** GCN2 also inhibits mammalian target of rapamycin complex 1 [mTORC1; **(G)**], inducing autophagy and **(H)** modulating the frequency and composition of stress granules during exogenous stress induction. **(I)** mTORC1 activity is reactivated during later-stage infection via *S. flexneri*'s OspB effector. **(J)** Concurrently, *S. flexneri*'s peptidoglycan is detected by nucleotide-binding oligomerisation domain-containing protein 1 (NOD1), **(K)** which induces dissociation of the chaperone heat shock protein beta-r 8 (HSPB8) from HRI, causing its activation and subsequent eIF2α phosphorylation. **(L)** This results in the activation of activating transcription factor 4 (ATF4), which along with ATF3, upregulated the expression and translation of HSPB8. **(M)** This nascent HSPB8 associates with NOD1 **(N)** leading to the activation of pro-inflammatory responses by nuclear factor kappa-light chain-enhancer of activated B cell (NF-κB) activation.

persistent GADD34 expression leads to eIF2α dephosphorylation during infection, and the consequential effects upon host cell viability and *S. flexneri* persistence and replication, is an area requiring further attention. Given that increased host cellular

replication during OspB-mediated mTORC1 reactivation is thought to provide a preferential niche for *S. flexneri* survival (Lu et al., 2015), it is entirely plausible that this potential ISR termination may feed into this, helping to create an even further

immune-privileged environment for *S. flexneri*. Furthermore, the effect of OspB only occurs later during the infection (Lu et al., 2015), whereas the initial phenotype of GCN2 activation and mTOR inhibition require internalisation and the resulting membrane damage (Tattoli et al., 2012; Abdel-Nour et al., 2019), which is intriguing as internalisation of OspB only requires extracellular contact between the bacteria and host cell (Hueck, 1998). Therefore, elucidation of the interaction between *S. flexneri* and these pathways may provide valuable insights into the pathogenic mechanisms of *S. flexneri* in chronic infections.

Salmonella enterica

Salmonella enterica serovar Typhimurium is an enteric pathogen primarily associated with food-borne gastrointestinal disease (Fabrega and Vila, 2013) and is thought to affect 1.3 billion people annually, leading to approximately 3 million deaths globally (Pui et al., 2011). During infection, *Salmonella typhimurium* adheres to the host's intestinal epithelium, resulting in extensive cytoskeletal rearrangements (Finlay et al., 1991). These modifications cause membrane ruffles, which eventually engulf the bacteria in large vesicles known as *Salmonella*-containing vesicles, creating an intracellular compartment in which *Salmonella* can survive and replicate (Steele-Mortimer, 2008). *Salmonella* infection is detected by host TLRs and NOD proteins, which initiates the NF-κB signalling cascade and results in cytokine and chemokine production, leading to an inflammatory state (Souvannavong et al., 2007; Spiller et al., 2008; Winter et al., 2009; Keestra et al., 2011).

As with *S. flexneri*, membrane damage induced by *Salmonella* invasion causes intracellular AA starvation with the activation of ISR through the eIF2α kinases GCN2 (Tattoli et al., 2012; **Figures 4A,B**). This AA starvation-induced activation of GCN2 also initially inhibits the activity of mTORC1, via the dispersal from LAMP2 and results in the activation of autophagy (Tattoli et al., 2012; **Figures 4C,D**). Interestingly, within 4 h of infection, the raptor/rag/regulator pathways can reactivate mTORC1, effectively saving *Salmonella* from autophagy; however, the mechanism by which this occurs has yet to be fully determined (Tattoli et al., 2012; **Figure 4E**).

In later stages of infection, e.g., 12–24 h post-infection, *Salmonella* has also been shown to induce ER stress (**Figure 4F**), robustly activating the UPR and leading to the activation of X-box binding protein 1 and transcription factor 6 (Antoniou et al., 2018; **Figure 4G**), which is known to increase lipid biogenesis and increase ER expansion (Sriburi et al., 2004, 2007; **Figure 4H**). During *Salmonella*-induced ER stress, human leukocyte antigen (HLA)-B27 becomes misfolded, causing SCV to move away from host golgi apparatus (Antoniou et al., 2018). This, coupled with ER expansion, is thought to allow for increased space for the SCV and is supported by observations that ER stress induction by thapsigargin and misfolded HLA-B27 increase intracellular *Salmonella* viability and replication (Antoniou et al., 2018; **Figure 4I**). Thus, *Salmonella* effectively utilises the ISR and UPR in two opposing ways, firstly by reversing the autophagic responses brought on by the ISR during early-stage infection via

mTORC1 reactivation and then inducing ER stress to allow for preferential replication conditions in later stages of infection.

Pseudomonas aeruginosa

Pseudomonas aeruginosa, a gram-negative, rod-shaped, mono-flagellated bacterium, is one of the most frequent causative agents for hospital-acquired infections resulting in loss of life (Buhl et al., 2015), with immunocompromised patient's survival rates being disproportionately lowered (Migiyama et al., 2013). Chronic lung infections caused by *P. aeruginosa* are a common cause of death in patients with cystic fibrosis and chronic obstructive pulmonary disease, with those affected often experiencing recurrent infections (Murphy et al., 2008; Yum et al., 2014).

During infection, *P. aeruginosa* secretes a wide variety of proteins including the extracellular adhesin CdrA (Borlee et al., 2010), the diffusible quorum-sensing molecule *N*-(3-oxododecanoyl)-homoserine lactone (HSL; Smith et al., 2002), and virulence factors [e.g., alkaline protease A (ArpA; Vasil and Ochsner, 1999) and HasAp (Létoffé et al., 1996)], all of which are known to induce ER stress (Grabiner et al., 2014; van't Wout et al., 2015; **Figure 5A**). In mouse embryonic fibroblasts (MEFs), HSL, which has a key role in *P. aeruginosa* cell-to-cell communication within the structurally ordered biofilm (Smith et al., 2002), induces ER stress via the release of Ca^{2+} from ER stores (**Figure 5B**). This causes an imbalance in ER homeostasis and activates PERK (**Figure 5C**), which phosphorylates eIF2α and results in a global shutdown of protein synthesis (Grabiner et al., 2014; **Figures 5D,E**). Interestingly, this translational stalling reduces the expression and secretion of the pro-inflammatory chemokine keratinocyte chemoattractant (KC), the mouse equivalent of interleukin 8 (IL-8; Grabiner et al., 2014; **Figure 5F**). Thus, HSL may lead to the suppression of KC secretion through eliciting the host ISR, aiding *P. aeruginosa* to evade host inflammatory and antibacterial responses during the early stages of infection (Grabiner et al., 2014). This observation contrasts with the robust expression of IL-8 seen in *S. flexneri* infection (Abdel-Nour et al., 2019) and suggests a species-specific response (Abdel-Nour et al., 2019). However, further studies are required to probe this and ascertain whether this response is species specific or cell dependent due to the differential approaches used with one study using mouse-derived MEFs (Grabiner et al., 2014) and the other using the human cervical epithelial cell line HeLa (Abdel-Nour et al., 2019).

Another example of *P. aeruginosa*-mediated ISR manipulation is through the secretion of ArpA (**Figure 5G**), a protease involved in, amongst other pathways, hosts siderophore-mediated iron scavenging (Vasil and Ochsner, 1999; Kim et al., 2006; van't Wout et al., 2015). Whereas HSL induces ER stress through activation of the p53 MAPK pathway, ArpA specifically activates HRI (**Figure 5H**), which induces the expression of GADD34 (van't Wout et al., 2015; **Figure 5I**) and is protective against *P. aeruginosa* cytotoxicity, allowing for prolonged host cell survival (van't Wout et al., 2015; **Figure 5J**). The mechanism by which HRI activation and GADD34 expression increase host viability is currently unknown, but increased GADD34 expression could lead to increased PP1 activity and consequently
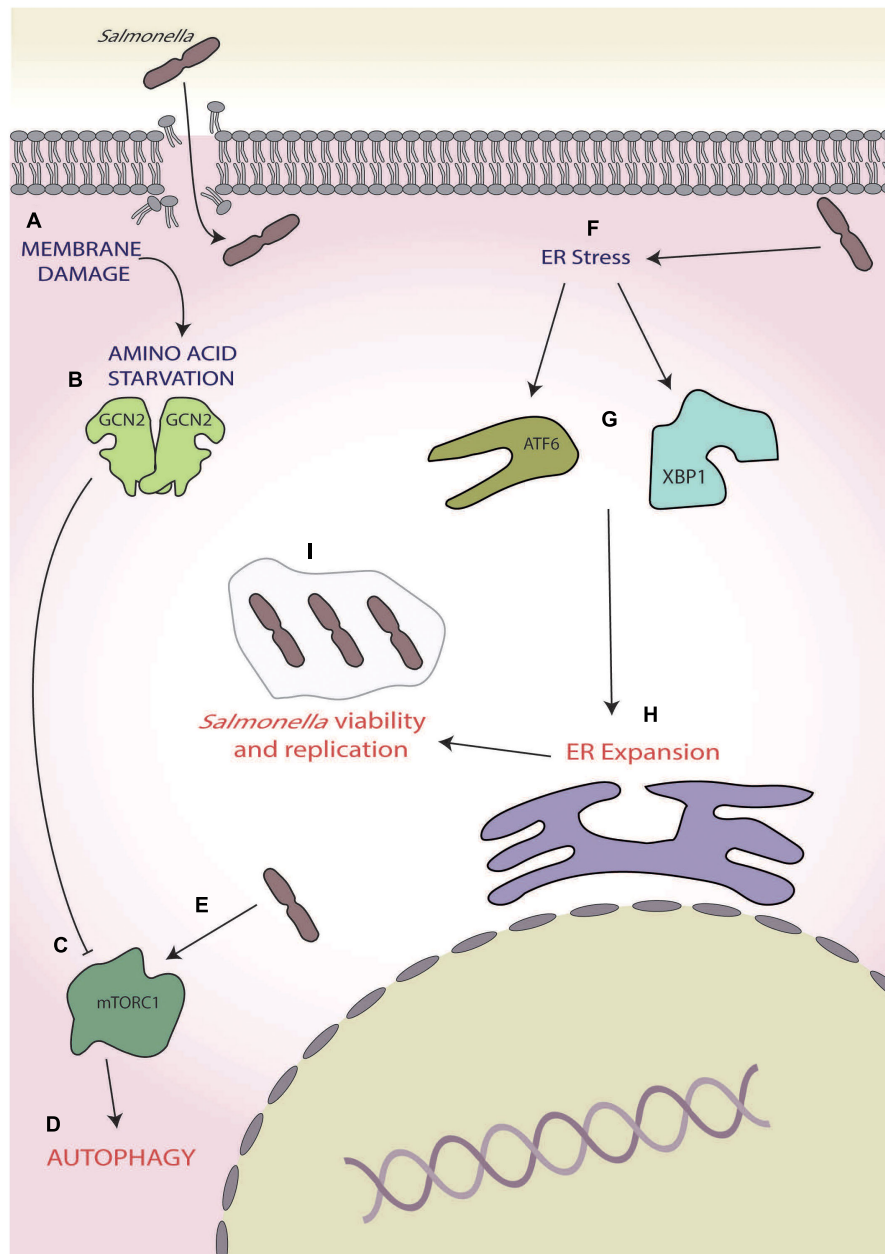
FIGURE 4 | *Salmonella*. **(A)** Membrane damage caused during *Salmonella* internalisation induces amino acid (AA) starvation, **(B)** leading to general control non-depressible 2 (GCN2) activation, and **(C)** inhibition of mammalian target of rapamycin complex 1 (mTORC1), and **(D)** ultimately inducing autophagy. **(E)** During later-stage infection, *Salmonella* reactivates mTORC1, thereby inhibiting autophagy. **(F)** *Salmonella* also induces endoplasmic reticulum (ER) stress, **(G)** activating the unfolded protein response (UPRs) ATF6 and X-box binding protein 1 XBP1 arms, **(H)** leading to expansion of the ER, and **(I)** which increases intracellular *Salmonella* viability and replication.

dephosphorylation of eIF2α (Connor et al., 2001; Novoa et al., 2001). This deactivation of the ISR could prove to be a promising system for *P. aeruginosa* to push the cell towards survival, thereby increasing viability. However, given the recent findings of Abdel-Nour et al. (2019), it is entirely plausible that activation of HRI by ArpA may activate the cUPR, which has been shown to be protective against *S. flexneri* infection. Whether this increased viability is due to eIF2α dephosphorylation or cUPR activation,

or a combination of both, remains to be elucidated and requires further attention.

Pseudomonas aeruginosa hereby displays a two-part manipulation of the host ISR, both dampening inflammatory responses and increasing host cell viability. These reduced inflammatory and immune responses may act as the critical tipping point, leading to the decreased survival of immunocompromised patients (who already have impaired

FIGURE 5 | *Pseudomonas aeruginosa*. **(A)** The quorum-sensing molecule *N*-(3-oxododecanoyl)-homoserine lactone (HSL) secreted by *P. aeruginosa* **(B)** induces endoplasmic reticulum (ER) stress, **(C)** activating protein kinase R-like ER kinases (PERKs), and **(D)** which leads to eukaryotic initiation factor 2 alpha (eIF2α) phosphorylation. **(E)** The consequential translational stalling **(F)** results in decreased expression of the pro-inflammatory cytokine interleukin 8 (IL-8) production. **(G)** A protease, alkaline protease A (ArpA), secreted by *P. aeruginosa* **(H)** activates heme-regulated inhibitor (HRI), **(I)** leading to the specific upregulation of growth arrest and DNA damage-inducible protein (GADD34), and **(J)** which increases host cell viability.

immune responses), as it could result in unregulated and therefore heightened *P. aeruginosa* growth.

Porphyromonas gingivalis

Porphyromonas gingivalis is a gram-negative, anaerobic bacterium and the "keystone pathogen" of the chronic oral inflammatory gum disease, periodontitis (Socransky et al., 1998).

Infection triggers host immune responses resulting in inflammation of the gingival tissues, which in some cases progresses to periodontitis, resulting in alveolar bone resorption and ultimately tooth loss (Pihlstrom et al., 2005). *P. gingivalis* is known to modulate several host cell responses including the inhibition of antimicrobial responses whilst leaving pro-inflammatory signalling active, thereby providing nutrients from

inflammatory spoils (Hajishengallis and Lambris, 2012). To achieve this, *P. gingivalis* employs a range of virulence factors including lipopolysaccharides (LPSs), fimbriae and lysine- and arginine-specific cysteine proteases, termed gingipains (Jia et al., 2019). Gingipains are cell surface-anchored proteins (Andrian et al., 2006), which can also be excreted in membrane-bound vesicles (Grenier et al., 1989) and therefore can account for up to 85% of proteolytic activity around the *P. gingivalis* infection site (De Diego et al., 2013).

A recent study using human umbilical vein cells as host cells suggested that the virulence of *P. gingivalis* (strain 381) may involve the UPR and ISR (Hirasawa and Kurita-Ochiai, 2018; **Figure 6**). In this study, the authors showed that whilst infection ultimately resulted in apoptosis after 21-h infection, early-stage infection (∼8 h) resulted in ER stress characterised by increased expression of CHOP and BiP at both the transcriptional and translational levels coupled with increased caspase-12 activity (Hirasawa and Kurita-Ochiai, 2018; **Figures 6A–C**). In addition, enhanced autophagy, characterised by the increased expression of autophagy markers Beclin-1, microtubule-associated protein 1A/1B-light chain 3, and acidic vesicular organelles (**Figure 6D**), was also observed. This response was inhibited by pretreatment with an ER stress inhibitor salubrinal, an inhibitor of PP1, that results in blockage of eIF2α dephosphorylation (Boyce et al., 2005). Furthermore, siRNA knockdown of LC3 resulted in increased apoptosis, thereby implicating ER stress-induced autophagy as a protective response against *P. gingivalis*-induced apoptosis (Hirasawa and Kurita-Ochiai, 2018). These results are corroborated by studies in mice where administration of *P. gingivalis* induced ER stress with increased expression of both CHOP and BiP (Yamada et al., 2015).

Interestingly, the lysine-specific gingipain of *P. gingivalis* has been shown to degrade mTORC1 and modulate levels of mTORC1-associated proteins in oral epithelial cells after 4 h of infection (Stafford et al., 2013; **Figures 6E,F**). However, this mTOR degradation requires *P. gingivalis* internalisation, indicating that these effects are probably not mediated by the secretory fraction of gingipains produced by extracellular *P. gingivalis* (Stafford et al., 2013). Inactivation of mTOR is known to induce autophagy (Jung et al., 2010), fitting with the early stage autophagy seen by Hirasawa and Kurita-Ochiai (2018; **Figure 6G**). Furthermore, mTOR inhibition by rapamycin suppresses tunicamycin-induced ER stress, resulting in autophagy (Dong et al., 2015). Therefore, gingipain-mediated degradation of mTOR may help dampen ER stress induced by *P. gingivalis* infection, aiding host cell survival in the early stages of infection by delaying the onset of apoptosis.

DISCUSSION AND FUTURE PERSPECTIVES

Infection by pathogens triggers concerted whole organism immune responses by the host, which are often initiated at the cellular level. In fact, individual host cells can respond independently to adverse conditions via a variety of intracellular signalling systems, with the ISR being a key mediator of these

responses and determining cellular fate (Costa-Mattioli and Walter, 2020). In recent years, it has become apparent that the ISR may have a wider role in host immune responses (Cláudio et al., 2013; Pulendran, 2015). Here, we discuss recent advances in understanding host–microbe interactions, which demonstrate that bacterial pathogens can interact with the host ISR during infection, directly manipulating cellular fate and immune signalling. This demonstrates that a comprehensive understanding of pathogenic interactions with the ISR is crucial for the elucidation of microbial disease progression.

Given the paradoxical role of the ISR, any change to a particular signal can have vastly different outcomes dependent upon the circumstance. For example, in neuronal cells, inhibition of PERK is protective during stress induction (Moreno et al., 2013); conversely in pancreatic cells, it induces type I interferon activation, proving to be fatal (Yu et al., 2015). These opposing outcomes of ISR dysregulation are also apparent during bacterial infection. Where PERK activation by STEC and *Porphorymonas gingivalis* infection ultimately leads to cell death (Tsutsuki et al., 2016; Hirasawa and Kurita-Ochiai, 2018), PERK activation by *P. aeruginosa* reduced the secretion of the pro-inflammatory cytokine KC, the mouse equivalent of IL-8, potentially aiding immune evasion (Grabiner et al., 2014). Conversely, *S. flexneri* infection and activation of both GCN2 and HRI showed the opposite of this phenotype, leading to robust upregulation of IL-8 (Abdel-Nour et al., 2019). Although it remains to be elucidated whether these changes are cell type or infection specific, these conflicting phenotypes demonstrate the range of outcomes that bacterial manipulation of the ISR can have on immune signalling and cellular fate; however, further work is required to evidence this.

Furthermore, Abdel-Nour et al. (2019) found that inhibition of eIF2 signalling via the knock-in eIF2α S51A mutant induced a significant decrease in intracellular *S. flexneri*, thereby implicating eIF2α signalling in the control of bacterial internalisation. These findings corroborate those of Shrestha et al. (2012), who reported that invasion of the Far East scarlet-like fever causing pathogen *Yersinia pseudotuberculosis* resulted in a 25-fold increase in MEFs containing the eIF2α S51A knock-in compared with wild type. The authors also identified functional eIF2α signalling as a prerequisite for cytokine expression and demonstrated that *Y. pseudotuberculosis* specifically dampens eIF2 phosphorylation during a range of cellular stresses through the action of a virulence factor YopJ, which is inserted directly into host cells via a T3SS. Ultimately, this resulted in decreased pro-inflammatory cytokine expression (Shrestha et al., 2012). These findings further demonstrate the potential for ISR manipulation as an immuno-evasive mechanism of bacterial pathogens.

Particularly striking is that both *S. flexneri* and *P. aeruginosa* infection result in the activation of HRI, with *S. flexneri* inducing HRI activation leading to initiation of immune signalling as demonstrated by NF-κB activation (Abdel-Nour et al., 2019) and *P. aeruginosa* inducing HRI activation leading to increased host cell viability (van't Wout et al., 2015). Interestingly, during monospecies bacterial infection, HRI activation is required for *Y. pseudotuberculosis* and *Listeria monocytogenes* to achieve their
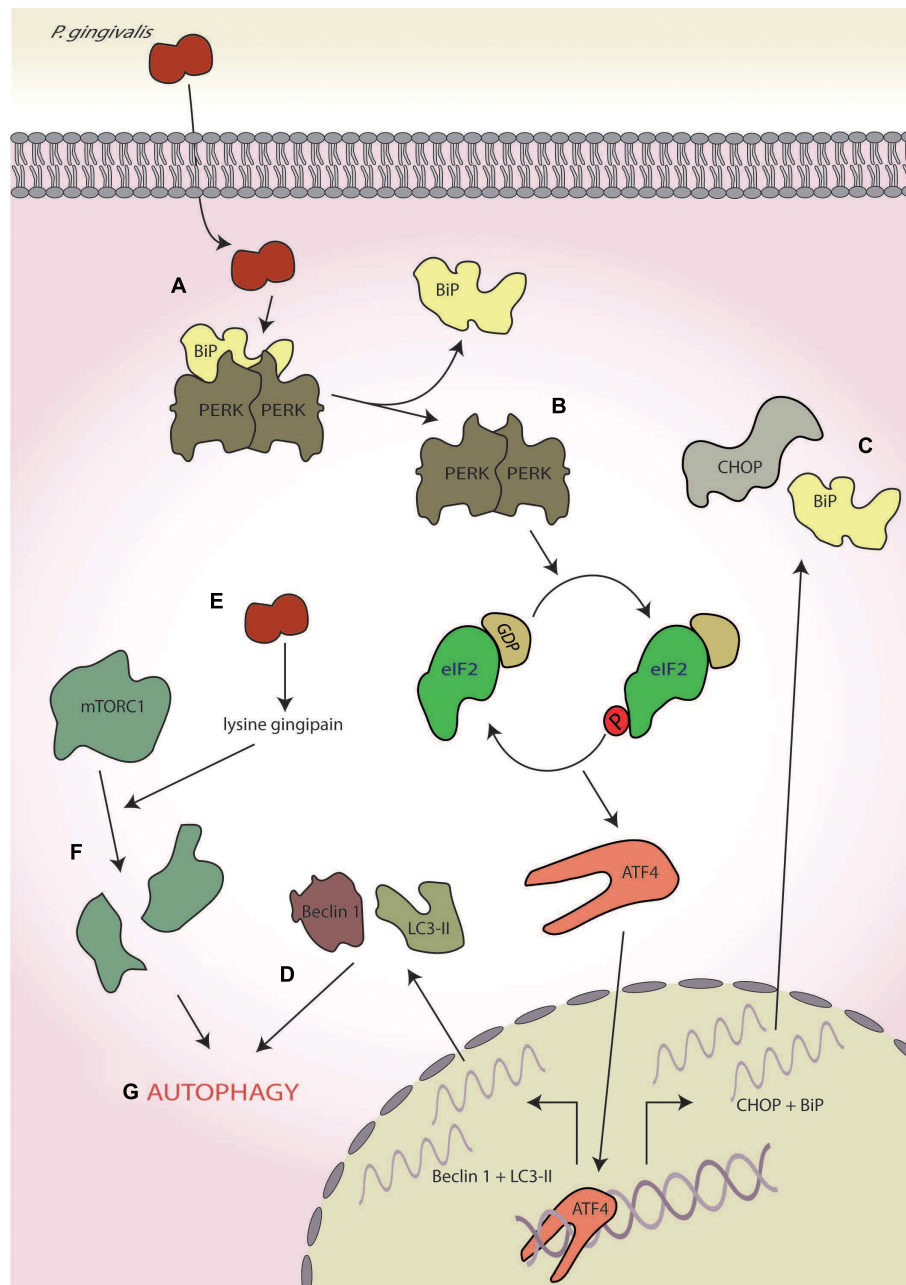
FIGURE 6 | *Porphyromonas gingivalis*. **(A)** *P. gingivalis* infection induces endoplasmic reticulum (ER) stress, **(B)** leading to protein kinase R-like ER kinase (PERK) activation, and **(C)** the expression of C/EBP homologous protein (CHOP) and binding immunoglobulin protein (BiP). **(D)** Concurrently, it also induces to autophagy as determined by the markers Beclin-1 and 1A/1B-light chain 3 (LC3-II). **(E)** *P. gingivalis* secretes a lysine-specific cysteine protease, termed the lysine gingipain, **(F)** which degrades mammalian target of rapamycin complex 1 (mTORC1), and **(G)** leading to the induction of autophagy.

virulence associated intracellular activities, where a lack of HRI interferes with the pathogens T3SS virulence factors (Shrestha et al., 2013). In contrast, at an organismal level, HRI-deficient mice have been shown to be more susceptible to *L. monocytogenes* infection and less able to mount a system-level cytokine response (Bahnan et al., 2018). This suggests potentially different outcomes depending on whether infection is monospecies or polymicrobial in nature, and hence, the exact role of HRI needs further

attention. To date, HRI-specific inhibitors have been identified (Rosen et al., 2009), and PERK inhibitors have already shown promise in combatting neurodegenerative diseases that impact upon the ISR (Moreno et al., 2013). Greater understanding of the exact role of HRI in bacterial infection is therefore an area that may allow for the targeting of HRI as a novel antimicrobial therapy using inhibitors or activators in an infection-specific manner. Findings that internalisation efficiency of *Chlamydia*

trachomatis was independent of HRI activity and that it was increased by loss of PKR (Shrestha et al., 2013) support PKR as another potential therapeutic target.

During prolonged ER stress, LPS is known to trigger a TLR-dependent reprogramming of the ISR (Woo et al., 2012). LPS is detected by TLRs, which triggers a signalling cascade mediated by the action of its downstream adaptor TRIF and results in decreased serine phosphorylation of eIF2Bε, thereby increasing eIF2B GEF activity in a manner independent of eIF2α phosphorylation (Woo et al., 2012). This increased eIF2B GEF activity results in suppression of CHOP, increasing cell survival, and increased translation of the pro-inflammatory cytokine TNF-α (Woo et al., 2012). Both *P. aeruginosa* and *P. gingivalis* induce the production of host TNF-α dependent on their LPS (Raoust et al., 2009; Nativel et al., 2017). Therefore, given that both *P. aeruginosa* and *P. gingivalis* infections are long term and chronic and can induce ER stress and PERK activation, it is entirely plausible that the TNF-α expression may be at least partially dependent on the TLR/TRIF eIF2B pathway (Grabiner et al., 2014; van't Wout et al., 2015; Yamada et al., 2015; Hirasawa and Kurita-Ochiai, 2018). Indeed, *P. gingivalis* survival is known to hinge upon increased inflammatory signalling, whilst dampening host antimicrobial responses, all in a TLR-dependent manner (Hajishengallis and Lambris, 2012). Here, investigation into this potential reprogramming of the ISR during infection may yield crucial information into *P. gingivalis* virulence and may point to the potential of therapeutic targeting of eIF2B activity during chronic infection. Given that Woo et al. (2012) only investigated the TLR-dependent ISR reprogramming under ER stress, it is entirely possible that this signalling cascade may also occur during other stresses; therefore, all of the bacteria discussed above may induce this response. The limiting factors would be host cell survival time and cytotoxicity of infection, as this cascade occurs primarily during long-term stress (Woo et al., 2012).

This review has highlighted a diverse range of cellular outcomes during bacterial manipulation of the ISR. It should be noted that most of the studies to date have investigated the role of a single species upon a single cell type, whereas most bacterial infections are polymicrobial (Brogden et al., 2005). As with host immune responses, pathogenic bacteria are known to interact with other microbes; indeed, the virulence and disease severity of both *P. gingivalis* and *Salmonella* infection are thought to be reliant upon their ability to manipulate the wider bacterial community (reviewed in Hajishengallis et al., 2012). *P. aeruginosa* is also known to secrete products, which have a community wide effect in cystic fibrosis patients, ultimately shaping microbial community dynamics within the lung (Reviewed in O'Brien and Fothergill, 2017). Whilst,

pyocyanin, a quorum-sensing molecule secreted by *P. aeruginosa* in response to gram-negative cell wall fragments, is thought to reduce microbial community diversity to select for a more pathogenic community (Norman et al., 2004; Korgaonkar and Whiteley, 2011; Korgaonkar et al., 2013), pyocyanin also functions to generate ROS (Xu et al., 2013), a known inducer of the ISR. Given that secretion of pyocyanin is governed by inter-bacterial communication, which is inherently non-linear (Dietrich et al., 2006), alterations in pyocyanin concentrations could induce differential ROS production over time. This could plausibly result in oscillation of host ISR activation, adding another layer of complexity to the ISR dampening interaction seen during monospecies *P. aeruginosa* infection. Furthermore, *Bifidobacterium* spp. protects mice from STEC toxicity via the production of acetate, which inhibits the subAB toxin produced by STEC (Fukuda et al., 2011, 2012). This lowering of toxicity may well be due dampening of STEC-mediated ISR activation in host tissues around the infection sites, especially as subAB self-internalises (Morinaga et al., 2007), which is likely be in contact with the extracellular acetate before the internalisation event. Therefore, given the role of the wider bacterial community upon virulence, studying the interactions between polymicrobial communities and the host ISR may lead to advances in the understanding of host–pathogen interactions, reflect physiological conditions and act as a platform for possible therapies.

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AK, SC, NC, and PS conceived and planned the manuscript. AK wrote the manuscript and designed the figures. AK, SC, NC, and PS edited and revised the manuscript. All authors approved and revised the final version of the manuscript.

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Article

Dysregulation of Stress-Induced Translational Control by *Porphyromonas gingivalis* in Host Cells

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Abstract: *Porphyromonas gingivalis* contributes to the chronic oral disease periodontitis, triggering the activation of host inflammatory responses, inducing cellular stresses such as oxidation. During stress, host cells can activate the Integrated Stress Response (ISR), a pathway which determines cellular fate, by either downregulating protein synthesis and initiating a stress–response gene expression program, or by initiating programmed cell death. Recent studies have implicated the ISR within both host antimicrobial defenses and the pathomechanism of certain microbes. In this study, using a combination of immunofluorescence confocal microscopy and immunoblotting, the molecular mechanisms by which *P. gingivalis* infection alters translation attenuation during oxidative stress-induced activation of the ISR in oral epithelial cells were investigated. *P. gingivalis* infection alone did not result in ISR activation. In contrast, infection coupled with stress caused differential stress granule formation and composition. Infection heightened stress-induced translational repression independently of core ISR mediators. Heightened translational repression during stress was observed with both *P. gingivalis*–conditioned media and outer membrane vesicles, implicating a secretory factor in this exacerbated translational repression. The effects of gingipain inhibitors and gingipain-deficient *P. gingivalis* mutants confirmed these pathogen-specific proteases as the effector of exacerbated translational repression. Gingipains are known to degrade the mammalian target of rapamycin (mTOR) and the findings of this study implicate the gingipain-mTOR axis as the effector of host translational dysregulation during stress.

Keywords: *Porphyromonas gingivalis*; Integrated Stress Response; mTOR; gingipains

1. Introduction

The oral cavity harbors a wide array of biofilm-forming bacteria, which form a symbiotic relationship with their host [1]. However, in some cases, the community becomes dysbiotic with an increased load of pathogenic bacteria, ultimately resulting in oral disease characterized by inflammation of gingival tissues [2,3]. In severe cases, disease progresses into the chronic condition known as periodontitis [3], the sixth most prevalent disease worldwide affecting ~743 million [4]. Periodontal disease has been associated with a range of diseases including cardiovascular disease [5], rheumatoid arthritis [6], diabetes [7], cancer [8], Alzheimer's disease [9], and Parkinson's disease [10].

Periodontitis is caused by a variety of pathogenic bacteria, the most prominent pathogens being *Porphyromonas gingivalis*, the keystone pathogen, as well as *Tannerella forsythia* and *Treponema denticola* [2,11]. Invasion of oral epithelial cells by *P. gingivalis* disrupts intracellular homeostasis in several ways [12]. One example is via the major virulence factor gingipains, extracellular cysteine proteases [13], which are known to degrade key host proteins, including the mammalian Target of Rapamycin Complex 1 (mTORC1) [14,15], a protein central to many cellular processes including protein synthesis and autophagy [16]. In addition, *P. gingivalis* inhibits host antimicrobial and phagocytic responses, which can create a favorable replicative niche [12].

Progression of periodontitis leads to an increasingly cytotoxic environment within the periodontal pocket with increasing levels of bacterial metabolites and oxidative stress due to neutrophil activation [17]. Under such stress conditions, host cells can activate several signaling cascades, one of which is a concerted cellular reprogramming system, termed the Integrated Stress Response (ISR), which functions to determine cellular fate [18].

ISR activation initially causes a global downregulation of protein synthesis, which sets out to conserve energy and allow the activation of a stress–response gene expression program thereby allowing cells to overcome the stress [18]. A variety of stresses, including bacterial infection, activate one or more of four stress response kinases; Protein Kinase R (PKR), Protein Kinase R such as ER Kinase (PERK), General Control Nondepressible 2 (GCN2), and Heme Regulated Inhibitor (HRI) (kinases reviewed by Donnelley et al. [19]; bacteria and kinases reviewed in Knowles et al. [20]). Once activated, these stress–response kinases converge upon the phosphorylation of the eukaryotic initiation factor 2 alpha sub-unit (eIF2α) at serine 51 [19,21]. eIF2α in its GTP–bound form binds the initiator methionyl tRNA, forming the ternary complex, a prerequisite for functional translation initiation [22]. During homeostatic translation, eIF2-GTP is hydrolyzed to eIF2-GDP, following which eIF2-GTP is regenerated by eIF2B, allowing for subsequent rounds of translation initiation [23,24]. Stress-induced eIF2α phosphorylation blocks the ability of eIF2B to regenerate eIF2-GTP resulting in the abrogation of global translation by inhibiting the formation of the active ternary complex [25,26]. Translation may be stalled independently of eIF2α through the eIF4E binding protein 1 (4E-BP1) [27] regulated by mTORC1 [28].

Independent of the upstream stimuli, translational shutoff pathways result in stalled messenger ribonucleoprotein particles (mRNPs), which are aggregated into cytoplasmic foci known as stress granules. These aid the sorting of mRNPs into those which will be degraded, or re-initiation if stress is overcome and translation resumes [29]. Stress granules form within minutes and dissolve at a similar pace [30]. Therefore, owing to the dynamic nature of their existence, ongoing retrograde transport of components along functioning microtubules is required [31].

In the context of infection, viruses have been well documented to dysregulate translational control and ISR function [32]. Recent studies have reported that bacterial species may also target the host translational control machinery and ISR function (Reviewed in Knowles et al. [20]). Several bacteria are known to activate host ISR stress–response kinases upon infection including *Shigella flexneri*, *Salmonella* [33,34], *Pseudomonas aeruginosa* [35], and Shiga toxin *Escherishia coli* (STEC) [36]. *E. coli* is known to decrease the frequency of cells expressing stress granules during exogenous ISR activation [37], whilst during similar conditions, *S. flexneri* infection increases stress granule frequency and alters their composition [38]. The mechanism by which *S. flexneri* manipulates stress granules is not fully elucidated, however, proposed mechanisms include dysregulation of the cellular microtubule network and inhibition of mTORC1, which both function to regulate the movement of certain stress granule components [34,38].

Intracellular *P. gingivalis* have been shown to degrade mTOR in a manner dependent on secreted lysine-specific gingipain [15]. However, when secreted, both the lysine- and arginine-specific gingipains elicit the downregulation of mTOR activity acting through the PI3K-AKT pathway [39]. Furthermore, *P. gingivalis* has been shown to induce activation of the Unfolded Protein Response (UPR) [40], which interlinks with the ISR [41]. These findings, together with the fact that periodontal infection produces stress through inflammation [12,17], suggest that *P. gingivalis* infection may also manipulate the host translational control pathways and stress granule formation. The overall aim of this study was to determine whether *P. gingivalis* dysregulates host translational control during oxidative stress and alters stress granule dynamics.

2. Materials and Methods

2.1. Reagents

All cell culture reagents unless otherwise stated were from Sigma/Merck Life Science UK LTD (Dorset, UK).

2.2. Cell Culture

The oral squamous carcinoma derived cell line (H357) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Fisher Scientific, Loughborough, UK) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Glu) in a humidified environment (5% CO_2, 37 °C). Cells were passaged when ~75% confluent by trypsinization and cell viability were assessed using trypan blue exclusion method as previously described [15].

2.3. Bacterial Strains and Culture

Bacterial strains used in this study include *P. gingivalis* NCTC11834, W50 (ACTC 53978) and the derivative W50 isogenic mutants K1A (*kgp::Em*), E8 (*rgpA::Em rgpB::Tet*) [42], and EK18 (*rgpA::Em rpgB::Tet kgp::Chlor*) [15]. All strains used were a kind gift from Professor G. Stafford (School of Clinical Dentistry, University of Sheffield, UK).

P. gingivalis were grown and maintained on fastidious anaerobe agar (Lab M, Bury, UK) containing oxylated horse blood (5% *v/v*); TCS Biosciences, Buckingham, UK) and supplemented with antibiotics as required under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) at 37 °C. Bacteria were subcultured every 3–4 days for maintenance. Throughout this study, bacteria were used to infect cells when no older than 3–4 days old post-subculturing. During infection, it was ensured that *P. gingivalis* were left no longer than necessary out of anaerobic conditions before cell treatment. *P. gingivalis* were grown as liquid cultures in brain heart infusion broth (BHI, Difco laboratories, East Molesey, Surrey, UK) supplemented with yeast extract (0.5% *w/v*), hemin (5 µg/mL), vitamin K (0.5 µg/mL), and cysteine (0.1% *w/v*). The purity of liquid cultures was confirmed by Gram staining before use.

2.4. Bacterial Infection, Oxidative Stress Induction, and Cell Treatments

H357 were seeded at a density of $6x10^4$ cells/cm^2 on coverslips or at 3.6×10^4 per cm^2 in tissue culture flasks in DMEM/Glu/FBS, following which cells were incubated (5% CO_2, 37 °C) and allowed to adhere overnight. After replacement of overnight media with fresh media, cells were challenged with *P. gingivalis* at a multiplicity of infection (MOI) of 100, harvested from solid agar, at the timepoints as detailed below. Oxidative stress was induced using sodium arsenite (SA, 250 µM) which was added for the final 30 min of infection. Cells were also treated with or without ISRIB (200 nM, 30 min), Nocodazole (200 nM, 30 min), Rapamycin (400 nM, t = 1 h), or Lipopolysaccharide (LPS) purified from *P. gingivalis* (NCTC11834; (Sigma/Merck Life Science, UK) at 1, 5, or 10 µg/mL, t = 2 h. Uninfected cells were included as control.

After treatment, for Western blotting, adherent cells were washed with phosphate buffered saline (PBS) before the addition of lysis buffer (PBS supplemented with 10% *v/v* PhosStop (Roche, Basel, Switzerland), 10% *v/v* complete EDTA-free protease inhibitors, and 0.1% *v/v* SDS). Total proteins were extracted using a cell scraper and cell lysates were stored at −80 °C for a minimum of one hour or overnight after which proteins were recovered by centrifugation (17,200× *g*, 14 min, 4 °C) and stored at −80 °C until required. Total protein extracts were quantified using the Qubit™ protein assay (ThermoFisher, Loughborough, UK) according to manufacturer instructions and expression levels of proteins of interest were probed by Western blotting. For immunofluorescence analysis, cells were fixed as detailed below.

2.5. Isolation of Crude Preparations of P. gingivalis Outer Membrane Vesicles (OMVs)

Crude preparations of *P. gingivalis* (NCTC11834) OMVs were extracted as previously described [43]. *P. gingivalis* were grown to a late exponential phase overnight in liquid culture as outlined above. The next day, cultures were adjusted to OD_{600} of 1.0 following which they were subjected to centrifugation (8000× *g*, 5 min, 4 °C). The resulting supernatant was filtered and sterilized (0.22 µM) and centrifuged (100,000× *g*, 2 h, 4 °C), after which the supernatant was discarded and the pellet was resuspended in PBS. Protein content was determined as outlined above and the resulting quantified OMVs were used to challenge H357 cells.

2.6. Generation of P. gingivalis Conditioned Media and Gingipain Inhibition

To determine the effect of *P. gingivalis* secreted components, H357 cells were infected (MOI of 1:100) as described above after which the conditioned media was recovered and filtered (0.22 µM) to remove bacteria and other particulate matter. Untreated adherent H357 cells were then challenged with the recovered conditioned media for 2 h. For gingipain inhibition studies, oral epithelial cells were challenged with conditioned media supplemented with either leupeptin (0.2 mM) or Na-Tosyl-Lysine Chloromethyl Ketone (TLCK, 0.5 mM) after which total protein was extracted and levels of proteins of interest were probed by Western blotting.

2.7. Western Blotting

Total protein extracts were separated by SDS page electrophoresis (4–20% polyacrylamide gradient gels; Bio-Rad, Watford, UK) before transferring to nitrocellulose membranes (Trans-blot Turbo transfer system, Bio-Rad). For blocking, membranes were incubated for 1 h at room temperature in Tris Buffered Saline (TBS; 37 mM NaCl, 20 mM Tris, pH 7.6) containing 0.1% *v/v* Tween 20 (TBST) and either bovine serum albumin (5% *w/v* BSA) or powdered milk (5% *w/v*) before incubation with primary antibodies overnight at 4 °C. Primary antibodies used include: puromycin (1:500; clone 12D10, MABE343, Merck), phosphorylated eIF2α (serine 51) (1:500, 44-728G, Invitrogen, Fisher Scientific), eIF2α (1:500, ab181467, Abcam, Cambridge, UK), G3BP (1:500, ab56574, Abcam), eIF3b (1:500, ab133601, Abcam), phosphorylated p70-S6 Kinase (Threonine 389) (1:200, 108D2, Cell Signaling, Leiden, The Netherlands), phosphorylated 4E-BP1 (Threonine 37/46) (1:200, 236B4, Cell Signaling), α-tubulin (1:500, 2144, Cell Signaling), acetyl-α-tubulin (1:500, 1215, Cell Signaling), GAPDH (1:10,000, G9545, Invitrogen, Fisher Scientific), and GAPDH (1:10,000, PL0125, Invitrogen, Fisher Scientific). Following incubation, membranes were washed with TBST (3 × 5 min) before incubation with the corresponding fluorescent conjugated secondary antibodies for 1 h (1:10,000, Li-Cor, Cambridge, UK). Proteins were visualized with a Li-Cor Odyssey infrared imager (Li-Cor) and quantified using Image Studio Lite software version 3.1.4 (Li-Cor).

2.8. Puromycin Incorporation Assay

The relative rates of protein synthesis were determined using the non-radioactive fluorescence–activated surface sensing of translation assay as previously described [44]. Briefly, post-treatment cells were incubated in culture media containing puromycin (91 µM) and emetine (208 µM) for 5 min (5% CO_2, 37 °C). Cells were then washed twice with PBS containing cycloheximide (355 µM) and total protein was extracted as detailed above following which puromycin uptake was probed by Western blotting.

2.9. Immunocytochemistry

Methanol-fixed cells were first washed with PBS supplemented with Tween 20 (0.5% *v/v*; PBST) before blocking in PBS supplemented with BSA (1% *w/v*) for a minimum of 1 h at room temperature. Cells were incubated with primary antibodies overnight. The following primary antibodies were used: G3BP (1:500, ab56574, Abcam), eIF3b (1:500, ab133601, Abcam), α-tubulin (1:500, ab6161, Abcam), and *P. gingivalis* (1:500, a kind gift from Prof.

G. Stafford, University of Sheffield Dental School). After washing with PBST (3 × 5 min), membranes were incubated with corresponding fluorescent Alexa fluor™ conjugated secondary antibodies for one hour at room temperature. Cells were washed with PBST, mounted using ProLong Gold™ antifade mountant containing DAPI (ThermoFisher), and protein localization was visualized using a Zeiss LSM800 microscope (Carl Zeiss, Cambridge, UK). Images were captured using ZenBlue software version 2.6, either a 40x or 63x plan-apochromat oil objective and a laser with a maximum output of 10 mW at 0.2% laser transmission. Stress granule frequencies, area, and co-localization were quantified using the analysis module of Zeiss ZenBlue software (Carl Zeiss).

2.10. Statistical Analysis

Significance between groups was analyzed using the StatsDirect software package version 3.3.5 (Statsdirect Ltd., Birkenhead, UK). Data was first subjected to a Shapiro–Wilks test where data was considered parametric if $p < 0.05$. All data was found to be non-parametric. Significance between unpaired groups was determined using a Kruskal–Wallis test, which if significant, was followed by a Conover–Inman post-hoc test. Significance was set at $p \leq 0.05$; **** $p \leq 0.001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$.

3. Results

3.1. P. gingivalis Infection Heightens Translational Repression and Modulates Stress Granule Formation during Exogenous Stress

Bacterial infection can lead to an oxidative stress environment which is known to activate the host-integrated stress–response. To determine if *P. gingivalis* (NCTC11834) can dysregulate the host ISR, the effect on protein synthesis during oxidative stress was monitored in H357 cells. Exposure to Sodium arsenite, a chemical inducer of oxidative stress, had no effect on H357 cell viability. While infection alone did not induce the ISR (Figure S1A–C), a heightened stress-induced translational inhibition of 2.1-fold was observed when cells were treated with both *P. gingivalis* and sodium arsenite (Figure 1A). As this increased inhibition of translation was observed at all infection timepoints, further studies were conducted after 2 h infection.
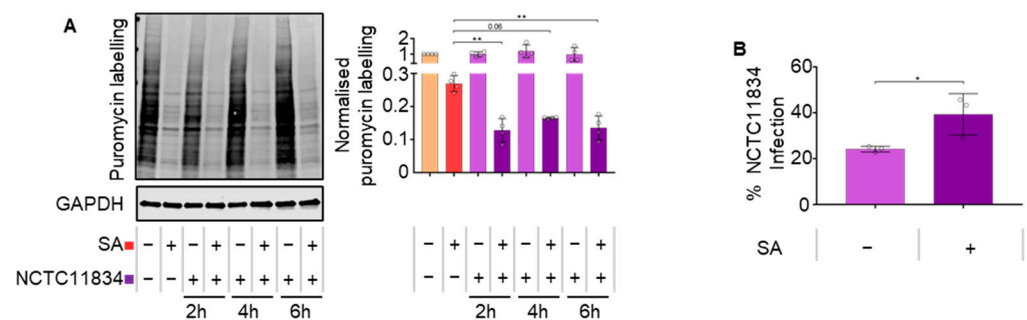

Figure 1. *P. gingivalis* infection heightens translational repression during oxidative stress. (**A**) Relative rate of protein synthesis in infected H357 cells following infection by *P. gingivalis* (NCTC11834, MOI of 100, t = 2–24 h) measured by puromycin uptake (left) and relative quantification by first normalizing to GAPDH and then to untreated sample (mean ± SD, *n* = 4). (**B**) Percentage of H357 cells displaying internalized antibody signal for *P. gingivalis* post-infection (NCTC11834, MOI of 100) after two hours (mean ± SD, *n* = 3). **, $p \leq 0.01$; *, $p \leq 0.05$ according to Kruskal–Wallis with Conover–Inman post-hoc.

The effect of oxidative stress on *P. gingivalis* invasion was next determined. In the absence of oxidative stress, *P. gingivalis* infected 24% of cells compared with 39% of total cells in the presence of oxidative stress (Figure 1B).

To establish whether *P. gingivalis* infection could impact the formation of stress granules, the number of stress granules was quantified in cells displaying internalized *P. gingi-*

valis (NCTC11834) (Figure S1D). Cells treated with oxidative stress induced on average the formation of 36.2 stress granules per cell with an average size area of 2.25 μm^2. Within the bacteria-treated population, neither uninfected nor infected cells showed evidence of stress granules (Figure 2A). In contrast, when *P. gingivalis* infection was coupled with oxidative stress, the frequency of stress granules increased on average to 59.4 per cell (Figure 2B). Differences between uninfected and infected cells within this population were further characterized and a decrease in stress granules frequency was observed in uninfected cells with the average area (2.2 μm^2) showing slight variance (Figure 2B).
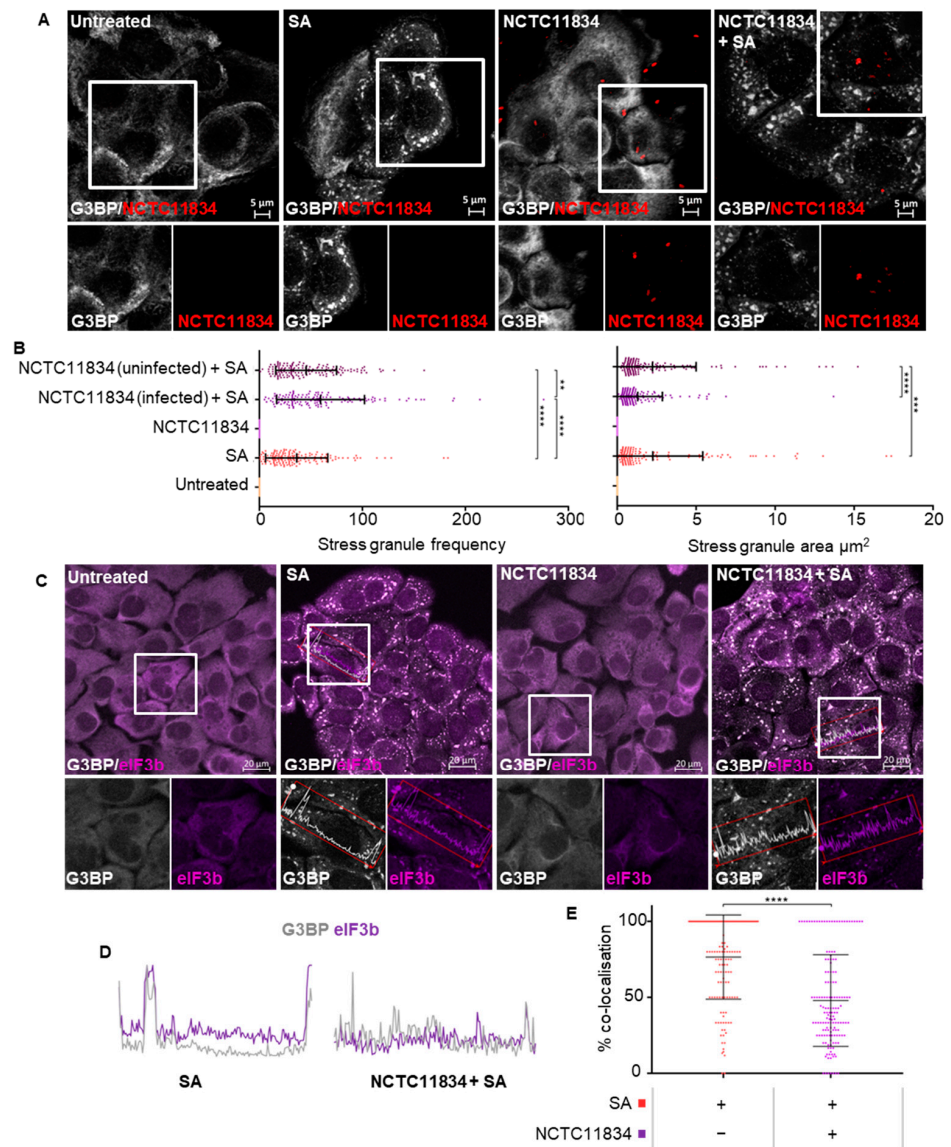

Figure 2. *P. gingivalis* infection modulates stress granule formation during oxidative stress in H357 cells. (**A**) Stress granule formation as visualized by G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks following *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h) challenge in the presence or absence of sodium arsenite. (**B**) Average area and frequency of stress granules determined in host cells (*n* = 3, 50 cells per biological replicate). (**C**) Co-localization of G3BP1 (white) and eIF3B (purple) in stress granules, as assessed by immunofluorescence. (**D**) Representative line segments of color profiles taken from H357 cells challenged with sodium arsenite with or without *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h) infection, where intensity peaks correspond to stress granules. (**E**) Percentage of colocalization of eIF3b and G3BP1 (*n* = 3, 50 cells per biological replicate). **** $p \leq 0.001$; ***, $p \leq 0.001$; **, $p \leq 0.01$ according to Kruskal–Wallis with Conover–Inman post-hoc.

As stress granule composition is known to be stress-dependent [45], the localization of eIF3b and G3BP in stress granules was analyzed (Figure 2C). During oxidative stress, eIF3b colocalized highly with G3BP positive stress granules (Figure 2D) (mean 75%). However, in the presence of *P. gingivalis* (NCTC11834) and oxidative stress, the mean percentage colocalization of eIF3b to G3BP decreased to 50% (Figure 2D). As *P. gingivalis* is known to degrade several host proteins, the potential for both G3BP or eIF3b degradation was investigated and no degradation was observed (Figure S2), thereby suggesting the ability of *P. gingivalis* to modulate host stress granule frequency and composition.

3.2. P. gingivalis Heightens Translational Repression Independently of eIF2α

Translational stalling during stress is classically mediated via the phosphorylation of alpha subunit of eIF2 at serine 51 [19,21]. The relative levels of total and p-eIF2α in *P. gingivalis* (NCTC11834)-infected cells treated with or without oxidative stress were determined by immunoblotting (Figure 3A). A similar basal level of p-eIF2α (Figure 3A) was observed in *P. gingivalis*-infected cells and the untreated control. Strikingly, despite the increased translational repression observed when *P. gingivalis* infection was co-treated with oxidative stress, a decrease in levels of p-eIF2α was observed compared with the oxidative–stress–only treatment (Figure 3A).
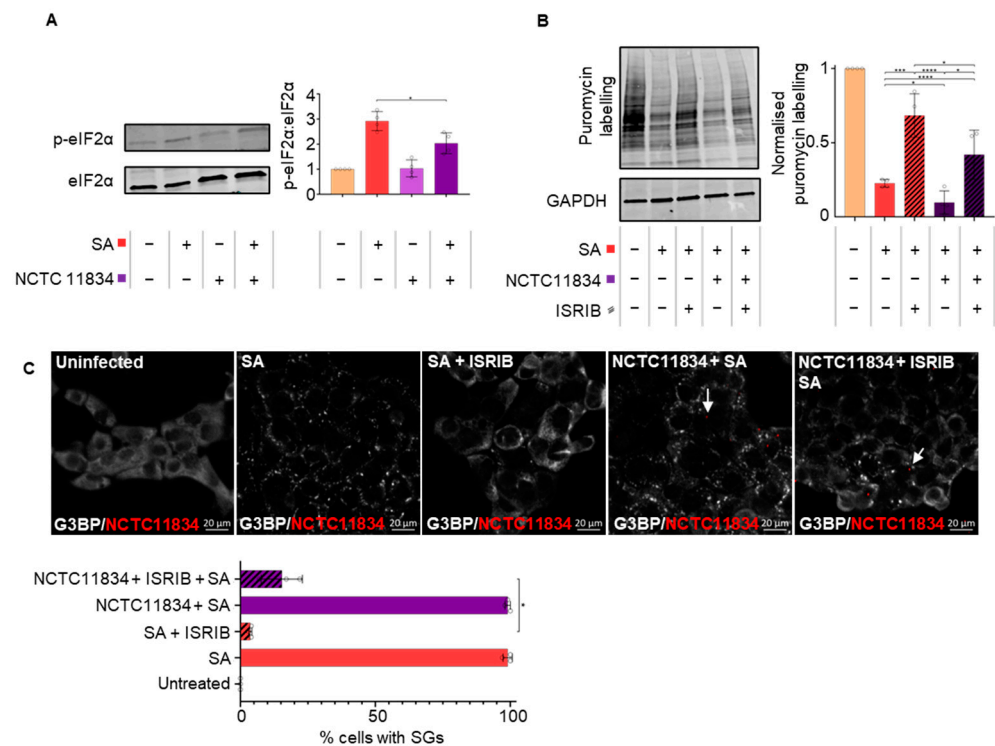

Figure 3. *P. gingivalis* heightens translational repression independently of eIF2α in the presence of stress. (**A**) Level of total and phosphorylated eIF2α (left) and ratio of phosphorylated eIF2α to total eIF2α after *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h) treatment of H357 cells in the presence or absence of sodium arsenite as determined by immunoblotting (right) (mean ± SD, *n* = 4). (**B**) Relative rate of protein synthesis when H357 cells were treated as above and with ISRIB as determined by puromycin uptake (left) and the concentration relative to GAPDH (right) (mean ± SD, *n* = 4). (**C**) Stress granule (SG) formation as visualization of G3BP1 (white) and *P. gingivalis* (red) using confocal microscopy and Z-stacks (*n* = 3, 100 cells per biological replicate). **** $p \leq 0.001$; ***, $p \leq 0.001$; *, $p \leq 0.05$ according to Kruskal–Wallis with Conover–Inman post-hoc.

The small molecular ISR Inhibitor (ISRIB) is known to reverse the effects of p-eIF2α on translational inhibition and stress granule formation [46]. Here, the ability of ISRIB to attenuate the heightened translational repression and modulation of SG formation during

P. gingivalis (NCTC11834) infection and oxidative stress was determined. As expected, during ISRIB treatment alone, protein synthesis remained at steady state rates (Figure S3). ISRIB was able to partially rescue stress-induced translation and this rescue was attenuated by *P gingivalis* (Figure 3B). *P. gingivalis* did not affect the proportion of cells containing stress granules (Figure 2B) whereas oxidative stress potently induced stress granule formation, which was inhibited with ISRIB. *P. gingivalis* partially but significantly reversed the ISRIB-induced reduction in stress granules (Figure 3C). Collectively, these data suggest that the heightened translational repression is independent of the ISR and cannot be rescued by ISRIB.

3.3. P. gingivalis Heightens Translational Repression via the Action of a Secretory Factor

As uninfected cells within the infected population displayed increased stress granule frequency during oxidative stress and infection, the effect of *P. gingivalis* (NCTC11834)-conditioned media was investigated to determine whether the observed effects were due to secreted bacterial components. In cells treated with conditioned media and oxidative stress, protein synthesis and eIF2α phosphorylation also decreased in line with infected cells, compared with the oxidative–stress–only treatment (Figure 4A,B). Taken together, these findings demonstrate that factors released by *P. gingivalis* can heighten oxidative stress-induced translational inhibition.
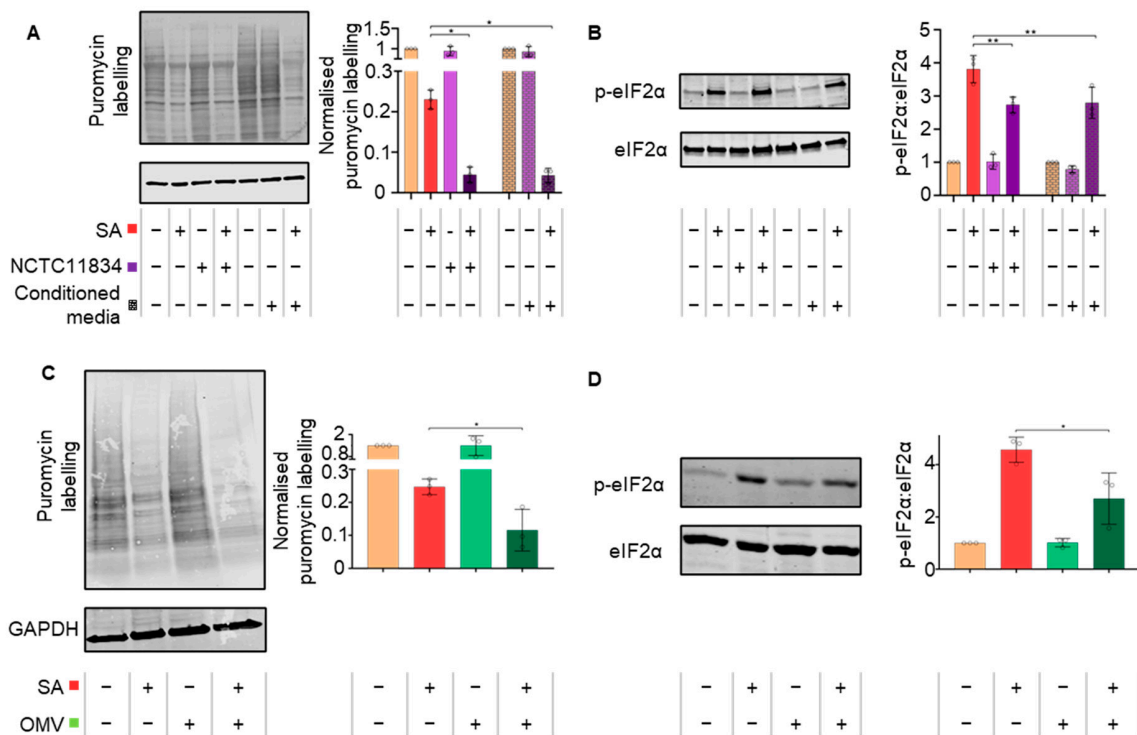

Figure 4. *P. gingivalis* heightens translational repression via the action of a secretory factor. (**A**) Relative rate of protein synthesis as determined by puromycin uptake (left) and quantification relative to GAPDH (right), when H357 cells were treated with filtered conditioned media recovered from cells previously treated with *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h) in the presence or absence of sodium arsenite. (**B**) Levels of phosphorylated eIF2α (left) and concentration of phosphorylated to total eIF2α (right) when probed using immunoblotting (mean ± SD, *n* = 3). (**C**) H357 cells were challenged with purified *P. gingivalis* (NCTC11834) OMV vesicles (100 µg/mL, t = 2 h) with or without sodium arsenite for the final 30 min and the relative rate of protein synthesis measured by puromycin uptake (left) and concentration relative to GAPDH (right) and (**D**) the levels of phosphorylated eIF2α (left) and the concentration of phosphorylated to total eIF2α (right) with OMVs were probed using immunoblotting (mean ± SD, *n* = 3). **, $p \leq 0.01$; *, $p \leq 0.05$ according to Kruskal–Wallis with Conover–Inman post-hoc.

To establish which secreted bacterial constituents elicited the heightened translational inhibition observed during stress, cells were challenged with crude preparations of *P. gingivalis* (NCTC11834) OMVs or purified lipopolysaccharide (NCTC11834 derived LPS). OMVs (1 µg/mL, 10 µg/mL and 100 µg/mL; t = 2 h) did not induce stress (Figure S4A,B). In the presence of oxidative stress, OMVs (100 µg/mL, t = 2 h) heightened translational repression 2.13-fold (Figure 4C) and decreased p-eIF2α 1.69-fold (Figure 4D).

Commercially purified *P. gingivalis* LPS (1, 5 and 10 µg/mL, t = 2 h) did not induce stress (Figure S4C,D). In the presence of oxidative stress, LPS (10 µg/mL, t = 2 h) did not alter translational repression or p-eIF2α (Figure S4E,F). This indicates that the heightened translational repression induced by *P. gingivalis* can be attributed to a secretory component distinct from LPS but present within the isolated OMV fractions.

3.4. P. gingivalis Dysregulates mTOR Signaling during Stress

Upon stress, mTORC1 has also been shown to contribute to translational control [47]. Previously, *P. gingivalis* has been shown to both inhibit and degrade mTORC1 through the activity of its gingipains [15,39]. As heightened translational repression during *P. gingivalis* infection and oxidative stress was independent of eIF2α signaling, the role of mTORC1 was evaluated using the selective mTOR inhibitor, rapamycin (400 nM, t = 1 h). Similar to *P. gingivalis* infection, rapamycin, in the presence of oxidative stress, heightened translational repression (Figure 5A) independently of p-eIF2α (Figure 5B).
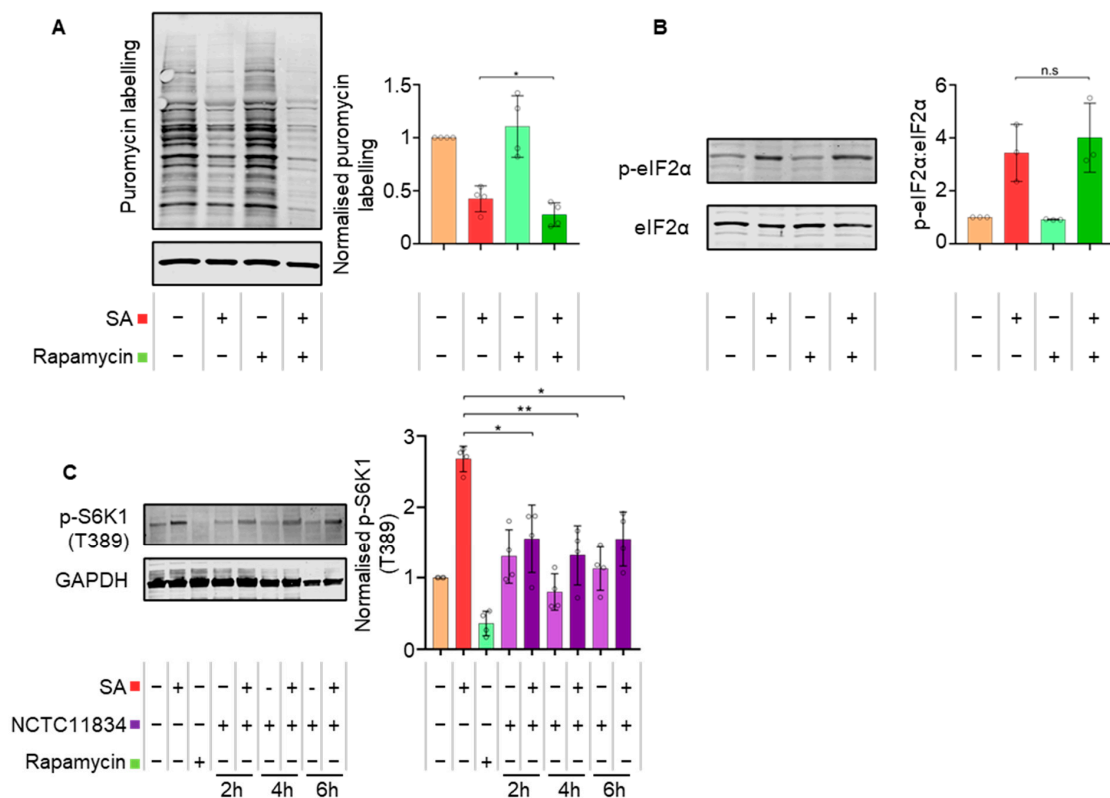

Figure 5. Rapamycin treatments exert the same effect on translation during oxidative stress as *P. gingivalis* and *P. gingivalis* attenuates stress-induced p-p70-S6-Kinase (T389). (**A**) Relative rate of protein synthesis as measured by puromycin uptake (left) and relative concentration compared to GAPDH in H357 cells treated with rapamycin and sodium arsenite as determined by immunoblotting (right). (**B**) Levels of phosphorylated eIF2α (left) and ratio of phosphorylated to total eIF2α as determined by immunoblotting (right) (mean ± SD, *n* = 3). (**C**) Levels of p-p70-S6K1 (T389) (left) and p-p70-S6K1 (T389) concentration relative to GAPDH in cells treated with *P. gingivalis* (NCTC11834, MOI of 100, t = 2–6 h) with and without sodium arsenite as determined by immunoblotting (right) (mean ± SD, *n* = 4). **, *p* ≤ 0.01; *, *p* ≤ 0.05 according to Kruskal–Wallis with Conover–Inman post-hoc.

To observe the impact of mTOR degradation on translation inhibition during oxidative stress, downstream mTORC1 targets were investigated. Rapamycin decreased the levels of phosphorylated p-p70-S6K1 (T389), whereas oxidative stress induced an increase. In contrast, whilst *P. gingivalis* (NCTC11834) infection alone did not result in altered levels of p-p70-S6K1 (T389), infection in the presence of oxidative stress caused a 1.73-fold decrease at all timepoints investigated (Figure 5C), suggesting that the phosphorylation activity of mTORC1 is downregulated by infection during stress.

3.5. Secreted P. gingivalis Proteases, Gingipains, Mediate Heightened Translational Repression during Stress

The findings thus far suggest that *P. gingivalis* can heighten translational repression during oxidative stress via a secretory factor. The impact of gingipains on translational control during oxidative stress and infection was therefore probed using gingipain-specific inhibitors TLCK (Lysine-specific, kgp) and Leupeptin (Arginine-specific, rgp). Both Leupeptin and TLCK, either alone or in tandem, inhibited the ability of the *P. gingivalis* (NCTC11834)-conditioned media to heighten translational stalling during oxidative stress (Figure 6A).

To confirm the role of gingipains in translational attenuation, a panel of isogenic gingipain null mutants (K1A, E8 and EK18) in *P. gingivalis* strain W50 were studied. Neither the wild type W50 strain nor the mutants induced a change in protein synthesis during infection in the absence of oxidative stress (Figure S5). Oxidative stress, together with W50, decreased puromycin incorporation (3.5-fold), compared with the oxidative–stress–only treated control. However, the mutants were unable to elicit this phenotype (Figure 6B).

The above data indicate that a role for gingipains in *P. gingivalis* mediated translational repression during oxidative stress. Hence, the effect of gingipains was investigated. During oxidative stress, an increase in stress granules comparable to what was seen with NCTC11834 was observed with wild-type W50 (Figure S6); neither wild-type W50 nor the gingipain mutants induced stress granules or inhibited their formation during oxidative stress (Figure 6C). In an oxidative stress environment, both the wild-type W50 and gingipain mutants E8 and EK18 induced an increase in stress granule frequency which was not observed in K1A infected cells. No change in the average stress granule area was observed with wild-type, K1A, nor EK18, whereas an increase in the area of stress granules was seen with the E8 mutant (Figure 6D). Taken together, these findings suggest that both lysine- and arginine-specific gingipains are accountable for *P. gingivalis*-mediated heightened translation repression during oxidative stress, with the lysine-specific gingipain inducing the increased stress granule frequency.

3.6. P. gingivalis Infection Dampens Stress-Induced Tubulin Acetylation

As changes to stress granule frequency and area were observed, the integrity of the microtubule network was determined since assembly of mature stress granules requires aggregation of components into smaller foci along polymerizing microtubules [31,48]. Visualization of α-tubulin showed no qualitative changes to the structure of α-tubulin following cell treatment with oxidative stress only or with *P. gingivalis* (NCTC11834) when compared with the total lack of structure observed with the positive control nocodazole (Figure 7A).
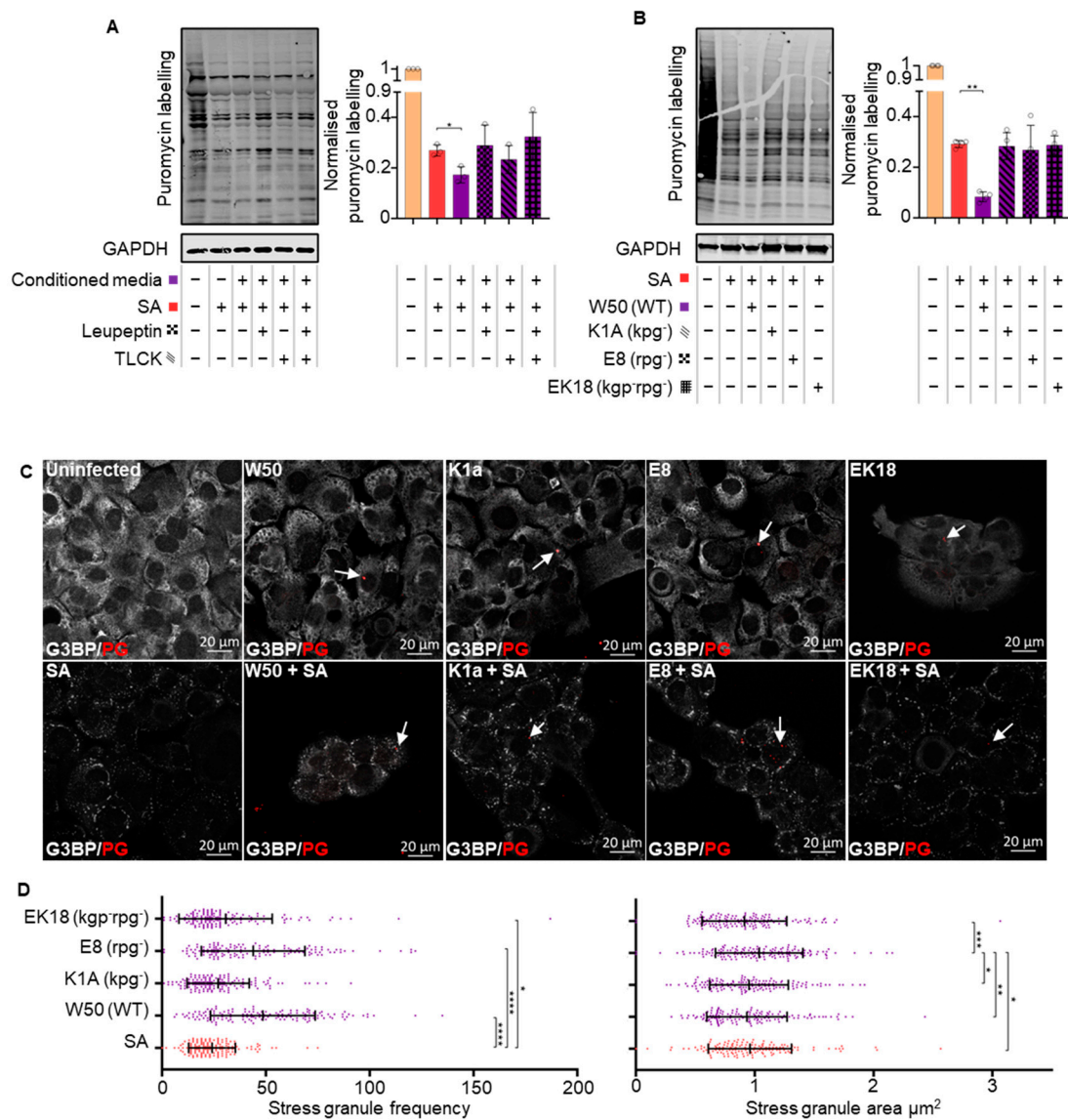
Figure 6. Secreted *P. gingivalis* proteases and gingipains mediate heightened translational repression during infection and stress. (**A**) Relative rate of protein synthesis as determined by puromycin uptake (left) and concentration relative to GAPDH (right) when H357 cells were treated with *P. gingivalis* (NCTC11834)-conditioned media in the presence or absence of leupeptin and TLCK and (mean ± SD, *n* = 3) (**B**) with *P. gingivalis* (W50, K1A (*kgp$^−$*), E8 (*rgp$^−$*), and EK18 (*rgp$^−$ kgp$^−$*), MOI of 100, t = 2 h); (mean ± SD, *n* = 4). (**C**) Stress granule formation was assessed by visualization of G3BP1 (white) and *P. gingivalis* (red) by confocal microscopy using Z-stacks. (**D**) Average area and frequency of SGs found in cells (*n* = 3, 50 cells per biological replicate). **** *p* ≤ 0.001; ***, *p* ≤ 0.001; **, *p* ≤ 0.01; *, *p* ≤ 0.05 according to Kruskal–Wallis with Conover–Inman post-hoc.

As the function of tubulin can be modified post-translationally, the levels of acetyl-α-tubulin were monitored. Both untreated cells and those infected with *P. gingivalis* (NCTC11834) displayed basal level acetylation. Oxidative stress resulted in a 3.6-fold increase in acetylation; a response which was dampened (1.42-fold) when cells were infected with *P. gingivalis* prior to the addition of oxidative stress (Figure 7B).
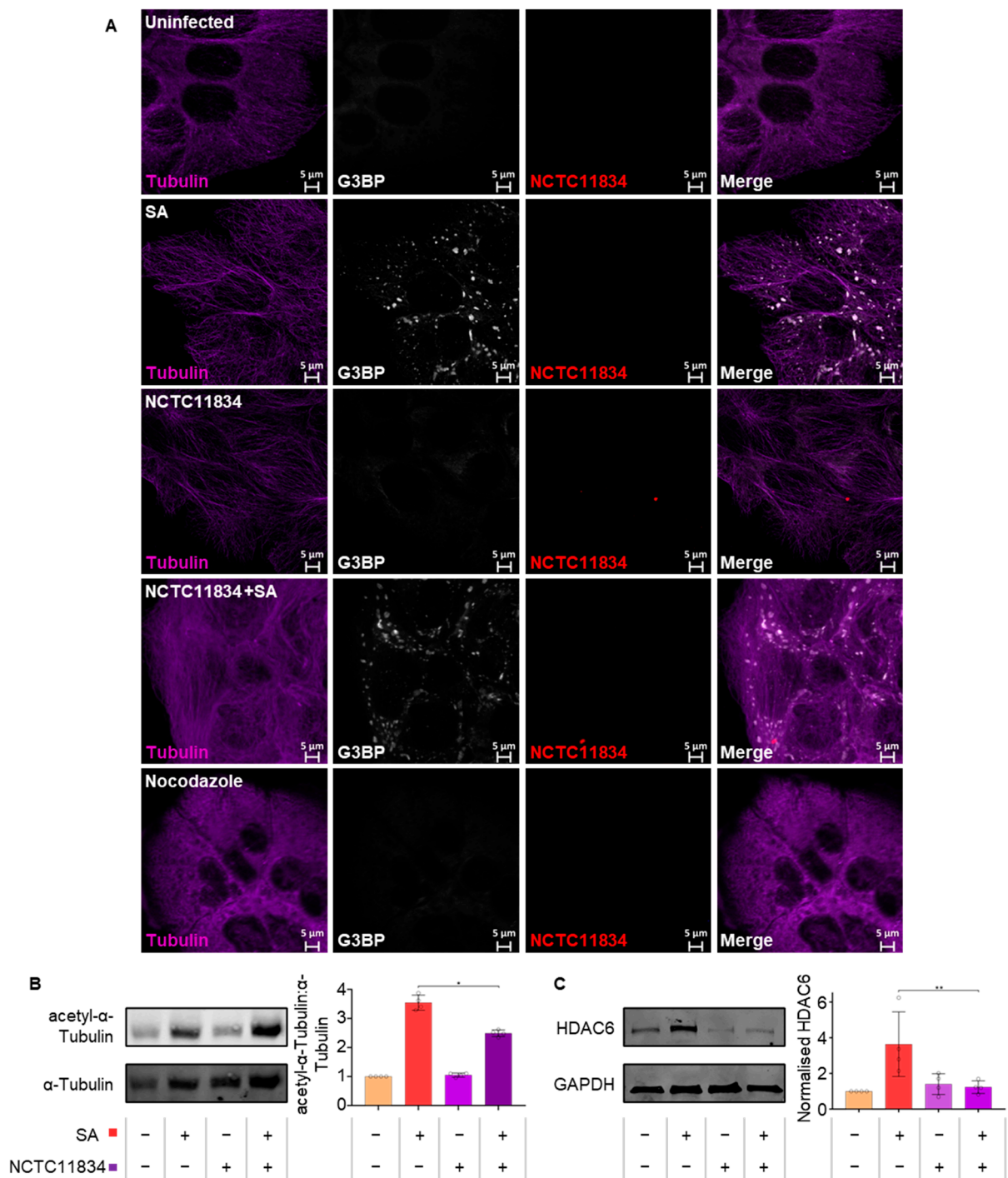
Figure 7. *P. gingivalis* infection dampens stress-induced tubulin acetylation. (**A**) Stress granule, α-tubulin integrity, and *P. gingivalis* were visualized using confocal microscopy following challenge of H357 cells with *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h) in the presence or absence of sodium arsenite. (**B**) Expression levels of α-tubulin (left) and ratio of acetyl-α-tubulin to α-tubulin (right; mean ± SD, *n* = 4). (**C**) Expression of HDAC6 (left) and concentration relative to GAPDH (right; mean ± SD, *n* = 3) as determined by immunoblotting. **, $p \leq 0.01$; *, $p \leq 0.05$ according to Kruskal–Wallis with Conover–Inman post-hoc.

To investigate the means of tubulin deacetylation during oxidative stress, the expression of the principal tubulin deacetylation enzyme, HDAC6, was determined (Figure 7C). *P. gingivalis* infection (NCTC11834) did not raise HDAC6 above basal levels whilst ox-

idative stress increased the levels of HDAC6 (3.64-fold). However, this phenotype was not observed when infection was coupled with oxidative stress (Figure 7C), suggesting that the lowered tubulin acetylation observed during *P. gingivalis* and oxidative stress was independent of increased HDAC6 expression.

4. Discussion

In recent years, ISR signaling and translational control during stress have garnered increased interest within the remit of host immune responses. These pathways, which can induce a wide variety of outcomes at cellular and systemic levels [49], offer a promising target for pathogens to manipulate. Both bacteria and viruses have been shown to influence the ISR, thereby reprogramming a variety of host responses and enabling the generation of a favorable replicative niche (Reviewed in [20,32]).

This study aimed to investigate the crosstalk between host translational control during stress and *P. gingivalis* infection and the potential wider impact upon periodontal disease progression. It was hypothesized that mTOR degradation following infection may impact translation control during stress, and as *P. gingivalis*-induced mTOR degradation was previously observed identically in both oral squamous carcinoma (H357) and immortalized oral keratinocytes (OK-F6) [15], H357 cells were chosen as the model for these studies.

Previously, *P. gingivalis* infection has been shown to activate the UPR in human umbilical cord endothelial cells [40]. Given that one arm of the UPR feeds into the ISR [41], it was hypothesized that *P. gingivalis* infection may also activate the ISR. However, over a period of 24 h, ISR activation was not observed, as evidenced by a lack of p-eIF2α or translational repression (Figure 1A,B), both core components of the active ISR [18]. Furthermore, infection over the same time did not result in the aggregation of G3BP into stress granules, a downstream marker of translational repression brought on by ISR activity (Figure 1C). Whilst it cannot be formally excluded that these responses might be cell type-specific, with human umbilical cord vein endothelial cells previously used [40] in contrast to the squamous oral epithelial cell carcinoma cells used here, it is possible that the UPR may have been active independent of translational attenuation (discussed further in [50]).

Given that *P. gingivalis* infection alone did not stimulate the ISR, the combined effect of infection and oxidative stress was investigated as reactive oxygen species are produced following neutrophil activation during host inflammatory responses [51]. Sodium arsenite, one of the most well-characterized ISR activating stressors, induces oxidative stress and inflammatory signaling via HRI kinase [52,53] and is known to reliably induce cellular stress [54]. Classically, studies challenge cells with 500 µM sodium arsenite [53,55], however, here, 250 µM (t = 30 min) was used as robust stress–responses without increased cell death as assessed by Hoechst 33342 and propidium iodide staining. The high levels of inflammation characteristic of periodontitis and caused by *P. gingivalis* infection [56] coupled with the expression of oxidative stress resistance genes by *P. gingivalis* [57] made sodium arsenite an attractive and relevant stress.

Previous studies [15,58] and analysis of *P. gingivalis* treated cells here show that 20% of the cells of a population are infected between two and four hours post infection (Figure 1B). In the presence of oxidative stress, bacterial invasion was found to increase 1.6-fold. Although the exact cause of this increase remains to be elucidated, *P. gingivalis* is known to express its own oxidative stress resistance genes [57] and whilst infection initially increases the production of reactive oxygen species [59], *P. gingivalis* later actively protects host cells against reactive oxygen species via the host antioxidant glutathione–response [60]. The layer of hemin present on the surface of *P. gingivalis* acts as a buffer against oxidative radicals and increases *P. gingivalis'* resistance to host oxidative stress [61,62]. *P. gingivalis'* ability to defend against oxidative stress whilst simultaneously upregulating host antioxidant pathways together with sodium arsenite's ability to decrease mammalian membrane integrity [63] may underpin the increased invasion observed during sodium arsenite induced oxidative stress.

Oxidative stress, as expected, resulted in translational repression [64]. *P. gingivalis*, in the presence of oxidative stress, exacerbated translational repression (Figure 1D) and increased stress granule frequency (Figure 2B). Previous studies looking at *S. flexneri* infection have implicated mTORC1 inhibition due to the membrane damage caused by bacterial internalization in stress granule modulation and translational dysregulation [34,38]. An increase in stress granule frequency has also been reported during chemical mTOR inhibition [65], suggesting that mTORC1 has a role in increased translational attenuation and stress granule frequency. This is supported by the involvement of mTORC1 as a key regulator of translation [66] with its inhibition leading to polysome disassembly and subsequent translational stalling [67]. Although *P. gingivalis* can inhibit and degrade mTOR [15,39], *P. gingivalis* alone did not lead to translational attenuation. A similar result was observed during rapamycin treatment. These differences in translational attenuation could reflect the variable outcomes of mTORC1 inhibition under different conditions [68]. The effects of *P. gingivalis*-mediated inhibition and degradation on translation may therefore only become apparent in the presence of another stress, as seen here where *P. gingivalis* heightened oxidative stress-induced translational attenuation.

Stress granules are formed by sequestration of stalled mRNPs into smaller foci, which in due course, fuse into larger aggregates [48]. The increased frequency of stress granules observed in this study may be due to *P. gingivalis* dysregulating stress granule aggregation and partially excluding eIF3b from the stress granules (Figure 2C,E). This is corroborated by reports that *S. flexneri* can selectively cause delocalization of eIF3b from stress granules during exogenous stress in a manner dependent on mTORC1 inactivation [38]. The movement of eIF3b is regulated by mTORC1, which phosphorylates S6K1 at T389, releasing S6K1 from eIF3b [69]. Oxidative stress-induced p-S6K1 (T389) [70] was decreased by *P. gingivalis* infection (Figure 5C), probably owing to inhibition or degradation of mTORC1 [15,39]. Therefore, decreased p-S6K1 (T389) could account for the lack of eIF3b in stress granules during oxidative stress and *P. gingivalis* infection (Figure 2E) and further supports the role of mTORC1 in the exclusion of eIF3b from stress granules.

Aggregation of stress granules requires constant retrograde transport along functioning microtubules [48,71]. Nocodazole, a chemical which disrupts microtubule assembly, increases the frequency of stress granules [71]. *P. gingivalis* is known to degrade cytoskeletal protein components including β-actin [15,72] and hence, whether the increase in frequency of stress granules observed during infection and oxidative stress was the result of tubulin degradation was determined. Infection did not result in visible changes to the microtubular network compared with nocodazole-treated cells (Figure 7A). However, microtubule network activity can also be controlled via post-translational modifications such as acetylation and phosphorylation [73] with hyper-acetylation of the cellular tubulin network at lysine 40 of α-tubulin reported during stress [74]. α-tubulin hyper-acetylation stimulates increased binding and activity of the microtubule motor proteins dynein and kinesin, which are involved in the movement of stress granules [75,76]. Here, *P. gingivalis* lowered the levels of α-tubulin acetylation during infection and oxidative stress (Figure 7B), which was independent of the increased expression of HDAC6, the major α-tubulin deacetylase [77]. Furthermore, HDAC6 is a critical stress granule component, ablation of which inhibits stress granule assembly [78]. Hence, the decreased tubulin acetylation and lowered HDAC6 expression may be influencing the modulated stress granule formation seen here during *P. gingivalis* infection and oxidative stress.

During ISR activation, translational attenuation, due to a range of stressors, is mediated by the phosphorylation of eIF2α [18]. Dysregulating eIF2α phosphorylation is also a mechanism by which many viruses hijack the host translational function (Reviewed in [79]). In this study, despite infection by *P. gingivalis* heightening translational repression during oxidative stress, no increase in p-eIF2α was observed (Figure 3A). These results were corroborated by the inability of ISRIB, a small molecule which antagonizes the inhibitory effects of p-eIF2 on eIF2B [80], to rescue translational function and to inhibit stress granule assembly during oxidative stress and infection (Figure 3B,C) [46]. Hence, the data points

towards a mechanism independent of eIF2α as the mediator of the heightened translational repression seen during *P. gingivalis* infection and oxidative stress.

As the heightened translational repression was eIF2α-independent and downstream mTORC1 targets were altered, rapamycin, a potent mTOR inhibitor, was used to further probe the pathway. During oxidative stress, rapamycin induced the same phenotype as *P. gingivalis* infection and heightened oxidative stress-induced translational stalling independently of p-eIF2α (Figure 5A,B), further supporting the contributory role for mTORC1. These findings are particularly relevant as *P. gingivalis* gingipains, which are known to degrade and inhibit mTOR [15,39], are expressed as cell surface-anchored proteins and in the secretome of *P. gingivalis*, where they exist both freely and packaged within OMVs [13,81]. With *P. gingivalis*-conditioned media and OMVs exhibiting a similar phenotype to internalized bacteria, the role of gingipains was investigated. Inhibition of secreted gingipains in conditioned media by gingipain-specific inhibitors inhibited the heightened translational attenuation observed with the conditioned media (Figure 6A). This inability to induce further translational repression was also observed using gingipain-knockout mutants (Figure 6B). Since secreted gingipains in conditioned media can enter cells in a clathrin-dependent manner [82], these findings implicate both the arginine- and lysine-specific gingipains in an extra- and intracellular manner and is possibly due to mTORC1 inhibition via the PI3K pathway, as reported by Nakayama and colleagues [39]. When the impact of these gingipain-knockout mutants on stress granule formation was investigated, the lysine gingipain-knockout (kgp) failed to increase stress granule frequency (Figure 6D), which could reflect the requirement of intracellular *P. gingivalis* secreted lysine-specific gingipains for mTOR degradation [15]. This may also account for the less marked increase in stress granule frequency in the *P. gingivalis*-negative cells of the exposed population, as OMVs containing gingipains only enter around 8% of cells [83]. In contrast, 40% of cells were infected in this study when exposed to *P. gingivalis* and oxidative stress (Figure 2B). Furthermore, as gingipains are secreted following *P. gingivalis* invasion and internalization of host cells [83,84], it could increase the concentration of intracellular lysine-specific gingipain, compared with conditioned media and OMV treatment. Taken together, these findings suggest that while both gingipains can heighten translational repression, the lysine-specific gingipain is the main effector of stress granule modulation and works most efficiently after invasion.

This study, for the first time, demonstrates that the periodontopathogen *P. gingivalis* dysregulates translational control and stress granule formation during oxidative stress, a condition phenotypic of the chronic inflammatory environment induced during periodontitis and caused by *P. gingivalis* [3] (Illustrated in Figure 8). These findings suggest a novel pathogenic mechanism employed by *P. gingivalis* to modulate host response, given that these pathways feed into cellular survival and the wider immune and inflammatory response [49], can contribute to the immune-subversive nature of *P. gingivalis*. Furthermore, dysregulation of the ISR, translational control, and stress granule dynamics have been implicated in a range of diseases from cancer to neurodegeneration [49,85,86].

While these findings implicate gingipains as defined effectors of translational dysregulation, studies into other *P. gingivalis* virulence factors including capsule, fimbriae, and various effector proteins may also converge onto these pathways. This study utilized a single-cell model and mono-species *P. gingivalis* infection, and to build on and extend these findings, it will be useful to look at the effect of polymicrobial infection on translational control. This is particularly relevant as induction of NOD1 signaling has been reported in periodontic mouse models [87] and NOD1 activation is known to induce ISR activation and NF-κB expression [33]. Furthermore, the impact of such polymicrobial infections of primary cells and 3D tissue models, as well as the effects of long-term infection and chronic stress environments, may offer key insight into the relationship between periodontitis, systemic *P. gingivalis* infection, and other diseases.
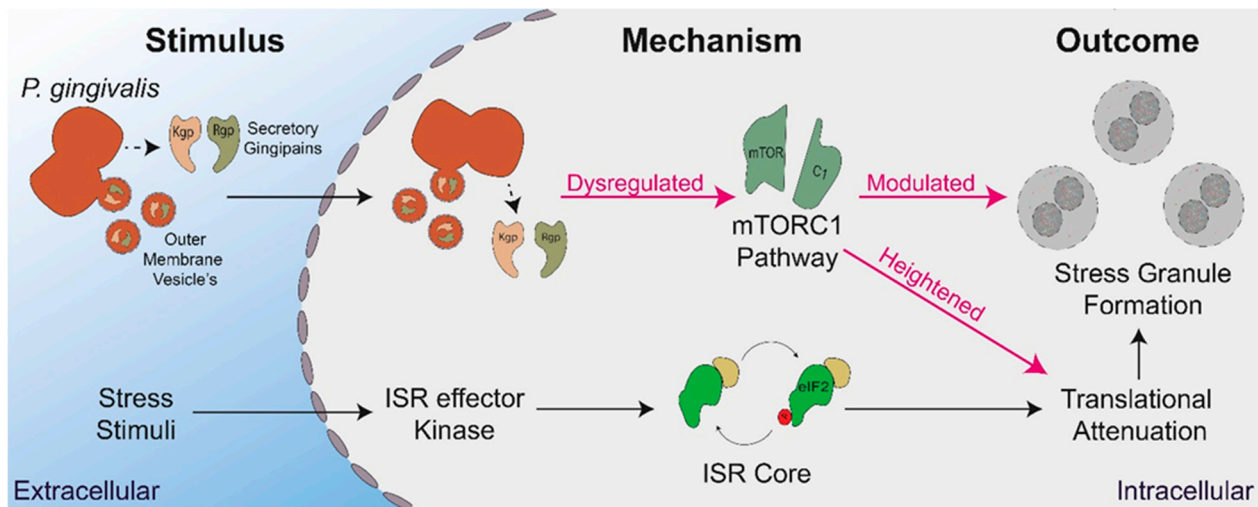
Figure 8. Summary of *P. gingivalis* interactions with host stress-induced translational control. Stress stimuli, such as oxidative stress, activate ISR effector kinases with phosphorylate eIF2α, resulting in translational attenuation and stress granule formation. *P. gingivalis* secretes gingipains and outer membrane vesicles in a both extra- and intracellular manner, which dysregulate the mTORC1 pathway, leading to heightened translational attenuation and modulated stress granule formation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11030606/s1, Figure S1. *P. gingivalis* does not induce ISR activation. (A) H357 cells were left untreated, infected with *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h to 6 h) in the presence or absence of sodium arsenite as shown. Relative rate of protein synthesis as measured by puromycin uptake (left) and concentration relative to GAPDH (right) (B) Levels of phosphorylated eIF2α (p-eIF2α) were probed using immunoblotting (left) and the ratio to phosphorylated to total eIF2α was determined (right). GAPDH was included as a loading control (mean ± SD, *n* = 3). (C) Stress granule formation was assessed by visualization of G3BP (white) and *P. gingivalis* (red). (D) H357 cells were infected with *P. gingivalis* (NCTC11834, MOI of 100, t = 24 h). G3BP (white) and *P. gingivalis* (red) were visualized using immunofluorescence confocal microscopy. No significant differences in means were found with a Kruskal–Wallis test. Figure S2. *P. gingivalis* and exogenous stress do not alter G3BP or eIF3B expression. H357 cells were left untreated or infected with *P. gingivalis* (NCTC11834, MOI of 100, t = 2 h to 6 h). Expression levels of (A) G3BP1 and (B) eIF3B were probed using immunoblotting. Concentration relative to the loading control GAPDH was first determined before being normalized to the untreated sample. Data are expressed as mean ± SD, *n* = 3. No significant differences in means were found with a Kruskal–Wallis test. Figure S3. ISRIB treatment does not alter translation in H357 cells. H357 cells were treated with either ISRIB or sodium arsenite, following which the relative rate of protein synthesis normalized first to the loading control GAPDH and then to control sample, was determined by immunoblotting for puromycin incorporation (mean ± SD, *n* = 3). No significant differences in means were found with a Kruskal–Wallis test. Figure S4. *P. gingivalis* outer membrane vesicles and lipopolysaccharide do not induce ISR activation. H357 cells were challenged with purified *P. gingivalis* OMV vesicles (1, 10 and 100 µg/mL, t = 2 h). Sodium arsenite was included as a positive control for ISR activation. (A) The relative rate of protein synthesis as measured by puromycin uptake and (B) the levels of phosphorylated eIF2α were probed using immunoblotting. To the right of each panel is a column graph of the quantified blot signals (mean ± SD, *n* = 2). (C) H357 cells were challenged with purified *P. gingivalis* LPS (1, 5 and 10 µg/mL, t = 2 h). Sodium arsenite was included as a positive control for ISR activation. Following which the relative rate of protein synthesis measured by puromycin uptake and (D) the levels of phosphorylated eIF2α were probed using immunoblotting. To the right of each panel is a column graph of the quantified blot signals (mean ± SD, *n* = 3). GAPDH was included as a loading control. (E) H357 cells were challenged with purified *P. gingivalis* LPS (10 µg/mL) with or without sodium arsenite for the final 30 min and the relative rate of protein synthesis measured by puromycin uptake (left) and concentration relative to GAPDH (right). (F) The levels of phosphorylated eIF2α (left) and the concentration of phosphorylated to total eIF2α (right) with LPS were probed using immunoblotting (mean ± SD, *n* = 3). No significant

differences in means were found with a Kruskal–Wallis test. Figure S5. *P. gingivalis* null mutants do not alter protein synthesis. H357 cells were left untreated or infected with *P. gingivalis* (W50, K1A (kgp^-), E8 (rgp^-) and EK18 (rgp^-kgp^-), MOI of 100, t = 2 h). Relative rate of protein synthesis was measured by immunoblotting for puromycin uptake. GAPDH was included as a loading control (mean $\pm$ SD, $n = 3$). No significant differences in means were found with a Kruskal–Wallis test. Figure S6. Comparison of stress granule frequency between NCTC11834 and W50 during stress. H357 cells were left untreated or infected by *P. gingivalis* (strains NCTC11834 and W50, MOI of 100, t = 2 h) and treated with or without sodium arsenite for the final 30 min. Stress granule formation was assessed by visualization of G3BP1 (white) and *P. gingivalis* (red) by confocal microscopy using Z-stacks. ($n = 3$, 50 cells per biological replicate). No significant differences in means were found with a Kruskal–Wallis test.

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