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# Host lncRNAs recognize invading bacteria and aid against infection by associating with polysomes


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# Declaration

The work described in this thesis was carried out at the Laboratory of Translational Architectomics, Institute of Biophysics (National Research Council), Trento (IT) and at the Department of Cell and Molecular Biology (Karolinska Institutet), Stockholm (SE) between September 2016 and November 2016. I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged. This work has not been submitted previously for any other degree at the University of Trento, Stockholm or any other university.

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# ABSTRACT

*BACKGROUND*: Infections mediated by pathogens, such as bacteria, often interfere with the host process of protein synthesis [1]. This process is the most energy consuming in cells, that is why cells fine-tune it to conserve energy and respond quickly to stress[2]. Thus, translation should be tightly regulated upon bacterial infection, yet the picture is still sketchy at best. Surprisingly, lncRNAs have recently been found to associate with ribosomes[3,4] and polysomes[5,6]; however, to date no research exists that addresses their role in translation regulation upon bacterial infection.

*AIM:* The key question I wanted to answer during my PhD is whether host produced lncRNAs rewire the cell's translation upon infection, inducing pathogen-specific and virulent factor-specific translational controls to cope with the infection.

*EXPERIMENTAL APPROACHES*: To address the abovementioned aim, I used human colon epithelial cells (Caco-2) and *Listeria monocytogenes* as a host-pathogen model to explore the host cell's response to infection at the translational level. By using a WT and a strain deficient for the expression of the main virulent factor Listeriolysin O (LLO-deficient (ΔLLO) strain). Taking advantage of these strains, I explored whether the pore forming toxin, LLO, is able to trigger a host toxin-specific translational controls. To address this question, I massively employed polysome profiling, a classical approach to study translation and Next Generation Sequencing (NGS) to monitored changes in the transcriptome and the translatome upon infection at early time-points after infection and studying the possible function and mechanism of two lncRNAs that I found to be over-expressed upon infection.

*RESULTS*: I showed that infection with either bacterial strain induced strong translational defects especially upon infection with WT *Listeria*. Cells responded to the infection by expressing numerous lncRNA and uploading them on polysomes. By comparing the transcriptome and the translatome of cells infected with either WT and ΔLLO I focused on two lincRNAs. The first, AC016831.1, is strongly upregulated upon infection with both strains of *Listeria* and exclusively associates with small active polysomes. In fact, my results show strong evidence that AC016831.1 is in fact actively associated with translating ribosomes and bioinformatics analyses of co-expressed genes showed its involvement in the innate immune response. The second, MIR181A1HG displayed a *Listeria*-specific and LLO-specific upregulation upon infection. I demonstrated that it is strongly associated with inactive

stalled small polysomes. Importantly, its expression upon infection exerted a protective role against bacterial replication in host cells. Considering the obtained results, I propose that MIR181A1HG is acting as a ribosome sponge, decreasing the number of available ribosomes, ultimately leading to translation down-regulation. This role may help cells to keep the overall protein production rate at a low pace during infection, allowing the host to properly activate the innate immune system and fight-off the pathogen.

In this research, using polysome profiling, I demonstrated for the first time that lncRNAs play a role in the host-pathogen crosstalk by rewiring translation. Evidence shows they might be even producing peptides, challenging their non-coding status and paving the way for understanding the possible role of short peptides in controlling bacterial infections.

The work performed during this PhD project contributed to publishing a Perspective article in the journal Toxins, titled: "The Unexpected Tuners: Are LncRNAs Regulating Host Translation during Infections?"[7] and a research paper (in preparation) for submission to peer review and publication in a scientific journal.

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# 1 Introduction

## 1.1 The innate immune response

The crosstalk between higher organisms and pathogens is well described with the evolutionary hypothesis known as the Red Queen's race. This hypothesis, first conceived by Leigh Van Valen in 1973, proposed that organisms constantly adapt to an ever-changing environment and evolve to gain reproductive advantages over each other. This continuous adaptation is needed in order for a species to survive among co-evolving organisms[8]. An example of this arms race is between hosts, that developed immune systems (defence mechanisms able to recognise and fend-off invading pathogens), and the pathogens, which developed elaborate ways to evade them. In vertebrates, we recognize two types of immunity: adaptive and innate.

The adaptive immune response is highly specific. It is able to examine a pathogen, build specific antibodies against it, in order to neutralize it, and is even able to remember previous encounters with pathogens. However, compared to the innate immune response the adaptive immune response is slow to develop, especially upon first exposure to a pathogen and can take days before the responses are effective. Therefore, during the very first few hours upon pathogen exposure, organisms like humans rely solely on the innate immune system to protect them from infection[9].

The innate immune system is the cell's first line of defence and it is genetically programmed to detect relatively invariable molecular components found in most microorganisms. These components are recognised by so-called pattern recognition receptors (PRRs), which are germline-encoded sensors of microbes that can induce antimicrobial defence mechanisms in order to maintain homeostasis. PRRs are constitutively expressed in all cells and can be broadly categorized into three classes[9,10]:

i) Secreted PRRs, such as collectins, ficolins, and pentraxins, which are found in plasma and tissue fluids and work by binding to components on microbial surfaces. Upon recognition, they activate classical and lectin complement pathways which attracts macrophages and neutrophils to the infection site and trigger phagocytosis of these pathogens[11].

ii) Transmembrane PRRs, such as Toll-like receptors (TLRs), found on cell endosomes and plasma membrane[12].

iii) Cytosolic PRRs, such as NOD-like receptors (NLRs).

Whichever way PRRs are activated, the end-result is the activation of signalling pathways including nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs), which are responsible for inducing transcription of pro-inflammatory genes[10] and translation regulation[2]. Because of these defensive measures, in order to survive, pathogens developed their own mechanisms by targeting these pathways and dampening the activation of the innate immune response of the host.

### 1.1.1 Gene expression regulation

Numerous studies have demonstrated that host cells strongly respond to bacterial infection by reprogramming their transcriptome and translatome in order to clear the infection[1,13,14].

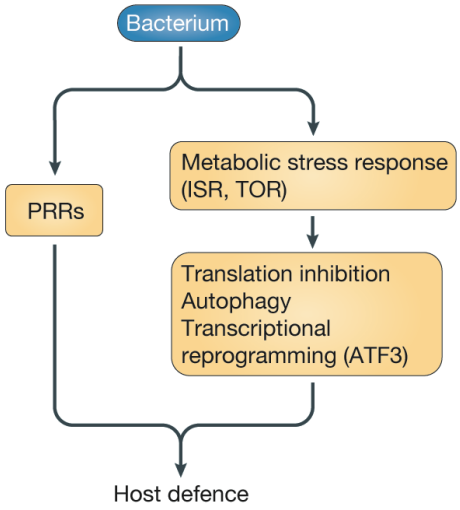


*Figure 1.1:* ***Host's response to invading bacterial pathogens***

*Host cells recognize invading bacteria via pattern recognition receptors (PRRs) and by detecting metabolic stress caused by pathogens. This in turn activates multiple pathways which regulate gene expression of proinflammatory genes, which leads to host defence against bacteria. (Figure adapted from Lemaitre and Girardin, 2013[14]).*

In order to survive and maintain homeostasis, cells strongly rely on the recognition and defence against invading pathogens.

Their first line of defence is their innate immune system, which is based on: i) recognition of specific molecules produced by pathogens via pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs). ii) modulation of metabolic stress through pathways such as the mammalian target of rapamycin (mTOR)[10,14] and the integrated stress response (ISR)[15] (**Fig. 1.1**).

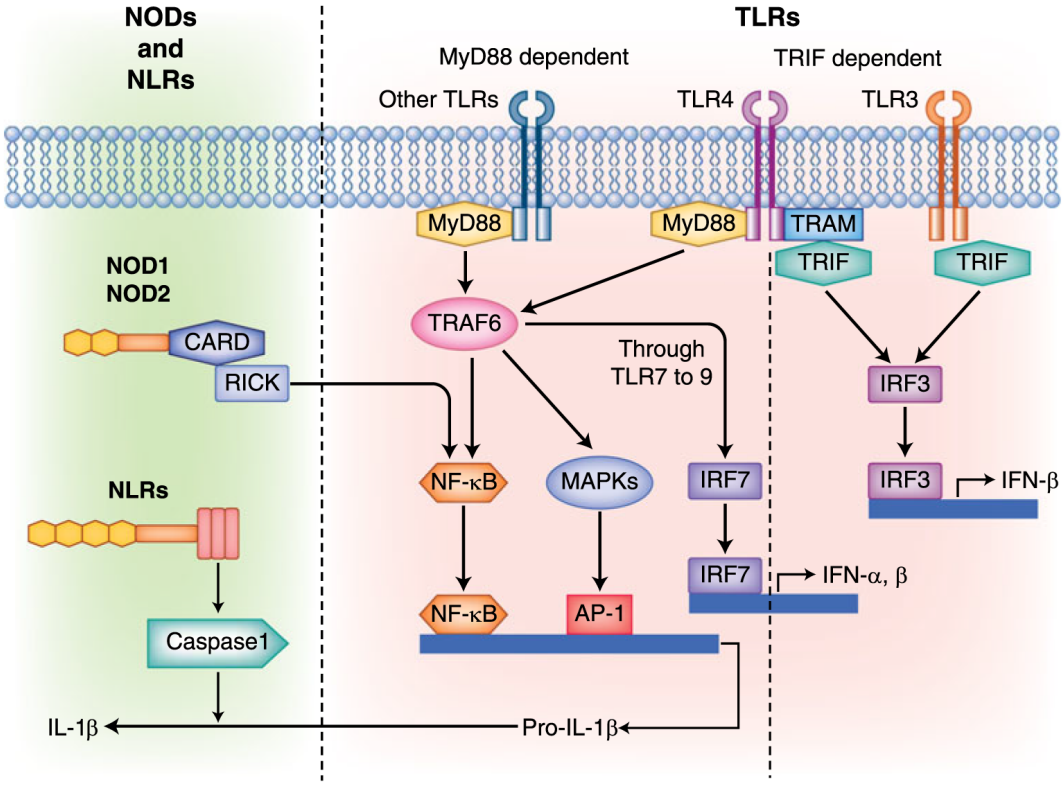


*Figure 1.2:* ***Signal transduction pathways of TLR and NLR***

*Pathogens are recognized by the host receptors, such as the transmembrane TLRs and cytosolic NLR. TLRs which are located on the plasma membrane and the membrane of endosomes, induce NF-κB and MAPK activation signalling cascades and production of pro-inflammatory genes. NLR on the other hand recognize pathogens in the cytosol and activate NF-κB signalling and Caspase-1, inducing the production of pro-inflammatory genes. (Figure adapted from Sotolongo et al., 2012[16]).*

Toll-like receptors (TLRs) are transmembrane proteins found on the plasma membrane as well as the endosome membrane[12]. They are responsible for recognizing pathogen-associated molecular patterns (PAMPs), which are microbial components like lipopolysaccharides (LPSs) of Gram-negative bacteria and lipoteichoic acids (LTAs) of Gram-positive bacteria. They are expressed on various immune cells and even on nonimmune cells such as epithelial cells[10]. Stimulation of TLRs results in NF-κB and MAPK activation signalling cascades and production of pro-inflammatory cytokines and chemokines[9].

NOD-like receptors (NLRs) are a specialized group of intracellular proteins, that are primarily involved in bacterial recognition and other forms of stress (such as UV irradiation). Upon pathogen recognition they activate NF-κB signalling as well as Caspase-1 which catalyses the production of mature cytokines[17] (**Fig. 1.2**).

A crucial part for the cell's survival is the detection of cellular damage triggered by invading pathogens[18] or perturbations caused by some microbial molecules, such as pore-forming toxins[19]. These signals, known as pathogen-associated molecular patterns (PAMPs) alert the innate immune system to microbial invasion and include calcium influx and potassium efflux, lysosomal damage, reactive oxygen species (ROS)[14,20]. In cells, PAMPs cause metabolic stress, which triggers autophagy against intracellular bacteria, transcriptional reprogramming[21] and translation inhibition[14]. These responses depend on the activity of mTOR, a master regulator of cellular metabolism[22].

#### 1.1.1.1 Translation regulation

Translation is the final step of gene expression and certainly the most energy-consuming and controlled mechanism in cells[2,23,24]. It dictates the physiological state of the cell by influencing protein abundance throughout initiation, elongation, termination phases and ribosome recycling[25]. Protein synthesis is carried out on ribosomes where, using an mRNA as a template, ribosomes catalyse peptide bond formation between amino acids to produce proteins. Translation is a cyclical process, meaning that after translation termination the ribosomes are recycled by dissociating into individual subunits and then reused for a new complete round of translation[26–28]. Protein synthesis is the most energy consuming cellular process, that is why cells fine-tune it to conserve energy and respond quickly to

environmental changes[2]. It has been demonstrated that about 40 % of the variation in protein abundance can be explained by processes related to translation rather than to transcription[29–32]. Thus, translation regulation should be tightly regulated especially upon bacterial infection through a very complex, yet poorly understood mechanisms.

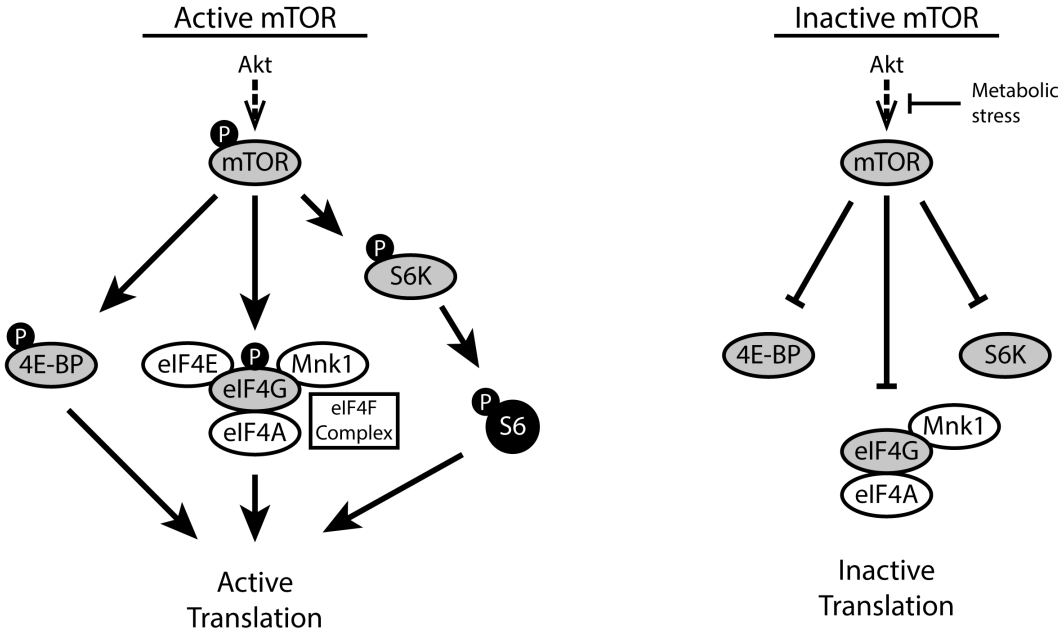


*Figure 1.3:* ***Translation modulation via mTOR signalling pathway***
*The mTOR signaling pathway. Phosphorylation of mTOR downstream effectors (4E-BP, RPS6 kinase (S6K) and eIF4G) involved in translation regulation when mTOR is either active (left scheme) or inactive (right scheme). Metabolic stress inactivates mTOR phosphorylation by Akt, resulting in inactive translation. (Figure adapted from Kudchodkar et al., 2004[33]).*

Indeed, the rewiring of translation to produce pro-inflammatory proteins is the very first step in the innate immune response which protects host cells against pathogens like bacteria[14]. Numerous research shows that bacterial PAMPs inhibit host translation, in order to adjust the metabolism to the energy status of the cells[34]. PAMPs like bacterial pore-forming toxins, produced by a plethora of bacteria, cause membrane damage in host cells and were demonstrated to trigger amino acid starvation[35,36] and the activation of the unfolded protein response (UPR)[37], thereby activating different signalling pathways as a response.

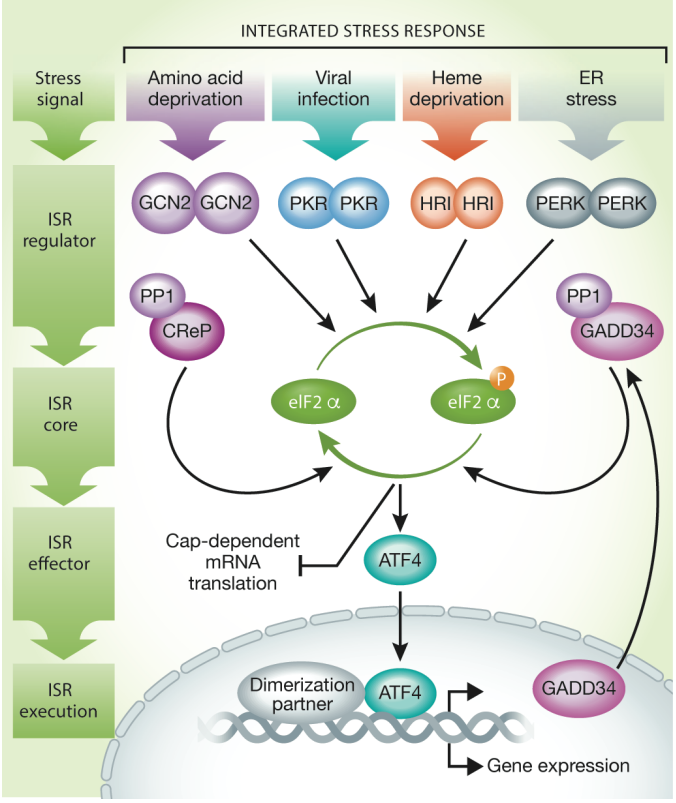


*Figure 1.4:* ***Integrated stress response signalling***

*Scheme of the activation of the integrated stress response (ISR) by different stress signals. ISR includes four pathways (PERK, HRI, PKR and GCN2) involved in eIF2α phosphorylation. Once phosphorylated, eIF2α induces a global protein synthesis inhibition. (Figure adapted from Pakos-Zebrucka et al., 2016[15]).*

In turn, the activation of these pathways results in translation inhibition, typically induced via regulatory proteins which are a part of stress-responsive pathways[14]:

i) mTOR inactivation, induced by metabolic stress, such as amino acid starvation[21,38]. mTOR is a downstream Serine/Threonine kinase that responds to extracellular stimuli, oxygen and energy status of cells and metabolic stress, such as amino acid starvation. It can directly phosphorylate several substrates relevant to translation, including eIF4G, the S6 kinases and also eIF4E binding protein (4E-BP1). Triggering the inhibition of mTOR consequently inhibits translation[39] (**Fig. 1.3**).

ii) ISR activation, triggering eIF2a phosphorylation, which has a strong inhibiting effects on translation initiation[40]. This process is mediated by four kinases that react to disturbances in cellular homeostasis[15] (**Fig. 1.4**):

a) double-stranded RNA-dependent protein kinase (PKR), activated during viral infection[41].

b) heme-regulated eIF2a kinase (HRI), activated during heme-deprivation[42].

c) PKR-like ER kinase (PERK) is a sensor of endoplasmic reticulum (ER) stress, activated by the UPR and perturbations of calcium homeostasis, or redox status[43,44].

d) General control non-derepressible 2 (GCN2), triggered by amino acid deprivation upon intracellular bacterial invasion [21,34,45].

## 1.2 Host – Pathogen interplay: focusing on intracellular bacteria

Bacteria have developed numerous mechanisms that help them evade the immune response in order to promote their survival. Cells of the immune system are capable of ingesting and destroying invading bacteria, and can activate highly-specific adaptive immune responses by producing antibodies against them, in order to be removed by phagocytes. A clever way bacteria developed to avoid recognition, is by invading the host cell. Even though this protects them from other immune cells, bacteria find themselves in vacuoles in which the host cell can direct molecules, such as hydrolytic enzymes, reactive oxygen species (ROS), or antimicrobial peptides which are able to eliminate them[46]. Furthermore, individual host cells also have PRRs that can detect intracellular bacteria and respond by activating pro-inflammatory signalling pathways. For this reason, bacteria produce virulent factors, which are able to subvert host defence mechanisms by manipulating host cell receptors that mediate internalization and signalling, membrane trafficking, autophagy and inflammasome activation[47] (**Fig. 1.5**).

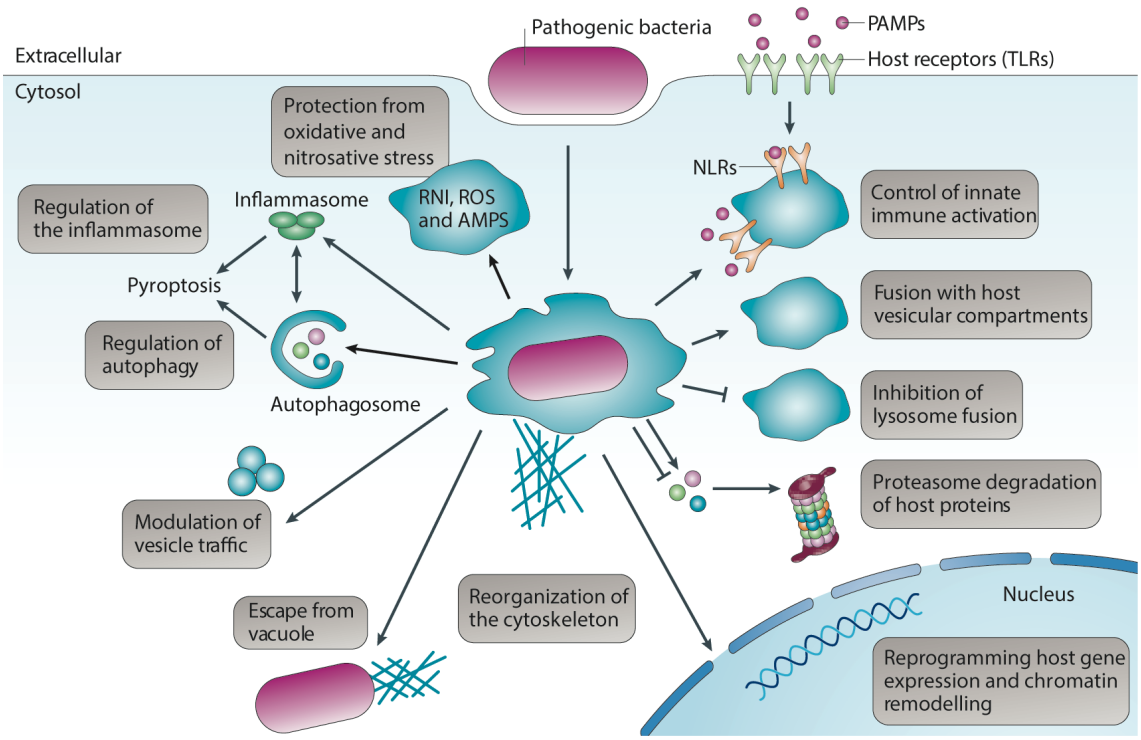


*Figure 1.5:* ***Manipulation of host innate immunity by intracellular pathogenic bacteria***
*Intracellular pathogens are recognized by the host's innate immune system via TLR and NLR, which activates antimicrobial defences, such as oxidative and nutrient stress, production of pro-inflammatory proteins, autophagy. Intracellular pathogens have evolved to control some of the signalling pathways activated by host receptors, interact with endocytic pathway, escape from the phagosome, inhibit fusion with lysosomes, manipulate vesicular trafficking and avoid autophagosome degradation and inflammasome activation. (Figure adapted from Diacovich and Gorvel, 2010[47]).*

Virulence factors (VFs) are crucial for bacteria to establish persistent infections and survive in a hostile environment. They aid bacteria at every step of its life-cycle and have therefore also very versatile functions[48]. A major category of VFs are toxins, which are bacteria-produced biological poisons and are categorized based on their target:

i) Membrane disrupting toxins, such as *Listeria monocytogenes's* pore-forming listeriolysin O[49];

ii) Intracellular-targeting toxins, such as *Salmonella enterica's* cytolethal distending toxin (CdtB), which displays DNase activity[50];

iii) Superantigens, like *Staphylococcus aureus's* staphylococcal enterotoxins A (SEA), that generates a massive non-specific immune response, resulting in the release of a large and sudden amount of cytokines[51].

Bacterial toxins were shown to alter signalling pathways which regulate the transcription of pro-inflammatory genes[52], among the most studied is *Yersinia pseudotuberculosis*'s YopJ, which can inhibit both the NF-κB and MAPK pathways, thereby blocking transcription of host proinflammatory genes[53]. Apart from transcription regulation, toxins produced by bacteria can also inhibition of the host's translation which limits the production of proteins involved in cellular recovery like cytokines[45]; however, this interference can also trigger a conserved innate immune response[1]. Toxins can act either indirectly through modulation of signalling pathways, such as activation of ISR or inactivation of the TOR pathway[34] or by directly inhibit host protein synthesis[1]. Toxins such as *Corynebacterium diphtheriae*'s Diphtheria Toxin (DT) and *Pseudomonas aeruginosa*'s Exotoxin A (ToxA) both inhibit translation by inactivating host translational elongation factor eEF-2, required for protein synthesis[14]. It has been demonstrated that some membrane disrupting toxins, such as pore-forming toxins, affect metabolic pathways, intracellular signalling, proteasome activity, transcription[19,34] and translation [34,54,55]. Interfering with host translation limits production of host defences, but on the other hand it can also trigger a conserved innate immune response[1], representing a good example of the host-pathogen interplay.

### 1.2.1 Listeria monocytogenes

*Listeria monocytogenes* is a widespread gram-positive bacterium that causes the disease listeriosis in humans and animals. This food-borne pathogen is able to counteract the innate immune system and is adapted to survive within macrophages and other host cells[56]. It can cross the intestinal, the blood-brain, and the fetoplacental barriers, causing meningitis in immunocompromised patients and abortion in pregnant women[56,57]. According to the European Food Safety Authority (EFSA), listeriosis infections reported in humans increased by 16% from 2013 to 2015 and have been rising since 2008. Although the overall number is relatively low, the continuous rise of reported cases is of concern since death rates are much higher than for other food-borne diseases. In 2015 alone 270 deaths were due to listeriosis, which is a 17.7% fatality rate and is the highest number reported since 2008[58].

*Listeria monocytogenes* is also one the most important paradigm in the study of intracellular host-pathogen interaction. It has been employed in multiple studies of the innate immune response, gene expression regulation and post-translational control upon infection, as well as

the mechanism of action of pore forming toxins[49,56,59]. At the cell level *Listeria*'s main stages of its life cycle are well established and are aided by a number of virulent factors (**Table 1.1**). The different stages can be summarized as follows: i) bacterial entry, ii) phagosomal escape, iii) actin-based motility, and iv) cell-to-cell spread (**Fig. 1.6**). The bacterium starts its intracellular life cycle by entering the host cell with the help of two internalin proteins. Internalin InlA can bind to host cell receptor E-cadherin and InlB can interact with the tyrosine-protein kinase Met. This interaction results then in the formation of an intracellular vacuole-containing the bacterium[60]. Once the bacterium is inside the host cell, it is located in this primary vacuole where it typically spends about 30 min before escaping into the cytosol[61]. Vacuolar destabilization occurs in concomitance with destabilization of the vacuole membrane. This step is mainly carried out by the pore-forming toxin listeriolysin O (LLO); however, *Listeria* also secretes two phospholipases C (PlcA and PlcB) which assist with membrane disruption, allowing the bacterium to enter the host's cytoplasm[62]. Once in the cytosol, the bacterium replicates and starts spreading to neighbouring cells. *Listeria* uses ActA, a protein located on its surface, to move around the cytosol by forming a polarized comet tail consisting of a dense array of cross-linked actin filaments. Using this actin-based motility the bacteria can induce the formation of protrusions in the host cell membrane and spread to neighbouring cells without host cell lysis[63,64]. When the bacterium enters neighbouring cells, it is located in a double membrane vacuole, called a secondary vacuole. The membrane of the secondary vacuole is then destabilized much like the primary, by LLO, assisted by the two PLCs. *Listeria* is then released into the cytosol and can start a new round of its life cycle[65].

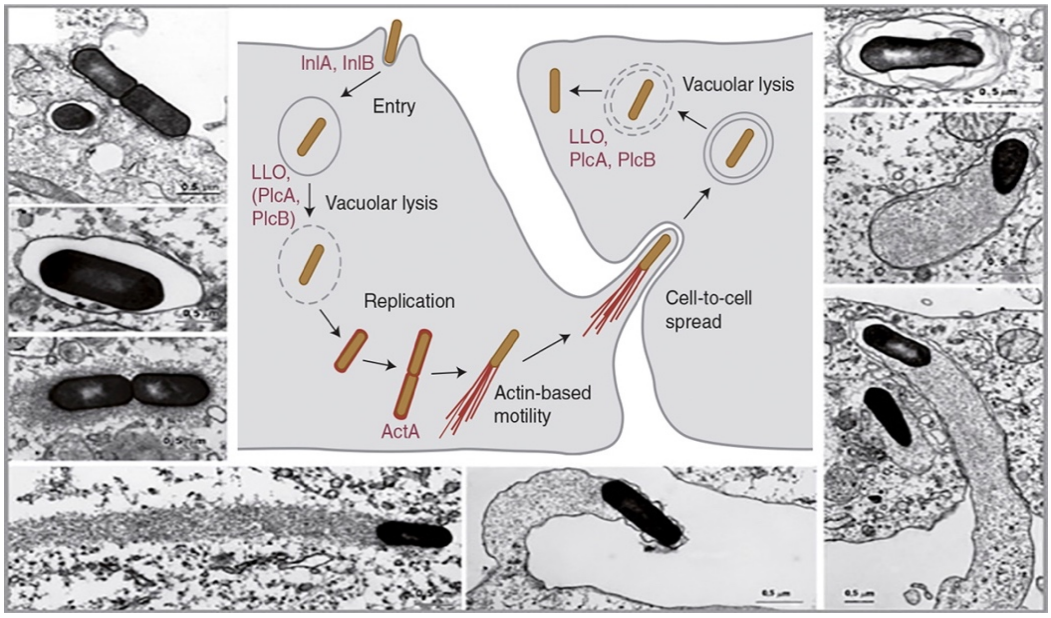


*Figure 1.6:* ***Intracellular life cycle of L. monocytogenes***

*Listeria uses two internalins (InlA and InlB) to facilitate host cell internalization. The bacterium is then located in a primary vacuole and uses a pore-forming toxin LLO, assisted by PLC to destabilize the vacuole's membrane and escape in to the cytosol. Listeria then replicates within host cells and uses ActA to cross-link actin filaments and propel itself and spread to neighbouring cells, where it starts a new round of its life cycle. (Figure adapted from Cossart, 2011*[56]*).*

*Listeria*'s transcriptional regulator, PrfA, regulates most virulence factors organized in a virulence gene locus of approximately 9 kb in size, comprised of 6 genes, called the virulence gene cluster (**Fig. 1.7**). Comparative genomics showed that this region is absent in the genome of non-pathogenic species like *L. innocua, L. welshimeri* and *L. grayi*, further pointing out their involvement in pathology[66,67]. The main actor in this cluster is *prfA*, encoding for the protein PrfA which is a transcriptional activator and the main switch which regulates the expression of all other virulence genes in this cluster[68]. PrfA expression is predominately regulated by an RNA thermosensor mechanism with a maximum activity at 37 °C[69]. However, this is not sufficient to induce PrfA-dependent gene expression[70], that requires the presence of host derived glutathione (GSH) for *prfA* to be activated[71]. PrfA activation leads then to expression of: *hly*, encoding the pore forming toxin listeriolysin O; *plcA* and *plcB*, encoding the two phospholipases C; *actA*, encoding for ActA protein needed for actin-based motility; *mpl*, encoding a zinc metalloprotease; *InlA* and *InlB* internalins and the secreted *InlC* internalin.

*Table 1.1:* ***Listeria monocytogenes's main virulent factors and their function***

| Virulence factor | Secreted/endogen | Function | References |
|---|---|---|---|
| LLO | Secreted | Vacuole escape; pore-formation | 56,72,73 |
| PLC | Secreted | Vacuole escape | 62,74,75 |
| InlA and InlB | Bacterial membrane | Host invasion | 76,77 |
| ActA | Bacterial membrane | Intracellular motility | 78 |
| mpl | secreted | Activation of PLC and ActA | 79–81 |

*Listeria's* virulence factors ultimately enable the bacterium to survive and replicate intracellularly and have been studied to understand which of these are in fact crucial and indispensable in *Listeria's* life cycle and which could potentially be disposable. To address this question, strains were made with deletion of virulent factors. *Listeria* strains lacking InlC, revealed that this virulent factor impairs phosphorylation of IκB, thereby inactivating NF-κB signalling and cytokine production[82]. Strains expressing non-functional forms of InlA lead to a reduced ability to invade host cells[83,84] and mutants lacking ActA retain immunogenicity but undergo ubiquitylation and finally autophagy[85].

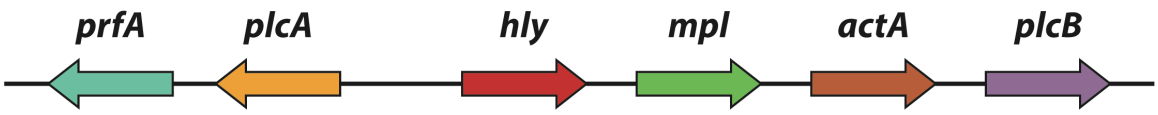


*Figure 1.7:* ***The virulence gene cluster of Listeria monocytogenes***

*Genomic map of Listeria's virulence gene cluster and their master expression regulator prfA. The cluster contains its most essential virulence genes: listeriolysin O (hly), phospholipase C (plcA and plcB), actin assembly-inducing protein (actA), metalloprotease (mpl).*

Interestingly, mutants lacking PLC were still able to escape the primary vacuole but were trapped in double-membrane compartments when spreading to neighbouring cells, suggesting that PLC may be involved in the dissolution of the inner membrane of the spreading vacuole, but it is not sufficient for disrupt of the outer membrane[62,75]. *Listeria* mutants lacking LLO were shown to be avirulent, unable to escape the primary vacuole of some cell types and proliferate in mammalian cells. Thereby LLO earned the title of *Listeria*'s main virulent factor[72,86].

#### 1.2.1.1 Host gene expression regulation

Upon host invasion, *Listeria* triggers several pathways aimed at pathogen recognition in host cells. Downstream results are in most cases responses by Toll-like-receptors (TLR)[87–89] and NOD-like-receptors (NLR)[90], triggering transcriptional responses and cytokine production. Despite these antimicrobial responses, *Listeria* is still able to survive within host cells, by inducing the reprogramming of genes involved in the innate immune response.

*Listeria* was shown to alter host transcription and modulate expression of proinflammatory genes upon infection, by either inactivating pathways, such as MAPK[91] and NF-κB[82] or under different conditions to activate them[92,93]. *Listeria* was also demonstrated to affect transcription, by inducing specific histone modifications in host cells, resulting in reduced expression of genes involved in the immune response[94]. Furthermore, *Listeria* infection induces indirect host translation regulation. It achieves this by acting on signalling pathways involved in translation, such as mTOR, PERK, GCN2 and MAPK. mTOR stimulates global protein synthesis by modulating downstream effectors like the eIF4E-binding protein (4E-BPs) and the ribosomal S6 kinase (S6K) [2,95]. Upon infection, *Listeria* triggers the host amino acid starvation responses which in turn causes mTOR signalling inhibition, GCN2 phosphorylation and consequently inhibition of the initiation stage of host translation[96]. Recently, the involvement of PERK pathway via eIF2α phosphorylation has been reported upon *Listeria* infection[45]. This in fact inhibits host translation and also activates NF-κB signalling[1] which *Listeria* is also able to modulate[82]. The host MAPK pathway has been shown to be either activated[92,93] or inactivated[91] upon *Listeria* infection was mainly studied in connection to transcription regulation upon *Listeria* infection. When inactivated a reduction in

phosphorylation of the ribosomal protein 6 (RPS6) phosphorylation and ultimately translation inhibition have been observed[2].

#### 1.2.1.2 Listeriolysin O

Listeriolysin O (LLO) is a pore-forming toxin (PFT) belonging to a family of cholesterol-dependent cytolysins (CDCs). These toxins are produced by Gram-positive bacteria as monomers or dimers which then bind to cholesterol-rich membranes and assemble into large pore complexes[97]. LLO is produced by *Listeria monocytogenes* and it uses it to mediate vacuolar escape during bacterial entry and cell to cell spreading, but there is now growing evidence that *Listeria* uses it also in other ways to gain advantage over the hosts innate immune system, by affecting both their transcription and translation through interference of signalling pathways[73].

*Listeria* continuously produces LLO during its lifecycle[98] and is able to tightly regulate its cytotoxicity in order to proliferate without killing the host cell[99]. Regulation of LLO was shown to be a combination of several processes including translational repression in the cytosol, degradation by the proteasome[100] and even its ability to regulate its own activity by exhibiting temperature- and pH-dependent stability. LLO rapidly and irreversibly aggregates to a non-functional form at temperatures above 30 °C and physiological pH. However, its thermal stability greatly increases at low pH 5.5, as found in late endosomes where *Listeria* finds itself upon host cell internalization[101,102]. Furthermore, two host factors were found that can modulate LLO's activity: i) a thiol oxidoreductase GILT, found to activate LLO by keeping its single cysteine residue in a reduced state[103] and ii) a chloride transporter cystic fibrosis transmembrane conductance regulator (CFTR) that can localize to the pathogen-containing vacuole, causing a chloride influx into the phagosome, which promotes LLO oligomerization and pore-formation[104].

The presence of intracellular LLO was shown to induce mTOR inhibition[96] as well as activate multiple signalling pathways, such as calcium signalling[105,106], the MAPK pathways[107], protein kinase C[108] and NF-κB signalling[109]. Despite it being responsible for activation of all these proinflammatory pathways, intracellular LLO strongly promotes bacterial survival by: i) controlling host autophagy, inhibiting the fusion of the *Listeria*-containing vacuole with

lysosomes, and disrupting the lysosome membrane releasing into the cytosol proteases, such as cathepsins, [110,111]; ii) supressing the production of reactive oxidative species (ROS) produced by macrophages, which play an important role in limiting bacterial replication[112]; iii) dampening the DNA damage response (DDR) during infection, through pore-formation induced degradation of the sensor Mre1. It was shown that *Listeria* induces host DNA brakes, and that dampening the DDR promotes bacterial replication[113].

In addition to the abovementioned effects, LLO, which is expressed both by extracellular and intracellular *Listeria,* was found to modulate the host processes also prior to bacterial infection[56,72,73]. Several studies have concluded that LLO, secreted by extracellular *Listeria*, interacts with the host before bacterial invasion. It perforates the host cell plasma membrane causing a rapid influx of extracellular $Ca^{2+}$, which is a universal secondary messenger that regulates a large array of cellular processes, one of which is counteracting bacterial infection[106,114]. Simultaneously, LLO causes a $K^+$ efflux, a secondary messenger for cellular effects like autophagy, protein phosphorylation, and transcriptional regulation[54]. This ionic imbalance caused by LLO produced extracellularly was shown to affect multiple host cell processes (**Fig. 1.8**):

i) Activate the inflammasome via signalling pathways like NFκB[109] and MAPK[54,115,116].

ii) Activate the unfolded protein response (UPR) in an LLO-dependent manner[37]. The mechanism is however not known, but a possible cause could be perturbation of the endoplasmic reticulum (ER) membrane by LLO and activation of signal transducer proteins PERK, ATF6, and IRE1[117].

iii) Histone modifications, stimulated by a $K^+$ efflux caused by extracellular LLO. This efflux was shown to trigger dephosphorylation of Ser10 on histone H3 and deacetylation of histone H4, causing an epigenetic silencing of genes in the affected region, with several genes involved in immunity downregulated[56,94,118].

iv) Dysregulation of the SUMOylation machinery, induced by a proteasome-independent degradation of Ubc9. This SUMOylation inhibition of key regulatory proteins and dampened the host response to infection[119].

v) Induction of bacterial entry, by activating the endocytic machinery[120].

vi) Activation of AMP-activated protein kinase (AMPK) and protein kinase receptor (PKR) activation, that cause early induction of autophagy[121].

vii) Transient mitochondrial fragmentation, induced by $Ca^{2+}$ influx mediated by LLO. This causes a temporary mitochondrial dysregulation, causing a decrease in cellular ATP[122] and AMPK activation[35].

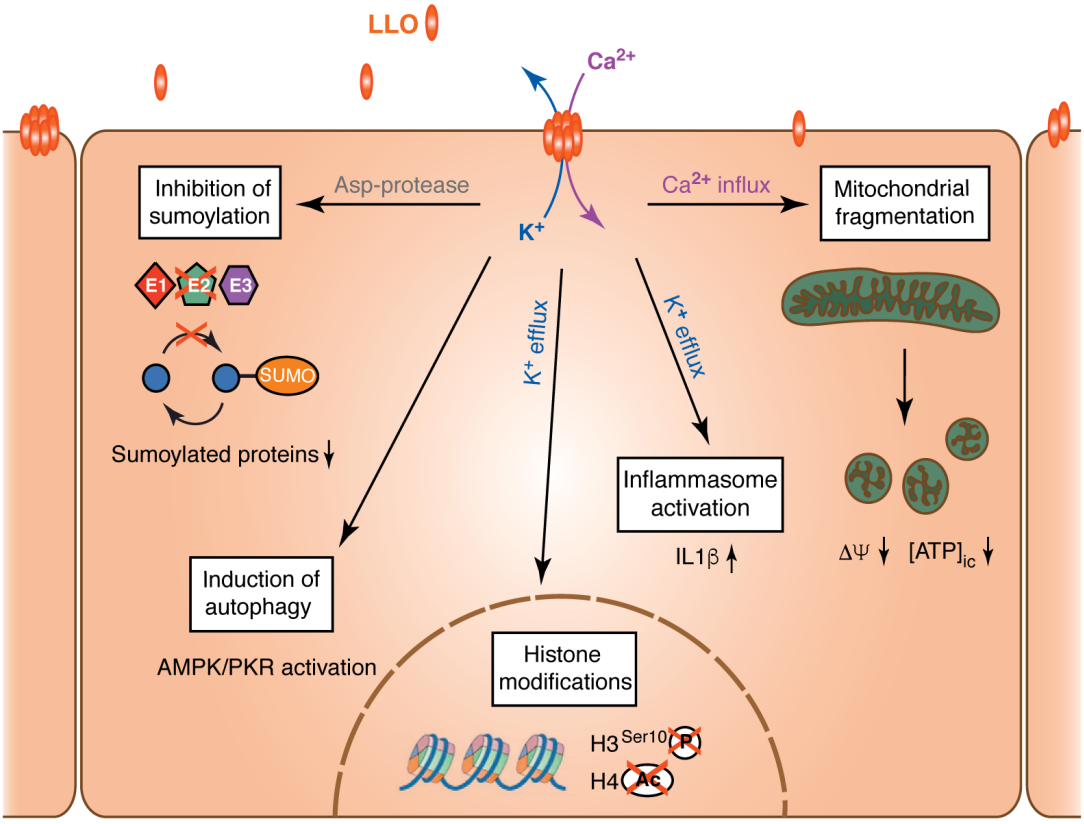


*Figure 1.8:* ***Host cell responses to extracellular LLO****.*

*Host cell responses to extracellular listeriolysin O (LLO). Downstream host cell effects induced by exposure to extracellular LLO. Pore formation in the plasma membrane causes calcium influx and potassium efflux, which induce mitochondrial fragmentation, specific histone modifications, inhibition of sumoylation, induction of autophagy and inflammasome activation. (Figure adapted from Hamon et al., 2012[72]).*

## 1.3 Non-coding RNAs

Interest in the field of non-coding RNA (ncRNA) has been largely driven by the finding that about 75 % of the human genome is at some level transcribed[123,124]. The Encyclopedia of DNA Elements (ENCODE) project, in which 32 institutions performed sequencing studies and computational analyses on 147 cell types, even claims that they were able to assign biochemical function to 80 % of the genome[125]. However, the scientific community is far from united on this matter, with many saying the term "functional" is misleading[126–128]. Reasons for these concerns are that the transcription machinery sometimes indeed produces spurious RNAs with no function[129] or they are generated as a result of transcriptional interference, where non-coding loci with overlapping regulatory regions are transcribed[130]. Another concern is that many ncRNAs are present in the cell at much lower levels when compared to mRNAs, which could mean that they are indeed not functional or are expressed only upon specific stimuli[126]. If the production of these RNAs imposes minimal fitness cost for the cell, it would be a reasonable assumption that instead of evolving a mechanism that would prevent them from being produced, the cell would simply tolerate them[131].

Despite these concerns, the fact that the majority of the human genome is transcribed, but only a small fraction of it codes for proteins, raises an important question: why would cells retain and continuously produce numerous non-functional RNAs? RNAs can in fact interact with numerous other biological molecules; they can regulate transcription of specific genes by base-pairing with DNA[132], base-pair with target mRNAs and direct their splicing, processing, translation and turnover[133], and form complex ribonucleoprotein structures such as ribosomes[134]. With this in mind, the production of these many different ncRNAs by accident could almost certainly interfere with at least some cellular processes. Furthermore, there is concrete evidence that some of these ncRNAs indeed play important roles in the cell, where they are mainly involved in the process of gene expression at the level of transcription, RNA processing and, as more recently demonstrated, translation[132,135,136]. A sensible way of approaching this vast new field of ncRNA research is to apply the null hypothesis, where one would need to disprove that there is no significant difference between two measured phenomena. Therefore, ncRNAs should be studied on a case by case basis and considered as truly functional only if they display significant non-random behaviour under certain conditions[126].

A non-coding RNA (ncRNA) is simply defined as lacking coding potential, but this vague definition consequently includes multiple types of RNA with very different functions and as such cannot be considered as a single group. Biologically functional ncRNAs, with no coding functions, have been known since the 1950s with the discovery of the highly abundant ribosomal RNA (rRNA) and transfer RNA (tRNA), but recent advances in sequencing enabled us to monitor low abundant ncRNAs at a genome wide level. ncRNAs specific sequences and natural structures allow them to employ RNA-RNA, RNA-DNA and RNA-protein interactions, making them the perfect candidates for gene expression regulation within the cell[137].

The "Central Dogma of Molecular Biology" describes the flow of genetic information within a biological system. It states that genetic information encoded in DNA is transcribed to messenger RNA (mRNA) by RNA polymerases, and mRNA is then translated to proteins by ribosomes. This model was first proposed by Francis Crick in 1958, describing only protein coding genes and regarding most of the remaining genome as "junk DNA". Since then, advances in sequencing revealed that in fact the vast majority of the genome is transcribed[123,138], mostly as non-coding RNA (ncRNA) and less than 2% is subsequently translated[124,139].

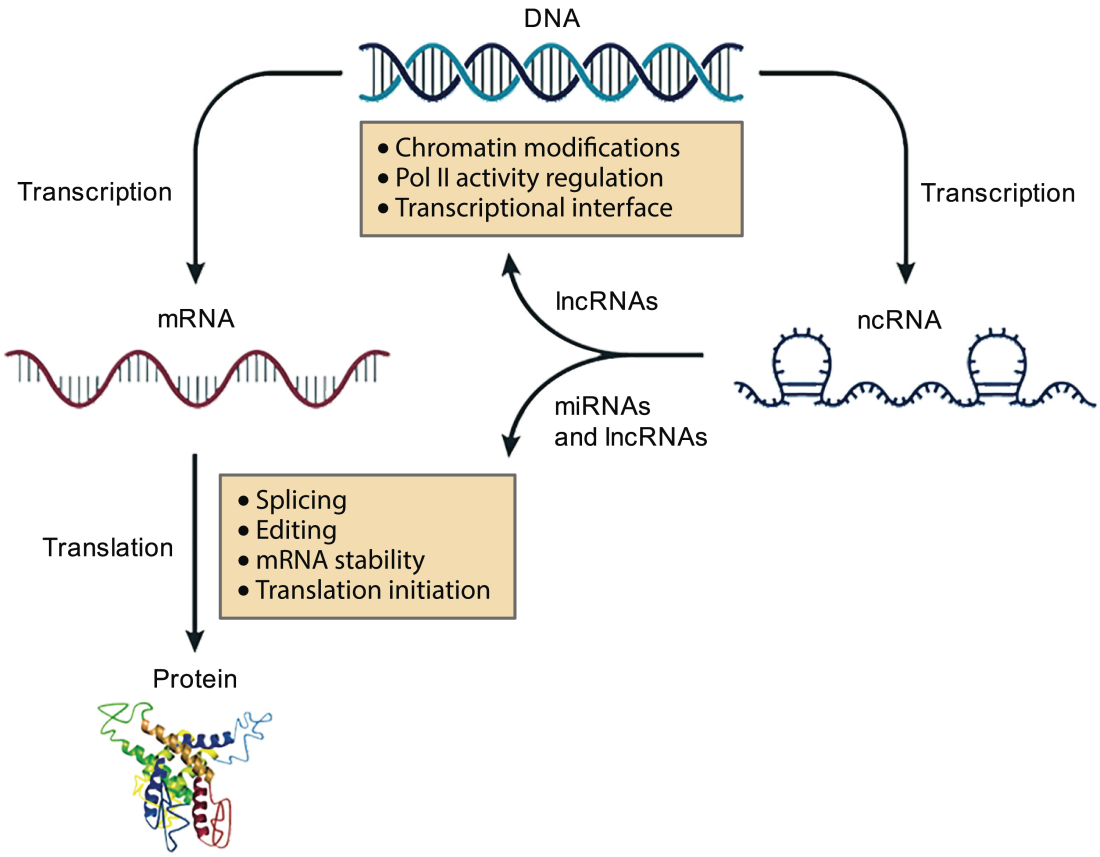


*Figure 1.9:* ***The "Central Dogma of Molecular Biology" in the context of regulatory non-coding RNAs***

*Non-coding RNAs such as lncRNAs and miRNAs have recently gained recognition in gene expression regulation at both transcriptional and translational level, therefore earning their position in the concept of the "central dogma". (Figure adapted from Wahlestedt, 2013[140]).*

At present, we know that some ncRNAs, such as micro RNA (miRNAs) and long non-coding RNAs (lncRNAs), indeed play important regulatory roles in the cell; however, the distinction between functional and non-functional RNA appears to be quite vague (**Fig. 1.9**). Therefore, a modified version of the Central Dogma was proposed, including ncRNAs as gene expression regulators[140,141] and as translation regulators in particular[135].

### 1.3.1 Long non-coding RNA

Long non-coding RNAs are found in every branch of life and are predicted, at least in humans, to be vastly more abundant than protein-coding genes[123]. They are considered to be primarily involved in gene expression regulation, mainly at a transcriptional level[132,142];

however, there is an ever-growing amount of evidence for their involvement in the regulation of post-transcriptional processes such as translation regulation[135,143]. Despite multiple research done in recent years, the function of most lncRNAs is still unknown and their mechanism of action remains elusive.

lncRNAs are a sub-group of non-coding RNAs, loosely defined as transcripts that are longer than 200 nt with no apparent protein coding potential[144]. Even if a significant fraction of them are 5'-capped and poly-adenylated[144–146] similarly to mRNAs, lncRNAs share common characteristics that distinguish them from *bona fide* protein coding mRNAs:

i) Lack of a single long (> 300 nt) open reading frame (ORF). In fact, lncRNAs have multiple small ORFs[147,148] and possess low protein coding capability [149,150,146];
ii) Low expression levels, compared to mRNAs[151,152];
iii) Longer but fewer exons than protein-coding genes, with a bias toward two-exon transcripts[153];
iv) exons with a significantly lower GC content, compared to protein-coding RNAs[150];
v) Paucity or absence of introns[150];
vi) Enrichment in nuclear localization, with 17 % occupying the chromatin fraction and only 4 % are enriched in the cytoplasm; compared to mRNAs, where 15 % are enriched in the nucleus and 26 % in the cytoplasm[153]. However, this distribution is likely biased towards high level in the nucleus due to still scarce information in the cytoplasm;
vii) high degree of tissue specificity, compared to protein-coding genes[151,153];
viii) Co-expression with neighbouring genes[151];
ix) Low evolutionary conservation. Despite the alteration is their sequences, it appears that lncRNA do possess a conserved RNA structure[154];
x) Promoter regions under strong purifying selective and are comparable to protein-coding genes[153,145];

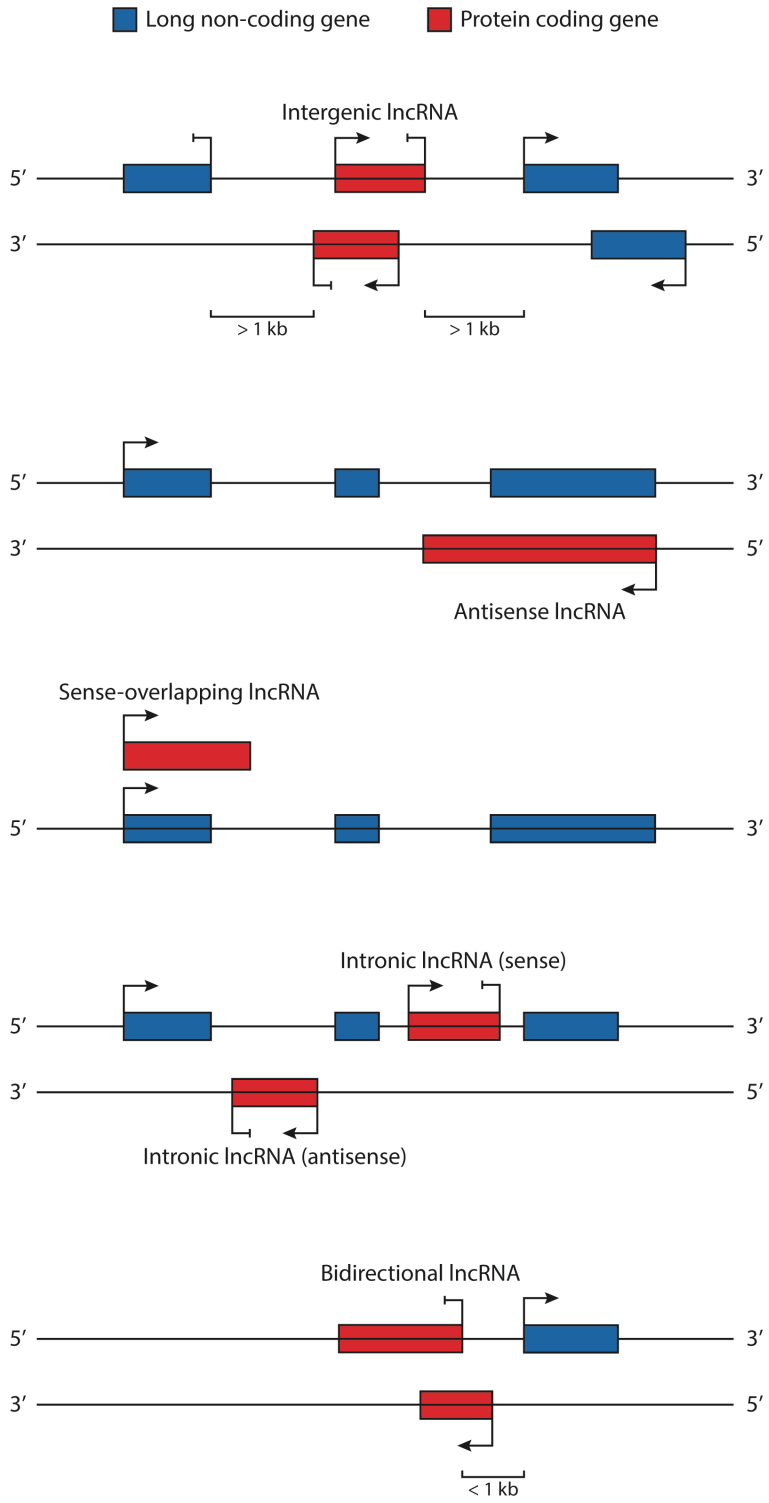


*Figure 1.10:* ***Classes of long non-coding RNAs base on their genomic location***

*The most general classification of lncRNAs (red) is based on their genomic location, specifically in relation to proximity to known protein coding genes (blue). This classification includes five distinct classes: intergenic, antisense, sense-overlapping, intronic and bidirectional lncRNAs. (Figure adapted from Arraystar*[155]*).*

Since the category of lncRNAs is so loosely defined and contains transcripts of varying sizes and distribution along the genome, the main classification used to describe lncRNAs is based on their genomic position[149,156] (**Fig. 1.10**):

i) Intergenic lncRNAs (lincRNAs) are lncRNA that do not overlap with any part of a protein coding gene and are at least 1 kb distant from it. This class is also considered the most likely to contain RNAs with the lowest coding potential.

ii) Antisense lncRNAs, which are transcribed from the antisense strand of a protein coding gene and in part overlap with at least one of their exons.

iii) Sense-overlapping or transcribed pseudogene lncRNAs that are considered transcript variants of protein coding mRNAs, since they overlap with a protein coding gene on the same genomic strand.

iv) Intronic lncRNAs, located in the introns of protein coding genes without overlapping with their exons.

v) Bidirectional lncRNAs, which are transcribed oriented head to head with a protein coding gene within 1 kb. They usually exhibit similar expression pattern as its protein coding counterpart.

The classification based on lncRNAs genomic position was useful in the past when the field was in its infancy; however, along the years we discovered multiple functional lncRNAs. Furthermore, the most recent efforts predict functionality of 69 % of known lncRNAs[157], therefore another possible classification of lncRNAs is based on their putative function or molecular mechanism used to exert that function:

i) Modulators of mRNA transcription and translation, by base pairing with other RNA molecules. lncRNAs like the antisense transcript for β-secretase 1 (BACE1-AS), protects the BACE1 mRNA from miRNA-mediated degradation by binding to a specific region and masking the miR-485-5p binding site[158]. Other lncRNAs function by first recognizing their target and then recruiting regulatory proteins to control expression. An example of such is ½-sbsRNA1 which base pairs with the mRNA of plasminogen activator inhibitor type 1 (SERPINE1) and FLJ21870 via an Alu element. The ½-sbsRNA1 then seems to facilitate the binding of STAU1 to the mRNA, which can be degraded via STAU1-mediated mRNA decay[159]. Moreover, knocking down the lncRNA was shown to increase the levels of SERPINE1 and FLJ21870. Similarly, the terminal differentiation-induced ncRNA

(TINCR) binds to a mRNA via a 25 nt long "TINCR box". TINCR also has a strong affinity for STAU1 protein and forms a complex which is able to mediate the stabilization of differentiation-related mRNAs[160].

ii) Competing endogenous RNAs (ceRNAs), also called miRNA "sponges", which participate in a microRNA-dependent cross talk. These lncRNAs share miRNA response elements (MREs) with some mRNAs, thereby sequestering miRNAs. This prevents miRNAs from binding to their target mRNAs, resulting in a higher concentration of protein-coding mRNAs[161,162]. The best example of such ceRNAs is the cerebellar degeneration-related protein 1 antisense transcript (CDR1as), a circular RNA with more than 70 miR-7 target sites[163]. Other examples of long non-coding ceRNAs are HULC and linc-MD1. HULC is able to sequester miR-372, regulating the expression of the kinase cAMP-activated catalytic subunit (PRKACB), involved in the phosphorylation of a transcription factor CREB[164]. linc-MD1 can actually sequester two miRNAs, miR-133 and miR-135, which control the expression of mastermind-like-1 (MAML1) and myocyte-specific enhancer factor 2C (MEF2C), respectively. These two proteins are important for activation of muscle-specific genes[165].

iii) Protein "sponges", which bind regulatory proteins, disabling them from interacting with their potential targets. This behaviour has been reported for a number of lncRNAs. An example of such is Gadd7, which is induced upon DNA damage and growth arrest[166]. directly interacts with TDP-43, decreasing the interaction between Cdk6 and TDP-43, which results in Cdk6 degradation and the inhibition of the cell cycle progession[167].

iv) Scaffolding lncRNAs. Probably the most studied lncRNA using this mechanism is the X-inactive specific transcript (Xist), which orchestrates X chromosome inactivation. The processed Xist transcript covers the future inactivated X chromosome. Xist acts as a scaffold for multiple chromatin remodelling complexes, including the Polycomb Repressive Complex 2 (PRC2) by Xist, finally silencing the Xist covered chromosom[168,169]. Scaffolding lncRNAs are essential in forming chromatin structures that modulate the topological organization of chromosomes[170] as well as other nuclear structures, such as paraspeckle[171].

v) SINEUPs, antisense lncRNAs that stimulate translation of target sense mRNAs through the activity of an embedded SINEB2 element, such is the antisense to Uchl1 (AS Uchl1), which was shown to trigger a cap-independent translation of the Uchl1 mRNA[172].

vi) Stress-induced lncRNAs (silncRNAs), such as the antisense CDC28 which is suggested to permit a faster recovery of the cell cycle delay caused by stress[173].

vii) Regulators of post-translational modification of proteins, such as ubiquitination and phosphorylation. NF-κB interacting lncRNA (NKILA) was shown to bind directly to IκB, inhibiting IKK-induced phosphorylation of IκB. NKILA seems to be therefore important for keeping the NF-κB pathway from over-activation[174]. Another protein modifying lncRNA is lincRNA-p21, reported to regulate the ubiquitination of a transcription factor HIF-1α. Under hypoxia condition, linc-p21 binds to HIF-1α and the tumour suppressor VHL, which blocks the VHL-mediated ubiquitination of HIF-1α[175].

Protein-coding genes and lncRNAs share some of the same features, such as polyadenylation and 5' capping[144–146], therefore it is difficult to determine which transcripts actually code for proteins and which are in fact non-coding, yet still functional. One approach is to use computational methods that can predict RNAs coding potential based on certain *bona fide* protein coding features (**Fig. 1.11**):

i) Prediction of ORFs. Coding regions tend to be much longer than expected by chance. The presence of an ORF with at least 300 nucleotides is commonly used to define a transcript as coding[147]. The ORF length alone is not enough to classify an RNA as non-coding, especially since well-known lncRNAs like Xist, Hotair, Meg3, H19 and Kcnqot1 all have putative ORFs longer than 300 nt[147].

ii) Non-random codon usage within ORFs. Amino acids are encoded by more than one codon and within coding regions we can find a greater usage of one codon over the other than expected by chance. The main hypothesis as to why this bias occurs is that cells preferably use codons that correspond to the greatest number of tRNAs available, which increases the efficiency of translation of highly expressed genes[176].

iii) Evolutionary conservation of coding sequences between different species. Recent report show that lncRNAs are indeed evolutionary conserved, just not to the same extent as many protein coding genes[145,177]. But this lack of conservation does not imply a lack of functionality, especially if the role of most lncRNAs is to facilitate an interaction with proteins or other RNAs an exact sequence would not be necessary[154].

iv) presence of known protein domains (e.g. the Pfam database). Since proteins are generally composed of one or more functional regions, the identification of these can provide insight into their function[178].

v) Using other known protein coding genes in the database to find similarities with a given transcript or a genomic sequence[179,180].

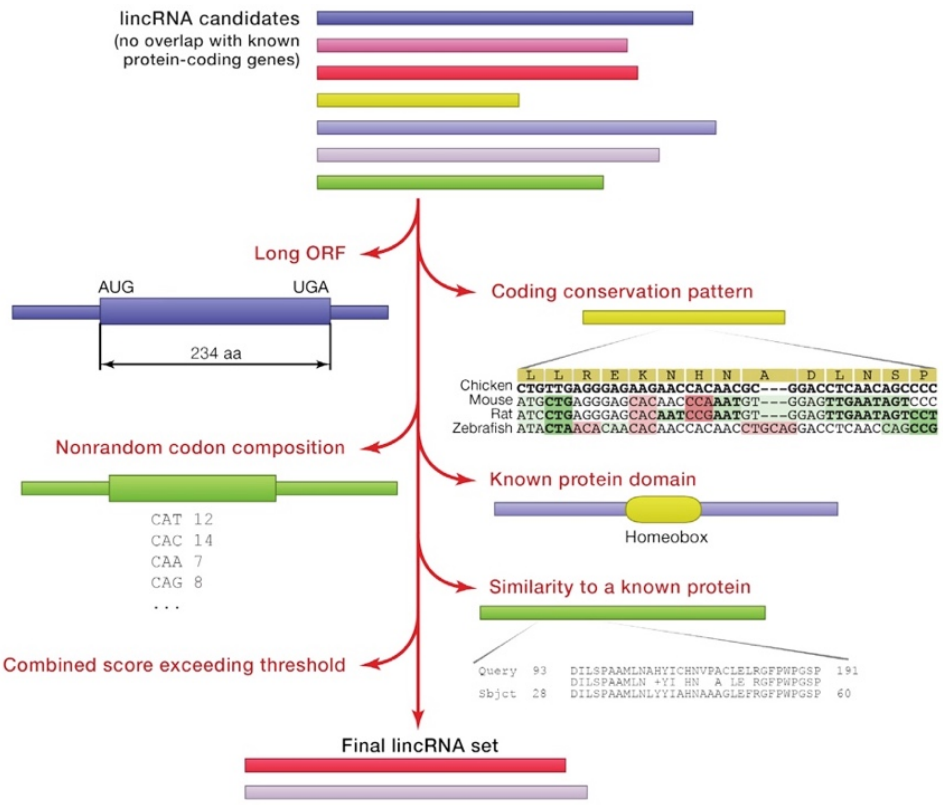


*Figure 1.11:* ***lncRNA annotation pipeline***

*Computational steps in lincRNA annotation, using criteria to filter potential mRNAs from the list of candidates. (Figure adapted from Ulitsky, 2013[131]).*

A combination of these criteria can produce more reliable annotations; however, in order to prove this beyond doubt, lncRNAs also need to be experimentally tested. This includes experimental testing, such as:

i) *In vitro* transcription translation systems, in order to definitively determine whether lncRNAs can produce peptides *in vitro*[181].

ii) Polysome profiling to determine whether lncRNAs associate with translationally active ribosomes[6]

iii) Ribosome profiling, which provides positional information of translating ribosomes on mRNA transcripts[3,4].

#### 1.3.1.1 lncRNAs: Translation regulation

Surprisingly, lncRNAs have been recently found to be associated with ribosomes[4,182] and polysomes[5,6], shown by ribosome profiling and polysomal profiling studies, respectively. As to what function they have is still a matter of debate. Ribosome profiling experiments demonstrated that they are in fact engaged by ribosomes as mRNAs are[4], raising questions about their classification as non-coding. In fact, they might produce short peptides[183], even though clear demonstrations of this ability has been hampered by experimental difficulties. Furthermore, they were shown to be associated with polysomal fractions, mainly containing one, two or three ribosomes[5,6]. One possible explanation is that these polysome-associated RNAs with short ORFs, currently annotated as non-coding are in fact coding for short peptides. Such an example was shown in the case of the polished rice (pri) transcript in *Drosophila* which was initially considered a lncRNA, but was demonstrated that it actually encodes four similar peptides, 11 to 32 amino acids in length, that play a role in embryogenesis[184]. Other explanations are indeed possible, such that they might act as scaffolds, associating with polysomes and serving a regulatory role rather than a coding one.

lncRNAs typically contain short ORFs (sORFs) and mRNAs have a similar feature in their 5' untranslated region (5'UTR), called upstream ORFs (uORFs), that inhibit expression of the main downstream ORF and are therefore able to regulate the translation of an mRNA and influence its stability[185]. Furthermore, uORFs are usually evolutionary less conserved[186], similarly to what has been observed with lncRNAs. With that in mind, the short ORFs in

lncRNAs might therefor act as uORFs, preventing the ribosome from reaching downstream regions and thereby protect binding factors from displacement by scanning ribosomes[131]. Other explanations could be that sORFs could function as tethers, bringing ribosomes and functional factors in close proximity, or modulate their stability by influencing RNA decay pathways[131]. lncRNAs can also interact with the ribosome by promoting or repressing translation of specific mRNAs.

Examples of known functional lncRNAs involved in translation regulation are presented in **Table 1.2.** Probably one of the most interesting lncRNAs that interacts with the ribosome is AS Uchl1[172]. The inhibition of mTORC1 triggers the transport of AS Uchl1 from the nucleus to the cytoplasm. This antisense lncRNA interacts with an embedded inverted SINEB2 element and a 73-nucleotide sequence on the 5'UTR of the Uchl1 mRNA to trigger its cap-independent translation. The precise mechanism underlying this cap-independent translation under stress condition still remains unknown. Other cytoplasmic lncRNAs, such as lincRNA-p21, known to negatively regulate CTNNB1 and JUNB translation[187], are able to depress the protein synthesis of specific transcripts. When the level of the RNA binding protein HuR drops, lincRNA-p21 forms a complex with both CTNNB1 and JUNB mRNAs by base pairing. This complex enhances the interaction with the protein FBRP and the translational regulator RCK, repressing translation through reduced polysome size and ribosome drop-off. Apart from regulating the translational efficiency of specific transcripts, lncRNAs such as BC1, found in Xenopus oocytes, represses general translation by targeting protein factors needed for effective initiation[188,189]. BC1 inhibits the assembly of the translation initiation complex by interacting with PABP and eIF4A, disrupting their mutual interaction. Another example is ZFAS1 lncRNA, found to be mainly associated with the 40S ribosomal subunit and with light polysomes. ZFAS1 indirectly regulates translation by controlling the phosphorylation state of RPS6 by a still unclear mechanism[190].

*Table 1.2:* ***Functions and mechanism of cytoplasmic lncRNAs involved in translation regulation***

| lncRNA | Organism | Function | Mechanism | Reference |
|---|---|---|---|---|
| AS Uchl1 | Mouse | Triggers cap-independent translation | Base-pairing between SINEB2 element of AS Uchl1and a 73-nucleotide sequence on the 5'UTR of Uchl1 mRNA | 172 |
| lincRNA-p21 | Human | Translation repression of CTNNB1 and JUNB | Base-pairing and forming a complex with CTNNB1 and JUNB mRNA and enhancing the interaction with FBRP and RCK | 187 |
| ZFAS1 | Human | Translation regulation | Proposed to modulate the abundance and phosphorylation of RPS6 | 190 |
| BC1 | *X. laevis* | Inhibits the assembly of the initiation complex | disrupting the interaction between PABP and eIF4A | 188,189 |
| tts-1 | *C. elegans* | Lifespan extension | unknown | 191 |

### 1.3.1.2 lncRNAs: Host-pathogen interaction

One way organisms can compete for survival is by exploiting their genetic code to produce molecules that can pivot the odds in their favour and RNA is a very good candidate, considering it can form complex structures and form specific interactions with DNA, proteins and other RNAs[137].

It is fair to say that when it comes to the involvement of ncRNAs in infection biology, the class of lncRNAs has taken the back seat to other classes. Most studies on host–pathogen crosstalk are focused on small ncRNAs, specifically miRNAs[192,193]. Studies that do address

lncRNAs are mainly restricted to their involvement in viral infection[194–196]. An example of a well-studied lncRNA expressed as a respond to viral infection is the lncRNA NEAT1. This particular lncRNA was the first one identified for its involvement in HIV-1 replication. Upon HIV-1 infection NEAT1 is upregulated and is able to decrease the nucleus-to-cytoplasm export of Rev-dependent instability element containing HIV-1 transcripts, thereby inhibiting virus production[197]. NEAT1 also indirectly regulates inflammation by increases the production of interleukin 8 (IL8) by sequestering the IL8-inhibitor SFPQ[198]. On the other hand, HIV-1 infection also causes a significant reduction of another lncRNA called NRON[199]. NRON is a repressor of NFAT, a transcription factor known to enhances HIV-1 gene expression in primary CD4 T cells[200]. This in term enhances HIV-1 replication because of an increased activity of NFAT[199].

Research shows that bacterial pathogens interfere with expression of host non-coding RNAs in order to modulate its response to infection[201–203]; however, the role of lncRNAs in bacterial infection is currently still very limited to only a handful of examples despite their undeniable biological functions and involvement in the host-pathogen crosstalk[204]. Studies are now shedding light on the possible roles of lncRNA expressed upon bacterial infection or induction with through Toll-like receptor ligands. Research shows that upon this kind of stimulation the large portion of lncRNAs expressed were closely located to expressed immune and inflammatory genes[205–207]. It is known that lncRNAs can regulate transcription of neighbouring protein coding genes[208] and this suggests that a significant amount of lncRNAs expressed upon bacterial infection is responsible for the regulation of the stress response proteins encoded in their genomic proximity. Following an infection, several lncRNAs were demonstrated to control the state of chromatin to control the gene expression relevant for infection. Most of which we know interact with the Polycomb Repressive Complex 2 (PRC2), repressing gene expression in that region by lysine trimethylation at position 27 of the Histone 3 protein (H3K27me3)[209]. Other lncRNA may interfere with NFκB signalling in order to modulate the innate immune response[210].These strategies can be exploited by either the pathogen, in order to promote its survival, or by the host organism, to fend of the pathogen.[204]

To date the function and mechanism of most lncRNAs expressed upon pathogen invasion remains largely unknown[204]; however, lncRNAs are now gaining recognition for their involvement in host gene expression regulation upon bacterial invasion (**Table 1.3)**. *Listeria*

*monocytogenes* is widely used in host-pathogen interaction studies and was used to determine the function of some stress-induced lncRNAs:

i) lincRNA-Cox2 expression was shown upon multiple stimuli. It was first reported in TLR4-stimulated mouse bone-marrow-derived dendritic cells and was shown to be induced directly via NFκB[145]. It was later demonstrated to be induced in intestinal epithelial cells exposed to TNF-α[211] and before also in mouse macrophages stimulated with TLR4 and TLR7/8 as well as upon *Listeria monocytogenes* infection[212]. lincRNA-Cox2 was shown to directly interact with multiple heterogeneous nuclear ribonucleoproteins (hnRNPs), but no direct target was identified. However, research shows that this lncRNA can induce the expression of immune related genes and probably even repress some pro-inflammatory cytokines like IL-12 via the Mi-2/nucleosome remodelling and deacetylase repressor complex (Mi-2/NuRD)[211].

ii) lincRNA-EPS is transcribed in mice bone-marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) and downregulated upon stimulation with TLR ligands. A similar repression was observed with cells infected with the Sendai virus or *L. monocytogenes*. It was demonstrated that lincRNA-EPS interacts with hnRNP-L which then localizes at regulatory regions of immune response genes, repressing their transcription[213].

iii) AS-IL1α was shown to be induced in mouse macrophages stimulated with a range of TLR ligands, LPS, as well as upon *L. monocytogenes* infection. AS-IL1α is expressed in a similar pattern as its partially overlapping protein coding gene for the cytokine IL1α. It has been demonstrated that it recruits the RNA polymerase ll (RNAPll) to the promoter of IL1α, enhancing its expression[214].

*Table 1.3:* ***Functions and mechanisms of lncRNAs expressed upon bacterial invasion or stimulation with bacterial molecular components***

| lncRNA | Organism | Pathogen/bacterial component | Function | Mechanism | Ref. |
|---|---|---|---|---|---|
| lincRNA-Cox2 | mouse macrophages, mouse bone-marrow-derived dendritic cells, epithelial cells | *L. monocytogenes,* TNF-α, TLR2-, TLR4-, TLR7/8-ligands | Induces expression of immune related genes, such as the cytokine IL-12 | Direct interaction with multiple heterogeneous nuclear ribonucleoproteins (hnRNPs) | 145,211,212 |
| lincRNA-EPS | Mouse bone-marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) | *L. monocytogenes,* Sendai virus, TLR2-, TLR3-, TLR4-ligands | Transcriptional repression of immune response genes | Interaction with hnRNP-L | 213 |
| AS-IL1α | Mouse macrophages | *L. monocytogenes,* LPS, TLR ligands | Enhances IL1α expression | Recruitment of RNA polymerase ll (RNAPll) | 214 |
| lincRNA-Tnfaip3 | Mouse macrophages | LPS | Transactivates and represses of a wide range of inflammatory genes | Scaffold for the assembly of a complex with the transcription factor NFκB and the chromatin protein Hmgb | 210 |
| lincRNA-IL7R | Human macrophages (THP-1) and peripheral blood mononuclear cells (PBMC) | LPS, TLR2-, TLR4-ligands | Reduces expression of proinflammatory mediators | Indirect chromatin remodelling by H3K27 trimethylation | 215 |
| THRIL | Human macrophages (THP-1) | TLR2-ligands | Stimulates TNF-α expression | Forming a complex with hnRNP-L and binding to TNF-α promoter | 216 |

| | | | | | |
|---|---|---|---|---|---|
| HOTAIR | Mouse cardiomyocytes | LPS | Promotes TNF-α production | NF-κB activation through NF-κB p65 subunit phosphorylation | [217] |
| NeST | Mice | *S. enterica* and Theiler virus | INF-γ accumulation | Binding with WDR5, a component of the histone H3K4 methyltransferase complex and trimethylation of the H3K4 histone | [218] |
| lincRNA-CD244 | Human and mouse CD8+ T cells | *M. tuberculosis* | Inhibits the expression of cytokines TNF-α and INF-γ | Interacting with the polycomb protein enhancer of zeste homolog 2 (EZH2) | [219] |
| MEG3 | Human monocytes (THP-1) | *M. bovis* BCG | Autpohagy | unknown | [220] |

At the moment, the field of lncRNAs, their functions and mechanisms remain elusive. However, the involvement of lncRNAs in gene expression regulation in undeniable and evidence points to lncRNAs playing an essential role in the host-pathogen crosstalk, aiding either the host to fend-off invading pathogens or the pathogen to avoid the host's immune system and proliferate. The number of studies demonstrating functional infection-induced host lncRNAs is currently restricted to only a handful of examples, none of which address their involvement in host translation regulation during bacterial infection. Further insight in this field will give us a better understanding of the host-pathogen crosstalk and may even yield novel ways to treat infections.

# 2 Materials and methods

## 2.1 Materials

**Cell culture:**

For the purposes of this research, human epithelial colorectal adenocarcinoma cells (Caco-2) were used, a widely used paradigm in host-pathogen interaction studies[113,221,222]. The cells were a kind gift from Prof. Gregor Anderluh from the Laboratory for Molecular Biology and Nanobiotechnology (National Institute of Chemistry, Slovenia). The cells were maintained in culture at 37 °C and 5 % $CO_2$ and in Cell Medium (see below). If not specified differently, cells were grown till reaching 80 % confluency before use. The media and buffers used for cell culture and treatments are the following:

- Cell Medium: Minimum Essential Medium Eagle (MEME) (Lonza) supplemented with 10 % (v/v) Fetal Bovine Serum (FBS) and 2 mM L-glutamine
- Cell Medium supplemented with antibiotics: Cell Medium, 10 µg/mL Gentamicin, 100 µg/mL Streptomycin, 100 U/mL Penicillin
- Phosphate-Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4

**Polysome profiling:**

- Polysomal cell lysis solution: 10 mM Tris pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 1 % (v/v) Triton X-100, 1 % (w/v) Sodium Deoxycholate, 5 U/ml DNase I, 200 U/ml RiboLock RNase Inhibitor, 1 mM DTT, 0.01 mg/ml cycloheximide.
- Polysome Buffer: 10 mM Tris pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$
- 15 % (w/v) sucrose solution in Polysome Buffer
- 50 % (w/v) sucrose solution in Polysome Buffer
- All buffers for polysome profiling were prepared in $H_2O$ treated with Diethylpirocarbonate (DEPC), which is an inhibitor of RNases.

**Protein extraction:**

- RIPA buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 1 % (v/v) Igepal CA-630, 1 mM EDTA, 0.5 % (w/v) Sodium Deoxycholate, supplementaed with protease inhibitors (1

mg/ml Pepstatin (Sigma Aldrich); Cocktail Inhibitor Phosphatase (cautharidin, bromotetramisde, mycrocystin LR in DMSO); Cocktail Inhibitor Phosphatase (Sodium Orthovadanate, Sodium Molybdate, Sodium Tertrade, imidazide in water) (Sigma Aldrich); Cocktail Inhibitor Protease (AEBSF, aprotinin, bestatin, E64, EDTA, leupeptin, in DMSO) (Sigma Aldrich)).

**SDS-PAGE and Western Blotting:**

- Running buffer: 25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS
- Transfer buffer: 25 mM Tris, 192 mM glycine
- Tris-Buffered Saline (TBS): 50 mM Tris, 150 mM NaCl, pH 7.5
- TBS Tween-20 (TBS-T): TBS, 0.1 % (v/v) Tween-20
- Blocking solution: TBS-T, 3 % non-fat milk

### 2.1.1 Bacterial strains

*Listeria monocytogenes:*

The pathogen used throughout this thesis to study the host-pathogen crosstalk was *Listeria monocytogenes* 10403S strain. For the purposes of my research I used a WT strain and an LLO-deficient (ΔLLO) strain with the *hly* gene deletion (Δ*hly*). Both strains (WT and ΔLLO) were transfected with p1522 plasmid carrying the resistance to tetracycline and constitutively expressing eGFP. Bacteria were grown at 37 °C with agitation in Luria Broth (LB) (Sigma) supplemented with 10 μg/ml of tetracycline. Both bacterial strains were a kind gift from Dr. Miha Mikelj from the Biotechnical Faculty's Department of Biology (University of Ljubljana, Slovenia).

*Salmonella enterica:*

The *Salmonella typhimurium* strain MC1 was transfected with a pEGFP-C1 plasmid, carrying the resistance to kanamycin and expressing either a wild-type typhoid toxin (TT) or a toxin carrying a deletion of the *cdtB* subunit (Δ*cdtB*). Bacteria were grown at 37 °C with agitation in LB (Sigma) supplemented with 50 μg/ml of kanamycin. The bacterial strains were

provided by the laboratory managed by Dr. Teresa Frisan at the Department of Cell and Molecular Biology (Karolinska Institutet, Sweden).

## 2.2 Methods

### 2.2.1 Infection protocol

Host-pathogen interactions were studied using host Caco-2 cells and the pathogen *Listeria monocytogenes*, either a wild-type strain (WT) or an LLO-deficient strain (ΔLLO). Depending on the assay, cells were seeded on 10 cm cell-culture dishes, 6-well or 12-well plates and kept in culture until reaching around 80 % confluency. In parallel, bacteria were grown overnight in LB supplemented with 10 µg/mL tetracycline at 37 °C under agitation. The next day bacteria were pelleted via centrifugation for 5 min at 4500 g and then re-suspended in Cell Medium to obtain an optical density at 600 nm ($OD_{600}$) of about 0.2. $OD_{600}$ was measured in a 1 cm cuvette, with Jasco V-670 spectrophotometer. The bacteria were then added to cells at a multiplicity of infection (MOI) of 40 bacteria/cell and incubated for 2 h (invasion time) at 37 °C and 5 % $CO_2$, depending on the assay. After the invasion time, the medium was removed, cells were washed with PBS. To kill extracellular bacteria that were not phagocytosed, Cell Medium supplemented antibiotics was added. Finally, cells were incubated at 37 °C and 5 % $CO_2$ for 2 h or 5 h, this incubation time is defined as the time post-invasion (POI). After the time POI, cells were either prepared for fluorescence imaging or lysed and used for polysome profiling or total protein extraction.

Control cells (non-infected cells) were treated at the same time as infected cells, except instead of the addition of bacteria, only Cell Medium was added. They were then incubated for 2 h at 37 °C and 5 % $CO_2$, washed with PBS and incubated in Cell Medium supplemented with antibiotics for further 2 h at 37 °C and 5 % $CO_2$.

Infection experiments involving *Salmonella*, were performed following the very same procedure described above.

### 2.2.2 Cell treatment with Puromycin

Cells were seeded on 10 cm dishes and kept in culture until reaching around 80 % confluency and infected according to the protocol described above. Cells were then treated with 100 µg/ml of puromycin 1 h before the end of the final incubation time, by directly adding it to the cell medium, and incubated at 37 °C and 5 % $CO_2$. Control samples were treated in the same way, using the non-infected control cells. After the final incubation time the cells were lysed and used for polysome profiling as described below.

### 2.2.3 Cell treatment with medium containing *Listeria*-released molecules.

Cells were seeded on 10 cm dishes and kept in culture until reaching around 80 % confluency and infected according to the previously described protocol. After the 2-hour invasion time in cells, the Cell Medium with the added bacteria was removed from the cell culture and centrifuged at 4500 g for 10 min to remove the excess of bacteria that did not invade cells. The supernatant was removed and centrifuged again in order to completely remove bacteria and cellular debris and to obtain a medium containing *Listeria*-released molecules (SUP). The SUP medium was then added to non-infected cells cultured in parallel in 10 mm dishes and incubated for 2 h at 37 °C and 5 % $CO_2$. After that, the SUP medium was removed and a regular Cell Medium supplemented with antibiotics was added. Cells were then incubated at 37 °C and 5 % $CO_2$ for 2 h and 5 h post SUP treatment. Control samples were treated in the same way, using the medium of non-infected control cells. After the final incubation time (post SUP treatment), the cells were lysed and used for polysome profiling as described below.

### 2.2.4 Cell treatment with recombinant Listeriolysin O

Cells were seeded on 10 cm dishes and kept in culture until reaching around 80 % confluency. Cells were then treated with a recombinant listeriolysin O (LLO), by adding it directly to cells in dishes, at a final concentration of 30, 3, 0.3, 0.003, 0.0003 nM or no addition (control cells). The recombinant LLO was produced and isolated in the Laboratory for Molecular

Biology and Nanobiotechnology (National Institute of Chemistry, Slovenia) and was a kind gift of Prof. Gregor Anderluh. Cells were then incubated for 2 h at 37 °C and 5 % $CO_2$, washed with PBS and incubated in Cell Medium supplemented with antibiotics for further 2 h at 37 °C and 5 % $CO_2$. After the final incubation, the cells were lysed and used for polysome profiling.

### 2.2.5 lncRNA silencing

For each selected lncRNA three different antisense LNA GapmeRs were designed using Exiqon's dedicated algorithm. Approximately 1 x $10^6$ Caco-2 cells were seeded on 10 cm dishes or on coverslips and kept in culture for 24 h at 37 °C and 5 % $CO_2$. The cells were then transfected with a combination of the three GapmeRs targeting the same lncRNA solubized in Oligofectamine Transfection Reagent (Thermo Fisher). The GapmeR/Oligofectamine transfection mixture was prepared according to the manufacturer's instructions and cells were transfected with a final concentration of 100 nM of each GapmeR in Cell Medium and incubated for 48 h at 37 °C and 5 % $CO_2$. As a control, cells were also transfected with Mission esiRNA targeting Renilla Luciferase (Sigma) and Oligofectamine. The treated cells were then infected according to the previously described infection protocol. After the incubation time POI, the cells were either prepared for fluorescence imaging or lysed and used for polysome profiling.

### 2.2.6 Counting of intracellular bacteria

To obtain the number of intracellular bacteria per cell upon infection, I used fluorescence microscopy. Cells were seeded on cover slips in 12-well plates and infected as described above. After 2 h or 5 h POI, the medium was removed and the coverslips were washed with PBS. To stain the plasma membrane, cells were first incubated with Wheat Germ Agglutinin (WGA) conjugated to Alexa Fluor 633 at 5 µg/ml in PBS for 10 min at 37 °C and 5 % $CO_2$. Next cells were fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min, washed with PBS and then mounted using ProLong Diamond Antifade Mountant with DAPI (Life Technologies). The samples were then left to dry at room temperature for 24 h in the dark and the images were acquired with a fluorescence microscope, Leica DMLA. Samples were

imaged using a 40x objective and detected using excitation / suppression filters: 380 nm / 445 nm for observing nuclei stained with DAPI, 620 nm / 700 nm for plasma membrane stained with WGA conjugate and 480 nm / 527 nm for observing the GFP expressed by bacteria. The images were analysed using ImageJ's Cell Counter plugin. To obtain the number of bacteria per cell, GFP dots, corresponding to individual bacteria, were counted within cell boundaries, visualized by WGA staining of the plasma membrane.

### 2.2.7 Polysome profiling

Cells seeded on 10 cm dishes were first incubated with 10 µg/mL of cycloheximide at 37 °C for 3 min, in order to trap ribosomes on mRNAs. The dishes were then immediately put on ice, washed twice with ice-cold PBS supplemented with cycloheximide (10 µg/mL). The cells were then lysed with 300 µl of polysomal lysis solution, removed using a cell scraper and collected in a 1.5 mL tube. The cell lysates were vortexed, incubated for 5 min on ice and centrifuged at 20800 g for 5 min at 4 °C to remove cell debris, nuclei and mitochondria. The supernatant was then added on a linear sucrose gradient (15-50 %) and ultra-centrifuged at 180000 g for 100 min in a Beckman Optima LE-80K Ultracentrifuge with a SW 41 Ti rotor, in order to separate cellular components according to their sedimentation coefficients. Using a density gradient fractionation system (Teledyne Isco, model 160), 1 mL fractions were collected. During the fractionation, the absorbance at 254 nm was continuously measured in order to obtain a polysomal absorbance profile along the gradient (**Fig. 2.1**). From the obtained profile, I calculated the fraction of ribosomes in polysomes, calculated as the ratio between the area under the polysomal peak and the sum of the 80S peak and the polysomal area[223]. For RNA extraction of total cytoplasmic and polysomal RNAs, I combined equal parts of either all sucrose fractions (Total) or fractions corresponding to polysomes (Poly). For fraction by fraction co-sedimentation profiling proteins or RNA, I extracted the RNA from each fraction individually.

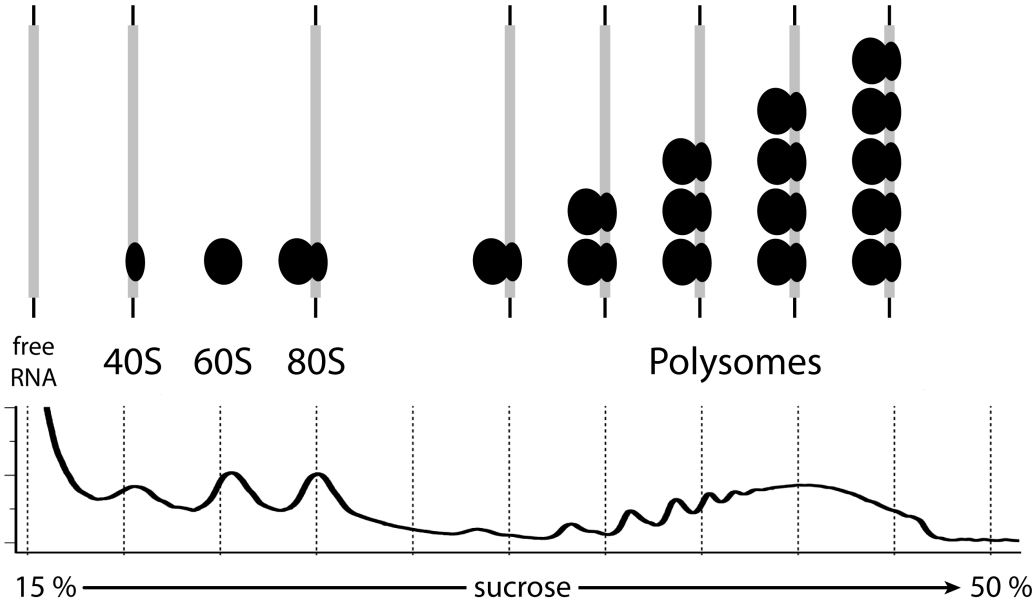


*Figure 2.1***: Example of a typical polysomal profile.**
*In the lower part of the image is shown a typical polysomal profile from a sucrose density gradient (15 – 50 %), obtained by measuring the absorbance at 254 nm. Individual peaks correspond to different parts of the translational machinery and above the profile is a representation of how these parts interact (free RNA, 40S and 60S ribosomal subunits, 80S ribosome and polysomes).*

### 2.2.8 Ribosome salt wash

The cellular lysates were obtained as described in the chapter Polysome profiling. Before the cell lysate supernatants were loaded on a sucrose gradient, a 5 M stock solution of NaCl was added to the lysates to obtain a final concentration of 500 mM NaCl and incubated on ice for 30 min. The high salt concentration is known to dissociates weakly bound molecules from ribosomes, such as translational factors[224,225]. After that the lysate was added on a sucrose gradient and processed as described in Polysome profiling.

### 2.2.9 Isolation of RNA from sucrose fractions

To analyse RNA associated to polysomes it is possible to consider: i) the total cytoplasmic RNA and RNA associated to polysomes, by pooling fractions comprised of all fractions (Total) or polysomal fractions (Poly), respectively or ii) the co-sedimentation of RNA along

the entire polysomal profile from individual sucrose fractions. In this thesis both approaches were used depending on the purpose of the assay. To extract RNA, each sample was supplemented with 1 % (w/v) SDS and 100 µg/mL of proteinase K (Euroclone) and incubated at 37 °C for 90 min to degrade proteins. RNA was then purified by adding per 1 ml of initial volume of sucrose: 250 µl/mL of Acid-phenol:Chloroform:Isoamylalcohol, pH 4.5 (Sigma Aldrich) and 250 µl/mL of 2M NaCl. The samples were mixed and centrifuged for 10 min at 4500 g and 4 °C. The aqueous phase was isolated, per 1 ml of initial volume of sucrose, 1 ml of isopropanol was added and kept at -80 °C overnight, for the RNA to precipitate. Finally, the samples were centrifuged for 45 min at 4500 g and 4 °C. The obtained RNA pellet was dried, re-suspended in nuclease-free water and the concentration of RNA was determined using a NanoDrop spectrophotometer.

### 2.2.10 Protein extraction from polysomal fractions

To monitor which proteins co-sediment with different parts of the translational machinery, it is possible to study the co-sedimentation of proteins along the sucrose gradient similarly to what performed for the abovementioned fraction by fraction analysis of RNA. Proteins were extracted from each sucrose fractions using the methanol/chloroform extraction. For each 1 ml of initial volume of sucrose, 600 µl of methanol, 150 µl chloroform and 450 µl of deionized water were added. The samples were mixed by vortexing and centrifuged at 20800 g for 1 min and the top aqueous phase was discarded. Then, for each 1 ml of initial volume of sucrose, 450 µl of methanol was added, the samples were mixed and centrifuged at 20800 g for 5 min to pellet the precipitated proteins. The protein pellets were solubilized in an appropriate volume of Electrophoresis Sample Buffer (Santa Cruz Biotechnology), typically 50 µl for each fraction, for SDS-Page and Western Blot analysis after denaturation at 99 °C for 10 min.

### 2.2.11 Total protein extraction

Cells were seeded in 6-well plated and infected according to the described protocol. After 2 h or 5 h POI, the cell medium was removed and the plates were put immediately on ice. The cells in each well were washed with PBS and then lysed with 40 µl of RIPA buffer (see

Material paragraph). The cells were collected and freeze/thawed three times in order to fully lyse cells and extract all proteins. The lysates were then centrifuged for 5 min at 20800 g, to remove cellular debris. The concentration of proteins in each lysate was then determined by using a Bradford assay and BSA standards. Proteins were then prepared for SDS-PAGE in appropriate volume of Electrophoresis Sample Buffer (Santa Cruz Biotechnology) and denatured at 99 °C for 10 min.

### 2.2.12 SDS-PAGE and Western blotting

Protein samples (15-30 µg for total protein analysis or 20 µl of protein fraction from polysomal profiles) were loaded on a 12 % polyacrylamide gel (PAG) and separated via electrophoresis in Running buffer at 80 V for 20 min and 120 V until the dye front reached the end of the gel. Proteins from the PAG were transferred to a nitrocellulose membrane in Transfer buffer at 200 mA and 4 °C for 90 min. The membrane was first passivated with Blocking solution (see Material section) at room temperature for 1 h and then incubated with the selected primary antibody (see **Table 2.1**) diluted in Blocking solution at room temperature for 1 h. Next, the membrane was washed with TBS-T (see Material section) and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (see **Table 2.1**). The chemiluminescent signal was detected using Clarity Western ECL substrate (BioRad) or Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and images were acquired with ChemiDoc MP Imaging System (BioRad). Semi-quantitative analysis of the obtained chemoluminescence images was performed with BioRad's Image Lab software.

*Table 2.1:* ***List of antibodies used for WB***

| **Antigen** | **Company** | **Product code** | **Description** |
|---|---|---|---|
| LLO | Abcam | ab43018 | Rabbit polyclonal |
| eIF2α | Santa Cruz | sc-11386 | Rabbit polyclonal |
| 4E-BP | Abcam | ab2606 | Rabbit polyclonal |
| RPS6 | Cell Signaling | 5G10 | Rabbit monoclonal |
| p-eIF2α (S51) | Abcam | ab32157 | Rabbit monoclonal |
| p-4E-BP (T46) | Abcam | ab27792 | Rabbit polyclonal |
| p-RPS6 (S240/S244) | Cell Signaling | D68F8 | Rabbit monoclonal |
| RPL26 | Abcam | ab59567 | Rabbit polyclonal |
| PABP | Santa Cruz | sc-32318 | Mouse monoclonal |
| Actin | Santa Cruz | sc-69879 | Mouse monoclonal |
| Anti-mouse HRP | Santa Cruz | sc-2004 | Goat polyclonal |
| Anti-rabbit HRP | Santa Cruz | sc-2005 | Goat polyclonal |

### 2.2.13 *De-novo* protein synthesis - Azidohomoalanine assay

Cells were seeded at 1.6 x $10^4$ cells/well in 96-well plates (CellCarrier, PerkinElmer), kept in culture for 24 h and infected according to the previously described infection protocol. For measuring *de-novo* protein synthesis, we employed the Click-iT AHA Alexa Fluor 594 Protein Synthesis Assay (Invitrogen). Cells were incubated for 30 min at 37 °C in a L-methionine-free medium supplied with 50 µM of Click-iT AHA, 10 % Fetal Bovine Serum (FBS), 2 mM L-glutamine and antibiotics (Gentamicin 10 µg/mL, Streptomycin 100 µg/mL and Penicillin 100 U/mL). Cells were then fixed with 4 % PFA in PBS for 15 min and permeabilized with PBS-Triton X-100 (0.5 %) for 20 min. After two washes with PBS supplemented with 3 % Bovine Serum Albumin (BSA), cells were incubated for 30 min at room temperature (RT) with the Click-iT Reaction cocktail. Cells were then washed once with PBS-BSA 3 % and incubated with 10 µg/ml Hoechst 33342 for 30 min at RT to stain the nuclei. After two washes with PBS-BSA 3 %, the plate was stored at 4 °C in PBS until the analysis. Plates were imaged using High Content Screening System Operetta (PerkinElmer) at the High Throughput Screening Facility (CIBIO, University of Trento). In each well, images were acquired in 8-12 preselected fields with LWD 20x objective over three channels with excitation/emission wavelengths: Hoechst, 380 nm / 445 nm; GFP, 475 nm / 525 nm; AHA Alexa Fluor, 535 nm / 615 nm. The images were analysed using the Harmony software version 3.5.2 (PerkinElmer). Based on the Hoechst dye and GFP fluorescence intensity, cell nuclei and internalized bacteria were identified, respectively. To detect *de novo* protein synthesis, the mean fluorescence intensity of AHA Alexa Fluor 594 (AHA) was quantified in the cytoplasm, from: i) infected cells that also contained the GFP signal from bacteria and ii) non-infected cells (control). The experiments were performed in 9 biological replicas and more that 1000 cells were considered for each condition. The percentage of AHA incorporation was calculated applying the formula:

$$\% \text{ AHA} = 100 \times \text{AHA}_{(\text{infected})} / \text{AHA}_{(\text{control})}$$

### 2.2.14 Library preparation and NGS data analysis

RNA extracted from Total and Polysomal sucrose fractions (see Paragraph 2.1.8) was first depleted from ribosomal RNA (rRNA), using Ribo-Zero rRNA Removal Kit (Human, Mouse,

Rat) (Illumina). cDNA libraries were then produced starting from 1 µg of Total or Polysomal RNA using the TruSeq Stranded Total RNA (Illumina), according to manufacturer's instructions. RNA-seq and POL-seq sequencing was performed with Illumina HiSeq 2000 (*Homo sapiens*, GPL11154). Barcoded libraries were pooled and sequenced in four lanes. Fastq files were checked with FastQC. Reads of 100 bp generated from each sample were aligned to the human genome (GRCh38.p3) with Tophat (version 2.0.14), using the Gencode 23 transcript annotation as transcriptome guide.

*Table 2.2:* ***Conditions and samples used for NGS***

| **Infection** | **Final incubation time (time POI for infected)** | **Total RNA / Polysomal RNA** | **Number of replicas** |
|---|---|---|---|
| Non-infected (control) | 2 h | Total | 4 |
| | | Polysomal | 4 |
| WT | 2 h | Total | 2 |
| | | Polysomal | 2 |
| | 5 h | Total | 2 |
| | | Polysomal | 2 |
| ΔLLO | 2 h | Total | 2 |
| | | Polysomal | 2 |
| | 5 h | Total | 2 |
| | | Polysomal | 2 |

All programs were used with default setting unless otherwise specified. Mapped reads (ranging from 80 % to 90 % of total reads) were subsequently assembled into transcripts guided by reference annotation (Gencode 23) with Cufflinks (version 2.2.1).

Expression levels were quantified by Cufflinks with normalized FPKM (fragments per kilobase of exon per million mapped fragments). Differentially expressed genes were detected

with CuffDiff with a double threshold on the log2 fold change (absolute value > 0.75) and the correspondent statistical significance (p-value < 0.05). Cluster analysis of DEGs was performed with the Affinity Propagation Clustering method[226], implemented in the APCluster Bioconductor package. Spearman correlation between scaled expression values was used to generate the similarity matrix for clustering. Resulting clusters were ranked according to gene size. Identification of co-expressed genes was performed with a correlation test implemented in R, using scaled expression values (one-tailed correlation test, $P<0.05$). Functional annotation of gene lists and enrichment analysis with Gene Ontology terms and KEGG pathways were performed with the topGO and clusterProfiler Bioconductor packages. Conditions and numbers of replicas used to perform NGS are listed in **Table 2.2**. All bioinformatics analyses were performed in collaboration with Dr. Toma Tebaldi (CIBIO, University of Trento).

### 2.2.15 RT-qPCR

Primers were designed using Primer3. Amplicon size of 70-150 bp was set. All the primers were tested for hairpin and primer-dimer formation with multiple primer analyzer (Life-Technology) and Oligo calc (Northwestern). Finally, the primers were checked in order to confirm their specificity for the selected genes with BLAST/BLAT from Ensembl. Primers that fit these parameters are listed in **Table 2.3** for the genes under study. Reverse transcription was performed from 1 μg of RNA from individual samples (Total RNA, Polysomal RNA or RNA from individual sucrose fractions), using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and each cDNA sample was then diluted 5 times with nuclease-free water. Samples for RT-qPCR were prepared with the Kapa Sybr Fast Universal qPCR Master Mix (Kapa Biosystems) in 96-well plates and the fluorescence intensity was read using CFX Connect Real-Time System (BioRad). Reaction conditions were as follow: 95 °C for 3 min and 40 cycles of: 95 °C for 2 s of denaturation and 60 °C for 25 s of annealing and extension. Reference genes used for the experiment were β-actin, GAPDH and 18S rRNA. All reactions were performed in technical duplicated and biological triplicates, unless stated otherwise.

*Table 2.3:* ***List of primers used for qPCR***

| Gene | Sequence (5'-3') | Strand | Amplicon size (bp) |
|---|---|---|---|
| CCL20 | ACTGTGGCTTTTCTGGAATGG | FW | 92 |
| | ACCCTCCATGATGTGCAAGT | REV | |
| CXCL8 | TGTTCCACTGTGCCTTGGTT | FW | 72 |
| | ACTGTGAGGTAAGATGGTGGC | REV | |
| CXCR4 | GGCACTTATAACCAAAGCCCA | FW | 75 |
| | TGCTGAAATCAACCCACTCCT | REV | |
| CYP1A1 | ACACAGTGATTGGCAGGTCA | FW | 99 |
| | AGGAAGAGTGTCGGAAGGTCT | REV | |
| ACTB | AAGTACTCCGTGTGGATCG | FW | 111 |
| | GGACTCGTCATACTCCTGCTT | REV | |
| GAPDH | ACATGGCCTCCAAGGAGTAA | FW | 137 |
| | AACTGTGAGGAGGGGAGATTC | REV | |
| BHLHE40 | AGCTGTCAGGGAGAAATGTCG | FW | 128 |
| | AAGCTGCGAAGACTTCAGGT | REV | |
| 18S | ATGGCCGTTCTTAGTTGGTG | FW | 132 |
| | AACGCCACTTGTCCCTCTAA | REV | |
| MIR181A1HG | ACACTTCTGCGTCTGACAGT | FW | 105 |
| | AATGGGGCGGGGAATAGAAA | REV | |

| | | | |
|---|---|---|---|
| AC016831.1 | TCCTACTGAACTGTCACGGCA | FW | 143 |
| | TGTAGTTAGCGACCTCTGCT | REV | |
| AC006552.1 | GGCCTGGCTAAAATTGGGTA | FW | 137 |
| | CTGGTGCCTTCTGTTTTTCC | REV | |
| LINC01558 | CACAGCGGGAAGGTTATCGA | FW | 79 |
| | GGATCAAGCTCTGTGGGCAT | REV | |

The obtained Ct values were used to calculate the log2 fold-change of expressed genes between infected and non-infected (control) cells applying the formula[227]:

$$\Delta\Delta Ct = [Ct_{(target)} - Ct_{(reference)}]_{(control)} - [Ct_{(target)} - Ct_{(reference)}]_{(infected)}$$

The fraction of RNAs in individual sucrose fractions along the gradient after polysome profiling was calculated applying the formula[228]:

$$2^{(40 - Ct_{(fraction)})} / \Sigma_{(all\ fractions)} (2^{(40 - Ct_{(fraction)})}$$

To determine the significance of change between different conditions (control vs. infected; WT vs ΔLLO; 2 h vs. 5 h) the statistical hypothesis t-test was used, with a significance threshold (p-value) of 0.05.

# 3 Results

## 3.1 Infection of human cells with wild-type or LLO-deficient *Listeria monocytogenes*

*Listeria monocytogenes* is a widespread food-borne intracellular pathogen, adapted to invade and counteracting the host cell's innate immune system in order to promote its survival[56]. Over the years this pathogen has been widely used as a model for bacterial infection and host-pathogen interaction studies[113,118,229]. The main virulence factor produced by this bacterium is the pore-forming toxin listeriolysin O (LLO), shown to be essential for it to escape from the host's vacuole and escape into the cytosol where it proliferates and spreads to neighbouring cells[230,231]. Furthermore, in the past years there has been a growing body of evidence that LLO also has many subtle and still poorly characterized activities during infection beneficial for the *Listeria*'s survival[73], for example its activity in the extracellular milieu[100,114]. The goal of my PhD research was to determine how host cells respond at the translational level upon infection with *Listeria monocytogenes* at early time post-infection. Furthermore, I wanted to investigate whether its main virulent factor, LLO, is able to induce specific translational controls during infection. Providing insight to this almost completely unexplored field would contribute to a better understanding of the host-pathogen crosstalk and potentially yield novel approaches in fighting-off bacterial infections, which still remain a major cause of death worldwide[58,232].

In order to achieve this goal, I used a wild type *Listeria* strain (WT) and a strain where the *hly* gene, encoding for LLO, was deleted (ΔLLO). Before starting with host-pathogen interaction studies, I checked the two *Listeria* strains for the presence of LLO and compared their growth rates in broth. Firstly, I used a purified recombinant LLO to demonstrate that our anti-LLO antibody worked properly. I performed 7 serial dilutions of LLO (1:2 ratio), ranging from 5 µg to 40 ng, and detected LLO by western blotting. I observed a strong band just above the 55 kDa marker, which is compatible with LLO's molecular weight of 56 kDa (**Fig. 3.1A**). At high LLO concentrations I also detected weak non-specific bands at lower molecular weights which are probably products of either LLO degradation or a result of non-specific binding of the antibody to impurities (**Fig. 3.1A**).

Then, to validate the ability of the WT strain to produce the toxin, I extracted proteins from the pellet of both WT and ΔLLO bacterial strains and their broth supernatants and tested them for the presence of LLO (**Fig. 3.1B**). I showed that indeed the WT strain was expressing LLO, while, as expected, the ΔLLO strain does not (**Fig. 3.1B**). Detectable amounts of LLO were also present in the WT broth, demonstrating that LLO is produced and released even before *Listeria* comes in contact with host cells.

Next, I compared the growth rate in broth of the two strains and I did not observe any differences between their growth, meaning that the lack of LLO does not affect *Listeria*'s replication (**Fig. 3.1C**).

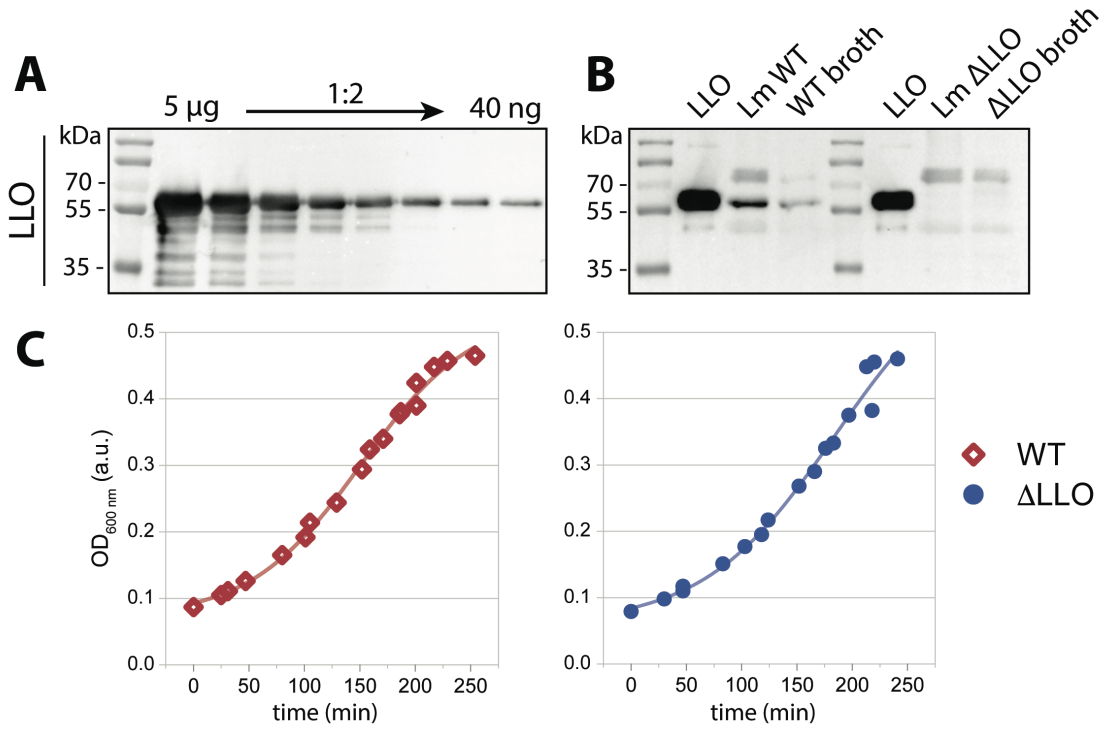


*Figure 3.1:* ***Comparison between WT and ΔLLO Listeria growth and LLO expression.***
*(**A**) Western blot of serial dilutions of purified recombinant LLO. (**B**) Western blot of WT and ΔLLO bacterial lysates and proteins extracted from their broths. (**C**) Growth curves of WT (left) and ΔLLO (right) Listeria in broth, showing the increase of optical density (600nm) in time. The red line represents a Sigmoidal fit of the data.*

As a host, I used the human epithelial colorectal adenocarcinoma cell line (Caco-2), that is a paradigm in host-pathogen interactions and is frequently used to study *Listeria* infections[113,221,222]. In studies on host-pathogen crosstalk, the infection is usually defined by

the multiplicity of infection (MOI), described as the number of added bacteria per cell and *Listeria* MOI usually ranges from 10 and up to 100 bacteria per cell[233,234].

For the purpose of my research, I used a WT and a ΔLLO strain of *Listeria* which were also transfected with a plasmid expressing the green fluorescent protein (GFP) in order to be visualized via fluorescence microscopy. This allows to monitor the presence of bacteria in cells. Taking advantage of the GFP expression, I optimized the WT *Listeria* infection protocol (see Methods for details) for Caco-2 by referring to the study done on the invasion assay using Caco-2 cells[235] and tested the infection at three different MOIs after a 2-hour invasion and a further 2 h or 5 h hour post-invasion (POI) (**Fig. 3.2A**). I exploited the fluorescence of the two *Listeria* strains to determine the number of bacteria per cell and the overall percentage of infected cells (containing at least one visible bacterium); examples of these images are presented in **Fig. 3.2B**. Having analysed fluorescence microscopy images, I observed that at MOI of 40 all cells were infected at 2 h and 5 h POI (**Fig. 3.2C**) with an average number of intracellular bacteria of 34 bac/cell at 2 h POI and 21 bac/cell at 5 h POI (**Fig. 3.2D**). Upon infection with the higher MOI (100), cells contain around 85 bac/cell at 2 h POI and 21 bac/cell at 5 h POI, while an infection with a lower MOI of 10 yielded around 80 % of infected cells, with around 6 bac/cell at 2 h POI and 4 bac/cell at 5 h POI.

In order to obtain the strongest response of host cells upon infection, I decided to use a MOI of 40 since at this condition all cells were infected. At MOI 100 all cells were also infected, but I dismissed this condition since Yamada *et al.* showed that with a higher MOI the bacterial recovery rate (i.e. the relationship between inoculated and recovered bacterial counts) drops, which they explained could possibly be due to competition between bacterial ligands and cellular receptors[235].

Next, I determined the number of bacteria per cell upon ΔLLO infection via fluorescence microscopy (example of 2 h POI shown in **Fig. 3.2E**), since for the MOI selection I used the WT strain. ΔLLO infected cells at MOI 40 contained around 25 bac/cell at 2 h POI and 24 bac/cell at 5 h POI (**Fig. 3.2F**) and the comparison between WT and ΔLLO infection 2 h POI showed a significant decrease in the number of intracellular bacteria, as expected since the presence of LLO indeed promotes bacterial invasion, as already demonstrated by Dramsi *et al.*[105].

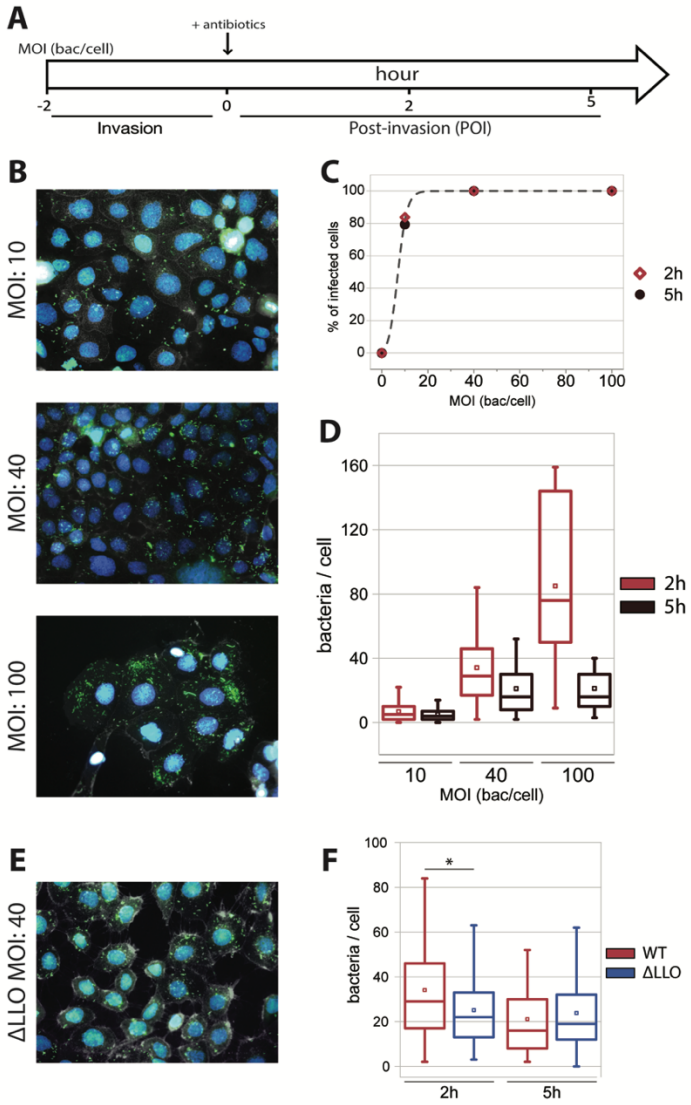


*Figure 3.2:* ***Selecting the multiplicity of infection.***

*(**A**) Infection protocol time-line. (**B**) Examples of fluorescence microscopy images of WT infected cells at the three different MOIs and 2 h POI. The images were obtained using DAPI to visualize the cell nucleus (in blue), WGA to dye the cell membrane (in grey) and the green dots are GFP-producing bacteria. (**C**) Calculated percentage of cells with at least one internalized WT bacterium at three different MOIs and two incubation times. (**D**) Listeria WT bacterial count using fluorescence microscopy of infected cells at three different MOIs, 2 h and 5 h POI. (**E**) Example of fluorescence microscopy images of ΔLLO infected cells 2 h POI and a MOI of 40. The images were obtained using DAPI to visualize the cell nucleus (in blue), WGA to dye the cell membrane (in grey) and the green dots are individual GFP-producing bacteria. (**F**) Bacterial count using fluorescence microscopy of WT and ΔLLO infected cells at a MOI 40, 2 h and 5 h POI. All experiments were performed in biological triplicates and for each condition, bacteria were counted in at least 150 cells. Significant changes between samples are represented by T-test P-value: * < 0.05.*

I then decided to set for all further experiments the following protocol: i) infection of cells monolayers with bacteria at a MOI 40 bac/cell; ii) 2 h invasion time, allowing the bacteria to invade host cells; iii) removal of the remaining extracellular bacteria and the addition of an antibiotic-containing medium; iv) further incubation of infected cells for either 2 h or 5 h POI.

## 3.2 Host cell translation is impaired upon infection with *Listeria monocytogenes*

Infections mediated by pathogens often interfere with the translation machinery and the process of protein synthesis. This process is the most energy consuming, that is why cells fine-tune it to conserve energy and respond quickly to stimuli if needed[2]. Thus, translation should be tightly regulated upon bacterial infection, but the picture is sketchy at best. Indeed, innate immunity protects cells against infection by triggering host protective and defence responses against pathogens[14]. Bacterial infection can result in inhibition of the translation machinery, either directly through bacterial toxin-mediated[236] or effector-mediated inhibition of translation elongation[237] or indirectly through activation of the GCN2 pathway or inactivation of the TOR pathway[34]. Few studies tackled translational controls in host stress response after exposure to virulence factor[238] and none of them used bacteria. Given these premises, my goal was to monitor host cell's early response upon infection with *Listeria monocytogenes* at a translational level and secondly, answering whether *Listeria*'s main virulent factor, LLO, can trigger translational controls and to characterize them.

In order to answer the first question, I took advantage of polysome profiling, a classical and powerful technique to study alterations in translation based on sucrose gradient ultracentrifugation of cellular lysates. From polysome profiling it is possible to : i) obtain the fraction of ribosomes in polysomes (FRP)[239], which gives indication on the general translational state of cells or tissues; ii) purify polysome-associated RNA[30,238] in order to monitor changes in mRNAs on the translation machinery; iii) purify total (cytoplasmic) RNA, which describes the whole set of mRNA produced by transcription and transported in the cytoplasm; iv) purify RNA from individual fractions along the sucrose gradient (**Fig. 3.3**), which permits monitoring changes in the localization of transcripts along the sucrose gradient.

I exploited the advantages of this technique to monitor transcriptional (RNA-seq, from total RNA) and in the translational (Pol-seq, from polysome-associated RNA) alterations in host cells upon bacterial infection at a genome-wide level by Next Generation Sequencing (NGS). From these pieces of information, I performed a variety of comparisons that helped me to better understand the host's response to infection: i) RNA-seq vs. POL-seq upon infection with the same *Listeria* strain; ii) RNA-seq upon WT vs. RNA-seq upon ΔLLO infection; iii) POL-seq upon WT vs. POL-seq upon ΔLLO infection; and iv) the relative distribution of specific RNAs along the sucrose gradient of non-infected, WT-infected and ΔLLO-infected cells.

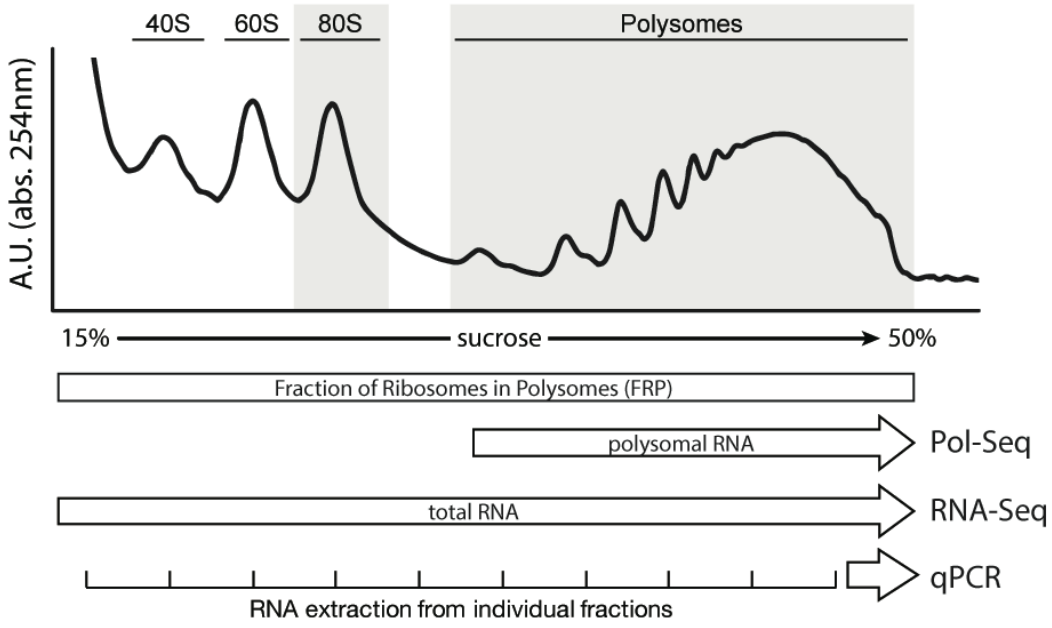


*Figure 3.3:* ***Experimental design using polysome profiling.***

*Example of a polysomal profile showing the distribution of different parts of the translational machinery, which are separated based on their sedimentation coefficient via centrifugation in a sucrose gradient. The FRP is calculated as the ratio between the area under the polysomal peak and the sum of the 80S peak and the polysomal area. After fractionation RNA can be extracted from individual fraction or it can be pooled into either polysomal or total cytoplasmic RNA, which can be used for analysis by NGS or qPCR.*

### 3.2.1 WT *Listeria monocytogenes* induces early translational defects in host cells

Polysomes are generally considered to be involved in the active translation of mRNAs in a growing system, whereas monosomes (80S) are regarded as newly assembled ribosomes positioned at the start codon and translationally inactive[26,27]. Considering this notion, we can calculate the Fraction of Ribosomes in Polysomes (FRP) in order to get a sense about the general state of translation. A decrease in the cell's FRP generally points to translational defects, which usually occur when cells respond to stress[240].

Examples of polysomal profiles upon *Listeria* infection are presented in **Fig. 3.4**, where one can observe a clear increase in the 80S peak after a 5 h POI with respect to the control in the case of WT (**Fig. 3.4A**) and ΔLLO (**Fig. 3.4B**) infection. The increase of the 80S peak area and the corresponding decrease of the polysomal area accounts for a lower FRP, which is indicative of translational defects as a result of bacterial infection.

Results obtained from polysome profiling show an impairment in translation upon infection, more so in the case of WT infection. I demonstrated that already 2 h POI, host cells are responding to *Listeria* infection at a translational level. This impairment is even clearer 5 h POI, showing that at these two time-points POI translation is strongly affected. Interestingly, the ΔLLO strain which had an overall similar effect on host translation as WT, seems to act in a somewhat delayed manner, since the Fraction of Ribosomes in Polysomes (FRP) value drops significantly only after the latter time point. This suggests that the presence of LLO during infection likely induces translational defects at an earlier stage than the LLO-deficient *Listeria* strain (**Fig. 3.4C**).

Further validation that the decrease in FRP represents a global protein synthesis downregulation upon infection was demonstrated by metabolic labelling, where I monitored the amount of L-azidohomoalaine (AHA) incorporation into *de-novo* synthetized proteins[241]. The decrease in FRP upon infection is strongly coupled with a significant decrease in the global protein production observed by metabolic labelling, suggesting that host cells slow-down translation upon bacterial invasion in order to respond to infection (**Fig. 3.4D**).

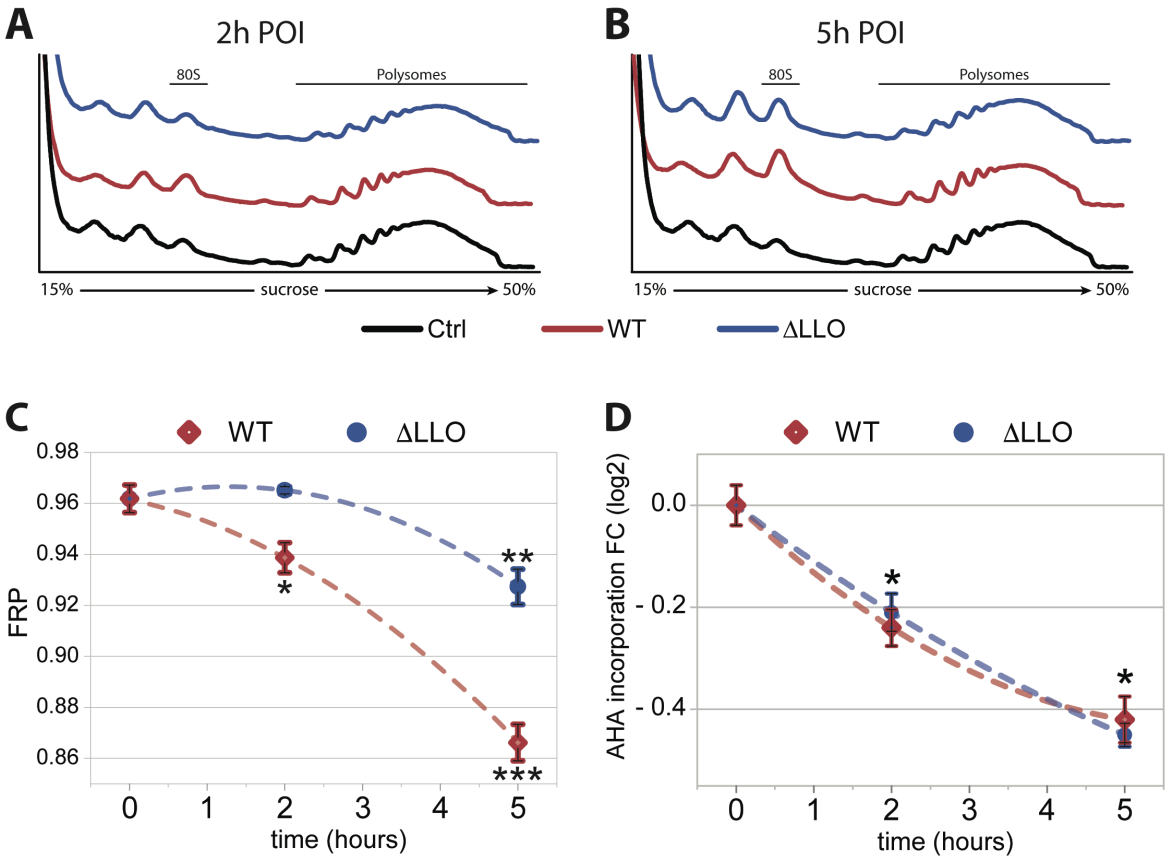


*Figure 3.4:* ***Translation is significantly impaired upon Listeria infection.***
*Polysomal profiles of non-infected control cells (black line) and Caco-2 cells upon infection with either WT (red line) or ΔLLO (blue line),* ***(A)*** *2h or* ***(B)*** *5h POI. The profiles have been stacked to facilitate their comparison.* **(C)** *FRP comparison of non-infected cells (0h) and cells upon infection with WT or ΔLLO strain after 2 h or 5 h POI.* ***(D)*** *De-novo protein synthesis analysis using metabolic labelling (AHA incorporation) at same conditions as for FRP calculation. upon infection with WT or ΔLLO strain after 2 h or 5 h POI. All experiments were performed in biological triplicates. Significant changes between samples are represented by T-test P-value: ns > 0.05; * < 0.05; ** < 0.01; *** < 0.001.*

Next, I wanted to determine whether the observed decrease in FRP upon infection could be the result of bacteria already acting on host cells before invasion, by releasing pathogen-associated molecular pattern (PAMPs). In fact, it is known that *Listeria* secretes its main virulent factor LLO already prior to host cell internalization and that this extracellular LLO is able to trigger host signalling pathways, such as NFκB[109] and MAPK[54,115,116].

To answer this question, I treated cells with medium containing *Listeria*-released molecules from WT and ΔLLO strain (SUP, see Methods for further details) and performed polysome

profiling. Examples of the obtained profiles are shown in **Figure 3.5**, where I observed an increase in the 80S peak only after WT SUP treatment (**Fig. 3.5A**), with the most obvious increase after 5 h POI.

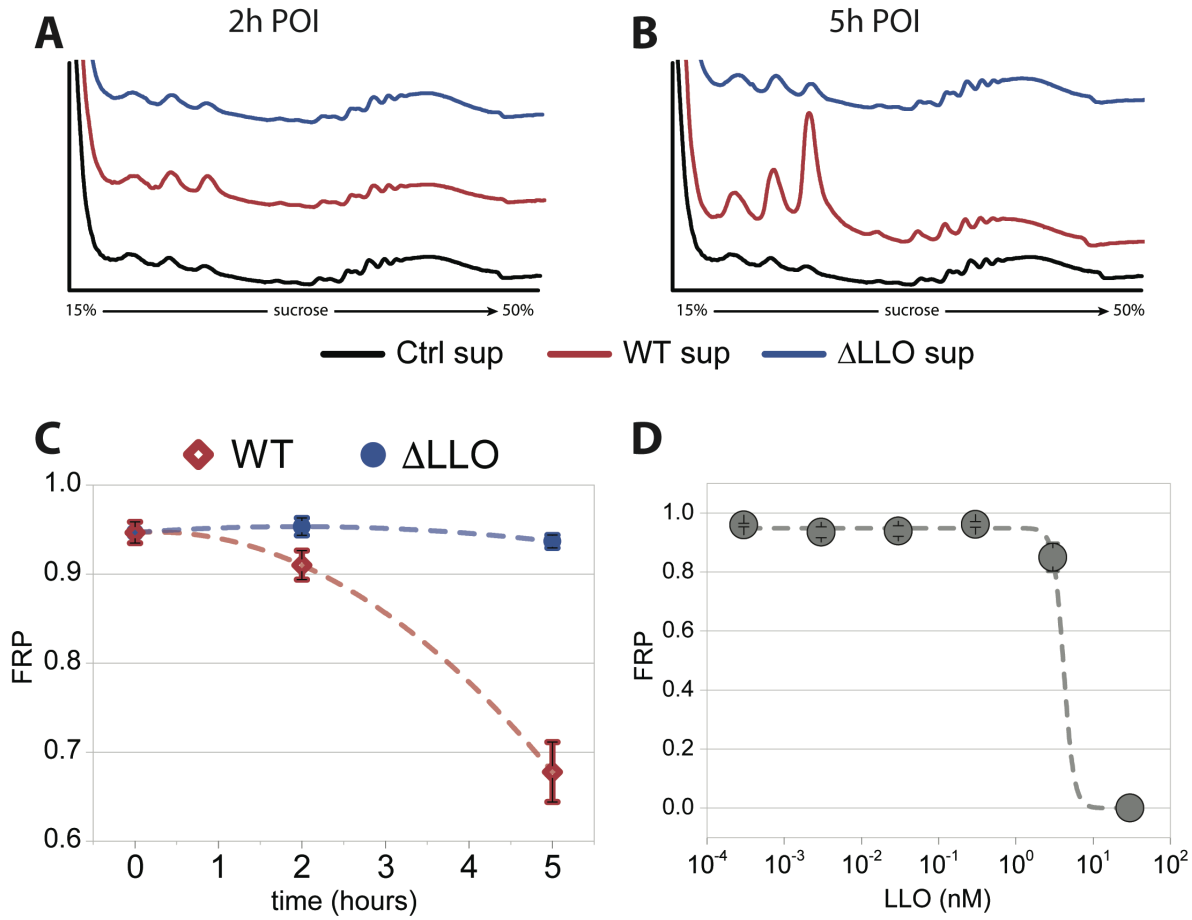


*Figure 3.5:* ***Translation is significantly impaired upon treatment with medium containing WT Listeria-released molecules.***

*Polysomal profiles of cells treated with medium from non-infected cells (Ctrl SUP) (black line) and medium containing either WT Listeria-released molecules (WT SUP, red line) or medium containing ΔLLO Listeria-released molecules (ΔLLO SUP) (blue line), (**A**) 2h and (**B**) 5h POI. (**C**) FRP comparison of cells treated with medium from non-infected cells (0h) and medium containing either WT Listeria-released molecules (WT) or medium containing ΔLLO Listeria-released molecules (ΔLLO), after 2 h or 5 h incubation time post-treatment. (**D**) FRP comparison of cells treated with different concentrations of recombinant LLO. All experiments were performed in biological triplicates. Significant changes between samples are represented by T-test P-value: * < 0.05; ** < 0.01.*

Comparing the FRP values after SUP treatment, I observed a strong and significant decrease only in cells treated with the WT SUP after a 5 h incubation time post treatment, showing that

the decrease in FRP upon WT infection is at least in part due to molecules secreted by the bacterium prior to host cell invasion (**Fig. 3.5C**). This effect was not evident in cells treated with ΔLLO SUP, which is why I speculated that the main molecule responsible for this effect might be LLO produced by extracellular bacteria.

To see whether this could be the case, cells were treated with a wide concentration range of recombinant LLO. I demonstrated that LLO concentrations above 0.3 nM induce translational impairments, shown by a decrease in the FRP value (**Fig. 3.5D**).

According to literature, *Listeria* is able to either trigger or inhibit signalling pathways in host cells upon invasion and even prior to it, by secreting virulence factors, such as LLO. *Listeria* was found to inactivate major pathways involved in translation regulation such as MAPK[91], mTOR[96] and activate pathways such as PERK[45], which in turn inhibit host translation. However, other reports demonstrated that under different conditions, *Listeria* can also activate MAPK and NF-κB[92,93], suggesting that its ability to modulate host pathways possibly depends on multiple factors, such as the origin and type of host cells, multiplicity of infection, time post-infection and the strain used in these studies.

To determine if the observed decrease in the fraction of ribosomes in polysomes might be at least in part ascribed to activation or inactivation of the abovementioned pathways, I extracted total proteins from infected and non-infected cells at different times POI. I then used western blotting to monitor the amount of proteins involved in MAPK, mTOR and PERK pathways (**Fig. 3.6**).

Looking first at the pathways induced upon WT infection, I observed a clear activation of the PERK pathway, shown by the increase in eIF2α phosphorylation, both at 2 h and 5 h POI compared to the control. Next, the phosphorylation state of 4E-BP shows minimal or no differences when compared to the control, suggesting that the mTOR pathway is not regulating translation at upon WT infection at these stages of infection. Since I can exclude the activation of the mTOR pathway, the clear phosphorylation of RPS6 suggests that this protein is modified post-translationally only by the activation of MAPK pathway at 2 h POI, while at 5 h POI its involvement in translation regulation is not evident any more.

Shifting to pathways induced upon ΔLLO infection, the PERK pathway is activated much like in the case of the WT, while the involvement of MAPK and mTOR are less clear in this instance. In fact, the changes in the total amount of 4E-BP at both time points make it difficult

to comment on the mTOR involvement upon ΔLLO infection. The MAPK pathways seems to be activated at 2 h POI, but a big variation between replicas shows a non-significant increase in RPS6 phosphorylation and at 5 h POI its involvement in translation regulation seems to diminish.

Considering the obtained results, a possible interpretation could be that infection with both *Listeria* strains induces a general inhibition of translation as is evident by the activation of the PERK pathway and is reported also in literature[45]. The activation of MAPK pathway at an early time-point (2 h POI) however, could be involved in translation rewiring in order to promote production of pro-inflammatory proteins, to fend-off invading bacteria.

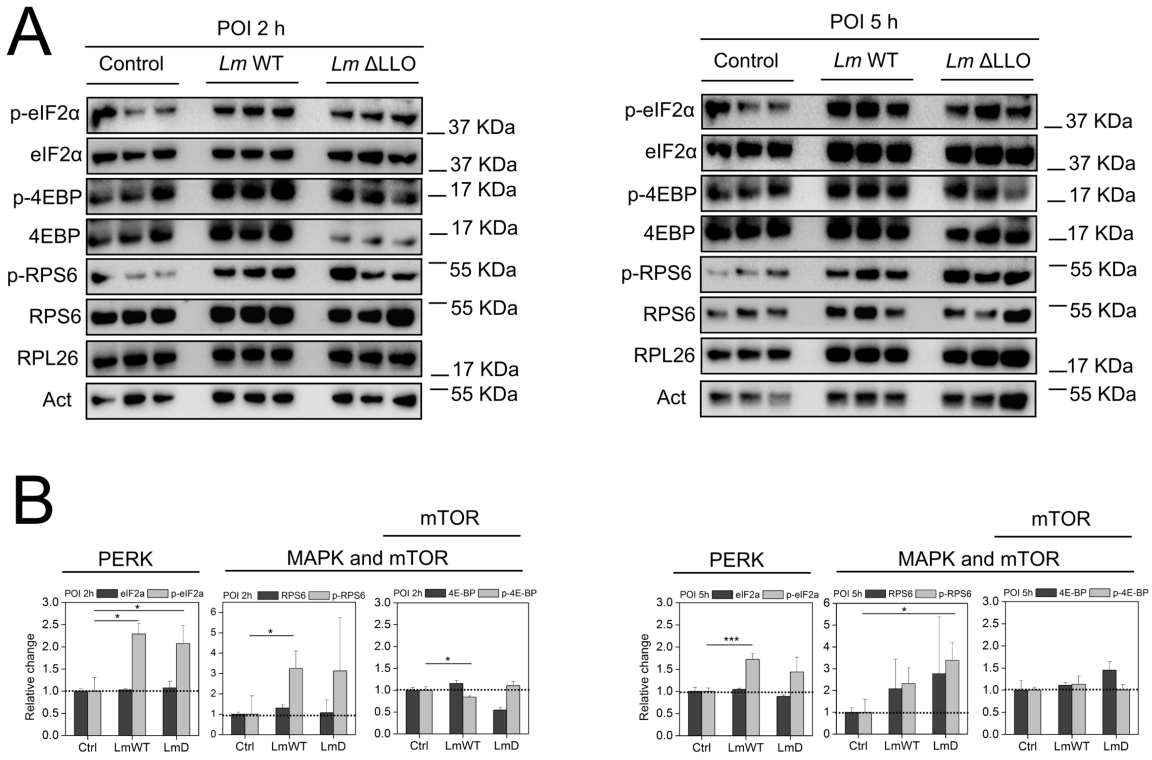


*Figure 3.6:* ***Translation is significantly impaired upon treatment with medium containing WT Listeria-released molecules.***

(**A**) *Western blots of proteins involved in signalling pathways that control the activity of translation, of non-infected (control) and either WT or ΔLLO infected cells, at 2 h (left) and 5 h (right) POI.*

(***B***) *Relative changes of total and phosphorylated proteins normalized to actin (Act). Shown are the signalling pathways associated with the analysed proteins: eIF2α - PERK; 4E-BP – mTOR; RPS6 – mTOR and MAPK. Significant changes between samples are represented by T-test P-value: * < 0.05; ** < 0.01; *** < 0.001.*

Using polysome profiling I was able to demonstrate that host cells respond to infection at a translational level upon infection with both *Listeria* strains; however, I show that at 2 h POI, WT already induces significant translational defects, whereas the ΔLLO strain exhibits a delayed response, since significant translational defects can firs be observed at 5 h POI. Furthermore, I demonstrated that molecules secreted by extracellular WT *Listeria* impair host translation and the evidence points to LLO being the main reason for this early effect. I also presented evidence that the main reason for the observed decrease in host FRP upon *Listeria* infection is probably the activation of the PERK pathway.

## 3.3 *Listeria* infection induces strong transcriptional and translational rewiring of gene expression

From sucrose fractions obtained by polysome profiling it is possible to extract total cytoplasmic RNA and RNA associated with polysomes and perform NGS in order to monitor changes in the transcriptome and the translatome at a genome-wide level. Given the observed translational defects at 2 h POI and the strong translational impairments at 5 h POI, shown as a decrease in FRP and global protein production, I wondered whether *Listeria* is able to induce a global rewiring of RNA uploading on polysomes and whether LLO is a part of this process. To answer this question, I performed RNA-seq and POL-seq from cells infected with either WT or ΔLLO at 2 h and 5 h POI. All the computational analyses of the sequencing data were performed in collaboration with Dr. Toma Tebaldi (Laboratory of Translational Genomics, CIBIO).

### 3.3.1 Transcriptome and translatome comparison upon infection with either WT or ΔLLO infection

After extraction of RNA, library preparation, sequencing and identification of differentially expressed genes (DEGs, see Methods for details), I first looked at the relative changes in the transcriptome (RNA-seq) and the translatome (POL-seq) upon WT and ΔLLO infection and at 2 h and 5 h POI, separately. This gave as a sense whether the RNAs that are up- or down-regulated as a consequence of the infection associate or dissociate with/from polysomes in a

proportional way with respect to mRNAs synthesis during transcription, according to the Central Dogma of Molecular Biology. Thus, I first considered the global changes in term of changes in just the number of DEGs at different conditions, identifying changes: i) only at the transcriptional level (total only); ii) only at the translational level (polysomal only); iii) both at transcriptional and translational level in the same direction (coupled); and iv) iii) both at transcriptional and translational level in the opposite direction (uncoupled), according to Tebaldi et al[30].

Firstly, comparing RNA-seq and POL-seq after infection with WT *Listeria* ***(Fig. 3.7)***, I observed that the DEGs belonging to transcriptionally and translationally coupled genes (green dots and bar in **Fig. 3.7**) account for 44 % of all genes upon WT infection at 2 h POI and 43 % at 5 h, showing a slightly decreased from 614 at 2 h POI to 568 at 5 h POI. Furthermore, I observed that coupled DEGs at 2 h POI have an overall higher fold-change of either up- or down-regulation when compared to 5 h POI, meaning that the strongest response to infection happens at an earlier time than 5 h POI. The amount of uncoupled RNAs, either transcriptionally (Total only, blue dots and bars in Fig.3.8) or translationally (Polysomal only, yellow dots and bars in Fig.3.8), account for the majority of changes, meaning that a large number of changes in transcription are not linearly correlated to changes in translation. These results suggest that translational controls are indeed involved in tightly modulating the interplay between transcription and translation, especially as early response to WT infection even if a large proportion of changes are coupled.

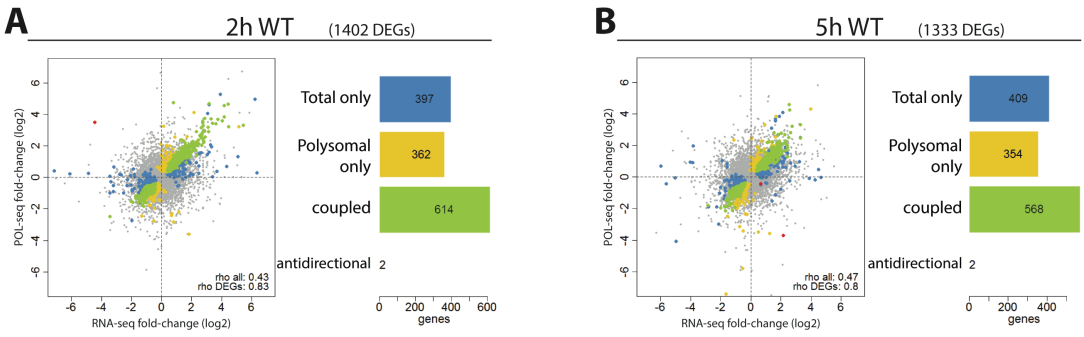


*Figure 3.7:* ***Transcriptome and translatome correlation upon WT infection.***
*Scatter plots comparing the transcriptome (RNA-seq, x-axis) and translatome (POL-seq, y-axis) DEGs upon infection with (**A**) WT Listeria after 2 h and (**B**) 5 h. On the right side of each plot is represented the number of DEGs belonging to one of four groups which are based on the correlation between each cytosolic and polysome-associated DEG upon WT infection. All experiments were perfomed in biological duplicate (see Methods for further details).*

Next, comparing RNA-seq and POL-seq after infection with ΔLLO (**Fig. 3.8**), I observed that the largest group of DEGs belongs to the coupled genes. At 2 h POI 57 % and at 5 h POI 54 % of all DEGs are transcriptionally and translationally coupled. Therefore, unlike upon WT infection, a higher overall coupling between transcription and translation can be observed, with a strong increase from 456 at 2 h POI to 721 at 5 h POI.

During the time-course of the infection, the number of "Total only" DEGs increased as well, from 172 at 2 h POI to 249 at 5 h POI and the Polysomal only DEGs increased from 177 at 2 h POI to 373 at 5 h POI. Interestingly, the overall amount of DEGs upon ΔLLO infection at 2 h POI summed up to 805 DEGs, compared to 1343 DEGs 5 h POI upon ΔLLO infection and also upon WT infection with 1402 at 2 h POI, 1333 at 5 h POI. This suggests that upon ΔLLO infection host cells have not yet fully responded to infection at 2 h POI, since after 5 h POI the amount of DEGs rises considerably and that WT infection induces more uncoupling between transcription and translation, suggestive of translational control mechanisms are playing a role in cells. Taking all observations into consideration, these results indicate that the expression of LLO (WT strain) induced an earlier response from host cells and a stronger translational control than in its absence and that it possibly induces different mechanisms of reply involving translational control of gene expression.

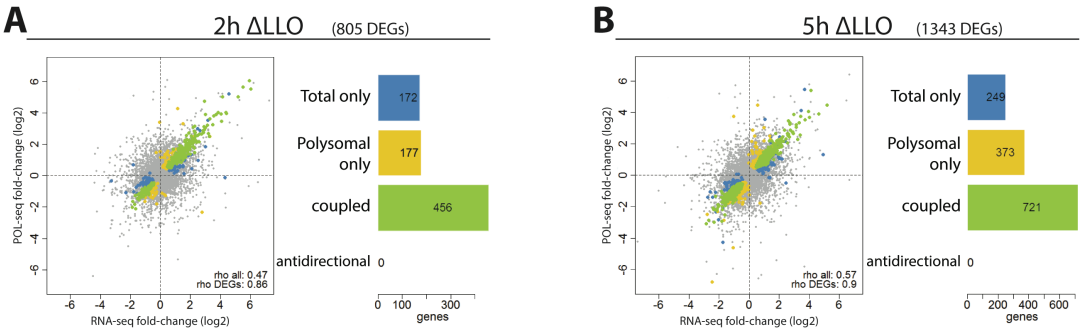


*Figure 3.8:* ***Transcriptome and translatome correlation upon ΔLLO infection.***
*Scatter plots comparing the transcriptome (RNA-seq) and translatome (POL-seq) DEGs upon infection with (**A**) ΔLLO Listeria after 2 h and (**B**) 5 h. On the right side of each plot is represented the number of DEGs belonging to one of four groups which are based on the correlation between each cytosolic and polysome-associated DEG upon WT infection. The experiment was performed in biological duplicate.*

### 3.3.2 Transcriptome and translatome comparison between WT or ΔLLO infection

After having shown that the bulk reply to WT and ΔLLO infections might be different at least in term of global changes in the number of differentially expressed genes, I wondered whether using NGS I was able to find genes specifically involved in the reply to infection with the WT bacterial strain. To do that, I obtained the DEGs comparing the transcriptome and translatome after host infection with the WT and the ΔLLO at both times POI. I considered the number of DEGs whose number changed: i) only in a WT-specific manner (WT only); ii) only in a ΔLLO-specific manner (ΔLLO only); iii) both upon WT and ΔLLO infection with changes in the same direction (coupled); and iv) both upon WT and ΔLLO infection with changes in the opposite directions (antidirectional).

Comparing cytoplasmic DEGs after a WT and ΔLLO infection (**Fig. 3.9**), I observed that the largest group at both time-points belonged to WT-specific DEGs. The number of this class of genes slightly decreases from 684 at 2 h POI to 589 DEGs at 5 h POI.

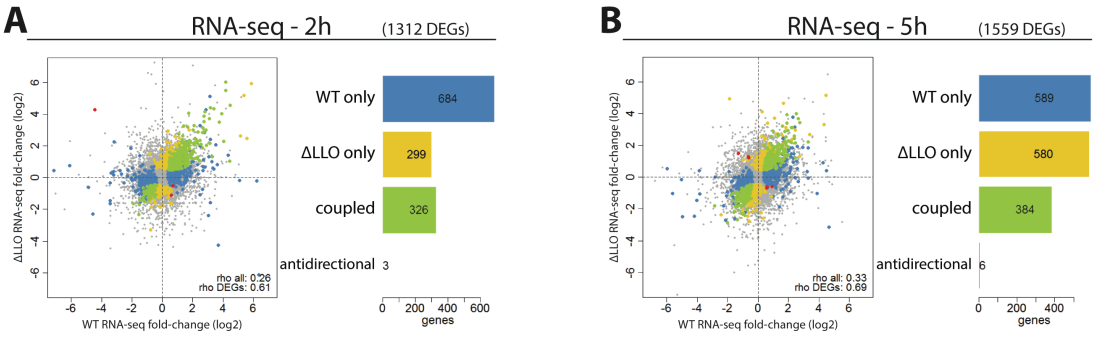


*Figure 3.9:* ***Comparison of cytoplasmic DEGs upon WT and ΔLLO infection.***
*Scatter plots comparing cytoplasmic DEGs upon infection with WT and ΔLLO Listeria after (**A**) 2 h POI and (**B**) 5 h POI. On the right side of each plot is represented the number of DEGs belonging to one of four groups which are based on the correlation between each RNA-seq DEG after WT and ΔLLO infection.*

The largest difference in the variation in the number of DEGs during time was observed for ΔLLO-specific genes, increasing from 299 at 2 h POI to 580 at 5 h POI and the group where the number of DEGs changed the least were the coupled DEGs with an increase from 326 at 2 h POI to 384 at 5 h POI.

Next, looking at the polysomal DEGs, WT-specific changes at 2 h POI represented the group with the largest number of DEGs and this number decreases from 612 at 2 h POI to 539 DEGs at 5 h POI (**Fig. 3.10**). The most stable group in terms of number of DEGs changing between the two time-points were the coupled genes, where the number again changed only from 363 at 2 h POI to 377 DEGs at 5 h POI.

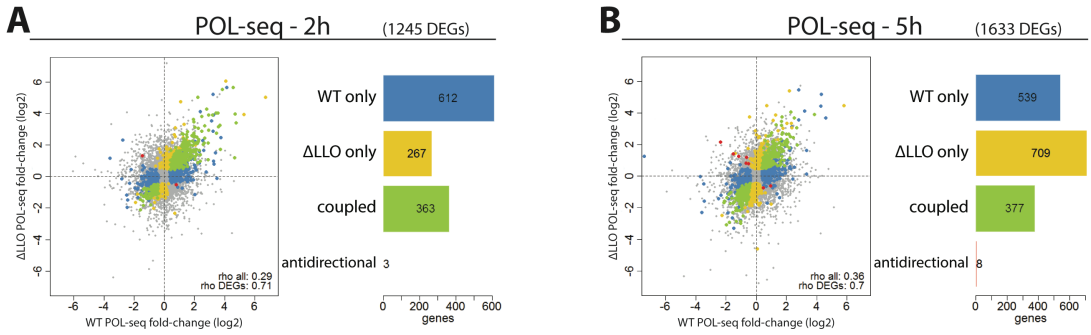


*Figure 3.10:* ***Comparison of POL-seq DEGs upon WT and ΔLLO infection.***
*Scatter plots comparing polysome-associated DEGs upon infection with WT and ΔLLO Listeria after* ***(A)*** *2 h POI and* ***(B)*** *5 h POI. On the right side of each plot is represented the number of DEGs belonging to one of four groups which are based on the correlation between each POL-seq DEG after WT and ΔLLO infection.*

As previously observed in the transcriptome, the biggest and an even more obvious difference I observed in the translatome was in the case of ΔLLO-specific DEGs, where the number noticeably increased from merely 267 at 2 h POI to 708 DEGs at 5 h POI. This big difference in ΔLLO-specific DEGs between the two time-points is possibly due to the difference in FRP (**Fig. 3.4C**), where I did not observe any significant translational defects at 2 h POI and then a strong drop in FRP at 5 h POI. Overall, these comparisons suggest that each *Listeria* strain induces the expression of a large number of specific genes with most profound changes upon WT infection.

### 3.3.3 Gene Ontology analysis of the translatome

To understand to what cellular processes the abovementioned DEGs are associated to and given the observed translatome sensitivity to *Listeria* infection, I performed GO enrichment analysis considering polysome-associated DEGs with a log2 fold-change difference > 1.

The GO analysis of WT-specific DEGs showed a significant enrichment of 18 genes involved in cancer development at 2 h POI (**Fig. 3.11A**) and 16 genes involved in cell growth and steroid biosynthesis at 5 h POI (**Fig. 3.11B**). This unexpected observation could potentially be due to LLO being a cholesterol dependent toxin, and suggests intriguing considerations about possible co-evolution of virulence factors and host-cell reply to infection.

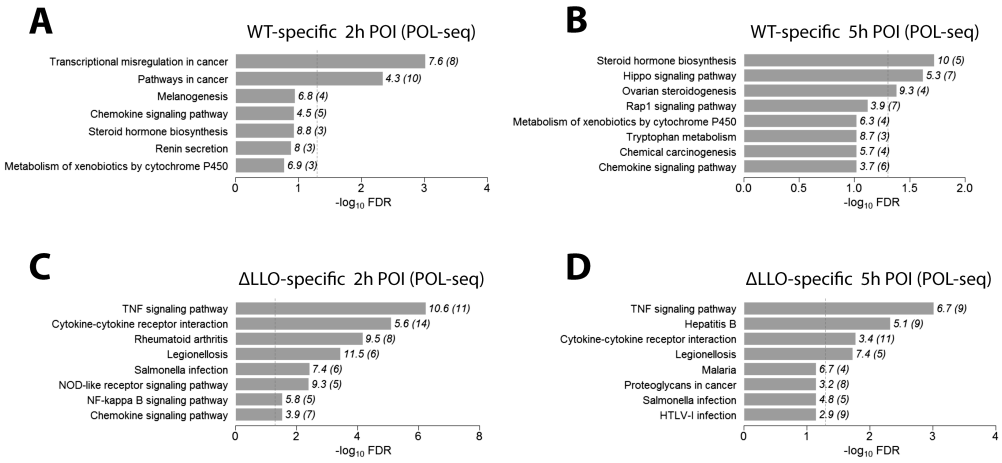


*Figure 3.11:* ***Gene Ontology analysis of WT-specific and ΔLLO-specific DEGs in the translatome.*** *Top enriched Gene Ontology terms among populations of translatome DEGs: (**A**) WT-specific after 2 h POI, (**B**) WT-specific after 5 h POI, (**C**) ΔLLO-specific after 2 h POI and (**D**) ΔLLO-specific after 5 h POI. Bar length is proportional to enrichment statistical significance (FDR). Fold-enrichment values and the number of DEGs associated to each term are provided beside each bar.*

Next, looking at the GO analysis of ΔLLO-specific DEGs, a much more obvious pattern emerged, where 62 DEGs at 2 h POI (**Fig. 3.11C**) and 34 DEGs at 5 h POI showed a significant enrichment for terms related to the immune response (**Fig. 3.11D**). The largest number of enriched genes at both time-points belonged to cytokine-cytokine receptor interaction and TNF signalling pathway, both of which are well known to be activated upon *Listeria* infection[242–246].

### 3.3.4 Validation of the NGS data

Since NGS is a high-throughput method, the reproducibility of the data obtained by it needed to be validated by a different technique, such as RT-qPCR.

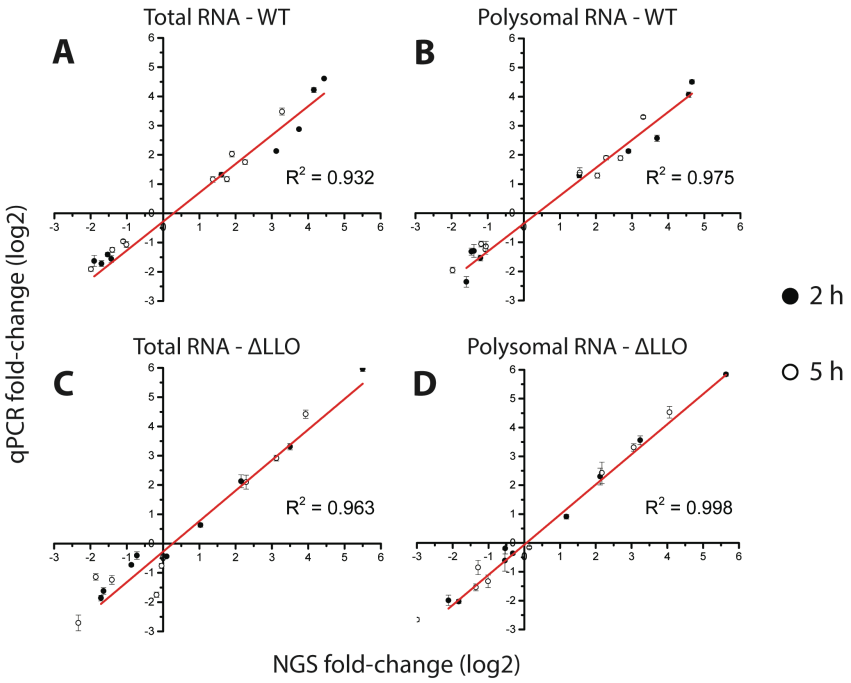


*Figure 3.12:* ***NGS gene expression validation****.*
*Scatter plots showing the log2 fold-change correlation of selected genes between values obtained from NGS (x-axis) and qPCR (y-axis) at 2 h and 5 h POI upon either WT infection for (**A**) Total and (**B**) Polysomal RNA or ΔLLO infection for (**C**) Total and (**D**) Polysomal RNA. How well the values obtained by the two techniques correlate is represented by a coefficient of determination ($R^2$). The mean value of the RT-qPCR biological triplicates and NGS duplicates is shown.*

From the NGS data I selected 13 transcripts among the most up- and down-regulated DEGs in all conditions and compared their NGS fold-change to the corresponding RT-qPCR fold-changes. The transcripts chosen for the validation were: CCL20, CXCL8, CXCR4, CYP1A1, BHLHE40, AC016831.1, AC006552.1, MIR181A1HG, LINC01558. I obtained a high correlation between the two techniques at all conditions, with correlation coefficients $R^2 \geq 0.932$ (**Fig. 3.12**), demonstrating the robustness of our NGS results.

## 3.4 Emergence of long non-coding RNAs associated to polysomes and connected to specific responses to infection with WT *Listeria monocytogenes*

Up until now, no research has yet addressed whether pathogens are able to induce specific gene expression regulation in host cells at a translational level, let alone whether they are triggered by a bacterial virulent factor[238]. After examining the data in terms of the number of DEGs, I then explored which RNA classes of DEGs are present in each condition. More importantly, to date no other research has reported on lncRNAs association with polysomes during bacterial infections, even if recent findings demonstrated that that lncRNAs can be indeed associated to ribosomes and polysomes[3,6,247].

### 3.4.1 Infection-induced lncRNAs associate with the translational machinery

First, looking at the classes of RNA (ncRNAs, protein coding RNAs, other RNAs) in the transcriptome DEGs, I observed that under all conditions the vast majority of DEGs belong to mRNAs, while the second biggest group was represented by long intergenic non-coding RNAs (lincRNAs) (**Fig. 3.13**).

As mentioned before, comparing the differentially expressed mRNAs, I came across an interesting observation when comparing 2 h with 5 h POI: upon WT infection, number of cytoplasmic mRNAs upon WT infection slightly decreased from 722 at 2 h to 652 at 5 h POI, while upon ΔLLO infection the number actually increases from 594 at 2 h to 858 at 5 h POI, which is also the highest number of differentially expressed mRNAs in the transcriptome. This behaviour could potentially mean that LLO is able to induce a downregulation of some protein coding genes that would otherwise be expressed as response to bacterial infection.

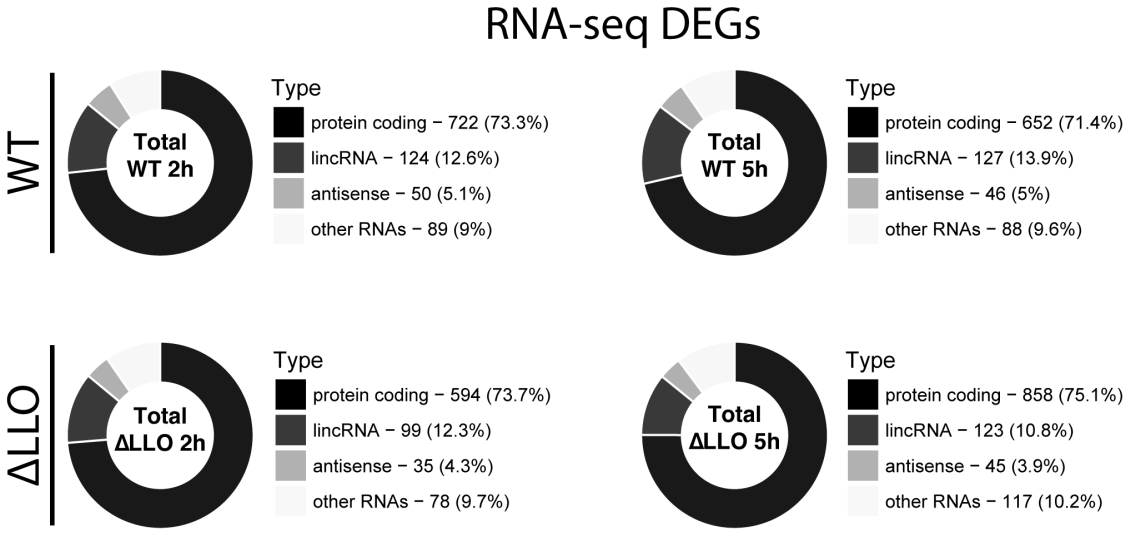


*Figure 3.13:* ***Classes of DEGs in the transcriptome upon WT and ΔLLO infection.***
*Doughnut charts representing the fractions of different classes of cytoplasmic DEGs (Total) upon either WT or ΔLLO infection, 2 h and 5 h POI. On the right side of each chart are the types, the number and the percentage of DEGs found at each condition. GO based on DEGs with log2 FC > 1.*

Surprisingly, looking at the classes of RNA in translatome DEGs, the largest type belonged to mRNAs, but the surprising realization was that the second largest group belonged again to lincRNAs, apart from 5 h POI upon WT infection, where the group of "other RNAs" contained only 4 DEGs more (**Fig. 3.14**). I observed the number of polysomal mRNAs decreased from 669 at 2 h to only 598 at 5 h POI and similar to what was observed in the transcriptome DEGs upon ΔLLO infection, the number of polysomal mRNAs increased from 638 at 2 h to a staggering 958 at 5 h POI. These results further enforce the hypothesis of an LLO-induced gene regulation upon infection.

Focusing on lincRNAs, I observed that at 2 h POI 124 differentially expressed lincRNAs were regulated upon WT infection and 99 upon ΔLLO infection, whereas at 5 h POI the numbers were similar, with 127 upon WT and 123 upon ΔLLO infection. Since in the case of ΔLLO infection the number of lincRNAs increases from 2 h to 5 h POI, I speculated that at the earlier time point cells have not yet fully responded to infection, especially because the number of lincRNAs in all other conditions appears to be similar. It appears that upon WT infection the number of polysomal lincRNAs is larger only by 6 genes after 2 h, when compared to 5 h POI. Upon ΔLLO infection I observed an increase of polysomal lincRNAs from 70 at 2 h to 109 at 5 h POI; however, it appears that upon ΔLLO infection more lincRNAs associate with polysomes at 5 h POI than upon WT infection, whereas in the

transcriptome DEGs I observed a similar number of lincRNAs upon ΔLLO at 5 h POI and upon WT infection at 2 h and 5 h POI. Furthermore, by comparing the transcriptome and translatome DEGs in each condition, I observed that the vast majority of transcribed lincRNAs upon infection are likely uploaded on polysomes; for example, upon WT infection, at 2 h POI 124 lincRNAs are transcribed and 86 are then found associated with polysomes.

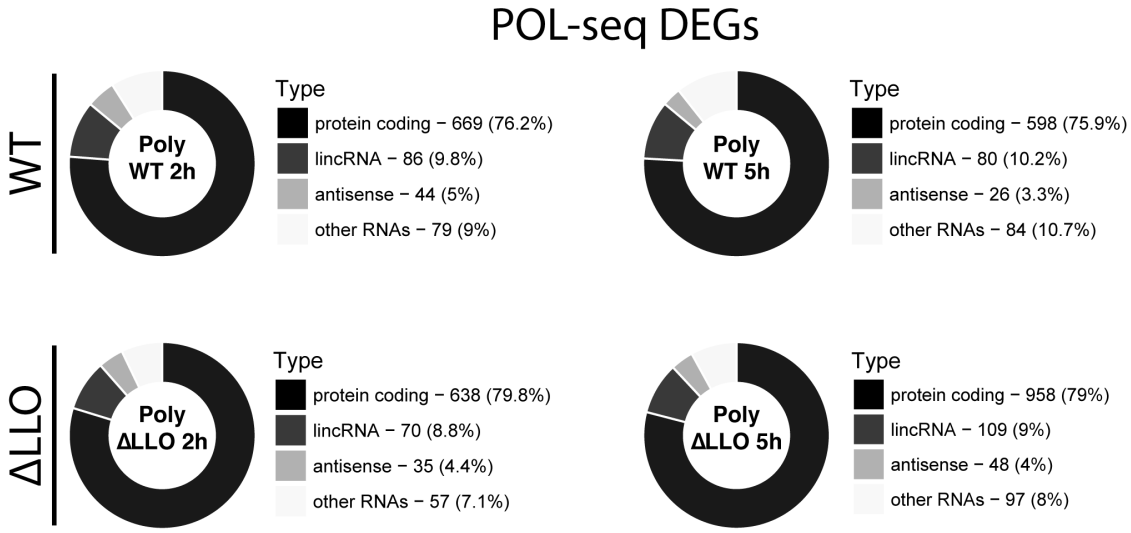


*Figure 3.14:* ***Classes of DEGs in the translatome upon WT and ΔLLO infection.***
*Doughnut charts representing the fractions of different classes of polysome-associated DEGs (Poly) upon either WT or ΔLLO infection, 2 h and 5 h POI. On the right side of each chart are the types, the number and the percentage of DEGs found at each condition GO based on DEGs with log2 FC > 1.*

Given the fact that lincRNAs emerged as an unexpectedly abundant group of polysomal RNAs and the fact that their function is mostly unknown, especially in host-pathogen studies, I decided to produce a scatter plot, comparing differentially expressed polysome-associated lincRNAs upon WT and ΔLLO infected cells at 2 h and 5 h POI to choose lincRNAs that are infection specific or strain specific (**Fig. 3.15**). Among the most differentially expressed lincRNAs, I selected two that were either significantly up- or down-regulated upon infection (AC016831.1 and AC006552.1, respectively), a lincRNA with minimal change in expression upon infection (LINC01558), and a lincRNA which appeared to be upregulated only upon WT infection (MIR181A1HG).

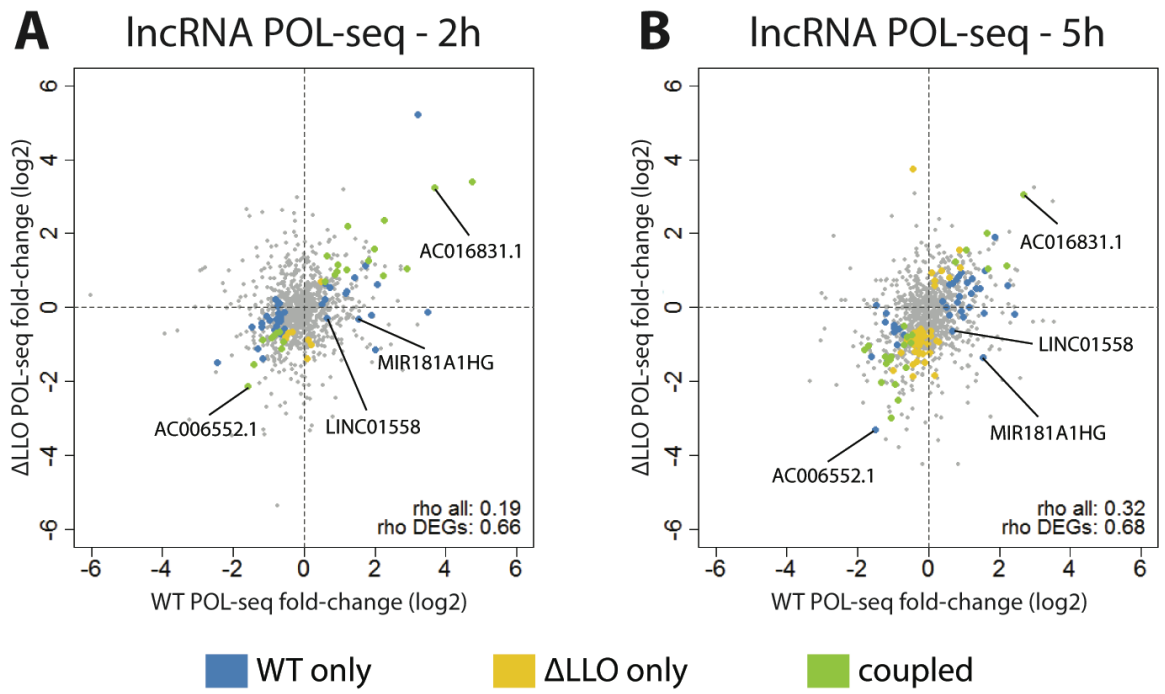


*Figure 3.15:* ***Selection of differentially expressed lincRNAs.***

*Scatter plots comparing polysome-associated lincRNA DEGs upon infection with WT and ΔLLO Listeria after (**A**) 2 h and (**B**) 5 h. The selected lncRNAs are pointed out on the plot.*

The expression of the selected lincRNAs and their association with polysomes was validated by RT-qPCR. For the selected lincRNAs, the transcriptome and the translatome are coupled, meaning that when these lncRNAs are transcribed they are also transported to the cytoplasm and immediately associated with polysomes (**Fig. 3.16**).

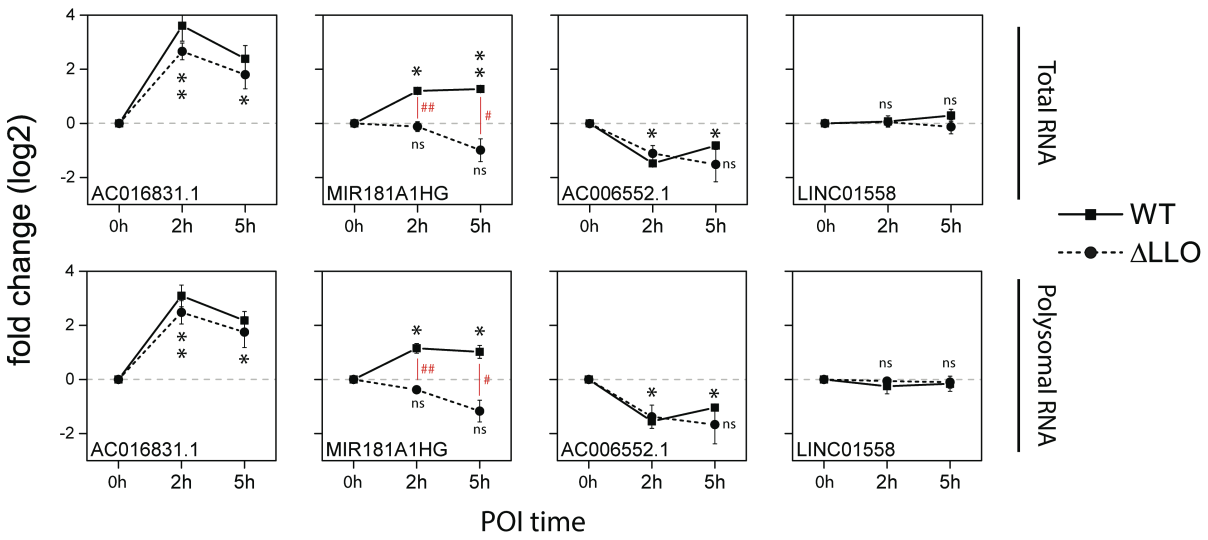

*Figure 3.16:* ***lncRNA is expressed upon infection as a result of the presence of LLO.***
*Plots showing differential expression in the transcriptome (RNA-seq) and the translatome (POL-seq) of the selected lncRNAs upon infection with either WT or ΔLLO at 2 h or 5 h poi. All experiments were performed with at least biological triplicates. Significant changes between samples are represented by T-test P-value: ns > 0.05; * < 0.05; ** < 0.01; # < 0.05; ## < 0.01.*

The lincRNA AC016831.1 showed a significant upregulation upon infection with either strains, at both time-points. The same result was obtained for AC006552.1, except it is downregulated upon infection; and LINC01558 which in this case is an example of a lincRNA whose expression is not altered upon infection. Probably the most interesting lincRNA is MIR181A1HG which was significantly upregulated upon infection with WT and not changing or even downregulated upon ΔLLO infection. This differential expression at both time-points upon infection demonstrates that MIR181A1HG exhibits a strain specific response, i.e. a virulent factor-specific response.

### 3.4.2 Co-expression analysis highlights association of infection-induced lincRNAs with specific response to bacterial infection

In order to understand a possible function of the selected lincRNAs with respect to other differentially expressed genes, I looked at which mRNAs are co-expressed upon infection.

The idea behind this method, known as "guilt-by-association"[248] is that genes with similar expression profiles could possibly be involved in similar processes upon infection. For that reason, I explored which mRNAs cluster together with our selected lincRNAs, and used Gene Ontology (GO) to unravel possible relevant biological processes they are involved in (**Fig. 3.17**).

Among the 16 different clusters showing coupled changes in the transcriptome or in the translatome and upon WT and ΔLLO infection, the biggest one was Cluster #1. In this cluster, DEGs exhibited a general upregulation, peaking at 2 h POI, showing that in our case cells responded stronger to infection at the earlier time-point POI (2 h) than at a later one (5 h). Cluster #1 contained 925 genes, which accounts for 25 % of all host cell DEGs upon infection

and among them was also the lincRNA AC016831.1 that I found strongly upregulated upon infection at both time-points and with either strain.

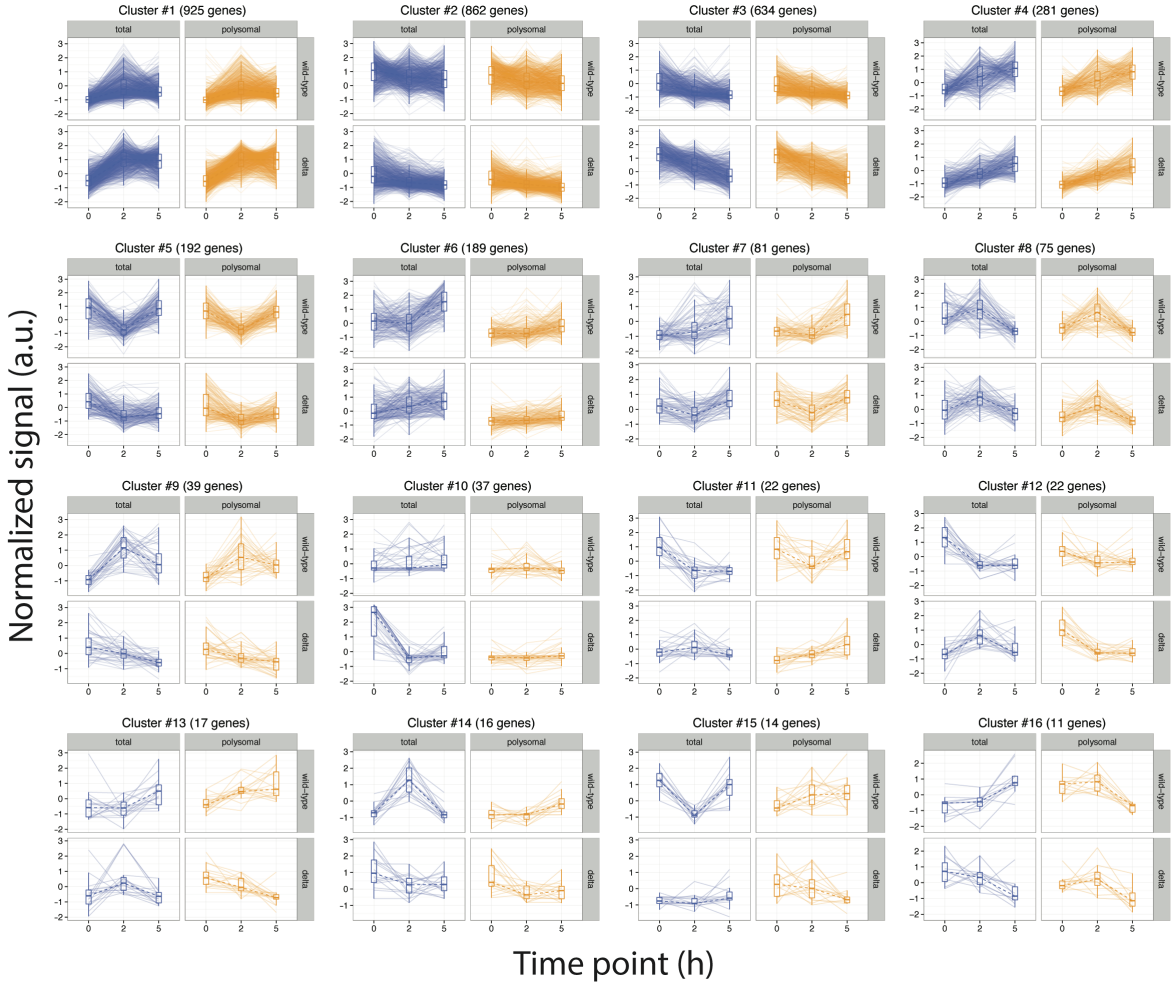


*Figure 3.17:* ***Clustering of DEGs based on their expression profile.***

*Panel of 16 distinct clusters containing DEGs with a similar expression profiles upon infection. In each cluster are represented the transcriptional (total) and translational (polysomal) trends of DEGs upon infection with either WT or ΔLLO, at 2 h and 5 h. The clusters are sorted according to the number of genes they contain.*

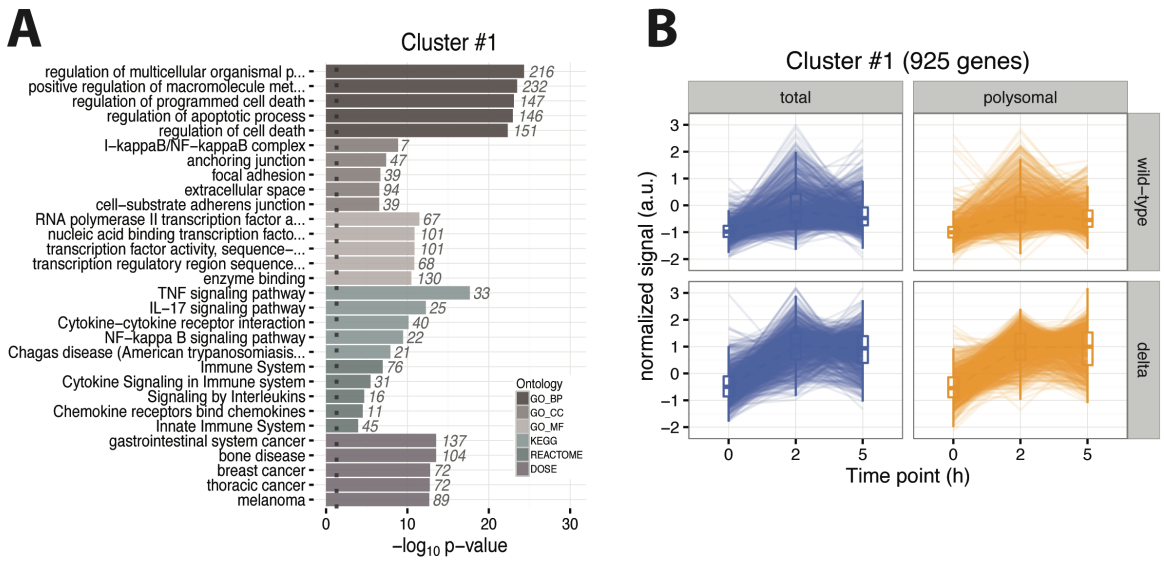


*Figure 3.18:* ***DEGs with a similar expression as AC016831.1. mainly involved in the innate immune response.***

*(**A**) Geno ontology analysis on transcripts with a similar expression profile as the lincRNA AC016831.1. 925 genes were used to build the different groups corresponding to terms of the ontology. (**B**) Expression profiles of all 925 genes stacked on top of one another, showing a general upregulated trend.*

Gene Ontology (GO) enrichment of Cluster #1 shows that these genes are mainly involved in the innate immune response (**Fig. 3.18**) and suggests that AC016831.1 could be involved in the innate immune response as other protein-coding genes from this cluster.

To further confirm this observation, I selected two most upregulated genes in this cluster, the chemokine CXCL8 and the cytokine CCL20, in order to validate their trend by qPCR. Both CXCL8[93] (also known as interleukin 8) and CCL20[249] are well known to be upregulated upon *Listeria* infection. The results show a strong coupling between the transcriptome and the translatome and a significant upregulation of all selected genes upon infection with either strain (**Fig. 3.19**). This confirmed a similar trend of behaviour as the lincRNA AC016831.1, suggesting its involvement in similar processes.

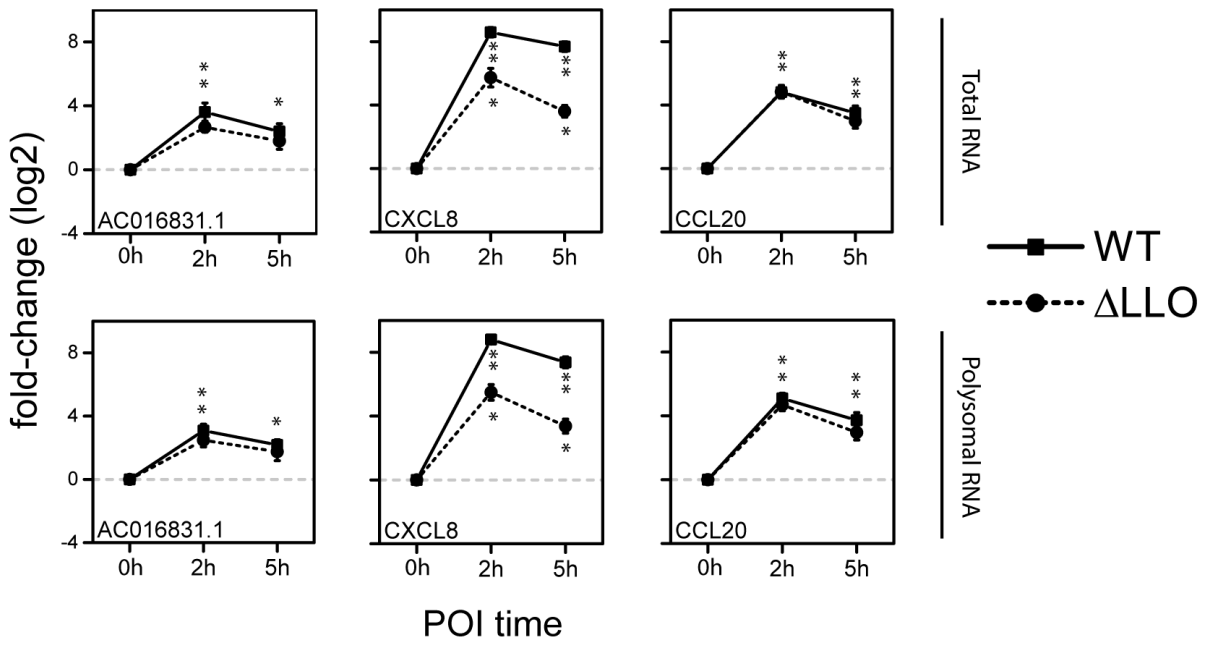


*Figure 3.19:* ***Innate immune related genes have a similar expression profile as lincRNA AC016831.1.***

*Expression of cytoplasmic (Total RNA) and polysome-associated (Polysomal RNA) RNAs, selected from Cluster #1. All experiments were performed with at least biological triplicates. Significant changes between samples are represented by T-test P-value: ns > 0.05; * < 0.05; ** < 0.01.*

Among the clusters containing genes with a different behaviour upon either WT or ΔLLO infection, the biggest one with 39 genes was Cluster #9, which also contained the lincRNA MIR181A1HG. Interestingly, despite the low number of genes comprised in this group, the GO enrichments are significant and contain terms related to vesicular transport (**Fig. 3.20**).

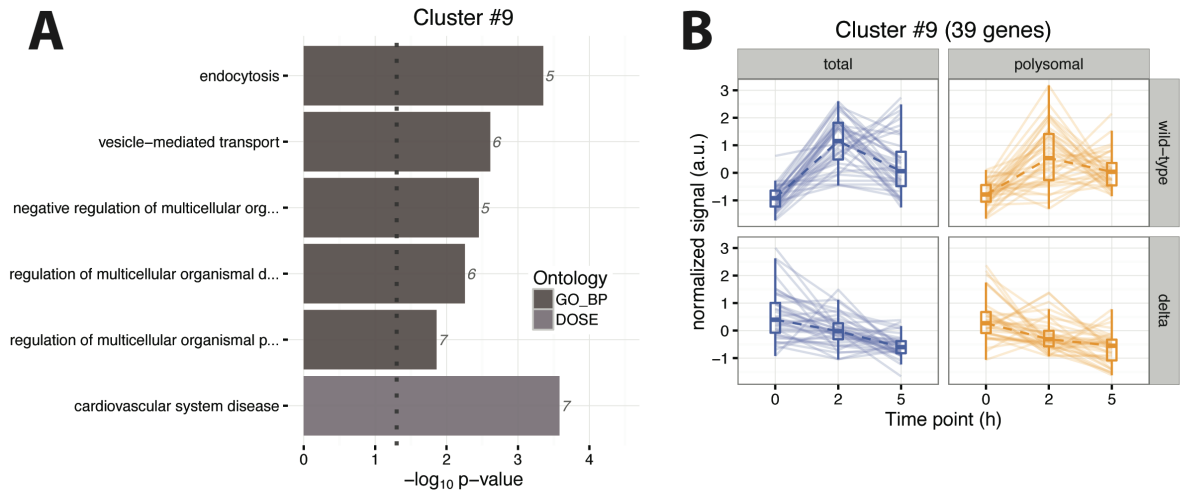


*Figure 3.20:* ***DEGs with a similar expression as MIR181A1HG are mainly involved in vesicle-mediated transport.***

*(**A**)Geno ontology analysis on transcripts with a similar expression profile as the lncRNA MIR181A1HG. 39 genes were used to build the different groups corresponding to terms of the ontology. (**B**) Expression profiles of the 39 genes stacked on top of one another, showing an upregulation upon WT and a downregulation upon ΔLLO infection.*

Summarising the results of this part, I found that, comparing and analysing both RNA-seq and POL-seq of cells infected with WT and ΔLLO strain of *Listeria* and at two time-points POI, host cells express a number of lincRNAs, which then associate with polysomes.

I was able to identify a polysome-associated lincRNA AC016831.1, which was significantly upregulated upon both WT and ΔLLO infection and at both time-points POI. I showed that around 25 % of all DEGs have a similar expression profile and the GO enrichment analysis of these co-expressed RNAs suggests its involvement in the innate immune response upon bacterial infection.

Moreover, I also identified a lincRNA MIR181A1HG, that is significantly upregulated exclusively upon WT infection. It is co-expressed with 38 other transcripts involved in vesicular transport. The results I obtained thus far suggest that the expression of MIR181A1HG might be induced by LLO. Hence, the fact that co-expressed mRNAs are involved in vesicular transport further suggests this hypothesis, given that LLO mainly interact with lipid membranes[49] and was even demonstrated to prevent the fusion of lysosomes with the endocytic vacuole in mammalian cells[111,250].

## 3.5 Protein coding potential of polysome-associated long non-coding RNAs

The data so far showed that lincRNAs indeed associate with polysomes upon bacterial infection; however, their function and mechanism remain completely unknown, despite the above-mentioned co-expression analysis. One possibility that needs to be considered is that these lncRNAs are in fact engaged by the ribosome and could be even producing short peptides. lncRNAs are by definition putative not coding for any protein; however, this assumption is heavily based on their lack of an ORF with at least 300 nucleotides in length[147]. Since our two upregulated lincRNAs do associate with polysomes the first assumption was that they could be engaged by the ribosome as other mRNAs.

To address this, I considered some characteristics from their sequence that may guide additional hypotheses. To understand whether they are associated with small or large polysomes, I then analysed in greater detail their association via polysome profiling, by studying their sedimentation along the sucrose gradient. Next, I assessed whether they are weakly associated to polysomes, as translation factors, or tightly associated to ribosomes as mRNAs. This approach allowed me to get some hints about the nature of their association with the translation machinery.

### 3.5.1 Bioinformatics evidence

I used the currently available online tools, which either predict or search through databases for certain features and interactions of lncRNAs, to get a better idea about the possible function of the two most interesting lincRNAs found to be uploaded on polysomes upon infection, MIR181A1HG and AC016831.1. I focused on the two largest isoforms of each lincRNA, since our NGS data shows that these isoforms are those predominantly expressed.

Concerning the ORF prediction, I used NCBI's ORFfinder, an online available tool for predicting potential ORFs and examined whether MIR181A1HG and AC016831.1 are predicted for potential ORFs with, with a minimal ORF length of 30 nt and an "AUG" start codon.

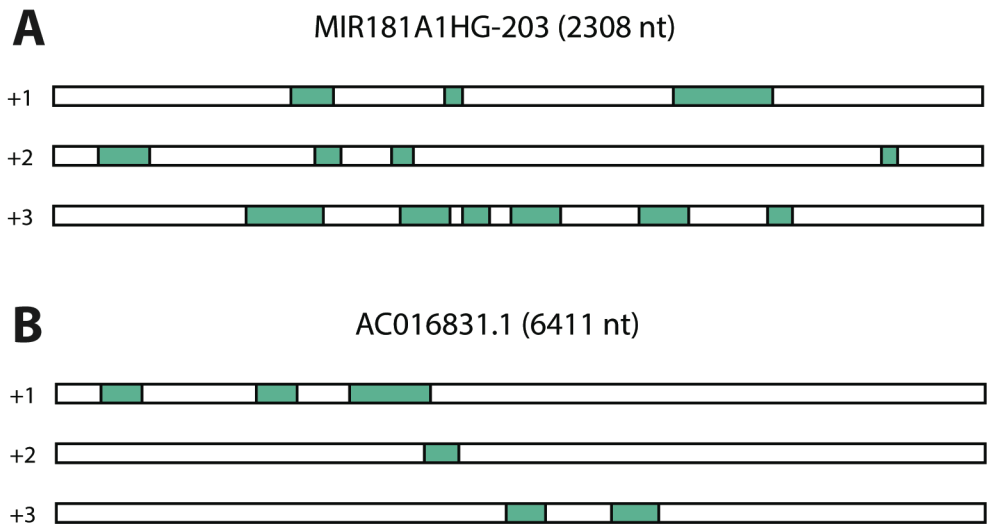


*Figure 3.21:* ***Predicted ORFs for MIR181A1HG and AC016831.1.***
*(A) Position and length of all predicted ORFs on MIR181A1HG, larger then 30 nt. (B) Position and length of the biggest 6 predicted ORFs, larger than 200 nt.*

The longest isoform of MIR181A1HG (2308 nt) was predicted to have altogether 13 ORFs on its positive strand. The biggest one was a 249 nt long ORF in the +1 frame and the average size of all predicted ORFs is around 105 nt (**Fig. 3.21A**). In the case of the longest isoform of AC016831.1 (6411 nt) the total number of ORFs was predicted at 56 on the positive strand and shown in (**Fig. 3.21B**) are only the 6 biggest that are at least 200 nt long. The biggest ORF was found in +1 frame with 519 nt and the second biggest in the +3 frame containing 315 nt. This lincRNA indeed has many predicted small ORFs, with an average size around 95 nt; however, it also has two large ORFs that could potentially be coding, considering the most general classification is based on the ORF length.

Considering the number of exons, I found that in all cases, the value is low, which is a well-known characteristic for defining a transcript a non-coding (**Table 3.1**).

***Table 3.1**: Details of the analysed lincRNAs transcripts and their fold-change expression value upon infection, obtained by qPCR.*

| Gene name | Number of transcripts | Length of longest transcript (nt) | Number of exons | POL-seq qPCR FC (log2) | |
|---|---|---|---|---|---|
| | | | | WT 2h | ΔLLO 2h |
| MIR181A1HG | 3 | 2308 | 2 | 0,95 | 1,01 |
| AC016831.1 | 3 | 6411 | 4 | 3,45 | 2,56 |
| AC006552.1 | 1 | 2216 | 2 | -1,55 | -1,04 |
| LINC01558 | 2 | 1652 | 4 | -0,25 | -0,16 |

Finally, also conservation is a parameter that is used to obtain insight into the protein coding hypothesis. In fact, high conservation is an indication of protein coding potential.

I used an online tool AnnoLnc which "enables a systematic annotation covering genomic location, secondary structure, expression patterns, transcriptional regulation, miRNA interaction, protein interaction, genetic association and evolution"[248].

lncRNAs are known to have sequences with a low evolutionary conservation[154], which can be represented with by the PhyloP score, which is a measure of conservation for every base. Predictions for MIR181A1HG (**Fig. 3.22A**) and AC016831.1 (**Fig. 3.22B**), showed a low level of exon conservation and a slightly higher level of promoter conservation within primates, mammals and vertebrates.

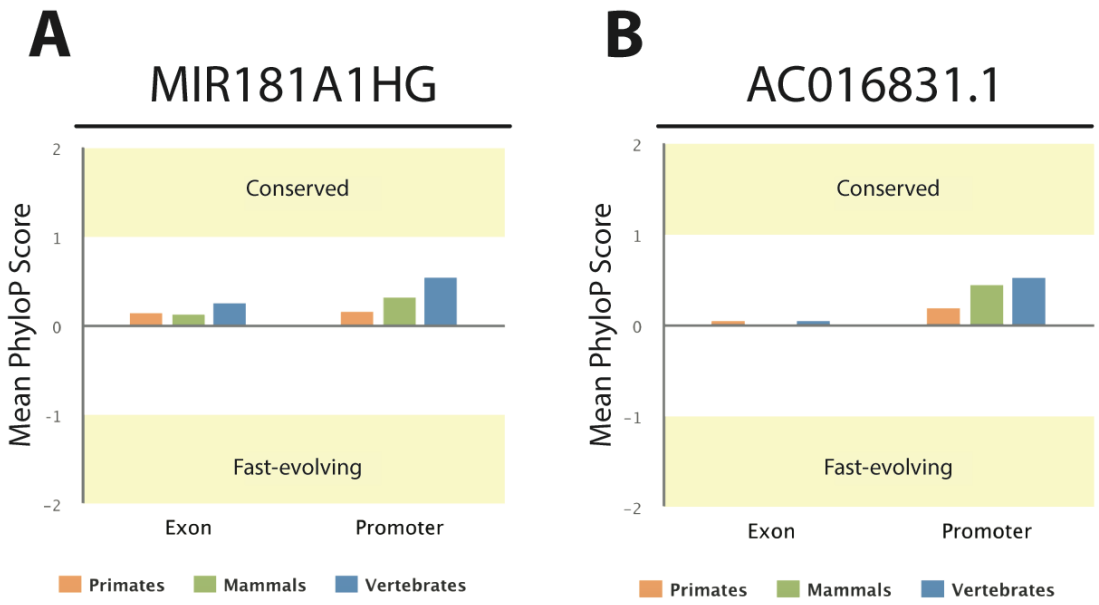


*Figure 3.22:* ***lncRNAs have a low level of conservation.***

*Predicted conservation scores of lncRNAs, represented by PhyloP score. Showing the mean PhyloP scores of (**A**) MIR181A1HG and (**B**) AC016831.1 exon and promoter regions in primates, mammals and vertebrates. A positive PhyloP score suggests transcript conservation (> 1) and a negative score suggests that the transcript is fast-evolving (< -1).*

In conclusion, by using bioinformatics analyses I found that these lncRNAs are *bona fide* long-intergenic RNAs, whose low conservation and low number of exons suggest that the probability of coding potential is low. The putative ORFs, nonetheless leave open the hypothesis that they might be bound by a small number of ribosomes to produce very short peptides.

### 3.5.2 Experimental evidence: lncRNAs strongly associate with small polysomes

Given the fact that the abovementioned indication from bioinformatics analysis of the two selected lincRNAs, where not conclusive, I search for experimental evidences supporting or disproving their putative protein coding role. An important piece of information that can help to understand their role in translation is knowing if they associate to small or large polysomes

and with what strength. In order to answer these questions, I first extracted RNA from individual fractions along polysomal profiles to observe the co-sedimentation with small or large polysomes. Next, to determine the strength of the lncRNA-ribosome interaction, the cellular lysates were treated with a high salt concentration before ultracentrifugation, to dissociate weakly bound molecules, such as translational factors[224,225].

First, by looking at the lincRNA co-sedimentation in non-infected cells and cells upon WT infection 2 h POI, I demonstrated that all analysed lncRNAs primarily associated with light polysomes (i.e. 2-3 ribosomes) (**Fig. 3.23**), while the mRNA GAPDH is mainly associated to large polysomes (i.e. ≥ 4 ribosomes) and the 40S subunit of the ribosome. Interestingly, this result is consistent with the putative small ORF prediction. Even though AC006552.1 is overall down-regulated upon infection, it was observed to co-sediment with the 40S upon WT infection 2 h POI. Surprisingly, MIR181A1HG also shows a co-sedimentation with the small 40S ribosomal subunit in both condition, suggesting that it might play a role in translation initiation or it could be associated to the 48S initiation complex as mRNAs.

Comparing the control polysomal profile (**Fig. 3.24A**) with the polysomal profile upon salt wash (**Fig. 3.24B**), that removes molecules loosely-bound to the ribosomes, the first observation was that in high salt conditions the polysomal profile is globally shifted towards lighter sedimentations values, i.e. towards lower sucrose concentrations along the profile. This is compatible with the effect exerted by the treatment, which indeed dissociated weakly bound molecules, producing lighter polysomes.

To validate the removal of loosely bound molecules from polysomes in the salt-treated samples, I extracted proteins from individual fraction and used WB to determine the location of a marker for the ribosomal protein RPL26, a protein belonging to the 60S ribosomal subunit, the Poly(A)-binding protein (PABP) and the initiation factor eIF2α.

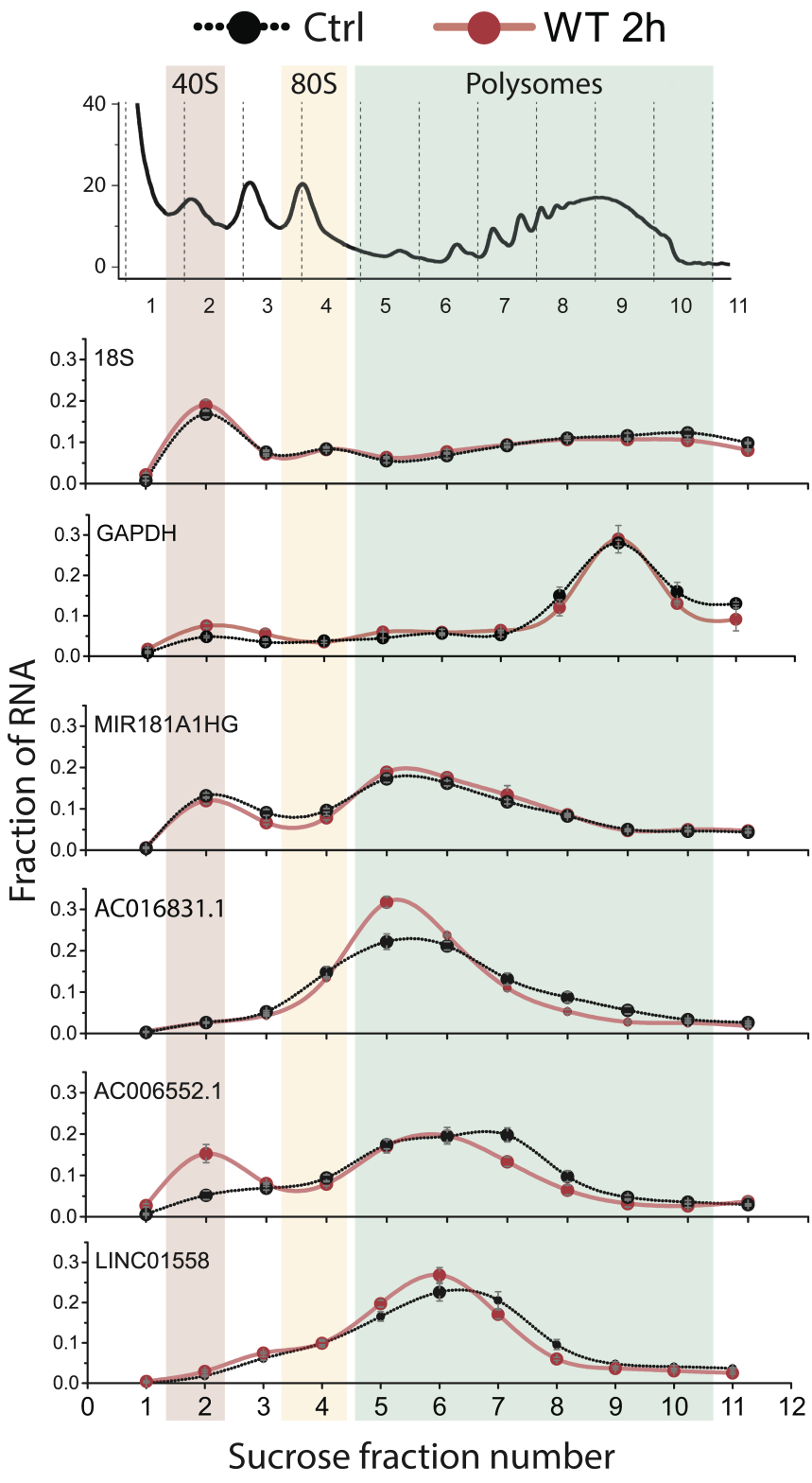


*Figure 3.23:* ***Differentially expressed lncRNAs upon infection are uploaded mainly on small polysomes.***

*Showing the relative distribution of individual lncRNAs along the sucrose gradient. The different coloured strips show which fractions correspond to the 40S (brown), the 80S (yellow) and polysomes (green). All experiments were performed in biological duplicates.*

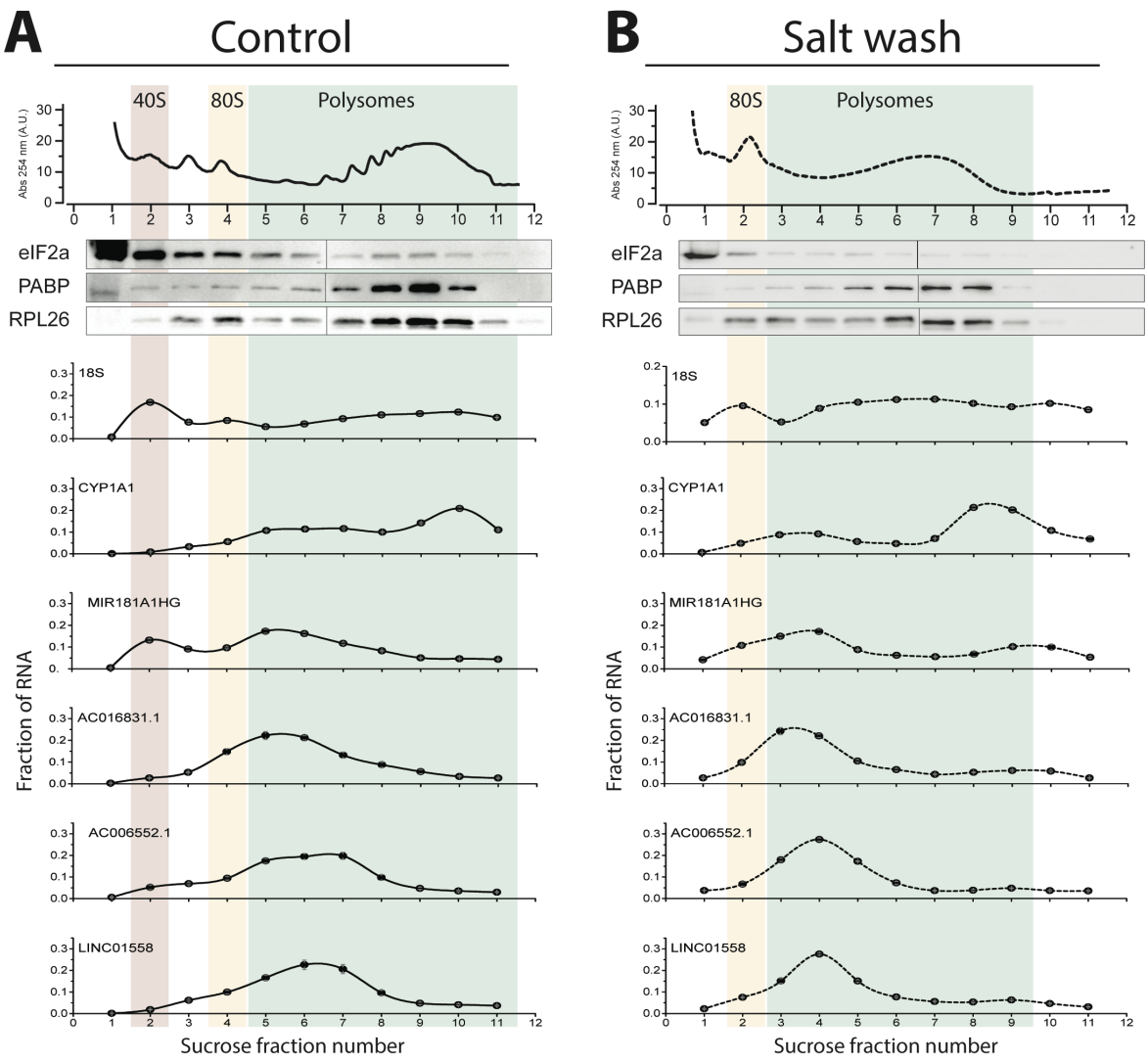


*Figure 3.24:* ***lincRNAs are strongly bound to small polysomes.***

*Showing the relative distribution of individual lncRNAs along the sucrose gradient of (**A**) non-treated cell lysates (Control) and (**B**) cell lysates upon high salt treatment (Salt wash). For this purpose, only non-infected cells were used, since I previously demonstrated that upon infection, the distribution or RNAs along the profile does not change. WBs of proteins (eIF2α, PABP and RPL26) extracted from individual fractions show their movements upon salt treatment. The different coloured strips show which fractions correspond to the 40S (brown), the 80S (yellow) and polysomes (green). The co-sedimentation profile of CYP1A1 serves as an mRNA reference. All experiments were performed in biological duplicates.*

I showed that the polysomal fractions in the control samples start with fraction number 5, while in the salt-treated samples the polysome fractions start with fraction number 3. As expected, the high salt treatment indeed removed weakly bound molecules such as the initiation factor eIF2α, which moved from polysomes to mostly the first fraction upon salt treatment. However, PABP that strongly interact with polysomes remains bound even upon salt treatment.

Next, I checked whether our lncRNAs dissociate or remain on polysomes upon salt wash. The co-sedimentation profile of the 18S rRNA, a component of the 40S ribosomal subunit, was used as a control for the sedimentation of the 40S, ribosomes and polysomes. The lincRNAs after the high salt wash shows that they are still co-sedimenting with fractions corresponding to small polysomes. The reference protein-coding RNA CYP1A1 remains bound to large polysomes. Upon salt treatment, MIR181A1HG also displayed a localization at the end of the gradient, suggesting it might interact also with other very heavy cellular structures such as pseudopolysomes[251].

### 3.5.3 Experimental evidence: lncRNAs might be involved in active translation

To determine whether the studied lncRNAs are involved in active translation or are possibly associated with stalled polysomes, I treated the cells with puromycin prior to polysome purification. Puromycin is a commonly used translation inhibitor which causes the dissociation of ribosomes into ribosomal subunits, by interfering with the elongation step of translation, thus affecting only active polysomes[252].

Comparing the polysomal profiles of puromycin-treated and non-treated cells (**Fig. 3.25**) after infection with WT *Listeria*, I observed a strong decrease in polysomes and an increase in the 80S, showing that puromycin indeed disrupted active polysomes.

The results I obtained by comparing the distribution of RNA along the polysomal profile of non-treated and puromycin-treated cells, suggest that only AC016831.1 is in fact associated with translationally active polysomes, since I observed a clear movement from light polysomes to the 80S upon treatment. All other lncRNAs, including MIR181A1HG, remained

associated with polysomes, suggesting they are either bound by stalled ribosomes or associated with silent polysomes in a non-conventional way.

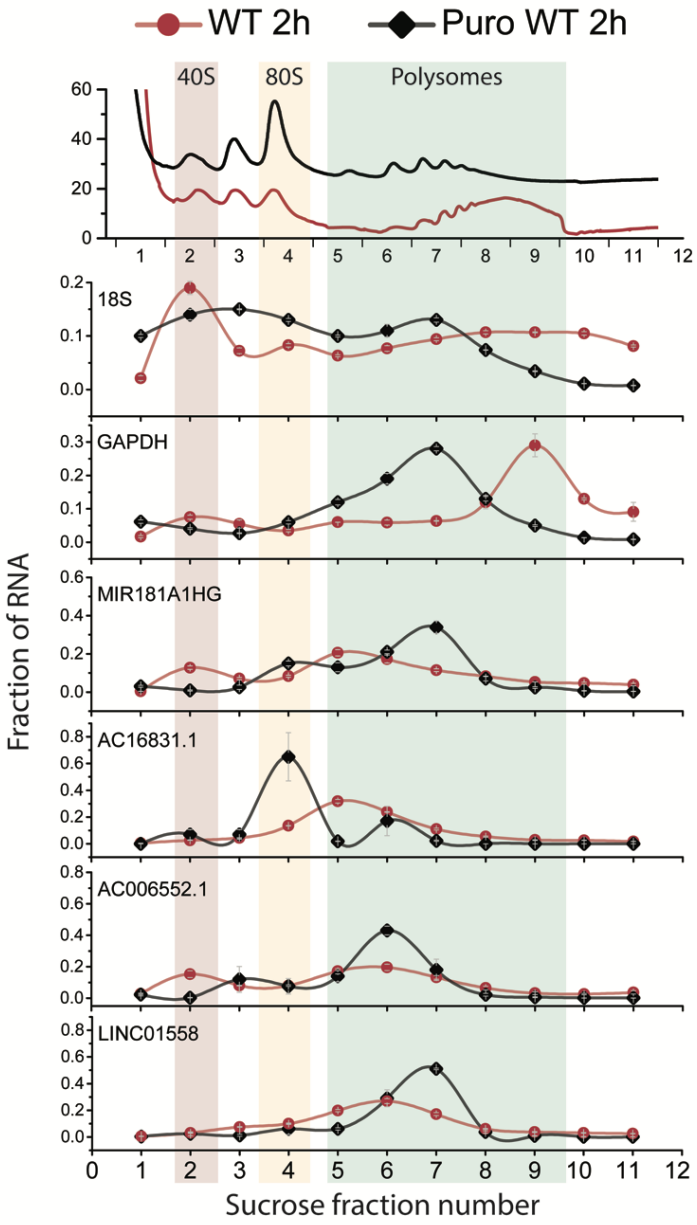


*Figure 3.25:* ***lincRNAs might be involved in active translation.***

*Showing the relative distribution of individual lncRNAs along the sucrose gradient of WT-infected cells 2 h POI and Puromycin-treated WT-infected cells 2 h POI. The different coloured strips show which fractions correspond to the 40S (brown), the 80S (yellow) and polysomes (green). The co-sedimentation profile of GAPDH serves as an mRNA reference. All experiments were performed with biological duplicates.*

These results are suggesting that it is unlikely that MIR181A1HG has a protein coding function. Indeed, its association to puromycin insensitive polysomes seems to suggest a possible role as a ribosome sponge.

## 3.6 MIR181A1HG is a pathogen and strain-specific polysome-associated lincRNA with host protective functions

I demonstrated that all selected lincRNAs associates with polysomes and that MIR181A1HG is expressed in a strain-specific manner, where the only difference was the presence or absence of the virulent factor LLO. At this point I also wanted to answer the question whether some of the selected lincRNAs exhibit a similar behaviour when host cells are infected with a different intracellular pathogen and exposed to a different virulent factor. This could help us to understand whether the lncRNAs in question are either general responders upon bacterial infection, or pathogen-specific responders.

### 3.6.1 MIR181A1HG is a pathogen-specific lncRNA

For this purpose, I used the same infection protocol and infected host cells (Caco-2) with two strains of another widely used intracellular pathogen, *Salmonella enterica*. One strain expresses the active typhoid toxin (TT), which is one of *Salmonella*'s main virulent factors and is known to cause cellular distension and DNA damage, the other strain expresses an inactive typhoid toxin (ΔcdtB). The experimental collection of polysomal lysates of Caco-2 cells infected with *Salmonella* was performed in collaboration with Dr. Teresa Frisan's research group at Karolinska Institutet during my period abroad.

To understand if these lincRNAs are general responders of infection, or *Listeria*-specific responders, I performed polysome profiling on Caco2 cells infected with *Salmonella* strains, observing that translation is affected upon infection with both *Salmonella* strains (**Fig. 3.26A)**; however, not nearly as much as upon *Listeria* infection (**Fig. 3.26B)**.

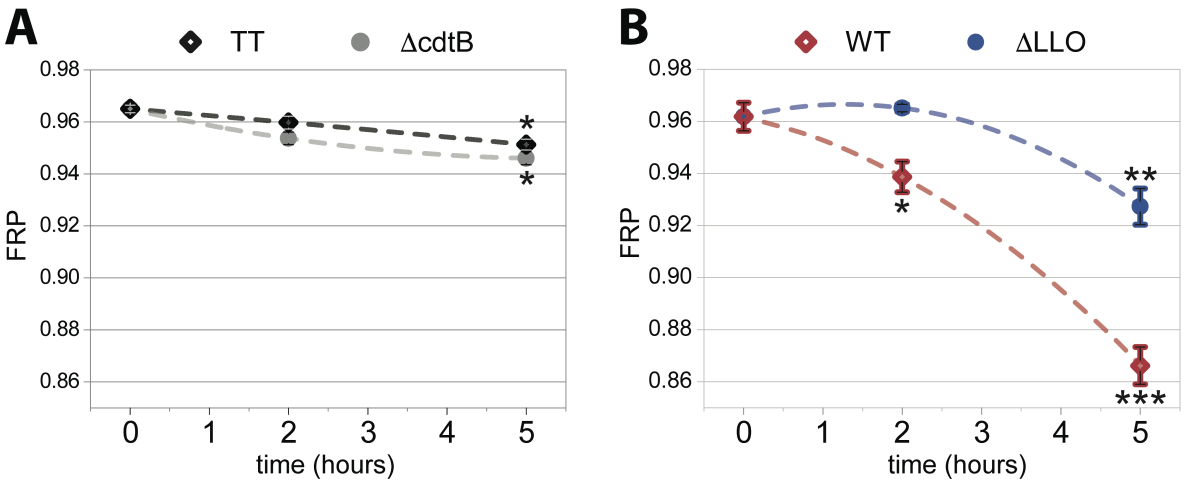


*Figure 3.26:* ***Salmonella induces a much smaller impairment in translation as Listeria.*** *Comparison of FRP upon infection with (**A**) Salmonella TT or ΔcdtB strain and after 2 h or 5 h POI and (**B**) Listeria WT or ΔLLO strain and after 2 h or 5 h POI. All experiments were performed in biological triplicates. Significant changes between samples are represented by T-test P-value: ns > 0.05; * < 0.05; ** < 0.01; *** < 0.001.*

Next, I extracted polysome-associated RNA and performed qPCR on the selected lincRNAs. The results show a different expression profile after *Salmonella* infection for the lncRNAs selected upon *Listeria* infection (**Fig. 3.27**). Interestingly, the lincRNA MIR181A1HG exhibits with *Salmonella* a behaviour similar to those observed with ΔLLO strain of *Listeria*, demonstrating that this lincRNA is up-regulated and uploaded on polysomes only as a result of infection with an LLO producing *Listeria*. And that this lincRNAs is not only virulent factor-specific but also pathogen specific.

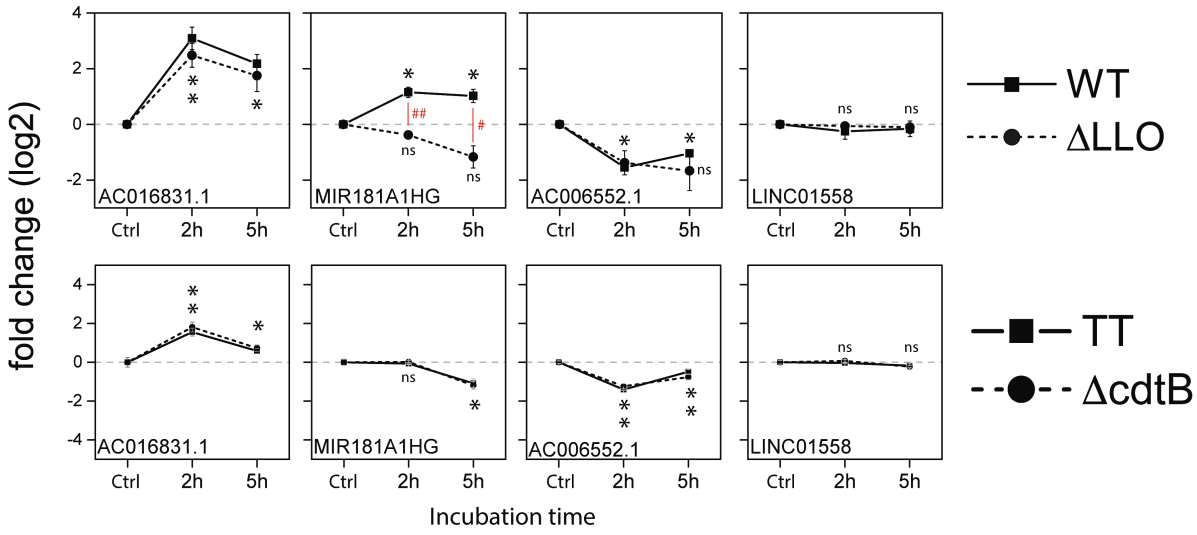


*Figure 3.27:* ***lncRNAs respond similarly when infected with a different intracellular pathogen.*** *Expression of polysome- associated differentially expressed lncRNAs upon infection with either Listeria (WT or ΔLLO) or Salmonella (TT or ΔcdtB) at 2 h or 5 h poi. All experiments were performed with at least biological triplicates. Significant changes between samples are represented by T-test P-value: ns > 0.05; * < 0.05; ** < 0.01; # < 0.05; ## < 0.01.*

### 3.6.2 Loss of MIR181A1HG expression is beneficial for *Listeria* replication in human cells

Considering the results I obtained up until now, the lincRNAs AC016831.1 and MIR181A1HG might be upregulated to either favour host or bacteria survival, suggesting a possible functional role for these lincRNAs in host-pathogen interaction. Therefore, I tried to silence the two upregulated lincRNAs (the virulent factor specific MIR181A1HG and AC016831.1) during infection with both *Listeria* strains. I then monitored any changes in the invasion ability of the bacteria in host cells after lincRNA silencing. To reduce the amount of the two lncRNAs I used Exiqon's Antisense LNA GapmeRs, which are antisense oligonucleotides used for highly efficient inhibition of mRNAs and lncRNAs. For each lincRNA I designed three different siRNAs in order to include all isoforms and treated the cells for either 24 or 48 hours (**Fig. 3.28**). By comparing their expression from control and silenced cells, I demonstrated that I succeeded to reduce the overall amount of MIR181A1HG, whereas the attempt to reduce the amount of AC016831.1 actually resulted in an increase of it. This result possibly suggests that this lncRNA might play an important role

in the cell's sensing or replying to stress. Following these results, I decided to proceed with the 48 hours antisense siRNA treatment for MIR181A1HG where I obtained the desired reduction.

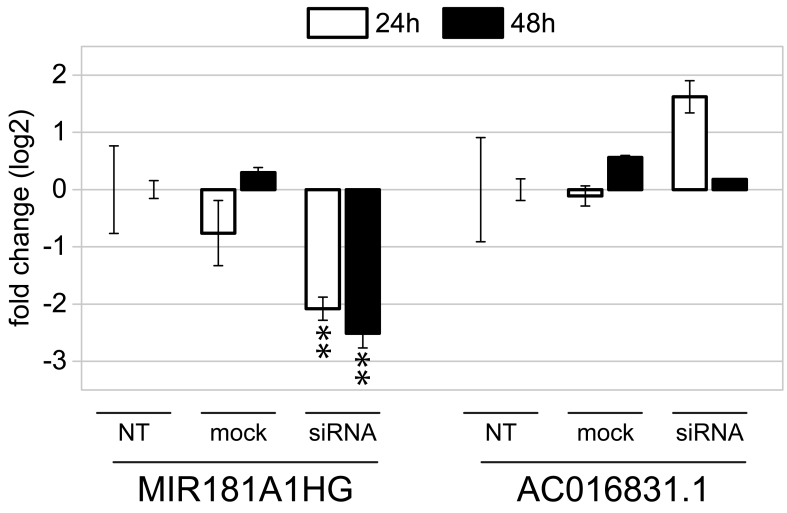


*Figure 3.28:* ***Cell treatment for inhibition of lncRNA function.***
*A) Quantification of MIR181A1HG (left) and AC016831.1 (right) in non-treated (NT), treated with a mock anti luciferase oligos (mock) and GapmeR anti MIR181A1HG oligos (siRNA), after either a 24- or 48-hour treatment. All experiments were performed in biological duplicates. Significant changes between samples are represented by T-test P-value: ns > 0.05; * < 0.05; ** < 0.01;*

To observe a possible difference in *Listeria*'s invasion and replication in antisense MIR181A1HG siRNA treated cells, I once again exploited fluorescence of the two strains used to count the number of bacteria per cell. I demonstrated that upon silencing the number of bacteria per cell significantly increases at both time-points only when cells were infected with WT *Listeria*, whereas infection with ΔLLO yielded no significant change (**Fig. 3.29A**). This result suggests that expression of MIR181A1HG, triggered by the presence of LLO, plays a protective role in host cells. In parallel, I monitored the global translational state of cells upon these silencing conditions. Result showed that the drop in FRP upon infection, as observed before, is similar in silenced and non-silenced cells; however, when MIR181A1HG is silenced and cells infected, the fraction of ribosome per polysome showed a robust decrease at both time-points only in the case of cells infected with the WT *Listeria* (**Fig. 29B**).

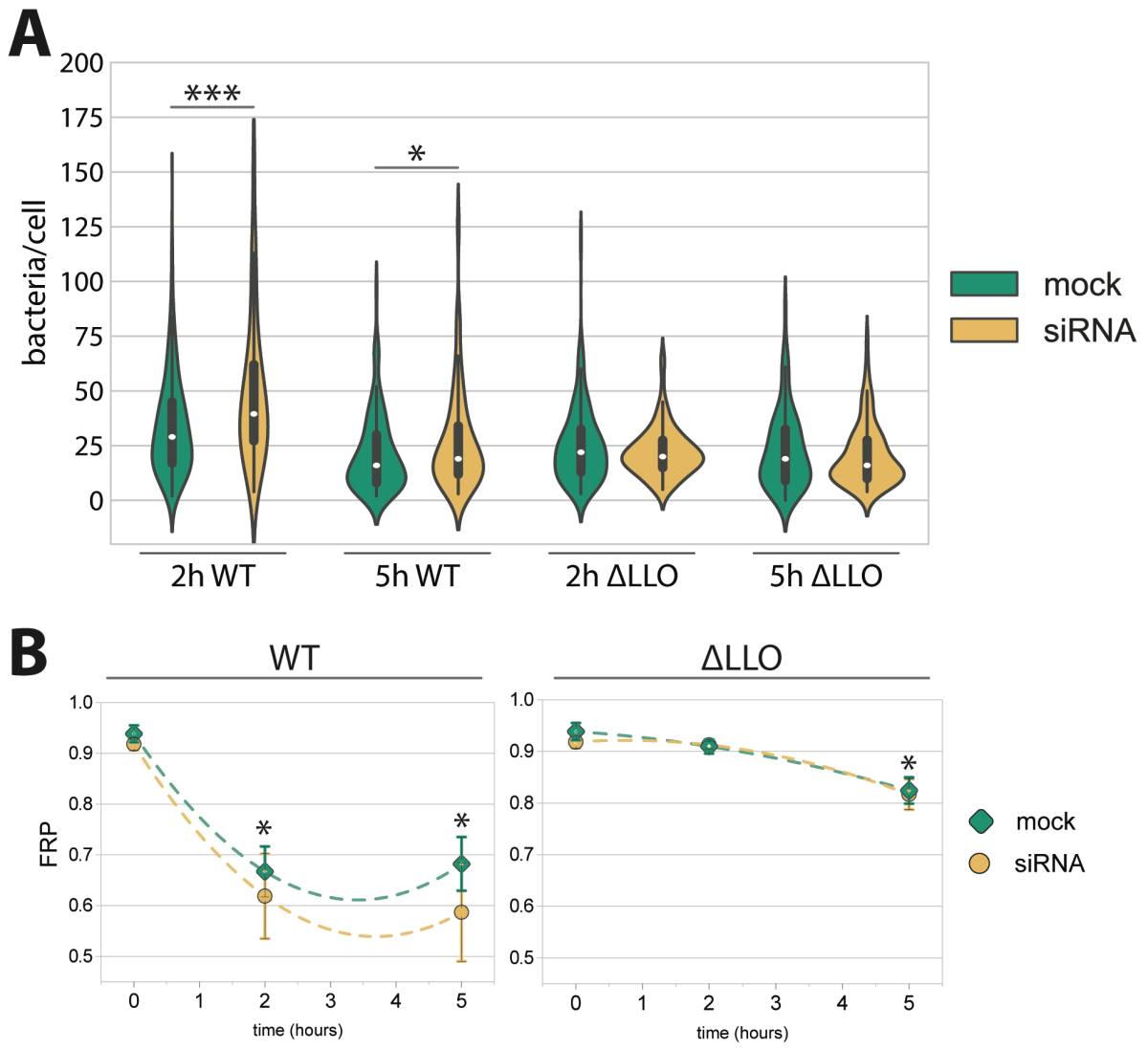


*Figure 3.29:* ***Reduction in the amount of MIR181A1HG upon infection significantly increases the amount of internalized WT bacteria.***

*(**A**) Violin plots of bacterial count using fluorescence microscopy in either silenced (siRNA) or non-silenced cells (mock, luciferase siRNA) infected with WT or ΔLLO strain at 2 h or 5 h poi. (**B**) FRP calculation of either silenced (siRNA) or non-silenced (mock) cells infected with WT or ΔLLO strain at 2 h or 5 h poi. All experiments were performed in biological triplicates. Significant changes between samples are represented by T-test P-value: * < 0.05; ** < 0.01; *** < 0.001.*

Summarising, I can conclude that cells express and upload MIR181A1HG on polysomes in order to regulate translation. The production of this lncRNAs is specific upon infection with WT *Listeria* and seems to promote host survival. Further studies are essential in order to determine its mechanism and exploit it to develop novel approaches to fend-off pathogens.

# 4 Discussion

In the past, lncRNAs were considered mostly as transcriptional noise and only recent developments in high-throughput genomic technologies such as Next Generation Sequencing (NGS), have enabled us to study their diverse mechanisms and functions. In recent years, numerous studies demonstrated that long non-coding RNAs in fact play key roles in the regulation of several cellular processes[144,162,204,253]. Despite the current interest and research done on lncRNAs, we still know surprisingly little about their function and whether or not they have any biological significance[254].

We know that they are predominantly enriched in the nucleus and mainly known to be involved in chromatin remodelling, either promoting[255] or inhibiting[168,169] transcription at targeted genomic positions. However, their functions probably extend beyond just the nucleus. In the cytoplasm lncRNAs are proposed to exert diverse functional roles, acting as signals, scaffolds or decoys, making at least some of them important post-transcriptional regulators[144,256]. They were shown to function as modulators of mRNA stability[158,159], by base pairing with specific motifs or indirectly as decoys by sequestering miRNAs that would otherwise induce mRNA degradation or translational inhibition[163]. They are able to regulate protein modification such as ubiquitination[175] and phosphorylation[174], by possibly acting as scaffolds, bringing relevant molecules in close proximity.

Surprisingly, lncRNAs have also recently been found to associate with polysomes[5,6], showing that they play a part in translation regulation and this is most probably their most mysterious role. Interestingly, some lncRNAs are in fact engaged by the ribosome as suggested by ribosome profiling data[3,4,6,247], which further blurs the line between coding and non-coding, i.e. between mRNAs and lncRNAs, presenting yet another challenge when investigating novel lncRNAs. However, the fact that we can find lncRNAs involved in multiple essential cellular processes, points to their importance in the cell.

It is known that bacterial pathogens interfere with expression of host non-coding RNAs in order to modulate the response to infection[201]. Indeed, the role of lncRNAs produced by the host during bacterial infection is currently very limited to only a handful of examples in regulation of gene expression via chromatin modulation[212,219]. Indeed, no research exist that connects them with translation regulation, which is a process known to be targeted by

pathogens[1]. This fact is particularly relevant, considering that only 2 % of the genome is coding for proteins[124] and that host-pathogen interaction studies have almost exclusively been exploring this minimal part of the host's genetic information as response to infection, while it was demonstrated about 75 % of the human genome is transcribed at some level[123,124].

To address this lack of information, I explored at a translational level the host cell's response to infection with an intracellular pathogen *Listeria monocytogenes.* Moreover, I also explored whether *Listeria*'s main virulent factor, the pore-forming toxin listeriolysin O (LLO), is able to trigger translational controls. To do that, I took advantage of a human epithelial cell line Caco-2, a widely-used model for *Listeria* infections[113,221,222] and two *Listeria* strains, a WT and an LLO-deficient strain for my experiments. To study translation during infection, I used polysome profiling of human cells which enabled me to get information on: i) the fraction of ribosomes in polysomes (FRP), that gives interesting insight into the general state of translation in cells, ii) isolate cytoplasmic and polysome-associate RNAs. I then performed transcriptome and translatome analysis of infected cells at different time-points post invasion (POI) and portray changes in total and polysomal RNA upon infection at a genome-wide level.

My data showed that infection induced expression of multiple lncRNAs which then associate with polysomes. Furthermore, I demonstrated that the presence of LLO during infection indeed induces a strain-specific expression of at least one lincRNA (MIR181A1HG). This seems to exert a protective role in host cells upon infection. Up until now, no research exists that addresses the issue of whether bacterial infections or bacterial virulent factors induce specific controls of translation. Therefore, this research is the first of its kind to tackle the question and potentially pave the way for deeper understanding as to how we can treat infections without the use of antibiotics.

A major part of my research was to answer whether *Listeria*'s LLO is able to induce translational controls and for that reason I used the ΔLLO alongside the WT strain in all experiments. The first difference I observed is that after infection the number of intracellular bacteria was significantly higher upon infection with WT, according to literature. In fact, LLO secreted by extracellular bacteria potentiates cell invasion, by inducing mobilization of extracellular $Ca^{2+}$ and activating downstream $Ca^{2+}$-dependent signalling[105].

Using polysome profiling and metabolic labelling of proteins, I observed the induction of translational defects in host cells already at 2 h POI in WT-infected cells and an even stronger impairment at 5 h POI. Infection with ΔLLO also induced a significant impairment in translation but only at the latter time-point. These finding show that in fact both strains affect translation, but in the absence of LLO this seems to be delayed. Using western blotting I showed that one reason for the global downregulation of translation upon infection was the activation of the PERK pathway, observed at both time-points POI and using both strains. The delayed effect observed upon ΔLLO infection could possibly be due to activity of *Listeria's* other virulent factors, such as the two membrane-active phospholipases C (PLC), which play a supporting role in bacterial escape[111]. It is known that LLO promotes *Listeria'*s escape from the vacuole and is essential for cell-to-cell spread[230]; however, PLC was found to be actually sufficient for bacterial escape from the primary vacuole, but with the absence of LLO the concentrations need to be higher[257–259]. A ΔLLO strain would therefor need more time to produce a sufficient amount of PLC to facilitate vacuolar escape, which could explain the delayed translational impairment upon ΔLLO infection when compared to WT.

Furthermore, LLO produced by extracellular bacteria was demonstrated to induce multiple host processes[56,72,73]. Therefore, I wondered if *Listeria* induces translation defects prior to host cell invasion, by secreting molecules that interact with the host, and whether LLO plays a role in this process. For this purpose, I treated cells with a medium containing either WT *Listeria*-released molecules (WT SUP) or ΔLLO *Listeria*-released molecules (ΔLLO SUP). Interestingly, I observed a strong translational impairment only in cells treated with WT SUP, suggesting that the presence of extracellular LLO indeed affects host translation, probably by activation of signalling pathways involved in translation regulation, such as PERK[45], NFκB[109] and MAPK[54,115]. The fact that the observed decrease in FRP was likely LLO dependent, was supported by the observation of the very same effect after treating cells with a recombinant LLO, which exerts translational defects at concentrations higher than 0.3 nM.

In order to characterize whether *Listeria* and LLO induce translational defects by triggering translational controls in host cells, I performed transcriptome (RNA-seq) and translatome (POL-seq) comparative analyses of either WT or ΔLLO infected cells at 2 h and 5 h POI. First, by comparing the transcriptome and translatome of infected cells, I observed that upon WT infection at both time-points POI, about 43 % of all DEGs are transcriptionally and

translationally coupled with the majority of changes occurring exclusively at a transcriptional or translational level. On the other hand, upon ΔLLO infection I observed a coupling between transcription and translation with 57 % of DEGs at 2 h POI and 54 % at 5 h POI changing at both levels. These results, indeed suggest that WT infection induces stronger translational control, because the majority of changes at the transcriptional level are not observed at the translational level. Interestingly, the overall amount of DEGs upon WT infection changed only from 1402 at 2 h POI to 1333 at 5 h POI, while upon ΔLLO infection the number of DEGs summed up to 805 at 2 h POI and then consistently increased to 1343 at 5 h POI. This suggests that upon ΔLLO infection host cells have most probably not yet fully responded to infection, given the massive increase of DEGs from 2 h to 5 h POI. This observation is in agreement with the fact that translation is not significantly impaired yet at 2 h POI upon ΔLLO infection, meaning that there must also be much less translational regulation present as a consequence of bacterial infection with this strain. Moreover, I also showed that 5 h POI the ΔLLO infection induces significant translational defects and indeed the global number of DEGs, as well as the number of "Polysomal-only" DEGs become comparable to those observed during the infection with the WT strain. On the contrary, the number of "Total-only" DEGs shows only a marginal increase from 2 h to 5 h POI. These findings suggest that translational controls upon bacterial infection modulate the interplay between transcription and translation, especially as an early response to WT infection. *In vivo* experiments demonstrated that the transcriptional response upon infection is in fact LLO-dependent. *Listeria* infections in mice demonstrated that the early transcriptional response of the host greatly differs when comparing infections with either *L. monocytogenes* WT or its ΔLLO mutant. Furthermore, the response upon ΔLLO mutant infection shares high similarity to an LLO-deficient *Listeria innocua* strain than to its isogenic *L. monocytogenes* WT strain[260].

To unravel a possible LLO specific rewiring of gene expression, I then focused on the translatomes of cells after WT and ΔLLO infection at 2 h and 5 h POI. The comparison at 2 h POI showed that the majority of DEGs associated with polysomes are in fact expressed as a consequence of WT infection and the GO analysis of these transcripts showed an enrichment in genes involved in cancer development. Research shows that some bacteria produce toxins that interfere with the host cell cycle by casing DNA damage and chronic infections with these bacteria were shown to cause cancer development[261,262]. This unexpected result could therefore potentially be caused by accumulated DNA damage within cells, since it was

demonstrated that *Listeria* induces a cell cycle delay via host DNA strand breaks[263] and uses LLO to dampen the DNA damage response in order to promote bacterial survival[113]. The smallest group at 2 h POI were DEGs expressed as a consequence of ΔLLO infection. GO analysis showed that these genes are mainly involved in the innate immune response, meaning that the lack of LLO upon infection with ΔLLO strain activates additional host defences against the invading pathogen.

Interestingly, the comparison at 5 h POI showed that ΔLLO-specific DEGs were enriched in terms related to the immune response, and WT-specific DEGs enriched in genes involved in cell growth and steroid biosynthesis. These finding are in line with previous findings, where *Listeria* was demonstrated to modulate cholesterol levels upon infection in order to prevent immune activation and to maintain a protected intracellular environment[264]. Numerous studies also showed that steroids interact with the cell membrane and membrane-bound receptors and can change its properties even activating signalling pathways such as MAPK[265–267]. Furthermore, LLO is a cholesterol-dependent cytolysin (CDC) and is able to form pores only in cholesterol-rich lipid membranes[49], opening the question for possible co-evolution of cellular and bacterial mechanisms and strategy of survival. Putting together all the pieces of evidence obtained from comparing the transcriptome and translatome of WT and ΔLLO infected cells at the two time-points, I can speculate that LLO can specifically modulate the host cell's innate immune response upon infection, in agreement with previous findings [106,268].

Looking at which classes of genes respond upon infection, I found lincRNAs can be detected as DEGs in the cytoplasmic transcriptomes and even, surprisingly, in translatomes with both strains. Interestingly enough, they are the second most abundant class after mRNAs. The observation that upon WT infection lincRNAs represent a bigger fraction of DEGs when compared to ΔLLO infection was intriguing. It is true that lncRNAs are classically thought to be enriched in the nucleus, but recent evidences demonstrated that they can be found also in the cytoplasm, where they possess mainly regulatory functions of different cell processes as previously discussed[131,158,163,174,175,187]. Very few studies demonstrated their association with ribosomes[3,4,191] and polysomes[5,6] and our study represents the first example for their involvement in translation upon bacterial infection, as I will discuss below.

With this in mind it was intriguing for me to search for some lncRNA that might regulate the host cell's response to infection. Moreover, what came as a surprise was to find a big fraction of DEG lincRNAs in the translatome, i.e. associated to polysomes. For example, in the transcriptome at 2 h POI 124 lincRNAs were differentially expressed upon WT infection and 99 upon ΔLLO infection, whereas in the translatome I found 86 upon WT infection and 70 upon ΔLLO infection. Comparing the translatomes of WT-infected and ΔLLO-infected cells at 2 h POI, I observed that in the case of WT infection, lincRNAs belonged to the second biggest group of DEG types, representing 9.8 % of DEGs upon infection, whereas in the case of ΔLLO lincRNAs represented 8.8 %. This raises the interesting hypothesis that the previously discussed translational defects at 2 h POI, might be associated in some way to the amount of polysome-associated differentially expressed lincRNAs.

In an attempt to predict in which cellular processes these differentially expressed lncRNAs might be involved in, I employ a co-expression analysis of DEGs. lncRNAs and mRNAs with a similar expression profile were first clustered together and then GO analysis was performed to predict a common function of transcripts in individual clusters[269–272]. Based on this prediction I found that the biggest cluster contained co-expressed genes mainly involved in the innate immune response and that more than 9 % of them were lncRNAs.

Considering the co-expression analysis and the intensity of differential expression, I first selected 5 protein-coding RNAs and 4 lncRNAs among the DEGs upon infection from the obtained NGS data. Their expression at the transcriptional and translational level was then validated using RT-qPCR. Furthermore, I tested their expression at the transcriptional and translational level upon infection with another intracellular pathogen: *Salmonella enterica* either expressing an active (TT) or an inactive typhoid toxin (ΔcdtB). By using two distinct intracellular bacteria (i.e. *Listeria* and *Salmonella*), I was able to determine whether the lncRNAs expressed upon infection are either pathogen-specific or a general response to invading bacteria.

To find a possible function and a mechanism of action of the infection-induced lncRNAs I focused my research on two lncRNAs that were among the most differentially expressed in a WT-specific manner or upon infection with either *Listeria* strain.

The lincRNA AC016831.1 showed an overall strong upregulation upon infection with either WT and ΔLLO strain and both TT and ΔcdtB *Salmonella* strains, making it a possible general

sensor of infection. These findings show AC016831.1 as a general sensor of infection and that cells possibly upregulate it to reply to invading bacteria or to general stress. A clue in favour of the first hypothesis is the fact that in our co-expression analysis it was clustered together mainly with genes involved in the innate immune response. It displayed a similar expression, at both transcriptional and translational level, as known pro-inflammatory genes such as CXCL8 (also known as interleukin 8) and CCL20, known to be involved in the response to *Listeria* infection[93,249].

The lincRNA MIR181A1HG displayed a much more interesting behaviour, since it was found to be significantly upregulated only upon infection with WT *Listeria* at both time-points and was found not changing at 2 h POI or be even downregulated at 5 h POI upon infection with ΔLLO or both *Salmonella* strains. The combination of these findings suggests that MIR181A1HG expression and polysome-association is in fact pathogen-specific and even strain-specific, possibly induced by the presence of LLO. Interestingly, the co-expression analysis also showed that it clusters together with genes involved in vesicle-mediated transport. Currently nothing is known about LLO's role in regulating vesicular transport in host cells; however, there is evidence that *Listeria* modulates endocytic and genes vesicular protein trafficking pathways[273].

Given these results, I focused on the possible function and mechanism of action of these lncRNAs. To better understand the possible mechanistic role of these lncRNAs on the translational machinery, I used polysome profiling to determine if they associate to small (1-3 ribosomes per transcript) or large polysomes (> 3 ribosomes per transcript). In fact, the association of lncRNAs with small polysomes, suggests protein coding function. I demonstrated that both AC016831.1 and MIR181A1HG associate with small polysomes in non-infected and WT-infected cells. In agreement with this observation are recent reports that the vast majority of lncRNAs in a human cells indeed associate with small polysomes[6]. Moreover, using ORF finder I predicted multiple short ORFs (< 300 nt) in both lncRNAs, meaning that if they are potentially bound by the ribosome as are mRNAs, they would be able to bind only a small number or ribosomes. Indeed, the abovementioned results raise the possibility that they could be in fact coding for short peptides. The main reason why lincRNAs are considered non-coding is their lack of a single ORF longer than at least 300 nt, yet our experiments are compatible with a putative coding role. Nonetheless, their association

to polysomal fractions does not rule out the possibility that they are acting as ribosome sponges, with non-translating ribosomes associated with them.

Therefore, I further explored their putative coding ability or ribosome sponge activity by analysing in deeper detail their association with polysomes by:

i) assessing the strength with which lncRNAs interact with polysomes. For this purpose, I treated polysomal lysates with a high-salt concentration, at which mRNAs are kept still associated to polysomes while weakly bound molecules, such as initiation and elongation factors[224,225] or loosely interacting ncRNAs, are dissociated from polysomes, ribosomes and their subunits.

ii) determining whether the selected lncRNAs could possibly be associated with actively translating ribosomes. To this aim I employed the ribosome drop-off assay that takes advantage of the inhibitor of translation elongation, puromycin, that is known to affect only active ribosomes, causing their drop-off from mRNAs[252].

From the high-salt ribosome wash experiment, I demonstrated that all studied lincRNAs are bound strongly to polysomes. The ribosome drop-off assay suggests that only AC016831.1 is associated with active polysomes, thus being potentially translated. Interestingly, this was not the case with MIR181A1HG, that is associated with either stalled ribosomes, possessing a possible role as a ribosome sponge, or with silent polysomes in a non-conventional way. The former hypothesis would be particularly in agreement with all my previous data showing an overall depression of translation. In fact, the data accumulated so far, are compatible with a model in which, upon infection with the WT strain, cells inhibit translation by simply subtracting ribosomes from the pool of coding RNAs in cells, giving in this way a rapid response of translational depression that would save cellular energy.

These results prompted me to focus on the LLO-specific MIR181A1HG and investigate its functional role in infection. To that aim, I silenced it and studied the cell reply in its absence during *Listeria* infection and using polysome profiling I compared the FRP of silenced and non-silenced cells. I observed a stronger decrease in FRP values upon both WT infection and silencing at both time-points, whereas no changes were observed upon ΔLLO infection. Next, I used fluorescence microscopy to observe whether the loss of MIR181A1HG yielded a difference in the number of bacteria per cell. Remarkably, in cells where I inhibited the

expression of MIR181A1HG, I observed a significant increase in the average number of intracellular bacteria upon WT infection at both time-points in association to a worst translational state, as described by the FRP. Cells infected with ΔLLO exhibited no change in the average number of bacteria per cell between silenced and non-silenced cells. These findings present concrete evidence that MIR181A1HG exerts a protective role and that the loss of MIR181A1HG is beneficial for *Listeria'*s replication in host cells through association with polysomes.

In summary, by using transcriptome and translatome analyses, I showed that human host cells respond to *Listeria* infection by expressing numerous lncRNA and uploading them on polysomes. From WT and ΔLLO comparisons of POL-seq data I found AC016831.1, a lincRNA that is strongly upregulated upon *Listeria* as well as *Salmonella* infection and is predicted to be involved in the innate immune response. AC016831.1 is exclusively and strongly associated with small polysomes and there is strong evidence that it is in fact coding for proteins. The product of AC016831.1 are possibly short peptides, as evident from sORFs found in its sequence, which could possibly possess antimicrobial effects or are produced in bulk in order to stimulate the host cell proteasome in order to efficiently clear the infection.

Furthermore, I was able to discover MIR181A1HG, a polysome-associated lincRNA that is upregulated upon WT *Listeria* infection in an LLO-dependent and pathogen specific manner. Evidence shows that it is unlikely that it is employed for translating peptides, yet it is still strongly associates with small polysomes. Importantly, the expression of MIR181A1HG upon *Listeria* infection appears as an early biomarker of WT *Listeria* infection, possessing a protective role for host cells. Undoubtedly the several experiments performed suggest that it aids host cells to fend-off invading bacteria. Considering the obtained results thus-far one hypothesis about the function of MIR181A1HG is it having a possible role in translation regulation upon infection, by acting as a ribosome sponge. This putative activity can allow cells to decrease the number of available ribosomes, thus keeping the overall protein production rate at a low pace during infections. Functionally speaking, this mechanism would allow cells to counteract bacterial replication, exerting a possible positive role on host-translation, given the fact that its absence negatively impacts translation upon infection.

In this study, I demonstrated for the first time that polysome-associated lncRNAs play a functional role in translation upon bacterial infection and furthermore, that a bacteria-produced pore-forming toxin in fact induces specific translational controls. In the very next stage of this research it would be essential to demonstrate the protective role of MIR181A1HG in Caco-2 cells upon WT *Listeria* infection, by overexpressing it. These experiments are ongoing in the lab. Moreover, it would be important to better address the hypothesis that MIR181A1HG acts as a ribosome sponge, using *in vitro* Transcription/ Translation systems that would allow to follow the synthesis of reporter protein in the presence or absence of MIR181A1HG.

It would also be important to understand the role of transcripts that are neighbouring to the two lncRNAs on which I focused on. In fact, since lncRNAs are known to be co-expressed with neighbouring genes[151] , I looked at the genomic position and neighbourhood of MIR181A1HG. First, I observed that no coding transcript are located in proximal regions, excluding the hypothesis that these lncRNAs can be a side-effect of transcription of other coding transcripts. Moreover, I observed that the MIR181A1HG gene contains two miRNAs sequences in its intron region, miR-181a1 and miR-181b1. Indeed, it is likely that the spliced intron can be used for the production of a mature miRNA. Interestingly, the miRNA-181 (miR-181) family is known to be differentially expressed upon inflammation, specifically upon TLR activation; furthermore, miR-181a was even demonstrated to regulate the expression of IL-8[274]. These observations could suggest a possible synergistic effect of the lncRNAs and the corresponding miRNAs, to cope with the infection, possibly even at the polysomal level. Nonetheless, the absence of miR-181 biding sites in MIR181A1HG, suggests that these ncRNAs are most probably involved at different levels of post-transcriptional control of gene expression. Further studies are required in order to better understand any functional or mechanistic connection between these ncRNAs.

Next, looking at AC016831.1, I found that its exon region is comprised of miR-29b1 and just outside the same exon miR-29a. Expression of miR-29 family was shown to be regulated by various transcriptional regulators and signalling pathways and studies have confirmed that they possess also NF-κB binding sites that regulate its expression[275]. Also in this case, no protein coding transcripts have been observed. Interestingly, another lncRNA called Pint, is present and coherently with the biological system we are studying, it has been associated to p53 mediated response to stress[276]. The close proximity of both MIR181A1HG and AC016831.1 to all these ncRNAs found to be regulated by signalling pathways involved in

maintaining homeostasis upon stress, are highly supporting the speculated functions of these lncRNAs in stress response.

Finally, discovering functions of other infection-induced lncRNAs and determining their mechanism of action could help deepen our knowledge of the host-pathogen interaction. The first step I propose towards reaching this goal is to silence other infection-induced lncRNAs and in parallel perform their overexpression. Comparing the host's response to invading bacteria at these conditions may give valuable insight on their purpose in the host-pathogen crosstalk. I believe that perusing research on lncRNAs able to modulate host translation during infection may yield novel approached in fighting bacterial diseases and at least partially address the crisis of an ever-increasing number of antibiotic-resistant bacteria.

The work performed during this PhD project contributed to publishing a Perspective article in the journal Toxins, titled: "The Unexpected Tuners: Are LncRNAs Regulating Host Translation during Infections?"[7] and a research paper (in preparation) for submission to peer review and publication in a scientific journal.

# 5 Bibliography


1. Mohr, I. & Sonenberg, N. Host translation at the nexus of infection and immunity. *Cell Host Microbe* **12,** 470–83 (2012).
2. Roux, P. P. & Topisirovic, I. Regulation of mRNA translation by signaling pathways. *Cold Spring Harb. Perspect. Biol.* **4,** a012252–a012252 (2012).
3. Guttman, M., Russell, P., Ingolia, N. T., Weissman, J. S. & Lander, E. S. Ribosome profiling provides evidence that large noncoding RNAs do not encode proteins. *Cell* **154,** 240–51 (2013).
4. Ingolia, N. T., Lareau, L. F. & Weissman, J. S. Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. *Cell* **147,** 789–802 (2011).
5. van Heesch, S. *et al.* Extensive localization of long noncoding RNAs to the cytosol and mono- and polyribosomal complexes. *Genome Biol.* **15,** R6 (2014).
6. Carlevaro-Fita, J., Rahim, A., Guigó, R., Vardy, L. A. & Johnson, R. Cytoplasmic long noncoding RNAs are frequently bound to and degraded at ribosomes in human cells. *RNA* **22,** 867–82 (2016).
7. Knap, P. *et al.* The Unexpected Tuners: Are LncRNAs Regulating Host Translation during Infections? *Toxins (Basel).* **9,** 357 (2017).
8. Van Valen, L. A new evolutionary theory. *Evol. Theory* **1,** 1–30 (1973).
9. Iwasaki, A. & Medzhitov, R. Regulation of adaptive immunity by the innate immune system. *Science* **327,** 291–5 (2010).
10. Akira, S. *et al.* Pathogen recognition and innate immunity. *Cell* **124,** 783–801 (2006).
11. Foo, S. S., Reading, P. C., Jaillon, S., Mantovani, A. & Mahalingam, S. Pentraxins and Collectins: Friend or Foe during Pathogen Invasion? *Trends in Microbiology* **23,** 799–811 (2015).
12. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int. Immunol.* **17,** 1–14 (2005).
13. Jenner, R. G. & Young, R. A. Insights into host responses against pathogens from transcriptional profiling. *Nat. Rev. Microbiol.* **3,** 281–294 (2005).

14. Lemaitre, B. & Girardin, S. E. Translation inhibition and metabolic stress pathways in the host response to bacterial pathogens. *Nat. Rev. Microbiol.* **11,** 365–9 (2013).

15. Pakos-Zebrucka, K. *et al.* The integrated stress response. *EMBO Rep.* **17,** 1374–1395 (2016).

16. Sotolongo, J., Ruiz, J. & Fukata, M. The role of innate immunity in the host defense against intestinal bacterial pathogens. *Current Infectious Disease Reports* **14,** 15–23 (2012).

17. Franchi, L., Warner, N., Viani, K. & Nuñez, G. Function of Nod-like receptors in microbial recognition and host defense. *Immunol. Rev.* **227,** 106–28 (2009).

18. Pedra, J. H., Cassel, S. L. & Sutterwala, F. S. Sensing pathogens and danger signals by the inflammasome. *Current Opinion in Immunology* **21,** 10–16 (2009).

19. Bischofberger, M., Iacovache, I. & van der Goot, F. G. Pathogenic pore-forming proteins: function and host response. *Cell Host Microbe* **12,** 266–75 (2012).

20. Tang, D., Kang, R., Coyne, C. B., Zeh, H. J. & Lotze, M. T. PAMPs and DAMPs: Signal 0s that spur autophagy and immunity. *Immunol. Rev.* **249,** 158–175 (2012).

21. Tattoli, I. *et al.* Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program. *Cell Host Microbe* **11,** 563–575 (2012).

22. Martin, S., Saha, B. & Riley, J. L. The battle over mTOR: an emerging theatre in host-pathogen immunity. *PLoS Pathog.* **8,** e1002894 (2012).

23. Buttgereit, F. & Brand, M. D. A hierarchy of ATP-consuming processes in mammalian cells. *Biochem. J.* **312,** 163–167 (1995).

24. Warner, J. R. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* **24,** 437–440 (1999).

25. Hershey, J. W. B., Sonenberg, N. & Mathews, M. B. Principles of Translational Control: An Overview. *Cold Spring Harb. Perspect. Biol.* **4,** a011528–a011528 (2012).

26. Jackson, R. J., Hellen, C. U. T. & Pestova, T. V. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat. Rev. Mol. Cell Biol.* **11,** 113–127 (2010).

27. Dever, T. E. & Green, R. The Elongation, Termination, and Recycling Phases of Translation in Eukaryotes. *Cold Spring Harb. Perspect. Biol.* **4,** a013706–a013706

(2012).

28. Kiel, M. C., Kaji, H. & Kaji, A. Ribosome recycling: An essential process of protein synthesis. *Biochem. Mol. Biol. Educ.* **35,** 40–44 (2007).

29. Plotkin, J. B. Transcriptional regulation is only half the story. *Mol. Syst. Biol.* **6,** 406 (2010).

30. Tebaldi, T. *et al.* Widespread uncoupling between transcriptome and translatome variations after a stimulus in mammalian cells. *BMC Genomics* **13,** 220 (2012).

31. Vogel, C. *et al.* Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol. Syst. Biol.* **6,** 400 (2010).

32. Schwanhausser, B. *et al.* Global quantification of mammalian gene expression control. *Nature* **473,** 337–42 (2011).

33. Kudchodkar, S. B., Yu, Y., Maguire, T. G. & Alwine, J. C. Human cytomegalovirus infection induces rapamycin-insensitive phosphorylation of downstream effectors of mTOR kinase. *J. Virol.* **78,** 11030–9 (2004).

34. Chakrabarti, S., Liehl, P., Buchon, N. & Lemaitre, B. Infection-induced host translational blockage inhibits immune responses and epithelial renewal in the Drosophila gut. *Cell Host Microbe* **12,** 60–70 (2012).

35. Kloft, N. *et al.* Pro-autophagic signal induction by bacterial pore-forming toxins. *Med. Microbiol. Immunol.* **199,** 299–309 (2010).

36. Kloft, N. *et al.* A subunit of eukaryotic translation initiation factor 2α-phosphatase (CreP/PPP1R15B) regulates membrane. *J. Biol. Chem.* **287,** 35299–35317 (2012).

37. Pillich, H., Loose, M., Zimmer, K.-P. & Chakraborty, T. Activation of the unfolded protein response by Listeria monocytogenes. *Cell. Microbiol.* **14,** 949–64 (2012).

38. Fontana, M. F. *et al.* Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent Legionella pneumophila. *PLoS Pathog.* **7,** e1001289 (2011).

39. Holz, M. K., Ballif, B. A., Gygi, S. P. & Blenis, J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123,** 569–80 (2005).

40. Hinnebusch, A. G. & Lorsch, J. R. The mechanism of eukaryotic translation initiation:

New insights and challenges. *Cold Spring Harb. Perspect. Biol.* **4,** a011544–a011544 (2012).

41. Lemaire, P. A., Anderson, E., Lary, J. & Cole, J. L. Mechanism of PKR Activation by dsRNA. *J. Mol. Biol.* **381,** 351–360 (2008).

42. Suragani, R. N. V. S. *et al.* Heme-regulated eIF2α kinase activated Atf4 signaling pathway in oxidative stress and erythropoiesis. *Blood* **119,** 5276–84 (2012).

43. Sonenberg, N. & Hinnebusch, A. G. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* **136,** 731–45 (2009).

44. Wang, M. & Kaufman, R. J. Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* **529,** 326–335 (2016).

45. Shrestha, N. *et al.* Eukaryotic Initiation Factor 2 (eIF2) signaling regulates proinflammatory cytokine expression and bacterial invasion. *J. Biol. Chem.* **287,** 28738–28744 (2012).

46. Kumar, Y. & Valdivia, R. H. Leading a Sheltered Life: Intracellular Pathogens and Maintenance of Vacuolar Compartments. *Cell Host and Microbe* **5,** 593–601 (2009).

47. Diacovich, L. & Gorvel, J.-P. Bacterial manipulation of innate immunity to promote infection. *Nat. Rev. Microbiol.* **8,** 117–28 (2010).

48. Chen, L., Xiong, Z., Sun, L., Yang, J. & Jin, Q. VFDB 2012 update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic Acids Res.* **40,** D641-5 (2012).

49. Kisovec, M. *et al.* Engineering a pH responsive pore forming protein. *Sci. Rep.* **7,** 42231 (2017).

50. Song, J., Gao, X. & Galán, J. E. Structure and function of the Salmonella Typhi chimaeric A(2)B(5) typhoid toxin. *Nature* **499,** 350–4 (2013).

51. Proft, T. & Fraser, J. D. Bacterial superantigens. *Clin. Exp. Immunol.* **133,** 299–306 (2003).

52. Tran Van Nhieu, G. & Arbibe, L. Genetic reprogramming of host cells by bacterial pathogens. *F1000 Biol. Rep.* **1,** 80 (2009).

53. Zhou, H. *et al.* Yersinia virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappa B activation. *J. Exp. Med.* **202,** 1327–32 (2005).

54. Gonzalez, M. R. *et al.* Pore-forming toxins induce multiple cellular responses promoting survival. *Cell. Microbiol.* **13,** 1026–1043 (2011).

55. Von Hoven, G. *et al.* Modulation of translation and induction of autophagy by bacterial exoproducts. *Medical Microbiology and Immunology* **201,** 409–418 (2012).

56. Cossart, P. Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 19484–91 (2011).

57. Lecuit, M. *et al.* Targeting and crossing of the human maternofetal barrier by Listeria monocytogenes: role of internalin interaction with trophoblast E-cadherin. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6152–7 (2004).

58. EFSA & ECDC. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA J.* **14,** (2016).

59. Pamer, E. G. Immune responses to Listeria monocytogenes. *Nat. Rev. Immunol.* **4,** 812–23 (2004).

60. Pizarro-Cerdá, J., Kühbacher, A. & Cossart, P. Entry of Listeria monocytogenes in mammalian epithelial cells: an updated view. *Cold Spring Harb. Perspect. Med.* **2,** 1–17 (2012).

61. Cossart, P. & Toledo-Arana, A. Listeria monocytogenes, a unique model in infection biology: an overview. *Microbes Infect.* **10,** 1041–50 (2008).

62. Smith, G. A. *et al.* The two distinct phospholipases C of Listeria monocytogenes have overlapping roles in escape from a vacuole and cell-to-cell spread. *Infect. Immun.* **63,** 4231–7 (1995).

63. Welch, M. D., Iwamatsu, A. & Mitchison, T. J. Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. *Nature* **385,** 265–9 (1997).

64. Skoble, J., Portnoy, D. A. & Welch, M. D. Three regions within ActA promote Arp2/3 complex-mediated actin nucleation and Listeria monocytogenes motility. *J. Cell Biol.* **150,** 527–38 (2000).

65. Cossart, P. & Lecuit, M. Interactions of Listeria monocytogenes with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. *EMBO J.* **17,** 3797–806 (1998).

66. Hain, T. *et al.* Pathogenomics of Listeria spp. *Int. J. Med. Microbiol.* **297,** 541–57 (2007).

67. Hain, T., Steinweg, C. & Chakraborty, T. Comparative and functional genomics of Listeria spp. *J. Biotechnol.* **126,** 37–51 (2006).

68. Chakraborty, T. *et al.* Coordinate regulation of virulence genes in Listeria monocytogenes requires the product of the prfA gene. *J. Bacteriol.* **174,** 568–74 (1992).

69. Johansson, J. *et al.* An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes. *Cell* **110,** 551–61 (2002).

70. Renzoni, A., Klarsfeld, A., Dramsi, S. & Cossart, P. Evidence that PrfA, the pleiotropic activator of virulence genes in Listeria monocytogenes, can be present but inactive. *Infect. Immun.* **65,** 1515–8 (1997).

71. Hall, M. *et al.* Structural basis for glutathione-mediated activation of the virulence regulatory protein PrfA in Listeria. *Proc. Natl. Acad. Sci. U. S. A.* **113,** 14733–14738 (2016).

72. Hamon, M. A., Ribet, D., Stavru, F. & Cossart, P. Listeriolysin O: the Swiss army knife of Listeria. *Trends Microbiol.* **20,** 360–8 (2012).

73. Osborne, S. E. & Brumell, J. H. Listeriolysin O: from bazooka to Swiss army knife. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **372,** 20160222 (2017).

74. Marquis, H. & Hager, E. J. pH-regulated activation and release of a bacteria-associated phospholipase C during intracellular infection by Listeria monocytogenes. *Mol. Microbiol.* **35,** 289–298 (2000).

75. Alberti-Segui, C., Goeden, K. R. & Higgins, D. E. Differential function of Listeria monocytogenes listeriolysin O and phospholipases C in vacuolar dissolution following cell-to-cell spread. *Cell. Microbiol.* **9,** 179–195 (2007).

76. Schubert, W. D. *et al.* Structure of internalin, a major invasion protein of Listeria monocytogenes, in complex with its human receptor E-cadherin. *Cell* **111,** 825–836 (2002).

77. Niemann, H. H. *et al.* Structure of the Human Receptor Tyrosine Kinase Met in Complex with the Listeria Invasion Protein InlB. *Cell* **130,** 235–246 (2007).

78. Gouin, E., Welch, M. D. & Cossart, P. Actin-based motility of intracellular pathogens. *Current Opinion in Microbiology* **8,** 35–45 (2005).

79. Forster, B. M. *et al.* The metalloprotease of Listeria monocytogenes is regulated by pH. *J. Bacteriol.* **193,** 5090–5097 (2011).

80. Bitar, A. P., Cao, M. & Marquis, H. The metalloprotease of Listeria monocytogenes is activated by intramolecular autocatalysis. *J. Bacteriol.* **190,** 107–111 (2008).

81. Alvarez, D. E. & Agaisse, H. The metalloprotease Mpl supports Listeria monocytogenes dissemination through resolution of membrane protrusions into vacuoles. *Infect. Immun.* **84,** 1806–1814 (2016).

82. Gouin, E. *et al.* The Listeria monocytogenes InlC protein interferes with innate immune responses by targeting the I{kappa}B kinase subunit IKK{alpha}. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 17333–8 (2010).

83. Olier, M. *et al.* Expression of truncated internalin A is involved in impaired internalization of some Listeria monocytogenes isolates carried asymptomatically by humans. *Infect. Immun.* **71,** 1217–1224 (2003).

84. Rousseaux, S., Olier, M., Lemaître, J. P., Piveteau, P. & Guzzo, J. Use of PCR-Restriction Fragment Length Polymorphism of inlA for Rapid Screening of Listeria monocytogenes Strains Deficient in the Ability to Invade Caco-2 Cells. *Appl. Environ. Microbiol.* **70,** 2180–2185 (2004).

85. Yoshikawa, Y. *et al.* Listeria monocytogenes ActA-mediated escape from autophagic recognition. *Nat. Cell Biol.* **11,** 1233–1240 (2009).

86. Portnoy, D. A., Jacks, P. S. & Hinrichs, D. J. Role of hemolysin for the intracellular growth of Listeria monocytogenes. *J. Exp. Med.* **167,** 1459–71 (1988).

87. Seki, E. *et al.* Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice. *J. Immunol.* **169,** 3863–3868 (2002).

88. Edelson, B. T. & Unanue, E. R. MyD88-Dependent but Toll-Like Receptor 2-Independent Innate Immunity to Listeria: No Role for Either in Macrophage Listericidal Activity. *J. Immunol.* **169,** 3869–3875 (2002).

89. Aubry, C. *et al.* Both TLR2 and TRIF contribute to interferon-β production during

listeria infection. *PLoS One* **7,** e33299 (2012).

90. Leber, J. H. *et al.* Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen. *PLoS Pathog.* **4,** 0084–0095 (2008).

91. Hashino, M. *et al.* Inactivation of the MAPK signaling pathway by Listeria monocytogenes infection promotes trophoblast giant cell death. *Front. Microbiol.* **6,** 1145 (2015).

92. Zhang, Y. *et al.* Microparticles released by Listeria monocytogenes-infected macrophages are required for dendritic cell-elicited protective immunity. *Cell. Mol. Immunol.* **9,** 489–96 (2012).

93. Opitz, B. *et al.* Listeria monocytogenes activated p38 MAPK and induced IL-8 secretion in a nucleotide-binding oligomerization domain 1-dependent manner in endothelial cells. *J. Immunol.* **176,** 484–90 (2006).

94. Hamon, M. A. *et al.* Histone modifications induced by a family of bacterial toxins. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 13467–72 (2007).

95. Hay, N. & Sonenberg, N. Upstream and downstream of mTOR. *Genes and Development* **18,** 1926–1945 (2004).

96. Tattoli, I. *et al.* Listeria phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures. *EMBO J.* **32,** 3066–78 (2013).

97. Cajnko, M. M., Mikelj, M., Turk, T., Podobnik, M. & Anderluh, G. Membrane interactions and cellular effects of MACPF/CDC proteins. *Subcell. Biochem.* **80,** 119–44 (2014).

98. Villanueva, M. S., Sijts, A. J. & Pamer, E. G. Listeriolysin is processed efficiently into an MHC class I-associated epitope in Listeria monocytogenes-infected cells. *J. Immunol.* **155,** 5227–33 (1995).

99. Glomski, I. J., Decatur, A. L. & Portnoy, D. A. Listeria monocytogenes mutants that fail to compartmentalize listerolysin O activity are cytotoxic, avirulent, and unable to evade host extracellular defenses. *Infect. Immun.* **71,** 6754–65 (2003).

100. Seveau, S. Multifaceted activity of listeriolysin O, the cholesterol-dependent cytolysin of Listeria monocytogenes. *Subcell. Biochem.* **80,** 161–95 (2014).

101. Schuerch, D. W., Wilson-Kubalek, E. M. & Tweten, R. K. Molecular basis of

listeriolysin O pH dependence. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 12537–42 (2005).

102. Bavdek, A. *et al.* pH dependence of listeriolysin O aggregation and pore-forming ability. *FEBS J.* **279,** 126–41 (2012).

103. Singh, R., Jamieson, A. & Cresswell, P. GILT is a critical host factor for Listeria monocytogenes infection. *Nature* **455,** 1244–1247 (2008).

104. Radtke, A. L. *et al.* Listeria monocytogenes exploits cystic fibrosis transmembrane conductance regulator (CFTR) to escape the phagosome. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 1633–8 (2011).

105. Dramsi, S. & Cossart, P. Listeriolysin O-mediated calcium influx potentiates entry of Listeria monocytogenes into the human Hep-2 epithelial cell line. *Infect. Immun.* **71,** 3614–3618 (2003).

106. Repp, H. *et al.* Listeriolysin of Listeria monocytogenes forms Ca2+-permeable pores leading to intracellular Ca2+ oscillations. *Cell. Microbiol.* **4,** 483–491 (2002).

107. Tang, P., Rosenshine, I., Cossart, P. & Finlay, B. B. Listeriolysin O activates mitogen-activated protein kinase in eucaryotic cells. *Infect. Immun.* **64,** 2359–61 (1996).

108. Wadsworth, S. J. & Goldfine, H. Mobilization of protein kinase C in macrophages induced by Listeria monocytogenes affects its internalization and escape from the phagosome. *Infect. Immun.* **70,** 4650–4660 (2002).

109. Kayal, S. *et al.* Listeriolysin O-dependent activation of endothelial cells during infection with Listeria monocytogenes: activation of NF-kappa B and upregulation of adhesion molecules and chemokines. *Mol. Microbiol.* **31,** 1709–22 (1999).

110. Malet, J. K., Cossart, P. & Ribet, D. Alteration of epithelial cell lysosomal integrity induced by bacterial cholesterol-dependent cytolysins. *Cell. Microbiol.* **19,** (2017).

111. Shaughnessy, L. M., Hoppe, A. D., Christensen, K. A. & Swanson, J. A. Membrane perforations inhibit lysosome fusion by altering pH and calcium in Listeria monocytogenes vacuoles. *Cell. Microbiol.* **8,** 781–92 (2006).

112. Lam, G. Y. *et al.* Listeriolysin o suppresses phospholipase c-mediated activation of the microbicidal NADPH oxidase to promote listeria monocytogenes infection. *Cell Host Microbe* **10,** 627–634 (2011).

113. Samba-Louaka, A. *et al.* Listeria monocytogenes dampens the DNA damage response.

*PLoS Pathog.* **10,** e1004470 (2014).

114. Gekara, N. O. *et al.* The multiple mechanisms of Ca2+ signalling by listeriolysin O, the cholesterol-dependent cytolysin of Listeria monocytogenes. *Cell. Microbiol.* **9,** 2008–21 (2007).

115. Arthur, J. S. C. & Ley, S. C. Mitogen-activated protein kinases in innate immunity. *Nat. Rev. Immunol.* **13,** 679–92 (2013).

116. Tang, P., Sutherland, C. L., Gold, M. R. & Finlay, B. B. Listeria monocytogenes invasion of epithelial cells requires the MEK-1/ERK-2 mitogen-activated protein kinase pathway. *Infect. Immun.* **66,** 1106–12 (1998).

117. Schröder, M. & Kaufman, R. J. The mammalian unfolded protein response. *Annu. Rev. Biochem.* **74,** 739–89 (2005).

118. Hamon, M. A. & Cossart, P. K+ efflux is required for histone H3 dephosphorylation by Listeria monocytogenes listeriolysin O and other pore-forming toxins. *Infect. Immun.* **79,** 2839–46 (2011).

119. Balagopal, V. & Parker, R. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Curr. Opin. Cell Biol.* **21,** 403–8 (2009).

120. Vadia, S. *et al.* The pore-forming toxin listeriolysin o mediates a novel entry pathway of L. monocytogenes into human hepatocytes. *PLoS Pathog.* **7,** e1002356 (2011).

121. Meyer-Morse, N. *et al.* Listeriolysin O is necessary and sufficient to induce autophagy during Listeria monocytogenes infection. *PLoS One* **5,** e8610 (2010).

122. Stavru, F., Bouillaud, F., Sartori, A., Ricquier, D. & Cossart, P. Listeria monocytogenes transiently alters mitochondrial dynamics during infection. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 3612–7 (2011).

123. Djebali, S. *et al.* Landscape of transcription in human cells. *Nature* **489,** 101–8 (2012).

124. Birney, E. *et al.* Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature* **447,** 799–816 (2007).

125. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature* **489,** 57–74 (2012).

126. Palazzo, A. F. & Lee, E. S. Non-coding RNA: what is functional and what is junk? *Front. Genet.* **6,** 2 (2015).

127. Niu, D.-K. & Jiang, L. Can ENCODE tell us how much junk DNA we carry in our genome? *Biochem. Biophys. Res. Commun.* **430,** 1340–1343 (2013).

128. Graur, D. *et al.* On the Immortality of Television Sets: 'Function' in the Human Genome According to the Evolution-Free Gospel of ENCODE. *Genome Biol. Evol.* **5,** 578–590 (2013).

129. Struhl, K. Transcriptional noise and the fidelity of initiation by RNA polymerase II. *Nat. Struct. Mol. Biol.* **14,** 103–105 (2007).

130. Shearwin, K. E., Callen, B. P. & Egan, J. B. Transcriptional interference--a crash course. *Trends Genet.* **21,** 339–45 (2005).

131. Ulitsky, I. & Bartel, D. P. lincRNAs: genomics, evolution, and mechanisms. *Cell* **154,** 26–46 (2013).

132. Chen, Y.-C. A. & Aravin, A. A. Non-coding RNAs in Transcriptional Regulation. *Curr. Mol. Biol. Reports* **1,** 10–18 (2015).

133. Sharma, E., Sterne-Weiler, T., O'Hanlon, D. & Blencowe, B. J. Global Mapping of Human RNA-RNA Interactions. *Mol. Cell* **62,** 618–26 (2016).

134. Anger, A. M. *et al.* Structures of the human and Drosophila 80S ribosome. *Nature* **497,** 80–85 (2013).

135. Pircher, A., Gebetsberger, J. & Polacek, N. Ribosome-associated ncRNAs: An emerging class of translation regulators. *RNA Biol.* **11,** 1335–9 (2014).

136. Wilusz, J. E. Long noncoding RNAs: Re-writing dogmas of RNA processing and stability. *Biochim. Biophys. Acta* **1859,** 128–38 (2016).

137. Engreitz, J. M. *et al.* RNA-RNA interactions enable specific targeting of noncoding RNAs to nascent Pre-mRNAs and chromatin sites. *Cell* **159,** 188–99 (2014).

138. Hangauer, M. J., Vaughn, I. W. & McManus, M. T. Pervasive Transcription of the Human Genome Produces Thousands of Previously Unidentified Long Intergenic Noncoding RNAs. *PLoS Genet.* **9,** e1003569 (2013).

139. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409,** 860–921 (2001).

140. Wahlestedt, C. Targeting long non-coding RNA to therapeutically upregulate gene expression. *Nat. Rev. Drug Discov.* **12,** 433–446 (2013).

141. Atianand, M. K. & Fitzgerald, K. A. Long non-coding RNAs and control of gene expression in the immune system. *Trends Mol. Med.* **20,** 623–31 (2014).

142. Rinn, J. L. & Chang, H. Y. Genome regulation by long noncoding RNAs. *Annu. Rev. Biochem.* **81,** 145–66 (2012).

143. de Andres-Pablo, A., Morillon, A. & Wery, M. LncRNAs, lost in translation or licence to regulate? *Curr. Genet.* **63,** 29–33 (2017).

144. Quinn, J. J. & Chang, H. Y. Unique features of long non-coding RNA biogenesis and function. *Nat. Rev. Genet.* **17,** 47–62 (2016).

145. Guttman, M. *et al.* Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* **458,** 223–227 (2009).

146. Bánfai, B. *et al.* Long noncoding RNAs are rarely translated in two human cell lines. *Genome Res.* **22,** 1646–57 (2012).

147. Dinger, M. E., Pang, K. C., Mercer, T. R. & Mattick, J. S. Differentiating protein-coding and noncoding RNA: Challenges and ambiguities. *PLoS Comput. Biol.* **4,** e1000176 (2008).

148. Okazaki, Y. *et al.* Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature* **420,** 563–573 (2002).

149. St Laurent, G., Wahlestedt, C. & Kapranov, P. The Landscape of long noncoding RNA classification. *Trends Genet.* **31,** 239–51 (2015).

150. Niazi, F. & Valadkhan, S. Computational analysis of functional long noncoding RNAs reveals lack of peptide-coding capacity and parallels with 3' UTRs. *RNA* **18,** 825–843 (2012).

151. Cabili, M. N. *et al.* Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* **25,** 1915–27 (2011).

152. Wang, Y. *et al.* Analyses of Long Non-Coding RNA and mRNA profiling using RNA sequencing during the pre-implantation phases in pig endometrium. *Sci. Rep.* **6,** 20238 (2016).

153. Derrien, T. *et al.* The GENCODE v7 catalog of human long noncoding RNAs: Analysis of their gene structure, evolution, and expression. *Genome Res.* **22,** 1775–1789 (2012).

154. Johnsson, P., Lipovich, L., Grandér, D. & Morris, K. V. Evolutionary conservation of long non-coding RNAs; sequence, structure, function. *Biochim. Biophys. Acta* **1840,** 1063–71 (2014).

155. Arraystar.com. LncRNA Classification. Available at: http://www.arraystar.com/reviews/v30-lncrna-classification/. (Accessed: 17th June 2017)

156. Ma, L., Bajic, V. B. & Zhang, Z. On the classification of long non-coding RNAs. *RNA Biol.* **10,** 925–33 (2013).

157. Hon, C.-C. *et al.* An atlas of human long non-coding RNAs with accurate 5' ends. *Nature* **543,** 199–204 (2017).

158. Faghihi, M. A. *et al.* Evidence for natural antisense transcript-mediated inhibition of microRNA function. *Genome Biol.* **11,** R56 (2010).

159. Gong, C. & Maquat, L. E. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3′ UTRs via Alu elements. *Nature* **470,** 284–288 (2011).

160. Kretz, M. *et al.* Control of somatic tissue differentiation by the long non-coding RNA TINCR. *Nature* **493,** 231–235 (2012).

161. Tay, Y., Rinn, J. & Pandolfi, P. P. The multilayered complexity of ceRNA crosstalk and competition. *Nature* **505,** 344–352 (2014).

162. Rashid, F., Shah, A. & Shan, G. Long Non-coding RNAs in the Cytoplasm. *Genomics. Proteomics Bioinformatics* **14,** 73–80 (2016).

163. Hansen, T. B. *et al.* Natural RNA circles function as efficient microRNA sponges. *Nature* **495,** 384–388 (2013).

164. Wang, J. *et al.* CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. *Nucleic Acids Res.* **38,** 5366–83 (2010).

165. Cesana, M. *et al.* A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* **147,** 358–69 (2011).

166. Hollander, M. C., Alamo, I. & Fornace, A. J. A novel DNA damage-inducible transcript, gadd7, inhibits cell growth, but lacks a protein product. *Nucleic Acids Res.* **24,** 1589–93 (1996).

167. Liu, X., Li, D., Zhang, W., Guo, M. & Zhan, Q. Long non-coding RNA gadd7 interacts with TDP-43 and regulates Cdk6 mRNA decay. *EMBO J.* **31,** 4415–4427 (2012).

168. Brown, C. J. *et al.* The human XIST gene: Analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* **71,** 527–542 (1992).

169. Zhao, J., Sun, B. K., Erwin, J. A., Song, J.-J. & Lee, J. T. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* **322,** 750–6 (2008).

170. Hacisuleyman, E. *et al.* Topological organization of multichromosomal regions by the long intergenic noncoding RNA Firre. *Nat. Struct. Mol. Biol.* **21,** 198–206 (2014).

171. Clemson, C. M. *et al.* An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. *Mol. Cell* **33,** 717–26 (2009).

172. Carrieri, C. *et al.* Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* **491,** 454–7 (2012).

173. Nadal-Ribelles, M. *et al.* Control of Cdc28 CDK1 by a Stress-Induced lncRNA. *Mol. Cell* **53,** 549–561 (2014).

174. Liu, B. *et al.* A cytoplasmic NF-κB interacting long noncoding RNA blocks IκB phosphorylation and suppresses breast cancer metastasis. *Cancer Cell* **27,** 370–81 (2015).

175. Yang, F., Zhang, H., Mei, Y. & Wu, M. Reciprocal regulation of HIF-1α and lincRNA-p21 modulates the Warburg effect. *Mol. Cell* **53,** 88–100 (2014).

176. Paulet, D., David, A. & Rivals, E. Ribo-seq enlightens codon usage bias. *DNA Res.* **166,** 679–90 (2017).

177. Ponjavic, J., Ponting, C. P. & Lunter, G. Functionality or transcriptional noise? Evidence for selection within long noncoding RNAs. *Genome Res.* **17,** 556–565 (2007).

178. López, D. & Pazos, F. Protein functional features are reflected in the patterns of mRNA translation speed. *BMC Genomics* **16,** 513 (2015).

179. Pearson, W. R. An introduction to sequence similarity ('homology') searching. *Curr. Protoc. Bioinforma.* **Chapter 3,** Unit3.1 (2013).

180. Bhagwat, M., Young, L. & Robison, R. R. Using BLAT to find sequence similarity in

closely related genomes. *Curr. Protoc. Bioinforma.* **Chapter 10,** Unit10.8 (2012).

181. Galindo, M. I., Pueyo, J. I., Fouix, S., Bishop, S. A. & Couso, J. P. Peptides encoded by short ORFs control development and define a new eukaryotic gene family. *PLoS Biol.* **5,** e106 (2007).

182. Ingolia, N. T. *et al.* Ribosome profiling reveals pervasive translation outside of annotated protein-coding genes. *Cell Rep.* **8,** 1365–79 (2014).

183. Ruiz-Orera, J., Messeguer, X., Subirana, J. A. & Alba, M. M. Long non-coding RNAs as a source of new peptides. *Elife* **3,** (2014).

184. Kondo, T. *et al.* Small Peptides Switch the Transcriptional Activity of Shavenbaby During Drosophila Embryogenesis. *Science (80-. ).* **329,** 336–339 (2010).

185. Calvo, S. E., Pagliarini, D. J. & Mootha, V. K. Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 7507–12 (2009).

186. Iacono, M., Mignone, F. & Pesole, G. uAUG and uORFs in human and rodent 5′untranslated mRNAs. *Gene* **349,** 97–105 (2005).

187. Yoon, J.-H. *et al.* LincRNA-p21 Suppresses Target mRNA Translation. *Mol. Cell* **47,** 648–655 (2012).

188. Wang, H. *et al.* Dendritic BC1 RNA in translational control mechanisms. *J. Cell Biol.* **171,** 811–21 (2005).

189. Kindler, S., Wang, H., Richter, D. & Tiedge, H. RNA transport and local control of translation. *Annu. Rev. Cell Dev. Biol.* **21,** 223–45 (2005).

190. Hansji, H. *et al.* ZFAS1: a long noncoding RNA associated with ribosomes in breast cancer cells. *Biol. Direct* **11,** 62 (2016).

191. Essers, P. B. *et al.* A Long Noncoding RNA on the Ribosome Is Required for Lifespan Extension. *Cell Rep.* **10,** 339–345 (2015).

192. Scaria, V., Hariharan, M., Maiti, S., Pillai, B. & Brahmachari, S. K. Host-virus interaction: a new role for microRNAs. *Retrovirology* **3,** 68 (2006).

193. Rederstorff, M. & Hüttenhofer, A. Small non-coding RNAs in disease development and host-pathogen interactions. *Curr. Opin. Mol. Ther.* **12,** 684–94 (2010).

194. Yan, B. & Wang, Z. Long Noncoding RNA: Its Physiological and Pathological Roles.

*DNA Cell Biol.* **31,** S-34-S-41 (2012).

195. Scaria, V. & Pasha, A. Long Non-Coding RNAs in Infection Biology. *Front. Genet.* **3,** 308 (2012).

196. Zhang, Y. & Cao, X. Long noncoding RNAs in innate immunity. *Cell. Mol. Immunol.* **13,** 138–47 (2016).

197. Zhang, Q., Chen, C.-Y., Yedavalli, V. S. R. K. & Jeang, K.-T. NEAT1 long noncoding RNA and paraspeckle bodies modulate HIV-1 posttranscriptional expression. *MBio* **4,** e00596-12 (2013).

198. Imamura, K. *et al.* Long noncoding RNA NEAT1-dependent SFPQ relocation from promoter region to paraspeckle mediates IL8 expression upon immune stimuli. *Mol. Cell* **53,** 393–406 (2014).

199. Imam, H., Bano, A. S., Patel, P., Holla, P. & Jameel, S. The lncRNA NRON modulates HIV-1 replication in a NFAT-dependent manner and is differentially regulated by early and late viral proteins. *Sci. Rep.* **5,** 8639 (2015).

200. Cron, R. Q. *et al.* NFAT1 enhances HIV-1 gene expression in primary human CD4 T cells. *Clin. Immunol.* **94,** 179–91 (2000).

201. Westermann, A. J. *et al.* Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. *Nature* **529,** 496–501 (2016).

202. Das, K., Garnica, O. & Dhandayuthapani, S. Modulation of Host miRNAs by Intracellular Bacterial Pathogens. *Front. Cell. Infect. Microbiol.* **6,** 79 (2016).

203. Kim, J. K., Kim, T. S., Basu, J. & Jo, E.-K. MicroRNA in innate immunity and autophagy during mycobacterial infection. *Cell. Microbiol.* **19,** e12687 (2017).

204. Zur Bruegge, J., Einspanier, R. & Sharbati, S. A Long Journey Ahead: Long Non-coding RNAs in Bacterial Infections. *Front. Cell. Infect. Microbiol.* **7,** 95 (2017).

205. IIott, N. E. *et al.* Long non-coding RNAs and enhancer RNAs regulate the lipopolysaccharide-induced inflammatory response in human monocytes. *Nat. Commun.* **5,** 3979 (2014).

206. Mao, A.-P., Shen, J. & Zuo, Z. Expression and regulation of long noncoding RNAs in TLR4 signaling in mouse macrophages. *BMC Genomics* **16,** 45 (2015).

207. Kaakoush, N. O. *et al.* Transcriptomic and proteomic analyses reveal key innate

immune signatures in the host response to the gastrointestinal pathogen Campylobacter concisus. *Infect. Immun.* **83,** 832–45 (2015).

208. Wang, K. C. & Chang, H. Y. Molecular mechanisms of long noncoding RNAs. *Mol. Cell* **43,** 904–14 (2011).

209. Khalil, A. M. *et al.* Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 11667–72 (2009).

210. Ma, S. *et al.* A long noncoding RNA, lincRNA-Tnfaip3, acts as a coregulator of NF-κB to modulate inflammatory gene transcription in mouse macrophages. *FASEB J.* **31,** 1215–1225 (2017).

211. Tong, Q. *et al.* LincRNA-Cox2 modulates TNF-α-induced transcription of Il12b gene in intestinal epithelial cells through regulation of Mi-2/NuRD-mediated epigenetic histone modifications. *FASEB J.* **30,** 1187–97 (2016).

212. Carpenter, S. *et al.* A long noncoding RNA mediates both activation and repression of immune response genes. *Science* **341,** 789–92 (2013).

213. Atianand, M. K. *et al.* A Long Noncoding RNA lincRNA-EPS Acts as a Transcriptional Brake to Restrain Inflammation. *Cell* **165,** 1672–85 (2016).

214. Chan, J. *et al.* Cutting Edge: A Natural Antisense Transcript, AS-IL1α, Controls Inducible Transcription of the Proinflammatory Cytokine IL-1α. *J. Immunol.* **195,** 1359–63 (2015).

215. Cui, H. *et al.* The human long noncoding RNA lnc-IL7R regulates the inflammatory response. *Eur. J. Immunol.* **44,** 2085–95 (2014).

216. Li, Z. *et al.* The long noncoding RNA THRIL regulates TNFα expression through its interaction with hnRNPL. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 1002–7 (2014).

217. Wu, H., Liu, J., Li, W., Liu, G. & Li, Z. LncRNA-HOTAIR promotes TNF-α production in cardiomyocytes of LPS-induced sepsis mice by activating NF-κB pathway. *Biochem. Biophys. Res. Commun.* **471,** 240–6 (2016).

218. Gomez, J. A. *et al.* The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon-γ locus. *Cell* **152,** 743–54 (2013).

219. Wang, Y. *et al.* Long noncoding RNA derived from CD244 signaling epigenetically

controls CD8+ T-cell immune responses in tuberculosis infection. *Proc. Natl. Acad. Sci. U. S. A.* **112,** E3883-92 (2015).

220. Pawar, K., Hanisch, C., Palma Vera, S. E., Einspanier, R. & Sharbati, S. Down regulated lncRNA MEG3 eliminates mycobacteria in macrophages via autophagy. *Sci. Rep.* **6,** 19416 (2016).

221. Conte, M. P. *et al.* Listeria monocytogenes infection of Caco-2 cells: role of growth temperature. *Res. Microbiol.* **145,** 677–82 (1994).

222. Hauf, N., Goebel, W., Fiedler, F. & Kuhn, M. Listeria monocytogenes infection of Caco-2 human epithelial cells induces activation of transcription factor NF-kappa B/Rel-like DNA binding activities. *FEMS Microbiol. Lett.* **178,** 117–22 (1999).

223. Bernabò, P. *et al.* Studying translational control in non-model stressed organisms by polysomal profiling. *J. Insect Physiol.* **76,** 30–5 (2015).

224. Vornlocher, H. P., Scheible, W. R., Faulhammer, H. G. & Sprinzl, M. Identification and purification of translation initiation factor 2 (IF2) from Thermus thermophilus. *Eur. J. Biochem.* **243,** 66–71 (1997).

225. Huch, S., Gommlich, J., Muppavarapu, M., Beckham, C. & Nissan, T. Membrane-association of mRNA decapping factors is independent of stress in budding yeast. *Sci. Rep.* **6,** 25477 (2016).

226. Frey, B. J. & Dueck, D. Clustering by passing messages between data points. *Science* **315,** 972–6 (2007).

227. Rao, X., Huang, X., Zhou, Z. & Lin, X. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat. Bioinforma. Biomath.* **3,** 71–85 (2013).

228. Darnell, J. C. *et al.* FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* **146,** 247–61 (2011).

229. Lebreton, A., Stavru, F. & Cossart, P. Organelle targeting during bacterial infection: insights from Listeria. *Trends Cell Biol.* **25,** 330–8 (2015).

230. Portnoy, D. A., Auerbuch, V. & Glomski, I. J. The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *J. Cell Biol.* **158,** 409–14 (2002).

231. Gedde, M. M., Higgins, D. E., Tilney, L. G. & Portnoy, D. A. Role of listeriolysin O in cell-to-cell spread of Listeria monocytogenes. *Infect. Immun.* **68,** 999–1003 (2000).

232. Toone, E. J. Bacterial infection remains a leading cause of death in both Western and developing world. Preface. *Adv. Enzymol. Relat. Areas Mol. Biol.* **77,** xi–xiii (2011).

233. Francis, M. S. & Thomas, C. J. Effect of multiplicity of infection on Listeria monocytogenes pathogenicity for HeLa and Caco-2 cell lines. *J. Med. Microbiol.* **45,** 323–30 (1996).

234. Rocha, E., Mol, J. P. S., Garcia, L. N. N. & Costa, L. F. Comparative experimental infection of Listeria monocytogenes and Listeria ivanovii in bovine trophoblasts. *PLoS One* **12,** 1–13 (2017).

235. Yamada, F. *et al.* Invasion assay of Listeria monocytogenes using Vero and Caco-2 cells. *J. Microbiol. Methods* **66,** 96–103 (2006).

236. Dunbar, T. L., Yan, Z., Balla, K. M., Smelkinson, M. G. & Troemel, E. R. C. elegans detects pathogen-induced translational inhibition to activate immune signaling. *Cell Host Microbe* **11,** 375–386 (2012).

237. Hoeffer, C. A. & Klann, E. mTOR signaling: At the crossroads of plasticity, memory and disease. *Trends in Neurosciences* **33,** 67–75 (2010).

238. Clamer, M. *et al.* Global translation variations in host cells upon attack of lytic and sublytic Staphylococcus aureus -haemolysin. *Biochem. J.* **472,** 83–95 (2015).

239. Francisco-Velilla, R., Fernandez-Chamorro, J., Ramajo, J. & Martinez-Salas, E. The RNA-binding protein Gemin5 binds directly to the ribosome and regulates global translation. *Nucleic Acids Res.* **44,** 8335–8351 (2016).

240. Liu, B., Han, Y. & Qian, S.-B. Cotranslational response to proteotoxic stress by elongation pausing of ribosomes. *Mol. Cell* **49,** 453–63 (2013).

241. Dieck, S. T. *et al.* Metabolic labeling with noncanonical amino acids and visualization by chemoselective fluorescent tagging. *Curr. Protoc. Cell Biol.* **1,** Unit7.11 (2012).

242. Maruyama, S., Shen, H., Kanoh, M., Matsumoto, A. & Asano, Y. Differential effect of Listeria monocytogenes infection on cytokine production and cytotoxicity of CD8 T cells. *Microbiol. Immunol.* **51,** 893–901 (2007).

243. Dussurget, O., Bierne, H. & Cossart, P. The bacterial pathogen Listeria monocytogenes

and the interferon family: type I, type II and type III interferons. *Front. Cell. Infect. Microbiol.* **4,** 50 (2014).

244. Torres, D. *et al.* Membrane Tumor Necrosis Factor Confers Partial Protection to Listeria Infection. *Am. J. Pathol.* **167,** 1677–1687 (2005).

245. Roach, D. R., Briscoe, H., Saunders, B. M. & Britton, W. J. Independent protective effects for tumor necrosis factor and lymphotoxin alpha in the host response to Listeria monocytogenes infection. *Infect. Immun.* **73,** 4787–92 (2005).

246. Torres, D. *et al.* Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection. *Infect. Immun.* **72,** 2131–9 (2004).

247. Dallagiovanna, B. *et al.* lncRNAs are associated with polysomes during adipose-derived stem cell differentiation. *Gene* **610,** 103–111 (2017).

248. Hou, M. *et al.* AnnoLnc: a web server for systematically annotating novel human lncRNAs. *BMC Genomics* **17,** 931 (2016).

249. Regan, T. *et al.* Identification of TLR10 as a key mediator of the inflammatory response to Listeria monocytogenes in intestinal epithelial cells and macrophages. *J. Immunol.* **191,** 6084–92 (2013).

250. Henry, R. *et al.* Cytolysin-dependent delay of vacuole maturation in macrophages infected with Listeria monocytogenes. *Cell. Microbiol.* **8,** 107–119 (2006).

251. Thermann, R. & Hentze, M. W. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. *Nature* **447,** 875–8 (2007).

252. Azzam, M. E. & Algranati, I. D. Mechanism of puromycin action: fate of ribosomes after release of nascent protein chains from polysomes. *Proc. Natl. Acad. Sci. U. S. A.* **70,** 3866–9 (1973).

253. Elling, R., Chan, J. & Fitzgerald, K. A. Emerging role of long noncoding RNAs as regulators of innate immune cell development and inflammatory gene expression. *Eur. J. Immunol.* **46,** 504–12 (2016).

254. Kung, J. T. Y., Colognori, D. & Lee, J. T. Long noncoding RNAs: past, present, and future. *Genetics* **193,** 651–69 (2013).

255. Bumgarner, S. L. *et al.* Single-cell analysis reveals that noncoding RNAs contribute to clonal heterogeneity by modulating transcription factor recruitment. *Mol. Cell* **45,** 470–

82 (2012).

256. Fang, Y. & Fullwood, M. J. Roles, Functions, and Mechanisms of Long Non-coding RNAs in Cancer. *Genomics. Proteomics Bioinformatics* **14,** 42–54 (2016).

257. Burrack, L. S., Harper, J. W. & Higgins, D. E. Perturbation of vacuolar maturation promotes listeriolysin O-independent vacuolar escape during Listeria monocytogenes infection of human cells. *Cell. Microbiol.* **11,** 1382–98 (2009).

258. Gründling, A., Gonzalez, M. D. & Higgins, D. E. Requirement of the Listeria monocytogenes broad-range phospholipase PC-PLC during infection of human epithelial cells. *J. Bacteriol.* **185,** 6295–307 (2003).

259. Marquis, H., Doshi, V. & Portnoy, D. A. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of Listeria monocytogenes from a primary vacuole in human epithelial cells. *Infect. Immun.* **63,** 4531–4 (1995).

260. Lecuit, M., Sonnenburg, J. L., Cossart, P. & Gordon, J. I. Functional genomic studies of the intestinal response to a foodborne enteropathogen in a humanized gnotobiotic mouse model. *J. Biol. Chem.* **282,** 15065–15072 (2007).

261. Mager, D. L. Bacteria and cancer: cause, coincidence or cure? A review. *J. Transl. Med.* **4,** 14 (2006).

262. Toller, I. M. *et al.* Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 14944–9 (2011).

263. Leitão, E. *et al.* Listeria monocytogenes induces host DNA damage and delays the host cell cycle to promote infection. *Cell Cycle* **13,** 928–40 (2014).

264. Zou, T., Garifulin, O., Berland, R. & Boyartchuk, V. L. Listeria monocytogenes infection induces prosurvival metabolic signaling in macrophages. *Infect. Immun.* **79,** 1526–35 (2011).

265. Cheskis, B. J. Regulation of cell signalling cascades by steroid hormones. *J. Cell. Biochem.* **93,** 20–7 (2004).

266. Chen, H. C. & Farese, R. V. Steroid hormones: Interactions with membrane-bound receptors. *Curr. Biol.* **9,** R478–R481 (1999).

267. Norman, A. W., Mizwicki, M. T. & Norman, D. P. G. Steroid-hormone rapid actions,

membrane receptors and a conformational ensemble model. *Nat. Rev. Drug Discov.* **3,** 27–41 (2004).

268. Abreu-Blanco, M. T., Verboon, J. M. & Parkhurst, S. M. Single cell wound repair: Dealing with life's little traumas. *Bioarchitecture* **1,** 114–121 (2011).

269. Yang, W., Li, Y., Song, X., Xu, J. & Xie, J. Genome-wide analysis of long noncoding RNA and mRNA co-expression profile in intrahepatic cholangiocarcinoma tissue by RNA sequencing. *Oncotarget* **8,** 26591–26599 (2017).

270. Hao, Y. *et al.* Prediction of long noncoding RNA functions with co-expression network in esophageal squamous cell carcinoma. *BMC Cancer* **15,** 168 (2015).

271. Feng, L. *et al.* Integrated Analysis of Long Noncoding RNA and mRNA Expression Profile in Advanced Laryngeal Squamous Cell Carcinoma. *PLoS One* **11,** e0169232 (2016).

272. Zhang, T. *et al.* Analysis of long noncoding RNA and mRNA using RNA sequencing during the differentiation of intramuscular preadipocytes in chicken. *PLoS One* **12,** e0172389 (2017).

273. Cheng, L. W. *et al.* Use of RNA interference in Drosophila S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 13646–51 (2005).

274. Galicia, J. C., Naqvi, A. R., Ko, C.-C., Nares, S. & Khan, A. A. MiRNA-181a regulates Toll-like receptor agonist-induced inflammatory response in human fibroblasts. *Genes Immun.* **15,** 333–7 (2014).

275. Kriegel, A. J., Liu, Y., Fang, Y., Ding, X. & Liang, M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiol. Genomics* **44,** 237–44 (2012).

276. Marín-Béjar, O. *et al.* Pint lincRNA connects the p53 pathway with epigenetic silencing by the Polycomb repressive complex 2. *Genome Biol.* **14,** R104 (2013).

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