Characterisation of the

L. pneumophila effectors LtpK and LtpM

Corinna Mattheis

Imperial College London

Division of Cell and Molecular Biology

This thesis is presented for the degree of Doctor of Philosophy of Imperial College London and Diploma of Imperial College London 2016

Abstract

Legionella pneumophila is an intracellular pathogen and the causative agent of a pneumonia called Legionnaire's disease or legionellosis. *L. pneumophila* translocates ~ 300 so called effector proteins into the host cells via a type IVB secretion system to enable its survival and replication. The role of most of these effectors is still unknown, however several have been characterised to modulate vesicle trafficking or phosphoinositol composition of the *Legionella* containing vacuole (LCV). Here we characterised two effector proteins: LtpK, which is conserved amongst clinical and environmental isolates, and LtpM, which is only found in a subset of *L. pneumophila* strains. LtpK was found to have a diffuse cytoplasmic localisation upon transfection, during infection it also locates diffusely in the cytoplasm but also shows nuclear localisation. Different potential cellular interaction partners for LtpK were identified in a Yeast-2-Hybrid screen, namely ASXL1, ACBD3 and VBP1. The role of these interactions requires further investigation. It should be noted, however, that it was not possible to confirm these interactions in a pBio-pulldown. LtpM on the other hand shows a vesicular cellular localisation upon transfection and appears on the membrane of the *Legionella* containing vacuole (LCV) and vesicular structures upon infection. These vesicular structures were identified as early endosomes by staining for the early endosomal marker EEA1. Furthermore, binding of LtpM to the phosphoinositide (PI) phosphatidylinositol-3-phosphate (PI3P) was characterized. LtpM binds to PI3P via its C-terminal domain ($LtpM_{455-639}$), which is 63% identical to the PI3P binding domain of the previously characterised *L. pneumophila* effector LtpD. Moreover, LtpM shares secondary structure homology in its N-terminus ($LtpM_{1-145}$) to a glycosyltransferase. It also harbours a DxN motif, which is as DxD or DxN motif characteristic of glycosyltransferases. It could be shown that LtpM has indeed mono-o-glycosyltransferase activity. LtpM inhibits growth of yeast after transformation and this growth inhibition depends on the enzymatic activity of the glycosyltransferase domain. As the enzymatic activity of LtpM could be resolved, however, a cellular target for LtpM could

not be identified. More research is necessary to find the target of LtpM that gets glycosylated and to therefore fully understand the role of LtpM for the pathogenicity of *L. pneumophila.*

Candidate declaration

I, Corinna Mattheis, hereby declare that the work contained within this thesis is my own and all other information is appropriately cited.

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List of abbreviations

AA	Amino acid
Ab	Antibody
ABC	ATP-binding cassette
Amp	Ampicillin
ATP	Adenosine triphosphate
AYE	Aces buffered yeast extract
BSA	Bovine serum albumin
CFU	Colony forming units
Cm	Chloramphenicol
CYE	Charcoal yeast extract
Cvs	coverslips
DDO	doube drop-out
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxyribonucleotide
Dot	defective in organelle trafficking
D-PBS	Dulbreccos phosphate buffered saline
EDTA	Ethylenediamine-tetraacetic acid
EEA1	Early endosomal antigen1
ER	Endoplasmic Reticulum
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GDP	Guanosine diphosphate
GFP	green fluorescent protein
gDNA	genomic Deoxyribonucleic acid
GST	Glutatione S-transferase
GT	Glycosyltransferase
GTP	Guanosine triphosphate
h	Hours
HA	Haemagglutinin (influenza epitope)
Icm	intracellular multiplication
IMP	Inositol monophosphate
IMPA1	Inositol monophosphatase 1

IPTG	Iso-propyl-β-D-thiogalactopyranoside
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
Kan	Kanamycin
l	Litres
LAMP	Lysosomal-associated membrane protein
LB	Luria Bertani
LCV	*Legionella* containing vacuole
LPS	Lipopolysaccharide
Ltp (D, K, M)	*Legionella* translocated protein (D, K, M)
min	Minutes
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p.i.	Post infection
PO	Phenoloxidase
PMA	Phorbol 12-myristate 13-acetate
PI/PtdIns	Phosphatidylinositol
PIP	Phosphatidylinositol-Phosphate
PTM	Post-Translational Modification
QDO	uadruple drop-out
RFP	red fluorescent protein
RIPA	Radio-Immunoprecipitation Assay buffer
RNA	Ribonucleic acid
rpm	rotations per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RS	Restriction Site
SDO	single drop-out
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sec/s	Seconds
TDO	triple drop-out
V	Volts
WT	Wildtype

Acknowledgements

I would like to take this opportunity and thank all the people who have contributed to the completion of this work.

I would like to thank Gadi for the opportunity to complete my PhD project in his group and to be able to work on this interesting topic. My gratitude and appreciation to work for Gadi in his group can hardly be put in words. I am very grateful that Gadi took me on as a PhD student, for the support and the providence of funding.

Furthermore, my special thanks go to Gunnar for the outstanding support during the whole time, for the many stimulating discussions and for all the advice. I am very grateful for the guidance through the project, the constant stimulations, suggestions and discussions which helped me to keep going and truly made a change at the end. I appreciate all the energy and effort Gunnar put into my project and the patience with which he constantly gave me new ideas. Vielen Dank!

I would also like to thank Prof. Aktories and Dr. Jank for investigating the glycosylation activity of LtpM and their support regarding that topic.

I also want to thank the others of "Team *Legionella*" for making it such a special experience. Thanks to Ernie for all the help with the Mass-Spec and analysis and to Dani for the support with the experiments and taking over where I had to leave. But to all of you even more for the fun in and out of the lab, which made it easier when it got tough.

I wholeheartedly thank the whole group for the great working environment, encouraging and stimulating conversation and the constant help! I truly enjoyed my time in the Frankel group and CMBI-1. It was an immense support to have an encouraging working environment with colleagues who are helpful, cooperative and encouraging.

I also want to mention and thank my fellow PhD students who went through the whole experience with me from the beginning to the end, Pamela, Bevin and Sujinna. I will not forget all the singing and laughter in the lab – and all the shared adventures outside the lab. I

count myself extremely lucky to have had such great colleagues who became true friends. At that point I also want to mention Marianne and thank her for the inspiring enthusiasm she brought along with her, but also for the help with the microscopy and image analysis. I truly hope we will manage to stay in touch wherever life and our careers take us in the next years.

My family deserves a very big grateful Thank You on these pages. My parents and my brother supported me all the way through my studies and my PhD, not only financially which was a great help, but more importantly for the mental support and backup. It is an immense support to always have a place to come home to and be greeted with open arms and happy smiles, and there were times when I took that for granted and forgot how special this actually is. I feel like I can always reach out to my parents and they have an open ear and arms for me, as proven through my write-up period which I was able to do back home. Thank you for your everlasting support and for having enabled me to this study. Thank you for always believing in me and for being there for me. Thank you for the wings and the roots.

I want to mention a few more people who helped me through the process of this PhD. I want to thank my London housemates, who became best friends and my family away from home. Thanks to Chris, Emma, Marissa, Tristan, Erin, Diana and Dan, and also Marina, for making a little Victorian house in West London a place to call home.

Last but not least, thanks to Dan. Thanks for all the kind words and for the immense support. I very much appreciate the patience with which you brought me back to reality and which gave me the necessary strength on the finishing line. Thank you for carrying me.

I could not have done it without anyone of you.

The sweet is never as sweet without the sour.

Chapter 1
Introduction

1. Introduction

1.1 The genus *Legionella*

Discovered 40 years ago, the genus *Legionella* in the family of the *Legionellaceae* belongs to the γ-Proteobacteria (Benson and Fields, 1998). These facultative intracellular bacteria are ubiquitous and are predominantly pathogenic (Meyer and Finegold, 1980). Currently there are over 60 species known (http://www.bacterio.net/legionella.html) with at least 73 serogroups (Luck, 2010) (http://www.dsmz.de; http://www.bacterio.cict.fr/). Most of the identified species were isolated from environmental samples and around half of them could also be isolated from infected patients. The species *Legionella pneumophila* is grouped in 16 serogroups, of which serogroups 1, 4 and 6 are most frequently associated with a human disease. The specificity of the serogroups of *L. pneumophila* is related to its lipo-polysaccharide (LPS) characteristics (Ciesielski et al., 1986), the LPS composition is a determinant of the serogroup specificity. Serotyping examinations are ususally done using The Dresden monoclonal antibodies (Mab) Panel, in which all serogroups of the *L. pneumophila* strains and subgroups of *L. pneumophila* Sg 1 strains are identified by 26 monoclonal antibodies (MAb) (Helbig et al., 2002). The strain with the highest clinical relevance is *Legionella pneumophila* serogroup 1, which is responsible for almost 84% of *Legionella* infections worldwide, 80% in Europe and 79% in Germany, (Brodhun and Buchholz, 2011, Beaute et al., 2013, Fields et al., 2002, Yu et al., 2002). So far, 19 other *Legionella* species have been classified as human pathogens (Muder and Yu, 2002, Diederen, 2008).

1.2 Ecology and habitats of *Legionella pneumophila*

Legionella pneumophila is a Gram-negative, aerobic bacterium with a length of 1 to 5 µm. It is ubiquitous in freshwater systems and can also be found in soil. *L. pneumophila* is an intracellular parasite of protozoa (Atlas, 1999, Fields, 1996) and it is believed that the ability to thrive in the professional phagocytes equipped the bacterium to replicate in human cells. For this reason, the bacteria are referred to as environmental pathogens. They can be found as intracellular pathogens in various protozoa, such as *Acanthamoeba, Hartmannella, Echinamoeba, Naegleria* and *Tetrahymenia* (Rowbotham, 1986, Fields, 1996, Fields et al., 2002, Rowbotham, 1980, Brieland et al., 1996, Brieland et al., 1997). The intracellular lifestyle allows *Legionella* to get access to nutrients and protects it from the external environment (Rowbotham, 1980, Fliermans et al., 1981, Fields, 1996). Despite their adaptation to a faculative intracellular life style, extracellular bacteria can survive as planktonic cells and endure low nutrient supply in a viable but non culturable (VBNC) state (Steinert et al., 2007). *Legionella* can also be detected in poly-microbial communities. These so-called biofilm systems represent a source of complex nutrients, therefore offering a possible niche and might enable replication outside of host cells (Rogers and Keevil, 1992).

Water, the main reservoir for *Legionella,* surrounds humans at all times. The increasing mechanization of water supplies has led to an increase of exposure of contaminated water and associated *Legionella* infections, mainly from man-made water reservoirs, such as water pipes, air conditioning systems, cooling towers, humidifiers or whirlpools (Fliermans et al., 1981, Mahoney et al., 1992, Atlas et al., 1995, Steinert et al., 1998, Benkel et al., 2000, Mouchtouri et al., 2010). Human pathogenic species such as *L. pneumophila* and *L. longbeachae* could also be detected in potting soil (Ruehlemann and Crawford, 1996, Velonakis et al., 2010). Infections occur in private households, hotels and hospitals (Woo et al., 1986, Woo et al., 1992, Steinert et al., 2001). Sources are often older water pipes with stagnant water flow and temperatures of 25-45 °C, which provide a place for *Legionella* to

survive and replicate. Heating of the water systems over 60 °C, copper-silver ionisation or hyperchlorination of the affected piping systems can effectively kill bacteria (Hentschel and Heudorf, 2011, Schaefer et al., 2011, Schaefer and Chorus, 2011). Ultimately, as infection with *Legionella* occurs via inhalation of contaminated water and leads to a respiratory disease (see chapter 1.3 for details about infection), all contaminated water sources that may produce aerosols are potential outbreak sources (Stout and Yu, 1997a). Therefore, outbreaks are often linked to human-made aquatic environments (e.g. air-conditioning water systems, swimming pools, spas) where *L. pneumophila* persists in amoeba and biofilms (reviewed in (Newton et al., 2010).

1.3 Legionnaire's disease or Legionellosis

1.3.1 The discovery of a new pathogen

Legionella pneumophila was first discovered 40 years ago. During the 58th meeting of the American Legion veterans of Pennsylvania on 21th - 24th July 1976 in the Bellevue Stratford Hotel in Philadelphia (USA) there was a momentous outbreak of pneumonia, caused by a previously unknown pathogen. 182 participants of the meeting were infected and 29 people died as a result. In addition, 39 people who had been in the vicinity of the hotel were infected, 5 of which with a fatal outcome. Following this outbreak, the disease was called Legionnaire's Disease. Six months after the epidemic a bacterium was identified as the etiologic agent, which was named *Legionella pneumophila*. Subsequently, it was demonstrated that the bacteria had spread via aerosols from the air conditioning of the hotel (Fraser et al., 1977, McDade et al., 1977, Brenner et al., 1979). Retrospective studies later showed that the oldest described case of infection by *Legionella* spp., in this case *L. micdadei*, could be dated to the year 1943 (Hebert et al. 1980). The first *L. pneumophila* strain had already been isolated in 1947, but was only identified and validated in 1979 (McDade et al., 1979).

1.3.2 Disease

Inhalation of *Legionella pneumophila* contaminated aerosols can lead to an infection, which can cause a severe form of pneumonia, called Legionellosis or Legionnaire's disease. Legionnaire's disease accounts for 2-8% of all cases of severe community acquired pneumonia and is thereby one of the most common causes (Khodr et al., 2016, Bartlett, 2008, Bartlett, 2011, Roig and Rello, 2003). Sporadic or epidemic infections occur mainly in the summer and autumn months, since growth of the pathogen is promoted by warm temperature. The route of infection is by inhalation of contaminated aerosols allowing the pathogen to reach the lungs and replicate in macrophages and epithelial cells (Swanson and Hammer, 2000). In addition, it was found that *Legionella* passaged with amoebae are more virulent than the ones cultured in medium, and that co-inoculations of both organisms (*Legionella* + amoebae) lead to serious diseases (Brieland et al., 1996, Cirillo et al., 1999). *Legionella* preferentially infects macrophages; more precisely, the bacteria replicate within the macrophages which are located in the alveoli (Cianciotto et al., 1989, Horwitz, 1992, Nash et al., 1988, Nash et al., 1984). The severity of the infection depends not only on the quantity of inhaled bacteria but also upon the immune status of the patient. Higher age, suppression of the immune system and respiratory capacity, heavy smoking, as well as male sex are risk factors for a *Legionella* infection (Broome and Fraser, 1979, Marston et al., 1994). Especially in elderly and immunocompromised people *L. pneumophila* can cause severe infections, making *Legionella* a major health risk in hospitals and communal facilities (e.g. nursing homes). On the basis of clinical symptoms, it is not possible to distinguish between a pneumonia caused by *Legionella* or pneumonia caused by pneumococcus. Non-specific symptoms such as fever, nausea, loss of appetite and headache or diarrhoea and mental confusion are common for Legionnaires' disease (Broome and Fraser, 1979, Stout and Yu, 1997b). It was long thought that *Legionella* spp. are an accidental pathogen to humans, as there was no man to man transmission reported and therefore humans represent the dead end of infection. However, a recent study reported the first person to person transmission of

L. pneumophila. It is reported that a 48-year-old man was infected with *L. pneumophila* and shortly after his infection, his mother who took care of him was infected as well (Correia et al., 2016). The strain causing the infection was identified as PtVFX/2014, which is from serogroup 1 and belongs to the *L. pneumophila* subspecies *fraseri* (Borges et al., 2016*)*.

1.3.3 Diagnosis, Therapy and Epidemiology

Diagnosis

The validation of a *Legionella* infection occurs primarily by culturing the bacteria. However, this method is very time consuming and labour intensive. A few years ago a urine antigen test for the detection of *Legionella* was developed (Berdal et al., 1979, Mangiafico et al., 1981, Couturier et al., 2014).

This has the advantage of rapid diagnostic turn around, but is less sensitive and specific, as it is biased toward dection of *L. pneumophila* serogroup 1. Since it is responsible for up to 90% of Legionellosis cases, it is regarded as a reliable diagnostic method (Fields et al., 2002, Helbig et al., 2002). In recent years, *Legionella* has also been increasingly detected by PCR from patient material, including non-invasive respiratory specimens (NIRS), which led to a better detection of *L. pneumophila* (Botelho-Nevers et al., 2016).

Furthermore, the bacteria can also be detected by immunofluorescence with direct fluorescent antibody testing (DFA) (sensitivity: 25-75%, specificity: 95%) (Luck and Steinert, 2006, Luck et al., 2008). For fine typing of *Legionella pneumophila*, the so-called SequenceBased-Typing (SBT) is the recognized gold standard. That SBT-test uses DNA sequences of seven virulence genes (*flaA*, *pilE*, *asd*, *MIP*, *MOMP*, *proA* and *Neua*) (Ratzow et al., 2007). In the near future, whole genome sequencing (WGS) might be used more frequently for the diagnosis of bacterial infections and the identification of the outbreak source (Reuter et al., 2013b). WGS has the advantage of being precise and providing in depth information, which helps to detect multidrug-resistant bacteria, and as it becomes faster and

less expensive, it might become more prominent for diagnostics and public health micriobiology (Koser et al., 2014, Reuter et al., 2013a).

Therapy

Legionnaires' disease is generally treatable by administration of antibiotics. However, shortly after the discovery of *L. pneumophila*, its resistance against beta lactams by a beta-lactamase activity was found in all *L. pneumophila* sero-groups (Fu and Neu, 1979).

An immediate start of the therapy is important because the mortality rate increases significantly if therapy is delayed (Heath et al., 1996). For this reason and because of the long duration for diagnosis of *Legionella* it is recommended to include antibiotics against *Legionella* in the treatment for severe pneumonia even without a defined diagnosis. To date, no multi-resistant *Legionella* strain has been isolated. However, there is evidence that *Legionella* might have gained resistance to some antibiotics (Fong et al., 2010). Resistance development has been considered rare, but recently resistances against fluoroquinolones, e.g. ciprofloxacin were reported (Shadoud et al., 2015, Bruin et al., 2014). Until some years ago the Legionnaires' disease was generally treated with erythromycin, which was replaced by other antibiotics with less adverse effects of ototoxicity (toxicity to the auditory nerve) and gastrointestinal distress. Today, usually other macrolide antibiotics (e.g. azithromycin, clarithromycin, roxithromycin) and broad-spectrum fluoroquinolones (e.g. ciprofloxacin, moxifloxacin, levofloxacin) are used because they have fewer toxic side effects and are more potent in penetrating into lung tissue and alveolar macrophages (Baldwin et al., 1990, Olsen et al., 1996).

Epidemiology

Legionnaires' disease is a notifiable illness in Australia, New Zealand, Japan, Singapore, Europe, USA and Canada and coordinated European surveillance has been in place since 1995 (Phin et al., 2014).

All recorded Legionnaire's disease cases in Europe are reported to the European Centre for Disease Prevention and Control (ECDC) and listed on their website (http://ecdc.europa.eu/en/healthtopics/legionnaires_disease/Pages/news.aspx). According to the ECDC in their latest annual report from 2014 published in January 2016, there have been 6941 cases of Legionnaire's disease reported in Europe. The notification rate of Legionnaire's disease in the EU/EEA in 2013 was 11.4 cases per million population, of which 787 cases where travel associated and which is within the range observed in the past five years. However, approximately 80% of the cases are reported from the UK, France, Spain, Italy, Germany and the Netherlands. In 2014 the number went up to 13.5 cases per million population, which is the highest ever observed. Germany, France, Italy, Spain and Portugal reported 74% of all cases in 2014, where a significant contribution to the high numbers was due to the community outbreak that occurred in Vila Franca de Xira near Lisbon, Portugal, in 2014. These countries represent only around two thirds of the EU population, indicating that Legionnaire's disease might be underdiagnosed in the remaining countries.

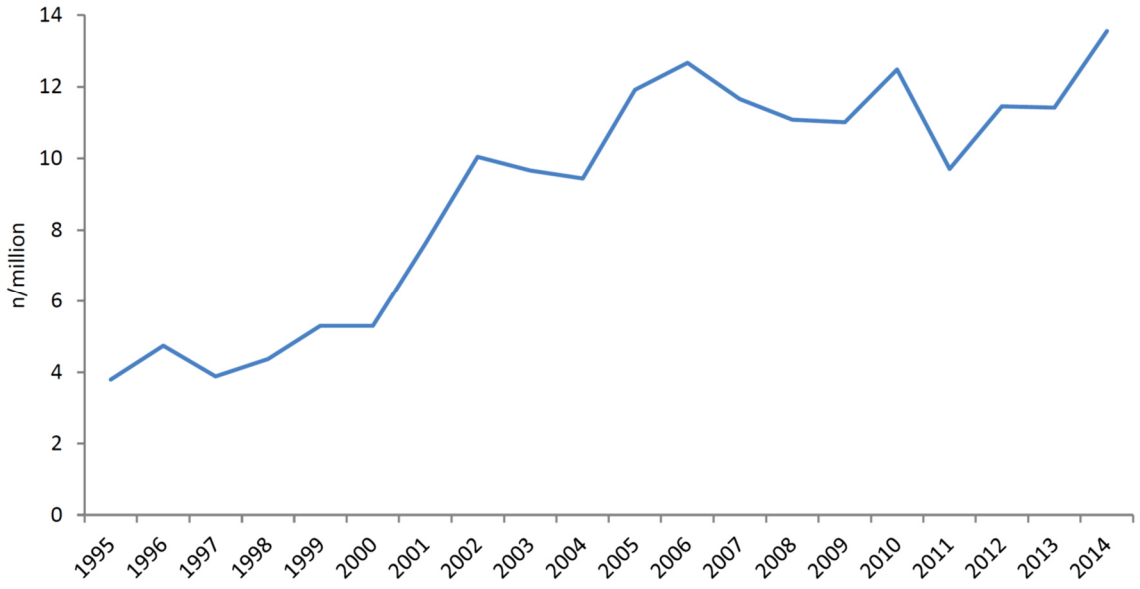


Figure 1 Notification rate of Legionnaires' disease in the EU by year of reporting
The reported cases of Legionnaires disease in the EU per million people in the years 1995-2014. Data from European Centre of Disease control (EDCD)

In the United Kingdom, the epidemiology of Legionnaire's disease is recorded by Public Health England (PHE) (https://www.gov.uk/government/collections/legionnaires-disease-guidance-data-and-analysis). According to their report, there were 342 cases of Legionnaire's disease in England and Wales in 2014, out of which 331 were confirmed. Of the confirmed cases, 69.5% were male individuals and 84.9% were at age 50 or older. The crude case fatality rate was 7.4% in 2014. *L. pneumophila* Serogroup 1 strains have the most important clinical relevance causing 90% of all cases worldwide. An approach was adopted by PHE in 2012 using real-time PCR specific for *L. pneumophila* and serogroup 1 combined with nested direct molecular typing. This led to a rapid investigation and characterisation of the outbreak strains in an average of 1.5 days, compared to 9.5 days with standard protocols (Mentasti et al., 2016). The increase of technology, but also the further improved *Legionella* diagnosis might lead to a continued increase of number of detected *Legionella* infections in the future.

1.4 Intracellular life cycle of *L. pneumophila*

In the environment, *L. pneumophila* infects and replicates within many different protozoan hosts. However, it should also be noted that *Legionella* is a source of food for many *Legionella*-resistant protists (Amaro et al., 2015). The selective pressure being applied by the protists forced *Legionella* to adapt and gain virulence factors which contribute to survival within these hosts. This battle between bacteria and amoeba in the environment is most likely the reason *Legionella* had to expand its virulence factor repertoire to very high numbers (for details, see chapter 1.6.6). Because these virulence factors manipulate fundamental cellular processes in protozoa, which are conserved in human macrophages, they also permit the bacteria to replicate inside human macrophages. In human and environmental hosts, the life cycle is divided into two phases, a replicative (non-virulent) and a transmissive (virulent) phase. This section presents a brief overview of infection/phagocytosis process, these phases and the definition and the regulation of the life cycle of *Legionella*.

1.4.1 Phagocytosis and creation of the *Legionella* containing vacuole

The infection of *L. pneumophila* in human macrophages and protozoa occurs in a similar fashion. After uptake of the bacteria by phagocytosis they remain in a phagosome, which does not enter the phagolysosomal pathway. *Legionella* prevents fusion with the lysosome by the function of effector proteins which are delivered through the secretion systems. *L. pneumophila* has three secretion systems, type I, type II and type IV, and a putative type V secretion system was described. However, only the type IV Dot/Icm system (defective in organelle trafficking/intracellular multiplication) is essential for intracellular lifestyle of *L. pneumophila*. The secretion systems will be described in detail in chapter 1.6. Thus, an acidification of the phagosome is prevented (Horwitz, 1983a, Horwitz and Maxfield, 1984, Roy et al., 1998, Clemens and Horwitz, 1993, Horwitz, 1983b, Horwitz and Silverstein, 1983).

Instead, the *Legionella* containing vacuole (LCV) is surrounded by mitochondria and recruits smooth vesicles (Figure 2) (Coers et al., 1999, Glavin et al., 1979). The smooth vesicles originate from the endoplasmic reticulum (ER). They attach and fuse with the LCV to generate a unique ER-like compartment (Robinson and Roy, 2006, Tilney et al., 2001, Kagan and Roy, 2002, Abu Kwaik, 1996, Swanson and Isberg, 1995, Swanson and Isberg, 1993).

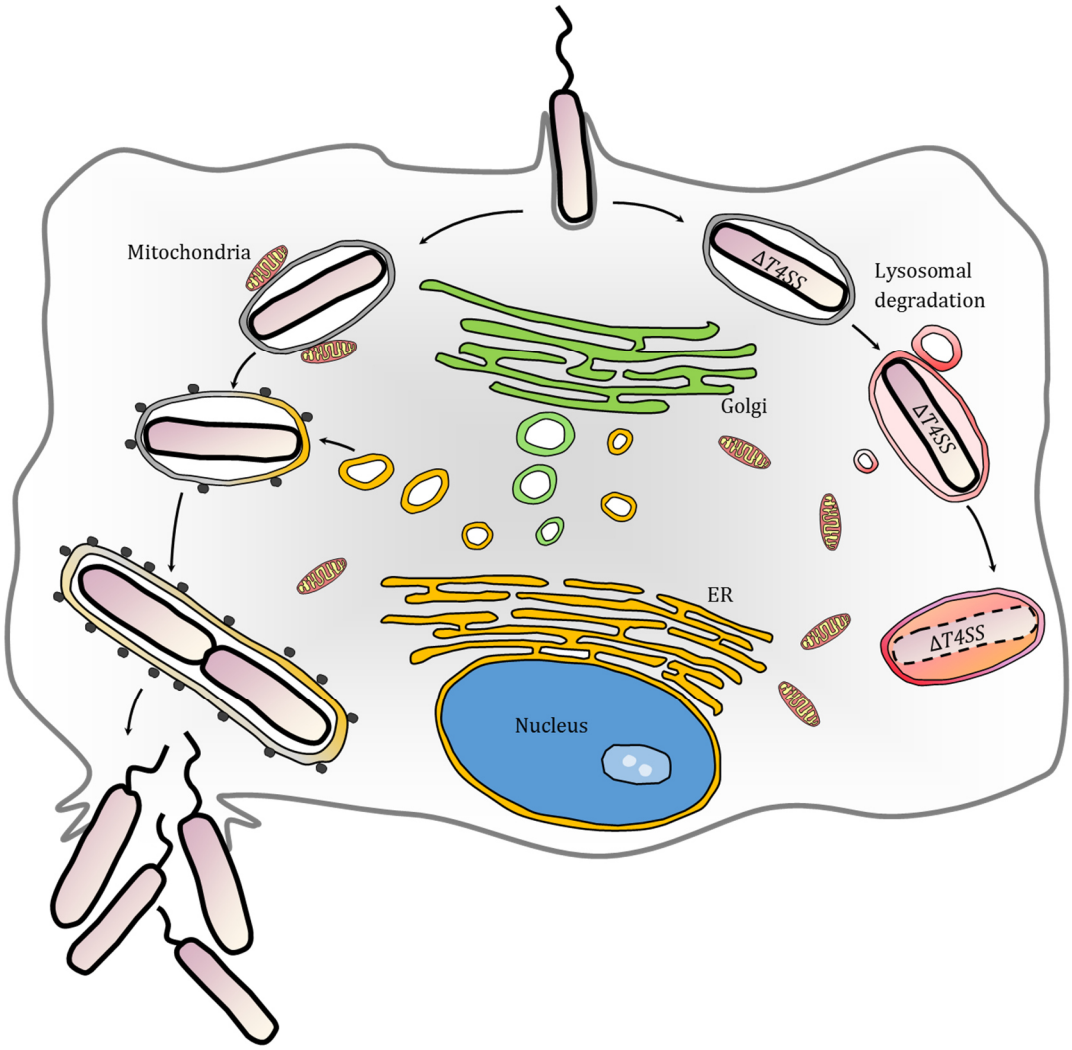


Figure 2 Overview of the intracellular replication and creation of the LCV
Once *L. pneumophila* has been phagocytosed by the host cell, it prevents phago-lysosomal delivery and replicates in a special compartment, the *Legionella* containing vacuole (LCV). The LCV associates with mitochondria and fuses with ER-derived vesicles. After replication inside the LCV, the bacteria transform into the transmissive phase. By lysing the host cell, the flagellated *Legionella* are released. Figure adapted from (So et al., 2015).

This unique compartment is the *Legionella* containing vacuole (LCV), which is completely isolated from the endosomal pathway, but is surrounded by the ER (Figure 3). The LCV provides nutrients and a safe niche for *Legionella* to replicate. In the late replicative phase the vacuole fuses with lysosomes, which may supply the bacteria with nutrients (Sturgill-Koszycki and Swanson, 2000). Nutrient levels control the number of replicative cycles and nutrient limitation eventually leads to the transition of *Legionella* in the transmissive phase (Byrne and Swanson, 1998).

Due to the large number of motile bacteria in the vacuole, the inner pressure increases greatly. It is believed that this is the reason for the collapse of the LCV and finally leads to the release of the bacteria and new host cells can be infected (Swanson and Hammer, 2000, Molofsky and Swanson, 2004, Swanson and Molofsky, 2005, Isberg et al., 2009). However, nutrient deficiency e.g. iron limitation, can trigger early abortion of replication and exit from the host cell (O'Connor et al., 2016).

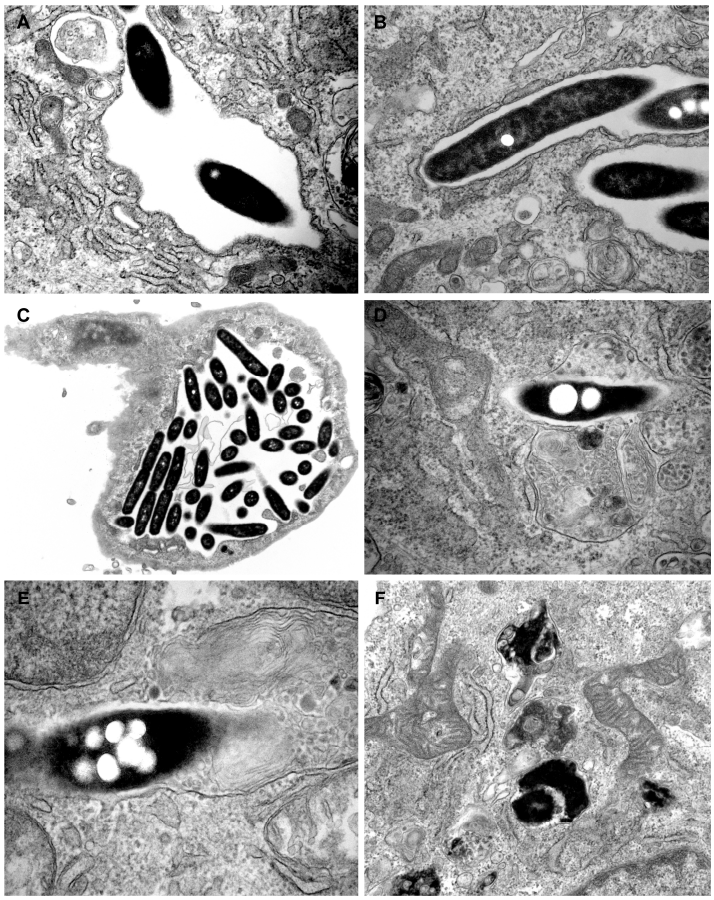


Figure 3 Transmission electron microscopy of endothelial cells infected with *L. pneumophila*
Human umbilical vein endothelial cells (HUVEC) were infected with *L. pneumophila* Lp01 wildtype (A, B, C) or a secretion system mutant (D, E, F). After 6 hours, the LCV is surrounded by rough endoplasmic reticulum (ER) (A, B). LCVs are filled with large numbers of bacteria after 24 hours (C). The mutated strain is not surrounded by smooth membranes not associated with ER (D, E) and partially degraded bacteria are present after 24 hours (F). (Chiaraviglio et al., 2008).

1.4.2 Characteristics of the biphasic life cycle

The biphasic life style of *Legionella* enables the bacteria to optimise resources for the environment in which they find themselves. The switch between the phases involves substantial shifts in the gene expression profile and is reflected by morphological and phenotypical changes. Replicative *Legionella* are not flagellated, salt-resistant and reside within the host cell. In contrast, bacteria of the transmissive phase are motile, salt sensitive, flagellated and more virulent, due to the upregulation of virulence factors (Bruggemann et al., 2006, Byrne and Swanson, 1998, Hales and Shuman, 1999b, Heuner et al., 1999, Sturgill-

Koszycki and Swanson, 2000, Joshi et al., 2001, Hammer et al., 2002, Zusman et al., 2002, Bachman and Swanson, 2004b, Bachman and Swanson, 2004a, Bachman and Swanson, 2001, Jacobi et al., 2004). Once *Legionella* has infected a new host cell, it transitions into the replicative phase. The biphasic life cycle can be simulated in liquid medium. In the medium exponential growth of the bacteria corresponds to the replicative phase and the subsequent stationary phase corresponds to the transmissive phenotype of *Legionella* (Byrne and Swanson, 1998). A recent characterisation of the *L. pneumophila* proteomes in exponential phase, postexponential phase and viable but non-culturable state cells illustrates a detailed phase-specific expression of many virulence proteins associated with the Dot/Icm type IV secretion system (Aurass et al., 2016).

However, this broth model does not account for several additional complex developmental forms which are observed intracellularly and in biofilms (Molofsky and Swanson, 2004, Robertson et al., 2014). To date, 14 developmental forms within a complex network have been reported for *L. pneumophila* (Robertson et al., 2014, Al-Bana et al., 2014, Faulkner et al., 2008, Faulkner and Garduno, 2002).

1.4.3 Regulation of the biphasic life cycle

The transition from the replicative to the transmissive phase in *Legionella* is controlled by a very complex regulatory network, in which not all steps are fully understood. In *E. coli*, the RNA-binding protein CsrA (carboxylic storage regulator A) and the regulatory RNAs CsrB and CsrC function as a global post-transcriptional, regulatory system (Romeo, 1998, Romeo et al., 2013). By binding to the mRNA CsrA can suppress the start of translation of different genes. The two regulatory RNAs bind to CsrA and support its activity (Majdalani et al., 2005). The expression of *csrB* is controlled by a two-component system of BarA and UvrY. With this autoregulatory loop the system remains stable. The regulatory RNAs are degraded through an RNase E-mediated pathway and CsrA is released (Suzuki et al., 2006). Homologous

systems were also found in other bacteria, e.g. *E. coli* or *Pseudomonas aeruginosa* (Majdalani et al., 2005, Van Assche et al., 2015).

For *Legionella* it is believed that a homologue of the *E. coli* CsrA supresses expression of the proteins important for the transmissive phase during the replicative phase (Fettes et al., 2001, Molofsky and Swanson, 2003). For the transition to the transmissive phase the suppression of mRNA translation by CsrA needs to be repealed. In *L. pneumophila* the 2-component system LetA/LetS appears to undertakes this task. These proteins are homologous to the BarA/UvrY system of *E. coli* or GacA/GacS of *Pseudomonas aeruginosa* (Hammer et al., 2002, Lynch et al., 2003). Nutrient deficiency triggers this shift between phases, resulting in the abolishment of CsrA mRNA binding in a mechanism downstream from LetA/LetS (Molofsky and Swanson, 2003). The expression of one or more regulatory RNAs is induced by LetA/LetS. These can then bind CsrA. It has been shown that in *Legionella* RsmY and RsmZ take over this role (Rasis and Segal, 2009, Edwards et al., 2010, Sahr et al., 2009).

The 2-component system LetA/LetS of *L. pneumophila* is responsive to the signalling molecule of the stringent response, second messenger guanosine 3',5'-bispyrophosphate (p)ppGpp (Hammer et al., 2002, Molofsky and Swanson, 2003, Bachman and Swanson, 2004b). This is produced by the major (p)ppGpp synthetase RelA under nutrient deficiency, specifically as response to amino acid starvation (Hammer and Swanson, 1999, Hammer et al., 2002, Zusman et al., 2002, Jain et al., 2006, Jain et al., 2007). In addition, fatty acid deficiency leads to the activation of the synthase/hydrolase SpoT, which also produces (p)ppGpp, although less efficiently than RelA (Dalebroux et al., 2009). Another factor which plays a role in the life cycle of *L. pneumophila* is the alternative sigma factor RpoS. Similarly to *E. coli,* it has been hypothesized that increase in (p)ppGpp levels lead to an increase in the amount of RpoS. However, this connection has not yet been fully established (Abu-Zant et al., 2006). It is speculated that there is a connection between LetA and RpoS instead, which

might be the response regulator LqsR (Gal-Mor and Segal, 2003, Bachman and Swanson, 2004a, Broich et al., 2006). It suppresses transition from the transmissive to the replicative phase and supports the cell-cell interaction between host and bacteria (Tiaden et al., 2008, Tiaden et al., 2010, Tiaden et al., 2007).

1.5 Virulence factors of *L. pneumophila*

Legionella produces a vast arsenal of virulence factors. The main virulence factors are the secretion systems of *L. pneumophila*, of which the Dot/Icm system plays the most pivotal role. The secretion systems are described in detail below.

Besides the secretion systems the major outer membrane protein MOMP plays an important role. In phagocytosis by human macrophages, MOMP binds the host C3R, a protein of the complement system. Complement receptor CR1 binds to C3R, resulting in uptake of the bacteria (Payne and Horwitz, 1987, Bellinger-Kawahara and Horwitz, 1990). *Legionella* can also be taken up independently of the complement system, whereby carbohydrate or lipid structures on the surface of the bacteria play a major role (Gibson et al., 1994). For phagocytosis by amoebae, type IV pili and the Hsp-60 (heat shock protein, 60 kD) were found to be important (Garduno et al., 1998, Stone and Abu Kwaik, 1998). Furthermore, LigA (*Legionella* infectivity gene A) influences the adhesion and the uptake of bacteria by an unknown mechanism (Fettes et al., 2000). The outer membrane located phospholipase PlaB was shown to have contact-dependent haemolytic activity. Although a *plaB* mutant was not impaired for growth in the macrophage and protozoan cell line models, PlaB seems to be important to establish infection in the guinea pig model (Banerji et al., 2008, Schunder et al., 2010).

1.6 The secretion systems in *L. pneumophila*

Pathogenic bacteria use secretion systems to transport virulence factors, such as toxins and other proteins, as well as single-stranded DNA, to the cell surface, into the medium or directly into the host cell. Especially for Gram-negative bacteria the transport across two membranes is a challenge. In addition to the protein secretion systems Sec and Tat which mediate translocation across the inner membrane, six types (I-VI) of secretion systems have been discovered in Gram-negative bacteria (Gerlach and Hensel, 2007, Costa et al., 2015). Substrates can be transported in two steps via the periplasm or directly across the two membranes. The latter applies to the systems I, III, IV and VI. Secretion via the type II and type V systems requires transport of the proteins via the Sec or Tat dependent transport from the cytosol to the periplasm.

L. pneumophila encodes all proteins of the Sec system but SecG (De Buck et al., 2007). In contrast to Sec-dependent transport, the TAT system allows the translocation of proteins already folded in the cytoplasm. All proteins that are secreted via the TAT system harbour a characteristic motif with two adjacent arginine residues in their N-terminal signal sequence (Berks et al., 2003). In the genome of the *L. pneumophila* strains Philadelphia and 130b, the proteins TatA, TatB and TatC are encoded, which are essential for the transport (De Buck et al., 2004, Rossier and Cianciotto, 2005). The proteins secreted via the TAT system include phospholipase C, the cytochrome c oxidase and LvrE (De Buck et al., 2008, De Buck et al., 2007). The TAT secretion system in *L. pneumophila* is involved in the formation of biofilms and contributes to fitness in both amoebae and macrophages, as well as for growth under low iron concentrations (De Buck et al., 2005, Rossier and Cianciotto, 2005).

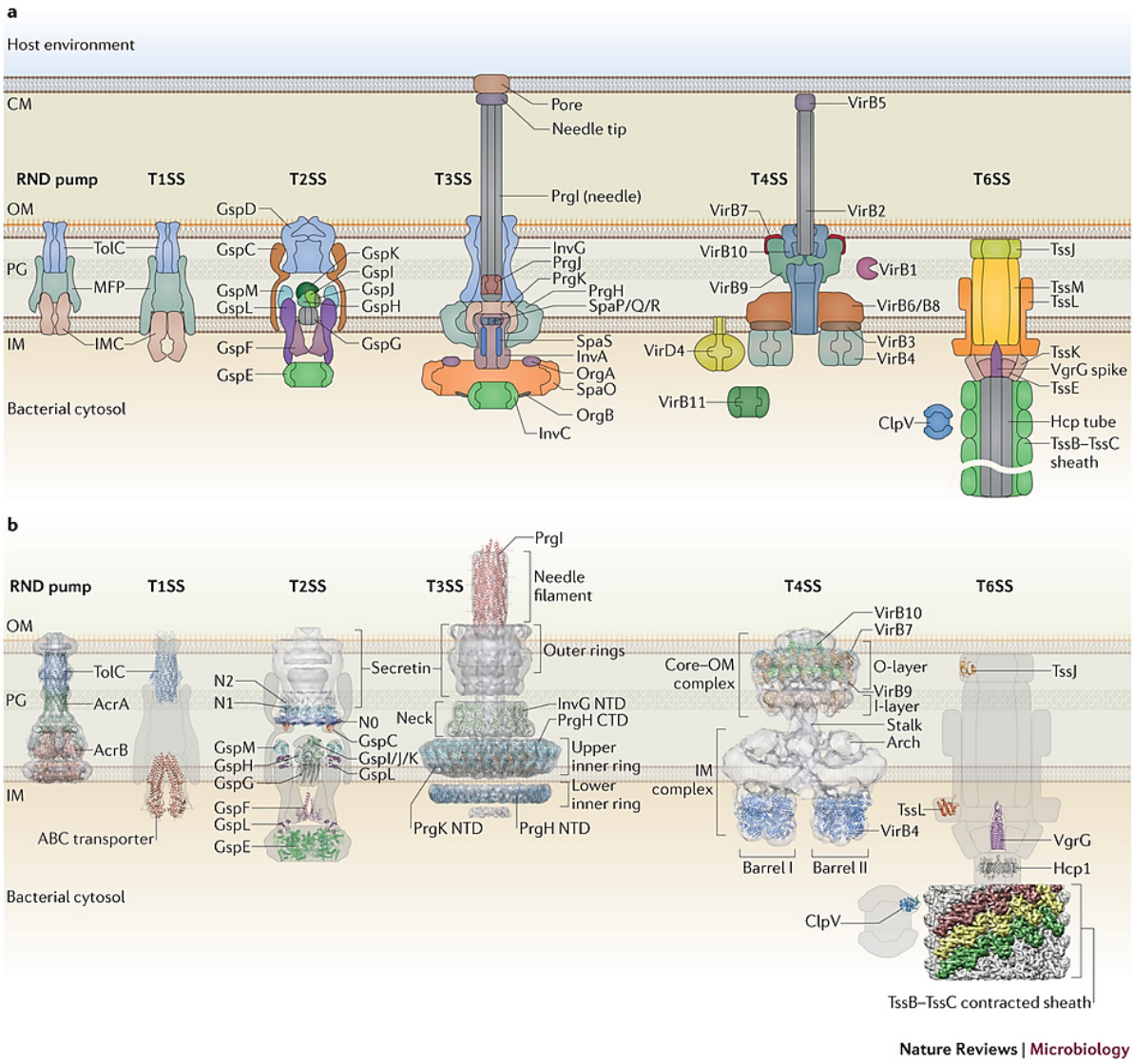


Figure 4 Putative structure of the secretion systems in Gram-negative bacteria
The putative locations of protein components of the T1SS, T2SS, T3SS, T4SS and T6SS are shown schematically (**a**) and as solved structures (**b**). Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (Costa et al., 2015).

So far the secretion systems I, II and IV have been found to be encoded by *L. pneumophila* strains. Furthermore, a putative type V secretion system was identified in *L. pneumophila* Paris. Although several secretion systems are described in *L. pneumophila* to date, mainly the roles of the Dot/Icm type IV secretion system and the type II secretion system Lsp in the virulence of *L. pneumophila* have been studied.

1.6.1 The LSS type I secretion system

The type I secretion system (T1SS) is an ATP-binding cassette (ABC) transporter. The proteins are transported in one step from the cytosol across the inner and outer membrane into the extracellular space. The type I secretion is determined by three proteins, two of which are located in the inner and one in the outer membrane (Thomas et al., 2014). The two proteins in the inner membrane form the ABC transporter and the outer membrane component is a porin-like protein (Lenders et al., 2013). The ABC transporter, an ATPase anchored in the inner membrane, provides the energy required for transportation by hydrolysis of ATP. The membrane fusion protein (MFP) spans the periplasmic space and mediates the transport of the substrate. The pore in the outer membrane is formed by the outer membrane protein (OMP) (Gerlach and Hensel, 2007). Toxins, proteases, enzymes and other virulence factors with a carboxy-terminal signal sequence are secreted through a T1SS in the extracellular milieu (Binet et al., 1997, China and Goffaux, 1999, Awram and Smit, 1998). In *L. pneumophila* a putative T1SS was identified. This so called "*Legionella* secretion system" (LSS) is encoded by the genes *lssXYZABD*. The genes *lssB* and *lssD* have a significant homology to genes of the T1SS of *V. cholerae* and *E. coli*. The gene *lssB* codes for the ABC transporters and *lssD* for the membrane fusion protein (MFP).

The functionality of the T1SS in *L. pneumophila* was reported recently (Fuche et al., 2015). It was shown that the repeats-in-toxin protein RtxA is secreted through the T1SS an LssB-LssD-TolC-dependent manner and that the T1SS seems to be important for the entry into the host cell. Additional substrates of this T1SS are yet to be identified (Jacobi and Heuner, 2003).

1.6.2 The LSP type II secretion system

The type II secretion system (T2SS) in Gram-negative bacteria mediates the transport of folded periplasmic substrates across the outer membrane. The secretion via the T2SS first

requires the translocation of proteins from the cytosol across the inner membrane by the Sec or TAT system. A functional T2SS was identified in *L. pneumophila* (Hales and Shuman, 1999a) and since then found in all sequenced strains of *L. pneumophila* (Costa et al., 2012, Rossier et al., 2004). The translocation system consists of 12 components and is encoded mainly by the LSP ("*Legionella* secretion pathway") genes *lspFGHIJK*, *lspDE*, *lspC*, *lspL* and *lspM*. The pseudopilins are encoded by *lspGHIJK* and *lspF* encodes for a conserved inner membrane protein. LspD forms the secretion pore in the outer membrane, whilst the ATPase LspE provides the energy that allows the pseudopilus to propel the substrates through the secretin. The components LspC and LspM help translocate the substrates (Rossier et al., 2004). In addition to the LSP proteins, the prepilin peptidase PilD is another essential component of the transport system. PilD is an enzyme that cleaves the signal peptides from the immature prepilins and prepseudopilins which allows them to be assembled into the fibres which make up the type IV pili and the assembly of the type II pseudopilus respectively (Liles et al., 1999, Liles et al., 1998). To date, approximately 25 proteins have been identified that are translocated by the T2SS from the bacteria, many of which have enzymatic activity (Cianciotto, 2009, Pearce and Cianciotto, 2009, DebRoy et al., 2006, Banerji et al., 2008)

Protein secretion by the T2SS plays an important role in both the colonization and biofilm formation, as well as for the growth of *L. pneumophila* at low temperatures (Soderberg et al., 2004, Lucas et al., 2006). Additionally, the system seems to be involved in the replication of *Legionella* in macrophages and amoebae (*Hartmannella vermiformis* and *A. castellanii*) (Rossier et al., 2009, Rossier and Cianciotto, 2001, Polesky et al., 2001).

1.6.3 The Type V secretion system (T5SS)

The Type V secretion system (T5SS) is an auto-transporter system. The protein catalyses its translocation across the outer membrane independently. The T5SS is Sec-dependent and proceeds in two steps. First, the auto-transporter protein is transported across the

cytoplasmic membrane into the periplasm. In the second step, the protein is translocated via autocatalytic translocation through the outer membrane. Here, the C-terminal domain of the auto-transporter forms a β-barrel pore in the outer membrane through which the active part of the protein is channelled through (type VA and C). In type VB the auto transporter pore is formed by a second protein (Desvaux et al., 2004b, Desvaux et al., 2004a, Henderson et al., 2004). In the genome of *L. pneumophila* Paris a gene (*lpp0779*) has been identified that encodes a putative auto transporter. The gene product of *lpp0779* has a N-terminal signal sequence that mediates a Sec-dependent translocation and a pore-forming C-terminal domain (Cazalet et al., 2004). However, function of this autotransporter still awaits experimental validation.

1.6.4 The Lvh and other type IVA secretion systems

Type IV secretion systems (T4SS) are a very diverse group of translocation systems which are phylogenetically widely distributed in Gram-negative and -positive bacteria as well as archaea (Berry and Pelicic, 2015, Alvarez-Martinez and Christie, 2009, Voth et al., 2012).

Type IV secretion systems (T4SS) play an important role in bacterial pathogenicity. Many pathogenic bacteria, including *L. pneumophila*, *Agrobacterium tumefaciens, Bordetella pertussis, Helicobacter pylori*, *Brucella* spp., *Rickettsia prowazekii* and *Coxiella burnetii*, use a T4SS for the secretion of virulence factors (Sexton and Vogel, 2002, Fischer et al., 2002). These systems not only translocate proteins, but they can also act as conjugative systems that mediate transfer of single-stranded DNA into other cells (Ding et al., 2003, Nester, 2000, Lacroix and Citovsky, 2016, Suzuki et al., 2015).

Similar to the Type I secretion system, the transport is carried out independently of the Sec or Tat system. In Gram-negative bacteria, the periplasmic part of the T4SS consists of a channel connecting the inner and outer membrane and a pilus-like structure, which makes contact to the host cell. The secretion system enables the transport of DNA and proteins from

the bacterial cytosol through three membranes into the host cell. The Type IV secretion systems had been divided into two categories, the type IVA (T4ASS) and the type IVB (T4BSS) secretion systems (Christie and Vogel, 2000), but are now categorised in three classes (Alvarez-Martinez and Christie, 2009, Bhatty et al., 2013)

First are conjugation systems, which deliver substrates (single-stranded DNA substrates and one or more proteins) in a contact-dependent manner to the target cells (de la Cruz et al., 2010). The second category are the effector translocators, systems which deliver effectors to eukaryotic target cells (Cascales and Christie, 2003). Third are DNA uptake and release systems, which are able to translocate DNA to and from the extracellular milieu (Zechner et al., 2012).

As a prototype of the T4ASS acts the Vir system, which is encoded by the Ti plasmid of *A. tumefaciens*. Therefore, all secretion systems which have a similarity to the Vir system are assigned to the group of T4ASS. Type IVB secretion systems, however, are homologous to the Tra/Trb systems of IncI plasmids (Christie and Vogel, 2000). The Dot/Icm system of *L. pneumophila* is a T4BSS (see 1.6.5 for more details).

In addition to the Dot/Icm T4BSS other secretion systems have been identified in the previously sequenced *Legionella* strains that are assigned to the T4ASS, one of which is the Lvh (*Legionella* vir homologue) system. The Lvh system is encoded through the 11 genes *lvhB2, B3, B4, B5, B7, B6, B8, B9, B10, B11* and *lvhD4*, which are homologous to the genes of the Vir system of *A. tumefaciens* (Segal et al., 1999). In contrast to the Dot/Icm system the Lvh system plays no essential role for intracellular growth of *L. pneumophila* in macrophages and amoebae (Segal et al., 1999). However, it is implicated in the replication of *Legionella* in host cells at lower temperatures and can complement some functions of the Dot/Icm system under specific environmental conditions (Bandyopadhyay et al., 2007, Ridenour et al., 2003). The genes of Lvh system have been identified in the sequenced *L. pneumophila* strains Philadelphia, Paris, Lens and 130b (Cazalet et al., 2004, Chien et al., 2004, Schroeder et al.,

2010). Possible substrates or proteins which are secreted by the Lvh system are yet to be identified. The Trb/Tra secretion system of *L. pneumophila* Corby is also a type IVA secretion system. Similar Trb/Tra systems have also been identified in the genomes of the *L. pneumophila* strains Alcoy and 130b, as well as in *L. longbeachae* (Cazalet et al., 2010, Schroeder et al., 2010, D'Auria et al., 2010).

1.6.5 The Dot/Icm type IVB secretion system

Out of all the secretion systems encoded by *Legionella*, the Dot /Icm is the most important one for its virulence (Brand et al., 1994, Berger and Isberg, 1993) and reviewed in (De Buck et al., 2007). Several recent studies demonstrated that the bacteria can translocate an extraordinary high number of more than 300 effectors proteins via the T4SS into host cells (Zhu et al., 2011). However, the function of the majority of these effectors remains unknown. The T4SS plays critical roles in *L. pneumophila*'s intracellular lifestyle, e.g. enabling the creation and maintenance of the LCV and preventing early cell death of the host to facilitate replication and ultimately inducing lysis of the host cells (reviewed in (De Buck et al., 2007, Xu and Luo, 2013).

The Dot/Icm system is essential for intracellular replication in amoebae and macrophages as well as the formation of the LCV (Andrews et al., 1998, Segal and Shuman, 1997). In addition, the Dot/Icm system is involved in uptake of *L. pneumophila* into cells, their release from the host cells and inhibition of apoptosis of host cells (Abu-Zant et al., 2007, Alli et al., 2000, Alli et al., 2003, Kirby et al., 1998, Molmeret et al., 2004). The Dot/Icm secretion system is encoded by 26 *dot/icm* genes (Segal and Shuman, 1998, Vogel et al., 1998). The *dot*/*icm* genes coding for the macromolecular complex of the secretion system are organized in two genomic regions. Region I includes the genes *icmVWX*, *dotA* and *dotBCD* and region II encodes an additional 18 genes that have partially both an Icm as well as a Dot nomenclature (Segal and Shuman, 1998, Segal and Shuman, 1997, Vogel et al., 1998).

Five proteins, DotC, DotD, DotF (IcmG), DotG (IcmE) and DotH (IcmK)] assemble the so called the core-transmembrane subcomplex, which was revealed by genetic analysis (Vincent et al., 2006b). The lipoproteins DotC and DotD are required for the insertion of DotH, which is presumed to build the outer membrane pore, into the outer membrane (Vincent and Vogel, 2006, Nakano et al., 2010). The proteins DotC/D/H in the outer membrane interact with DotF and DotG, which are inner membrane proteins (Luo and Isberg, 2004, Vincent et al., 2006b). The model of that complex is shown in Figure 5.

Another complex is stably formed in the inner membrane independently of the other Dot/Icm proteins by DotI and DotJ (Kuroda et al., 2015). DotI is a structural homolog of VirB8, which is an essential inner membrane component of type IV secretion systems (Sivanesan and Baron, 2011).

In addition to the core-transmembrane complex, data from Vincent *et al*. implicated the existence of a second subcomplex composed of the proteins DotL (IcmO), DotM (IcmP), DotN (IcmJ), IcmS and IcmW (Vincent et al., 2006a, Vincent et al., 2012). This second subcomplex is of particular interest because DotL has been proposed to function as the type IV coupling protein (T4CP) for the Dot/Icm secretion system (Sexton et al., 2004a, Sexton et al., 2004b, Sexton et al., 2005, Buscher et al., 2005). T4CP function as inner-membrane receptors for substrates and it was discovered that DotL requires a stable interaction to IcmSW in order to translocate a major class of substrates (Sutherland et al., 2012).

To date, it is believed that if a “supercomplex” of these proteins exists, it might be assembled dynamically only under certain conditions or it is not stable enough to be purified.

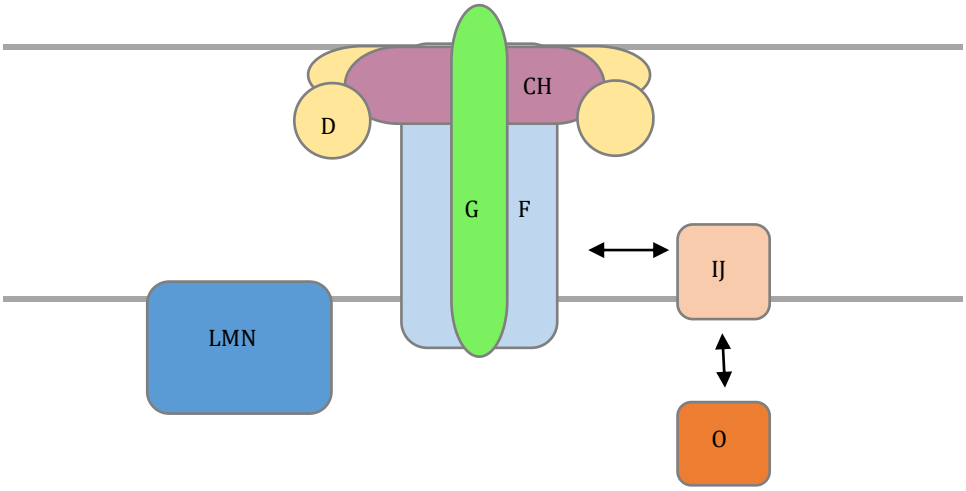


Figure 5 Model of the Dot/Icm Type 4 Secretion System core transmembrane complex
The core transmembrane complex of the Dot/Icm Type 4b secretion system consists of the proteins DotC, DotD, DotF, DotG and DotH. This complex interacts with the inner membrane complex DotI/DotJ and the cytoplasmic ATPase DotO. The coupling proteins DotL, DotM and DotN are proposed to form a complex and deliver substrates to the core complex. (Buscher et al., 2005, Vincent et al., 2006a) Figure adapted from (Kubori and Nagai, 2016).

1.6.6 Substrates of the Dot/Icm Type IVB secretion system

Substrates of the secretion system are called effector proteins or effectors. It is well established that most of the effectors have a translocation signal in the carboxy-terminus which enables translocation through the Dot/Icm system (Nagai et al., 2005).

However, research by Jeong *et al*. revealed an internal translocation signal, which is necessary for SidJ translocation(Jeong et al., 2015). The exact mechanism of how carboxyl-terminal signals are recognised is not completely understood, no consensus sequence can be specified within the carboxyl-terminus. However, a few characteristics were identified, such as a preference for small residues containing a hydroxyl group (serine/threonine), negatively charged and hydrophobic residues in a position-dependent manner (Huang et al., 2011). It was also found that translocated substrates harbour regions with 6-8 residues called E Blocks that are rich in glutamates.

Genetic deletions of single effectors usually do not show obvious phenotypes in most infection models, suggesting that functional redundancy exists. Even when several gene clusters are deleted, resulting in a mutant missing 31% of the known effectors, the resulting strain showed no growth defect in bone marrow-derived mouse macrophages. Notably, growth of some deletion mutants is impaired in the amoeba hosts *D. discoideum, H. vermiformis,* and *A. castellanii* (O'Connor et al., 2011). This indicates that amoeba are a more selective, restrictive environment for *Legionella* than macrophages and that the acquisition and evolution of effectors occured to specifically target different hosts.

A characteristic of the *L. pneumophila* effector arsenal is the presence of a high number and wide variety of eukaryotic like proteins. Several effectors contain eukaryotic protein-protein interaction motifs, including ankyrin- and leucine-rich repeats (LRR), suggesting that *L. pneumophila* effectors subvert host cell processes via functional mimicry of host proteins (Cazalet et al., 2004, de Felipe et al., 2008). The effectors which have been characterised so far exploit and subvert posttranslational modification systems, immune responses and cell death signalling as well as cellular trafficking and lipid signalling pathways (reviewed in (Newton et al., 2010, Rolando and Buchrieser, 2012, Ensminger and Isberg, 2009, So et al., 2015).

1.6.6.1 Modulation of phosphatidylinositol phosphates by effectors

Immediately after uptake of *L. pneumophila* in the host cell, the lipid composition of the LCV changes. Lipids serve as anchors for proteins and therefore determine the recruitment of selected proteins, which as a result direct membrane fission and fusion. Especially phosphatidylinositol-phosphates (PIPs), the phosphorylated derivatives of phosphatidylinositol (PI), negatively charged membrane lipids, were found to play important regulatory roles in cell physiology and trafficking of vesicles and organelles (Di Paolo and De Camilli, 2006, Hokin, 1985). They are key players in trafficking and signalling processes in

eukaryotes that control membrane–cytoskeleton interactions and vesicle trafficking. PIs comprise less than 1% of cell lipids (Rameh et al., 1997, Martin, 1998, Fruman et al., 1998), yet they play very important roles in major signal transduction pathways, serving as docking sites for signalling effectors and as precursors of secondary messengers (Di Paolo and De Camilli, 2006). Reversible phosphorylation of the inositol ring at positions 3, 4 and 5 results in the generation of seven phosphoinositide species PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 (Liu and Bankaitis, 2010)(Figure 6).

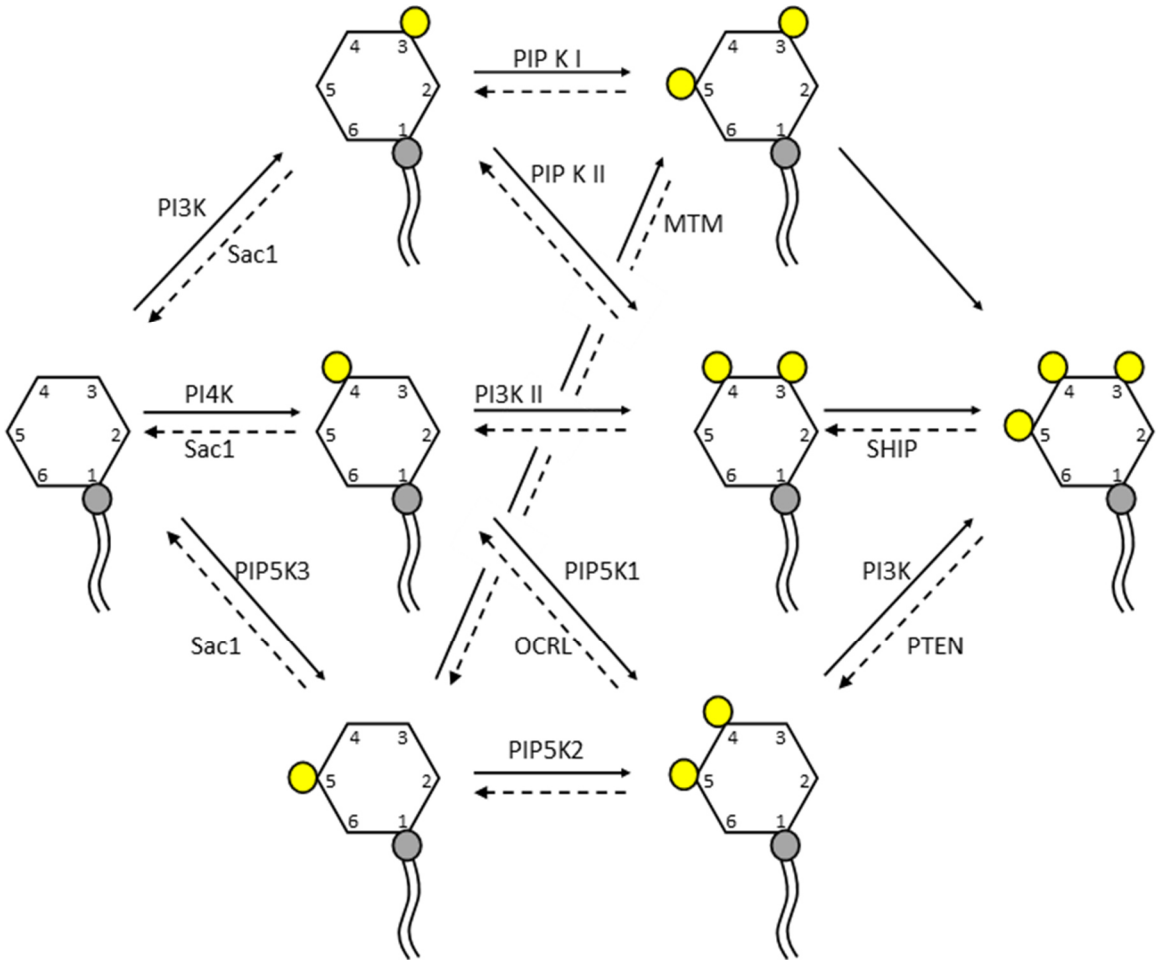


Figure 6 Metabolism of the seven phosphoinositide lipids
PI lipid synthesis and metabolism is highly regulated by different kinases and phosphatases. Seven different PIPs are generated by the reversible phosphorylation at position 3, 4 or 5 of the inositol ring. The yellow circles represent the reversible phosphorylation on the positions three, four and five of the inositol ring, the grey circle represents the phosphorylation of the phosphatidylinositol which links the inositol ring to the two fatty acid tails.

Each of the seven PIPs has a unique subcellular distribution with a predominant localization in subsets of membranes. The levels and turnover of PIPs are tightly controlled by a large set of PI-specific enzymes. To invade and survive within a host cell, prokaryotic and eukaryotic pathogens communicate across several membrane barriers and are able to modulate host endomembrane systems. Consequently, intracellular microbes develop strategies to modulate host membrane trafficking (Jiang et al., 2013). For endocytic trafficking, phosphatidylinositol-3-phosphate (PI(3)P) plays a crucial role in conferring identity to endosomes and regulating vesicle fusion on the cytoplasmic face of the membrane (Behnia and Munro, 2005). Pathogens take advantage of these properties to establish intracellular infection. The *Salmonella* effector protein SopB is a phosphatase whose activity recruits the host Rab5 and PI(3)P kinase Vps34 to the *Salmonella*-containing vacuole (SCV) (Mallo et al., 2008). In contrast, *Mycobacterium tuberculosis* uses the phosphatase SapM (secreted acid phosphatase M) to decrease PI(3)P levels on the vacuole and thus arrests maturation of the vacuole at the early endosome stage in which *M. tuberculosis* is able to replicate (Deretic et al., 2006).

L. pneumophila avoids fusion of the LCV with lysosomes, while it communicates with the endosomal, secretory and retrograde vesicle trafficking pathways (Isberg et al., 2009, Hubber and Roy, 2010). *L. pneumophila* subverts the host's PIPs in several different ways (Hilbi et al., 2011). Multipe Dot/Icm effectors anchor to the LCV membrane by interacting with different PIs. For example the effector SidC binds specifically PI(4)P through its 20 kDa P4CSidC domain and recruits ER vesicles to the LCV (Ragaz et al., 2008). Also PI(4)P binder are Rab1 guanine-nucleotide exchange factor (GEF)/AMPylase SidM (or DrrA) and the Rab activator/interactor LidA (Brombacher et al., 2009). PI(3)P is bound by LtpD (Harding et al., 2013a), the retromer interactor RidL (Finsel et al., 2013) , the glycosyltransferase SetA (Jank et al., 2012), and LidA, which also binds to PI(4)P (Neunuebel et al., 2012), as well as by the virulence factor LpnE (Weber et al., 2009a). It should be noted that the effector PIP binding domains do not have primary sequence homology to eukaryotic PIP-binding domains. Live

cell imaging of PI dynamics during *L. pneumophila* infection of *Dictyostelium discoideum* (Weber et al., 2013) showed that upon uptake PI(3,4,5)P_3 transiently accumulated on early phagosomes which was converted to PI(3)P within 1 min after uptake. Wild-type LCVs gradually lost PI(3)P within 2 hours, while a rearrangement of PI(3)P-positive membranes to the cell centre was observed. PI(4)P transiently localized to early phagosomes and was cleared within minutes after uptake and then later steadily accumulated only on wild-type LCVs.

The PIP composition of the host cell is directly modulated by *L. pneumophila* in several ways for its benefit (Hilbi et al., 2011). The two effectors SidF and SidP are both PI 3-phosphatases which were identified during a bioinformatics screen searching for the CX5R signature motif characteristic for the active site of PI phosphatases. Both effectors directly modify the PIPs on the LCV. SidF hydrolyses phagosomal/endosomal PI(3,4)P_2 and PI(3,4,5)P_3, eventually yielding PI(4)P (Hsu et al., 2012), and SidP hydrolyses PI(3)P as well as PI(3,5)P_2 *in vitro* (Toulabi et al., 2013). Significantly less PI(4)P anchored SidC is found on the LCV in absence of SidF, which indicates the importance of SidF to enrich PI(4)P on the LCV.

Additionally, host cell PIP converting enzymes are recruited to the LCV. RalF is an ADP ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) which activates members of the ARF family and is required for Arf1 localization to the *Legionella* phagosome (Nagai et al., 2002). Although RalF localizes to the LCV independently of PI lipids, it might indirectly recruit PI4KIIIβ through its interaction with Arf1 (Nagai et al., 2002, Hilbi et al., 2011, Godi et al., 1999). OCRL1, which dephosphorylates PI(4,5)P_2, yielding PI4P, (Weber et al., 2009a), is bound to the LCV by LpnE and potentially another redundant effector, as a LpnE null mutant still recruits OCRL1 to the LCV. Alternatively, the recruitment and activation of the host GTPase Rab1 by SidM could lead to the recruitment of OCRL1 as a downstream effect (Lichter-Konecki et al., 2006, Hyvola et al., 2006).

Some *Legionella* effectors have been found to modulate the phospholipid composition of the LCV themselves, instead of recruiting PI-modulating host proteins. LpdA was found to potentially act as phospholipase D (Schroeder et al., 2015) and LecE as activator of Pah1, the host cell phosphatidic acid phosphatase. This in turn could lead to the breakdown of phospholipids to phosphatidic acid and then diacylglycerol (Viner et al., 2012). Furthermore, the effector LtpD was shown to interact with the host enzyme inositol (myo)-1 (or 4)-monophosphatase 1 (IMPA1), which is further described in chapter 1.8.

1.6.6.2 Evasion of the phago-lysosomal pathway using effectors

L. pneumophila is confronted with the problem to avoid the endocytic pathway after entry into the cell. This usually proceeds via acidification of the phagosome and ultimately fusion with lysosomes. *L. pneumophila* avoids this phago-lysosomal pathway and instead maintains a neutral pH in its phagosome, the LCV (Roy et al., 1998).

The effector SidK inhibits the mammalian vacuolar H+-ATPase (vATPase) by blocking ATP hydrolysis of VatA, which inactivates the vacuolar proton pump and the phagosome cannot be acidified (Xu et al., 2010). However, the pH of the LCV was not effected when SidK is deleted, indicating a redundant function of other effectors.

Several other effectors which could participate in the decoupling from the endo-lysosomal pathway have been identified based on their ability to disrupt the endosomal system in yeast (de Felipe et al., 2008, Heidtman et al., 2009, Shohdy et al., 2005), but similar roles in infection have only been validated for few effectors. What has become clear from these few validated examples is that effectors target membrane traffic at multiple levels. They target vesicle traffic by modulating the key proteins involved as well as the membrane lipids and phosphatidylinositol-phosphates. To block and manipulate the vesicle trafficking, the effectors target the endosomal network throughout the cell and also act on the LCV.

Throughout the cell, vesicles move along the cellular cytoskeleton, which consists of microtubules, intermediate filaments and actin polymers (microfilaments). This movement of the vesicles along the cytoskeleton is necessary for the phago-lysosomal maturation (Lee et al., 2003, Fairn and Grinstein, 2012, Flannagan et al., 2012).

It was discovered that immediately after uptake the LCV moves through the cell prior coming to rest in a perinuclear position. This movement was found to depend on the effector LegG1 (Rothmeier et al., 2013), which localises to the LCV and stimulates the small GTPase Ran, which enhances LCV motility and migration by stabilizing the microtubules (Rothmeier et al., 2013, Simon et al., 2014).

Another way to escape the phagosomal pathway is by disrupting the cellular vesicle traffic. *Legionella* translocates the effector VipD. VipD binds Rab5 and Rab22 on early endosomes, which in turn activates the phospholipase activity of VipD. This phospholipase A1 (PLA1) alters the PIP and also protein composition on early endosomes by removing PI(3)P and therefore preventing fusion of these vesicles with the LCV (VanRheenen et al., 2006, Ku et al., 2012, Gaspar and Machner, 2014, Lucas et al., 2014). *L. pneumophila* VipD deletion mutants exhibit significantly more Rab5 on the LCV, which leads to the assumption that VipD has a direct impact on the evasion from the phago-lysosomal pathway.

Other effectors have been identified with potential roles in inhibiting endosomal maturation. VipA is a coiled-coil protein, which was found to associate with early endosomes but not the LCV in infection (Franco et al., 2012). Ectopically expressed VipA leads to defects in the MVB (multi vesicular body) pathway in yeast (Franco et al., 2012). *In vitro*, VipA binds and polymerizes actin (Bugalhao et al., 2016); however how these *in vitro* observations translate into a function in infection is still to be determined. Similarly, the effectors LegC3 and LegC7/ YlfA, with potential roles in inhibiting endosomal maturation by *Legionella,* have been identified based on their ability to cause aberrant endosomal traffic or cytotoxicity in yeast (de Felipe et al., 2008, Bennett et al., 2013, Yao et al., 2014, O'Brien et al., 2015, Campodonico

et al., 2005, Shohdy et al., 2005); however their exact location and targets in infection remain to be characterised.

1.6.6.3 Autophagy during intracellular replication

Autophagy is a cellular process to clear damaged proteins, organelles or pathogenic invaders by encapsulation into membranes, which subsequently fuse with lysosomes to degrade and recycle the content (Huang and Brumell, 2014). Pathogens including *L. peumophila* evolved diverse mechanism to avoid this pathway.

The ER is presumed to serve as one of the membrane sources for autophagosome formation (Axe et al., 2008, Simonsen and Stenmark, 2008, Simonsen and Tooze, 2009).

L. pneumophila produces the effector LpSPL (*lpp2128, lpg2176,* or *legS2*) and translocates it into the host cell during infection. LpSPL was discovered as a eukaryotic like protein in a bioinformatics screen and phylogenetic analysis revealed that it was likely acquired early in evolution by horizontal gene transfer from a protozoan host (Degtyar et al., 2009, Rolando et al., 2016a) . It is similar to the eukaryotic protein SGPL1 (sphingosine-1-phosphate lyase 1), which controls sphingosine-1-phosphate (S1P) levels in the cell by catalysing its synthesis, dephosphorylation or degradation (Rolando et al., 2016a, Rolando et al., 2016b). S1P levels in the cells are important regulators to mediate the balance between autophagy and cell death (Young et al., 2013). It was recently reported by Rolando and colleagues that LpSpl indeed confers S1P lyase activity, which leads to the specific reduction of cellular levels of sphingosine and thereby delays autophagy during *L. peumophila* infection (Rolando et al., 2016a, Rolando et al., 2016b).

Several, so called Atg proteins, mediate the formation of an autophagosome. The protein Atg8 is required for the acquisition of autophagosomal membranes and therefore particularly for maturation of the autophagosome. Similar to autophagosomes, the LCV first acquires and then loses Atg7 and Atg8 (Amer and Swanson, 2005). The *L. pneumophila*

effector RavZ, a cysteine protease, was found to specifically deconjugate Atg8 proteins from phospholipid membranes thereby inhibiting the maturation of the autophagosome (Choy et al., 2012).

Recent research found that the effector LegA9, which was discovered via a bioinformatics screen, might label the LCV for p62 recognition followed by autophagosome fusion and lysosome targeting (Khweek et al., 2013). The *legA9* deletion mutant replicated in mouse BMDMs and in human macrophages significantly more compared to the WT strain. However, LegA9 does not seem to induce autophagy but rather label the LCV for recognition by the autophagic machinery.

However, after recognition by and fusion with autophagosomes, the fusion with lysosomes is delayed. Depending on the host cell, *L. pneumophila*'s ways to overcome and circumvent autophagy are more or less efficient, as shown in autophagosome recruitment and subsequent quick delivery to lysosomes in *L. pneumophila* restrictive mice. Whether *L. pneumophila* is targeted to the autophagsome and/or lysosome therefore depends on the host's ability to deliver it down this pathway before other effectors modulate the cellular responses. The fate of the pathogen therefore depends on the efficiency of the effector arsenal to abolish autophagosome maturation and on the other hand how quick and efficient the host's autophagy pathway delivers the bacterium to the lysosome. Further research is required to elucidate the role of autophagy for intracellular replication of *L. pneumophila* in the different host cells.

1.6.6.4 Modulation of transcription by effectors

Post-translational modification of histones by methylation is a mechanism to regulate transcription and found to be modulated by the *L. pneumophila* effector RomA/LegAS4 (Li et al., 2013b, Rolando et al., 2013). RomA/LegAS4 was discovered to have a eukaryotic-like SET

domain. SET domains catalyse lysine methylation in eukaryotes and this histone modification leads to a change in chromatin structure and gene transcription.

RomA/LegAS4 catalyses the methylation of H3K14, which leads to transcriptional inhibition. H3K14 is usually acetylated upon transcriptional activation and it is believed that the methylation of the same residue competes with acetylation. RomA/LegAS4 acts genome wide, but was particularly active on promoters of immune genes, such as TNF (tumour necrosis factor) or IL6 (interleukin-6). In addition, it was shown that RomA/LegAS4 also methylates H3K4 which is suggested to have a role in rDNA transcription (Li et al., 2013b, Rolando and Buchrieser, 2014, Rolando et al., 2013).

Moreover, *L. pneumophila* was found to modulate transcription in the host cell by activating the transcription factor NF-κB (Losick and Isberg, 2006, Abu-Zant et al., 2007, Bartfeld et al., 2009, Teruya et al., 2007, Shin et al., 2008). The effectors LegK1 and LnaB activate NF-κB which leads to the activation of transcription of anti-apoptotic genes resulting in an elongated life-span of infected macrophages (Ge et al., 2009, Losick et al., 2010). While the mechanism of LnaB remains to be discovered, LegK1 was found to mimic the host IkB kinase and phosphorylates IkB family proteins, which targets them for ubiquitination and proteasomal degradation. Thus, NF-κB is no longer inhibited and can activate transcription in the nucleus.

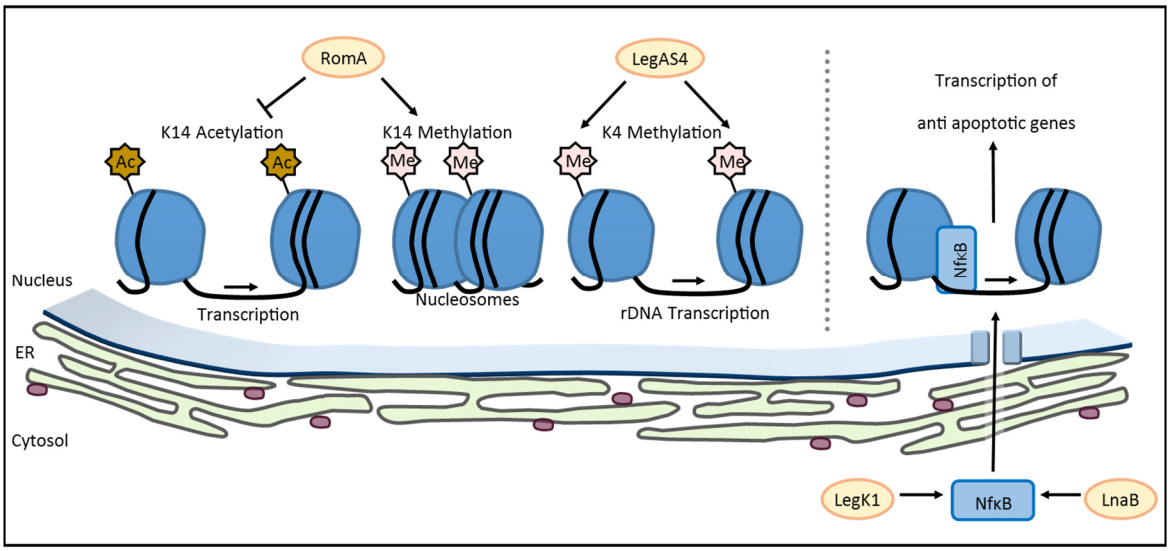


Figure 7 Manipulation of transcription by *L. pneumophila* effectors
Transciption is modulated by the effector RomA/LegAS4 by methylating Histone H3 on position K14, which interferes with acetylation on K14 and thereby prevents transcription, or by methylating Histone H3 on position K4 activating rDNA transcription. NF-κB signaling is activated by LegK1 and LnaB leading to transcription of NF-κB-regulated genes.

1.7 Glycosyl-transferase toxins of bacteria

Post-translational modifications modulate the function of proteins. Glycosylation is a frequent and important modification. Probably more than half of all proteins are modified by a glycosylation (Apweiler et al., 1999). Sugar moieties from activated sugar donors containing a phosphate-group are transferred to a substrate by glycosyltransferases (Breton et al., 2006, Lairson et al., 2008). The various types of glycosylation can be divided by the type of the bond between the protein and the appended saccharide. The most common modifications of proteins with glycans are N- and O- glycosylation. In N-glycosylation, the saccharide is linked via an N-glycosidic bond with the nitrogen nucleophile of the amide group of an asparagine residue of the protein, whereas in O-glycosylation, the sugar is transferred to the oxygen nucleophile of the hydroxyl group of a serine or threonine side chain (Lehrman, 1991, Chou et al., 1995, Hart, 1997, Cole and Hart, 1999, Cheng et al., 2000).

O- and N-glycosylation often results in attachment of chains of multiple sugars. In contrast, if N-acetylglucosamine (GlcNAc) is transferred to the hydroxyl group of a threonine or serine residue, only a single GlcNAc is transferred and not elongated (Hart, 1997, Comer and Hart, 2000).

Various bacterial effector proteins and toxins modulate the function of the host cell processes by glycosylation (Belyi and Aktories, 2010). Toxins A and B of *Clostridium difficile*, for example, glycosylate small Rho GTPases at specific threonine residues (Jank et al., 2007), locking them in an inactive state. As a consequence of this, downstream signalling is inhibited, which results in the disruption of the cell cytoskeleton and apoptotic cell death, ultimately leading to fluid accumulation in the large intestine (Voth and Ballard, 2005). Another example is the effector protein NleB1 of Enteropathogenic *Escherichia coli* (EPEC), which catalyses the glycosylation of Fas associated death domain protein (FADD) with GlcNAc in a N-glycosidic linkage to Arg117 (Li et al., 2013a, Pearson et al., 2013), which prevents interaction of FADD with Fas and therefore induction of cell death. The rhamnosylation of the translation elongation factor P by EarP is another example for arginine glycosylation of a bacterial glycosyltransferase (Lassak et al., 2015b, Lassak et al., 2015a).

1.7.1 *L. pneumophila* glycosyl-transferases inhibit translation and might interfere with vesicular trafficking

Legionella translocates multiple effectors to target host protein synthesis. This presumably prevents the host cell from mounting an immune response against the infection as well as maximising the potential nutrients available to be taken up by *Legionella*.

Through primary sequence homology, a series of glucosyltransferase effectors, 13 in total, was identified, which are grouped in the Lgt (*Legionella* glycosyl-transferases)-families Lgt1, Lgt2 and Lgt3 (Belyi et al., 2013, Belyi et al., 2011, Belyi et al., 2006, Belyi et al., 2009, Belyi et

al., 2008, Belyi et al., 2012, Lu et al., 2010). All Lgts were found to be glucosyltransferase enzymes and glucosylate eEF1A, a key protein involved in elongation of nascent polypeptide chains. Modification by Lgt leads to the inhibition of eEF1A and ultimately arrest of protein synthesis (Figure 8). However, although all Lgt proteins appear functionally equivalent, they are differentially regulated by *Legionella*. Lgt1 and Lgt2 are expressed during the stationary phase whereas Lgt3 is produced in the lag (pre-log) phase of growth (Belyi et al., 2008).

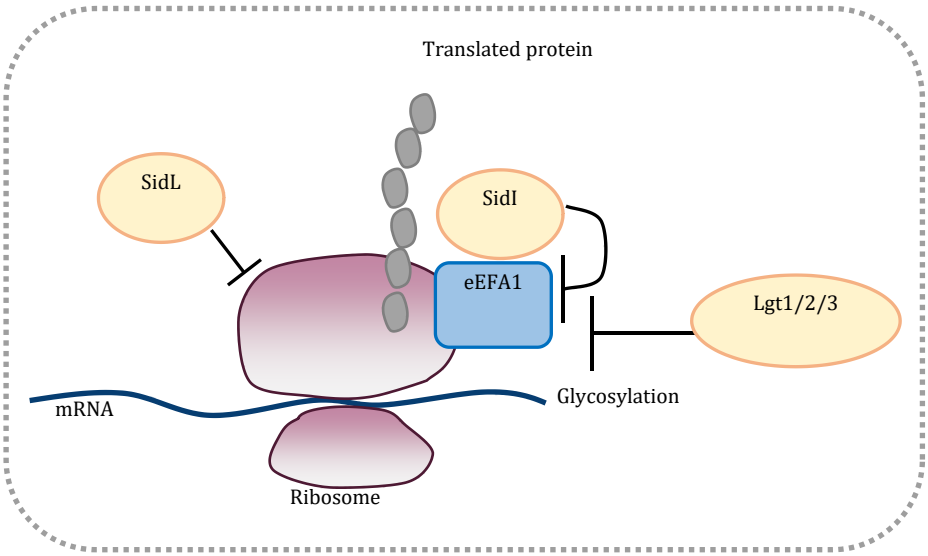


Figure 8 *L. pneumophila* effectors modulate host cell protein translation
The glycosyltransferases Lgt1, 2 and 3 inhibit protein translation by glycosylating the elongation factor eEFA1. Two other effectors, SidI and SidL, were found to inhibit protein translation. SidI directly inhibits eEFA1 whereas the mechanism of SidL remains to be clarified. Figure adapted from (So et al., 2015).

In addition to the glucosyltransferase Lgt proteins are the effectors SidI and SidL. SidI, like the Lgt proteins, targets protein translation by binding to eEF1A. However, it also has affinity for eEF1Bg. In contrast, although SidL is able to inhibit protein translation, it does so via an unknown mechanism (Fontana et al., 2011). No growth phenotype was observed with Lgt deletion mutants, which might be due to the presence of SidI.

Another glycosyltransferase in *L. pneumophila* is the effector SetA (subversion of eukaryotic vesicle trafficking A) was shown to use UDP-glucose as a donor substrate and mono-O-glucosyltransferase activity. A host target protein for SetA remains unknown. SetA interferes with vesicel trafficking and localises to endosomes via a PI3P binding domain localised in its C-terminal domain (Jank et al., 2012).

1.8 The effector LtpD

The effector LtpD was recently identified in *L. pneumophila* strain 130b (Schroeder et al., 2010). In previous studies it was shown that LtpD localises to the surface of the LCV during infection (Harding et al., 2013a). The C-terminal domain $LtpD_{472-626}$ is sufficient for localisation to the LCV.

PIP (phosphatidylinositol phosphate)-binding arrays showed that $LtpD_{472-626}$ binds to PI(3)P, suggesting that LtpD is bound to the LCV via PI(3)P. This conclusion was supported by ectopic expression experiments, in which LtpD co-localises with PI(3)P marker-proteins, but not with a PI4P marker. Although the contribution of PI4P to the LCV membrane is well established (Brombacher et al., 2009, Hsu et al., 2012, Weber et al., 2009b), the role of PI3P has not been well investigated. However, the results suggest that PI3P is present in the LCV membrane.

Inositol (myo)-1 (or 4)-monophosphatase-1 (IMPA1) was identified as the cellular interaction partner for LtpD using a Yeast-2-hybrid assays and co-immunoprecipitation experiments. In addition to PI(3)P binding this interaction was mediated by the C-terminus of LtpD.

1.9 Homologues of the effector LtpD

Bioinformatics searches revealed several LtpD homologues in *L. pneumophila* isolates and indicate that it appears to be a chimera of two separate domains, both of which are found in other translocated effectors. The N-terminus of LtpD ($LtpD_N$) shares 33% sequence identity with a protein of unknown function (Lpw_27671, named LtpK), which is conserved in all the sequenced *L. pneumophila* strains. The C-terminus of LtpD ($LtpD_C$) shares 63% identity with the C -terminus of a unique uncharacterized ankyrin-repeat protein, Lpp0356, named LtpM, present in *L. pneumophila* Paris (Table 1, Figure 9). LtpM is also present in the recently sequenced OLDA strain (Mercante et al., 2016) as well as in isolates from a hospital outbreak in Australia (Graham et al., 2014) and environmental isolates (Watanabe et al., 2015). Genome analysis of the isolates from the hospital outbreak in Australia revealed that they were highly similar to the Paris strain. With more *L. pneumophila* genomes getting sequenced, Table 1 only shows a subset of the strains where the LtpD homologues are found. Furthermore, two additional homologues, Lpp0303 and FdumT_02243, which also have a chimeric structure but include different C-termini than LtpD, are found in *L. pneumophila* Paris and *L. dumoffii*. The protein Lpp0303 shows 27% homology to LtpK. The activities of these proteins as well as the role of the N-terminal domain of LtpD remains elusive.

Table 1 The LtpD, LtpK and LtpM homologues in different *L. pneumophila* strains

Strain	Gene		
	ltpD homologue	*ltpK homologue*	*ltpM homologue*
130b	*ltpD (LPW_03701)*	*ltpk (LPW_27671)*	
Philadelphia		*lpg2538* (97%)	
Paris		*lpp2604* (96%)	*ltpM (lpp0356)*
Corby		*LPC_1930* (97%)	
Alcoy		*lpa03706* (99%)	
Lens		*lpl2459* (99%)	
Lorraine	*LPO_0332* (92%)	*LPO_2746* (97%)	
HL06041035	*LPV_0373* (92%)	*LPV_2874* (96%)	
OLDA		*A9P84_13125* (96%)	*A9P84_01760* (100%)
C10_S		*A9E86_12555* (97%)	*A9E86_01415* (100%)
ST42	*EUL120_00196 (100%)*	*EUL120_02449* (100%)	
Leg01/11		*N751_08280* (95%)	*N751_13150* (100%)
Leg01/20		*N749_13155* (95%)	*N749_05790* (100%)
Bnt314		*lpbnt_02639* (96%)	*lpbnt_00395* (100%)
Ofk308		*lpofk_02640* (96%)	*lpofk_00396* (100%)
Ymg289		*lpymg_02780* (94%)	*lpymg_00304* (100%)

Homologous genes identified by nucleotide blast (blastn) as well as hypothetical proteins by protein blast (blastp) (NCBI); only homologues with 100% query cover are shown; percentage of identity is shown behind each gene.

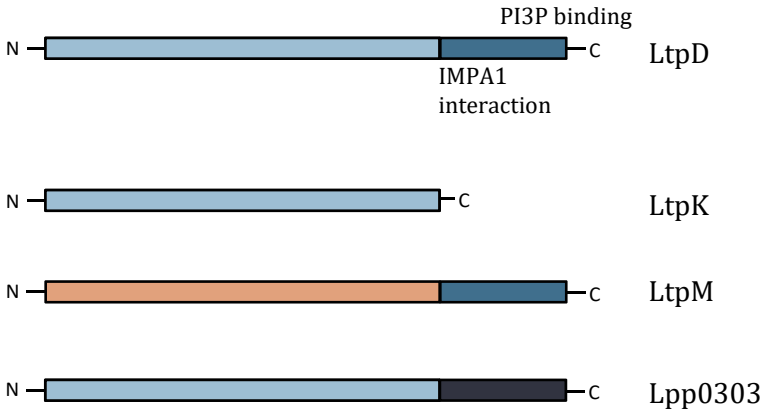


Figure 9 Domain organisation of the LtpD homologues LtpK, LtpM and Lpp0303
The effector LtpD has a PI3P binding domain in the C-terminus. The interaction with IMPA1 was also characterised to take place in the C-terminus. The N-terminus of LtpD, which harbours homology with the conserved effector LtpK as well with the N-terminus of Lpp0303, could not be functionally characterised yet. Lpp0303 harbours

a different C-terminus. The effector LtpM has no homology in the N-terminus, but 63% homology with the C-terminus of LtpD.

1.10 Aim of this study

Significant advances have recently been made in our knowledge of the intracellular lifestyle of *L. pneumophila* and the function of selected effectors. Nevertheless, our understanding of the interaction of specific *Legionella* virulence factors with host cell proteins and the cellular basis of their action remains superficial. In depth understanding of how virulence factors enable *Legionella* to persist in amoeba in the environment and in the human host is the necessary prerequisite for development of prevention and treatment strategies.

The aim of this study is to further characterise the LtpD-family of effector proteins. In particular, we aimed to determine the functions of the effectors of LtpK and LtpM which seem to share domains with the effector LtpD, promising to give insight in the function but also the evolution of this effector family in *Legionella* spp.

Specific objectives are to

- Confirm LtpK and LtpM as translocated effectors
- Determine the localisation of LtpK and LtpM in infection as well as in ectopic expression
- Determine and confirm host cell binding partners for LtpK and LtpM or cellular pathways affected by the effectors
- Determine the role for LtpK and LtpM in intracellular bacterial replication

Chapter 2

Characterisation of the effector LtpK

2. Characterisation of the effector LtpK

2.1 The effector LtpK

The effector repertoire between *Legionella* isolates varies and was found to be largely non-overlapping (Burstein et al., 2016). In this study, surprisingly, only 7 core effectors were found to be completely conserved. Between the analysed *Legionella* species, *L. pneumophila* is the one with the highest number of unique effectors. Between the *L. pneumophila* strains Philadelphia-1, Lens, Paris, Corby, Alcoy, and 130b, only 35% of effectors are conserved (Schroeder et al., 2010). One of the conserved effectors is the effector LtpK (Lpw_27671).

LtpK is conserved in all the sequenced *L. pneumophila* strains suggesting that it has important functions specifically for *L. pneumophila* - the most virulent strain towards humans (for more details on the homology between LtpK and LtpD, see chapter 2.2).

The conservation of LtpK across all *L. pneumophila* strains suggests that this group of homologues plays an important role in the *Legionella* life cycle. The LtpK homologue in *L. pneumophila* Philadelphia, Lpg2538, has already been shown to be important in infection in two different studies. Lpg2538 was identified by Aurass *et al*. in a transposon mutagenesis screen for *Legionella* mutants attenuated in the infection of *A. castellanii* (Aurass et al., 2009). Also, Lpg2538 was shown to be required for bacterial replication in *D. melanogaster* phagocytes when Bet5, a protein involved in the tethering of ER-derived vesicles to target membranes, was depleted (O'Connor et al., 2012). However, the biological function of LtpK is still undefined.

2.2 Bioinformatic sequence analysis of LtpK

LtpK is homologous to the N-terminal part of LtpD (Figure 10), but lacks the C-terminus of LtpD which binds to PI3P and interacts with IMPA1. No function has been discovered for the N-terminus of LtpD (Harding et al., 2013). A bioinformatics analysis for conserved domains as well as structural homology modelling in LtpK did not reveal similarity to proteins of known functions.

There are a few characteristic motifs in the sequence of LtpD which were partially characterised previously and are conserved in LtpK. A mitogen-activated protein kinase (MAPK) docking site (conserved motif F-F-X-P) and an adjacent serine phosphorylation site are found in both effectors, and LtpD was shown before to be serine- and tyrosine-phosphorylated. Mutation of the MAPK docking site $LtpD_{FF335AA}$ or the phosphorylation site $LtpD_{S338A}$ was shown to not affect localisation of LtpD in infection of A549 lung epithelial cells or during ectopic expression in HeLa cells (Thesis of Dr. C. Harding, 2013).

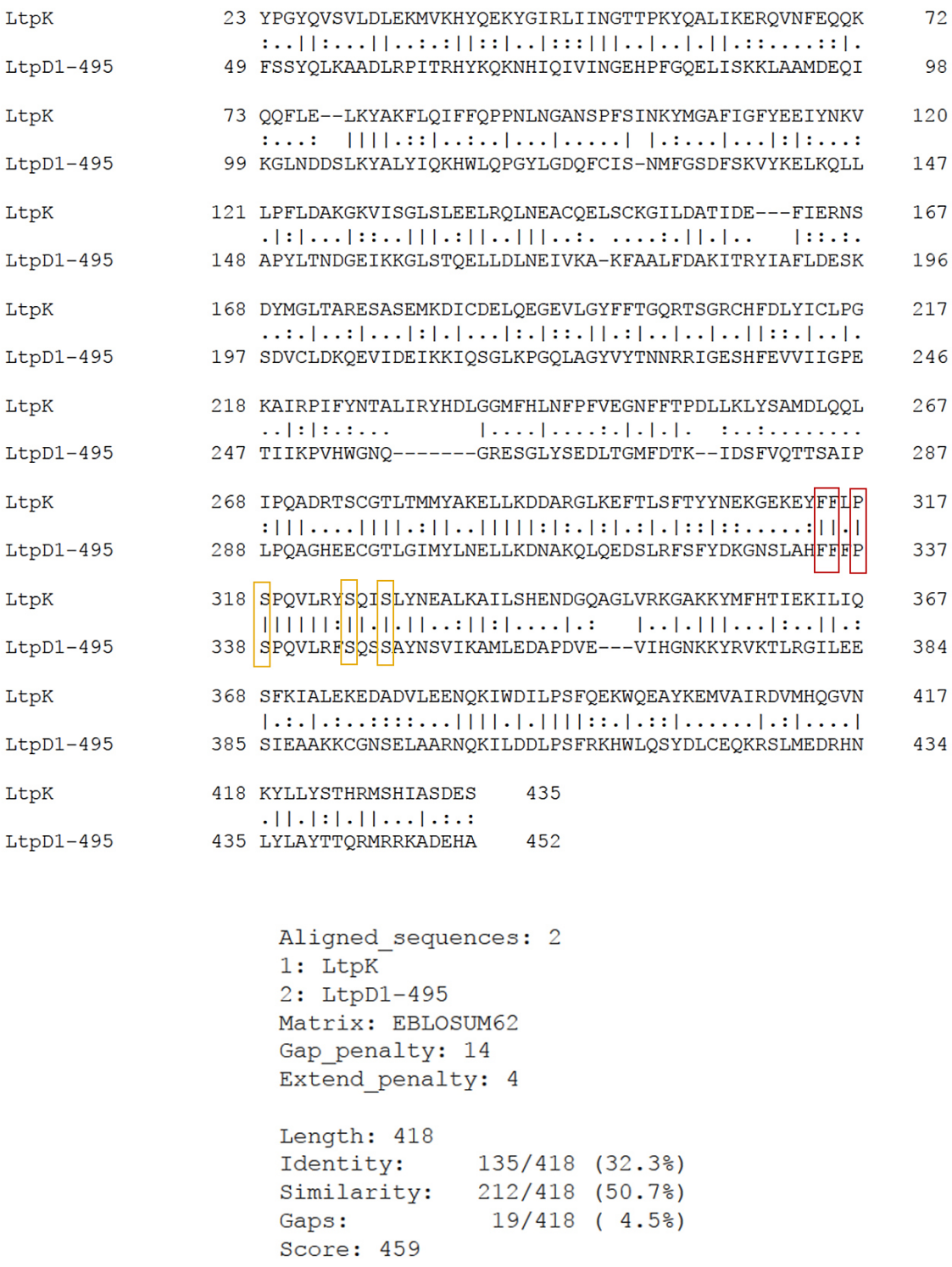


Figure 10 Homology between the effector LtpK and the N-terminal domain of $LtpD_{1-495}$

The N-terminal domain of the effectors LtpD (AA 1-495) and full length LtpK were aligned using the emboss-matcher and sequence homology and similarity was calculated by an algorithm based on Bill Pearson's align application, version 2.0u4 (Feb. 1996). Alignment shows a conserved MAPK docking site (red) and phosphorylation sites (yellow).

2.3 LtpK is a translocated effector

As the T4SS translocation signal is encoded in the C-terminus of the effectors and LtpK lacks the C-terminal domain of LtpD it was first investigated whether the putative *L. pneumophila* effectors LtpK and another homolog of LtpD, Lpp0303, are translocated into the cytoplasm of infected cells in a type IVB secretion system dependent manner, using a translocation assay as previously described (de Felipe et al., 2008). The putative effector proteins were fused to an N-terminal TEM1 (β-lactamase) tag using the *Legionella* expression vector pXDC61, transformed into wild-type and *ΔdotA L. pneumophila* and used to infect RAW 264.7 macrophages. The *ΔdotA L. pneumophila* deletion mutant is defective in the Dot/Icm translocation of effectors and therefore used as a translocation deficient control throughout this study (Berger et al., 1994).

Translocation was measured by loading the cells with a substrate for β-lactamase, CCF2/AM. When the fusion protein is translocated into the cytoplasm, the β-lactamase cleaves the substrate and a change in fluorescence from 520nm to 447nm can be detected with a spectrofluorometer.

LtpD was used as a positive control, while the *L. pneumophila* protein FAB, a cytosolic Enoyl-acyl CoA reductase, was used as negative control. As seen in Figure 11, neither protein was translocated by the *ΔdotA* mutant; also, FAB is not translocated by the wild-type, whereas LtpD is translocated. For LtpK, there was a strong increase in the translocation ratio, a measure of the relative turnover from beta-lactamase substrate to product, but no significant increase was observed for Lpp0303, showing that LtpK is a translocated effector. For this reason, Lpp0303 was not studied any further and work was focussed on LtpK.

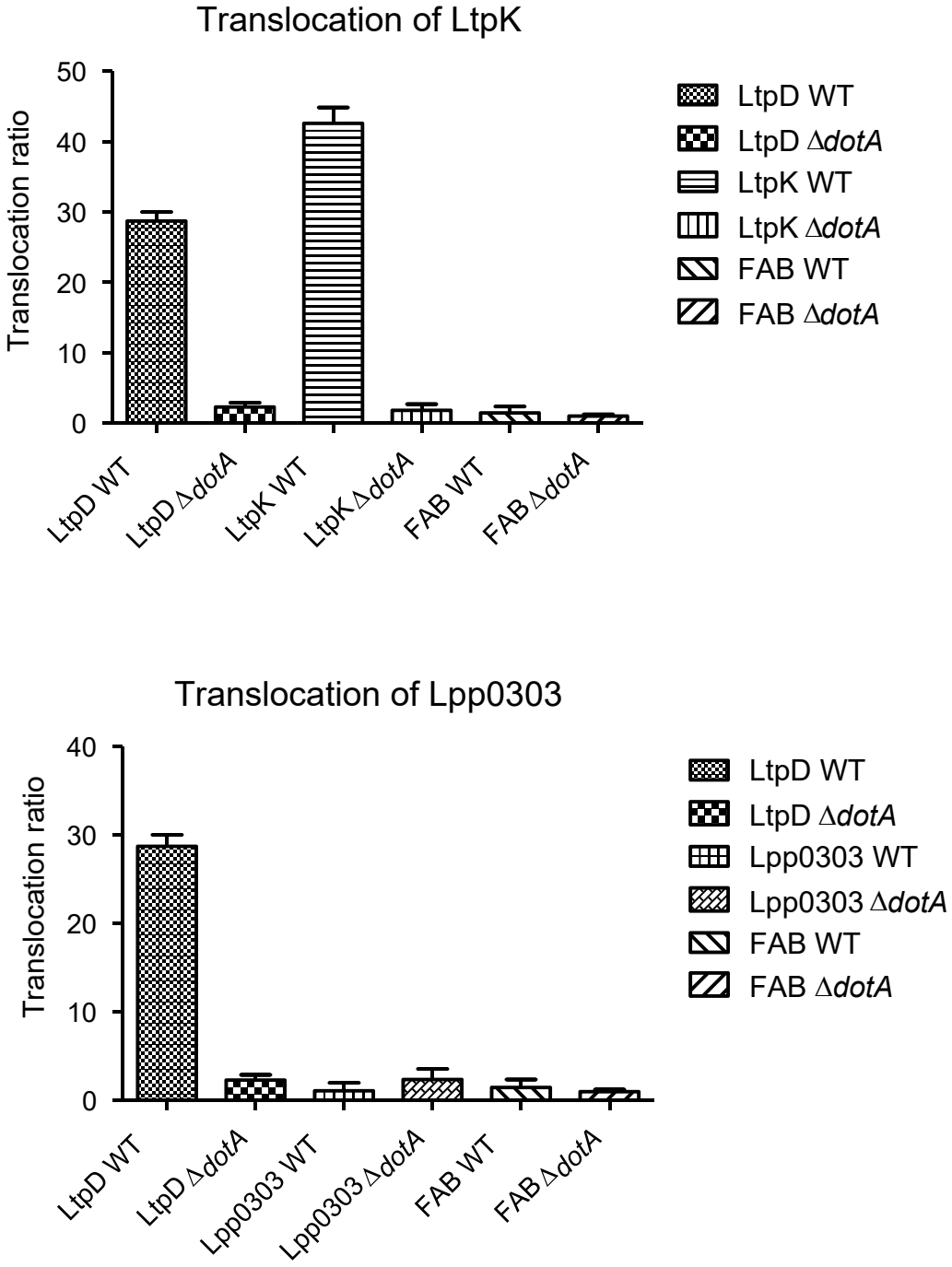
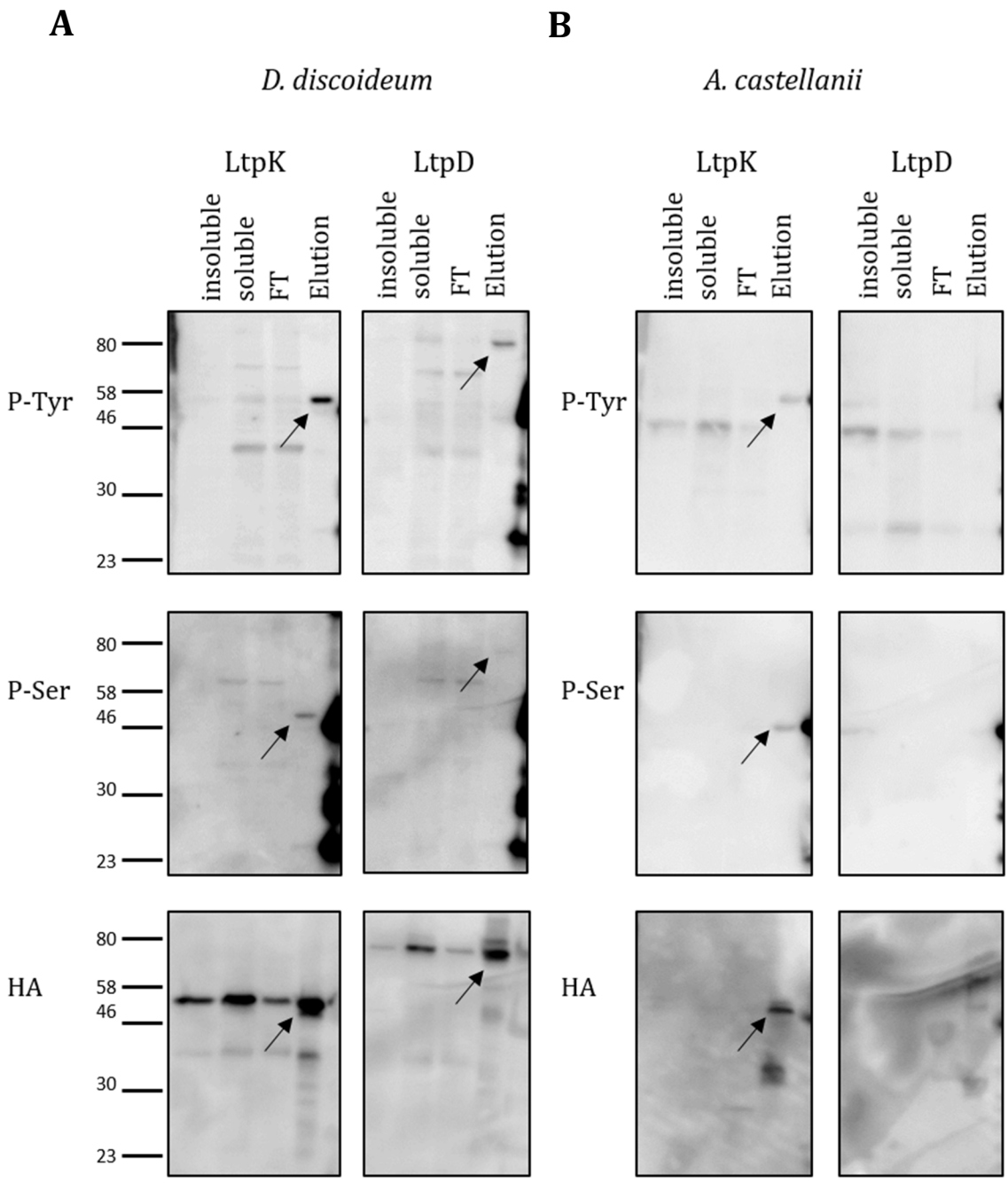


Figure 11 Translocation of LtpD and its homologues
RAW264.7 macrophages were infected with *L. pneumophila* 130b WT or *ΔdotA* overexpressing the putative effectors fused to β-lactamase. Translocation of the putative effectors (A) LtpK and (B) Lpp0303 was compared to the previously confirmed effector LtpD as positive and the reductase FAB as negative control. Translocation was measured calculating the ration of cleaved (520nm) to uncleaved (447nm) substrate CCF2/AM. Performed in triplicates; Error bars represent +/- SD; Graph representative for three independent experiments.

2.4 Translocated LtpK is phosphorylated

As noted previously in section 2.2 analysis of LtpD revealed that it contains a predicted mitogen-activated protein kinase (MAPK) docking site (conserved motif F-F-X-P) with an adjacent serine phosphorylation site. Like LtpD, LtpK harbours this conserved MAPK docking and phosphorylation site. As other effectors of *L. pneumophila* were found to manipulate the MAPK-pathway (Fontana et al., 2012), LtpK was investigated for phosphorylation during infection of A549 lung epithelial cells*, D. discoideum* as well as *A. castellanii*, and LtpD was used as a positive control.

The cells were infected for 18 hours with wildtype *L. pneumophila* expressing 4HA-LtpK or 4HA-LtpD. 18 hours post infection, the HA-tagged effectors were immunoprecipitated from the cells using anti-HA agarose beads and the insoluble, soluble, flow-through (FL) and elution fraction run on SDS gels for Western blot analysis. Antibodies against P-serine and P-tyrosine were used to investigate the phosphorylation status of LtpK, as LtpD was shown previously to be serine- and tyrosine-phosphorylated in A549 lung epithelial cells, but not threonine-phosphorylated. LtpK was recognised by anti-P-serine and anti-P-tyrosine antibodies in the amoeba hosts *D. discoideum* and *A. castellanii* (Figure 12 A and B). In contrast to the amoeba hosts, in A549 lung epithelial cells, LtpK was recognised only by the anti-P-serine antibody, but shows a considerably weaker signal than LtpD (Figure 12 C). LtpD, however, does not seem to be phosphorylated in *A. castellanii*, which might be due to a low expression of LtpD and therefore the signal is below the detection level.


A
B
D. discoideum
A. castellanii
LtpK
LtpD
insoluble
soluble
FT
Elution
P-Tyr
P-Ser
HA
80
58
46
30
23

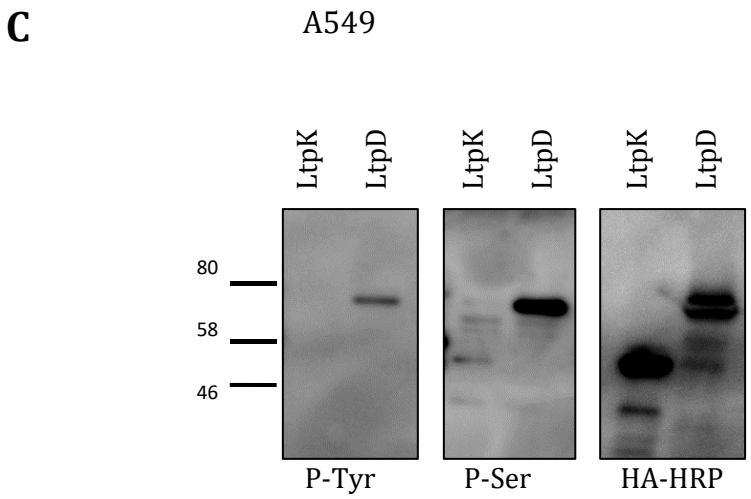


Figure 12 4HA-LtpK is phosphorylated in infection of A549 lung epithelial cells, *D. discoideum* and *A. castellanii*
The amoeba hosts *D. discoideum* (A) and *A. castellanii* (B) as well as the lung epithelial cell line A549 (C) were infected with *L. pneumophila* expressing 4HA-LtpK or 4HA-LtpD. After 18 h, cells were lysed and 4HA-effectors immunoprecipitated using anti-HA agarose. HA-LtpK and HA-LtpD were eluted from the resin using HA-peptide. Analysis by Western-blot shows a recognition of 4HA-LtpK by anti-p-tyrosine and anti-p-serine antibodies in the amoeba hosts *D. discoideum* and *A. castellanii* as well as recognition by anti-p-serine in A549 cells. Results representative of at least two separate experiments for each cell line. (FT = Flow through: supernatant after incubation with resin). Expected sizes: 4HA-LtpK: 55kDa; 4HA-LtpD: 80kDa; Errors represent bands corresponding to the sizes of LtpK and LtpD.

2.5 LtpK has a diffuse cytoplasmic or nuclear localisation in infection

To investigate the localisation of LtpK during infection, the gene was fused to four HA tags in the *Legionella* expression vector pMMB4HA and transformed into *L. pneumophila* WT and the *ΔdotA* mutant. 1 hour prior infection, expression of the HA-tagged effector protein was induced. A549 lung epithelial cells were infected with a multiplicity of infection (MOI)=40. Cells were fixed and stained after 6 hours and after 24 hours with anti-HA- and *Legionella* LPS-antibodies. Up to six hours, LtpK can only be detected inside the bacteria (data not shown), whereas after 24 hours it is detected within the bacteria, diffusely in the cytoplasm and in some cells it is present in the nucleus (Figure 13).

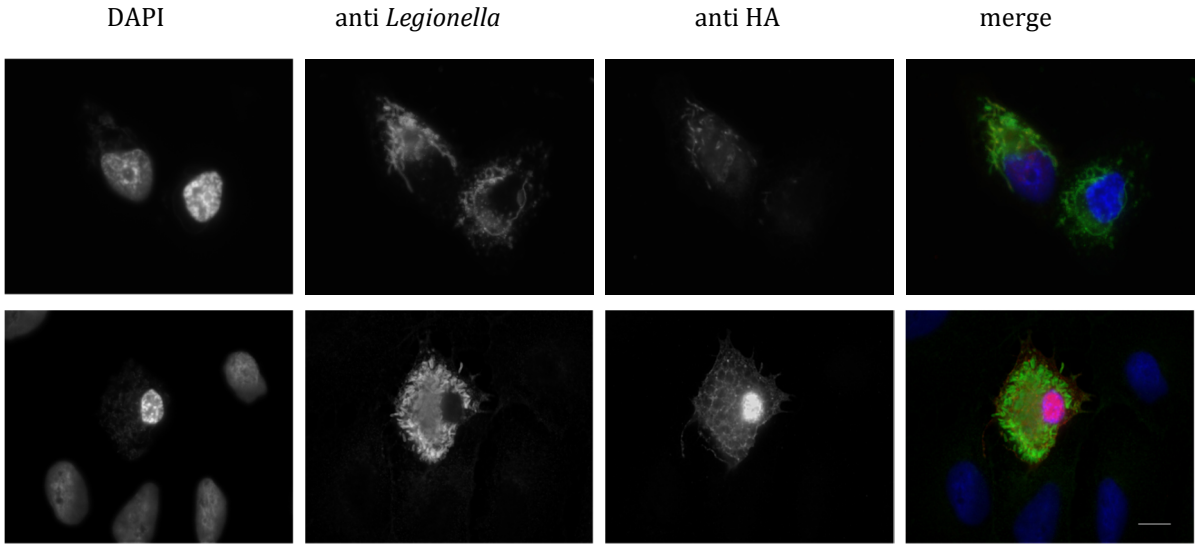


Figure 13 Localisation of LtpK 24h after infection of A549 cells
A549 cells were infected with wild-type *L. pneumophila* overexpressing LtpK fused to four HA tags (4xHA). After 24h, cells were washed, fixed and stained for *Legionella*-LPS and Myc using specific antibodies. Representative for three independent experiments. Scale bar 10 µm.

2.6 LtpK localises to the cytoplasm or nuclear structures upon transfection

To determine the role of LtpK in absence of interference by 300 other effector proteins, human cells ectopically expressing LtpK were analysed. LtpK was cloned in the mammalian expression plasmid pRK5Myc which allows expression of the protein fused to a Myc-tag. HeLa cells were transfected with the plasmid for Myc-LtpK expression or with the empty plasmid as control. After 24 hours, cells were fixed and stained for immuno-fluorescence microscopy using specific antibodies. As can be seen in Figure 14 LtpK shows a diffuse cytoplasmic or nuclear localisation, which agrees with the results from 2.5 (Figure 14).

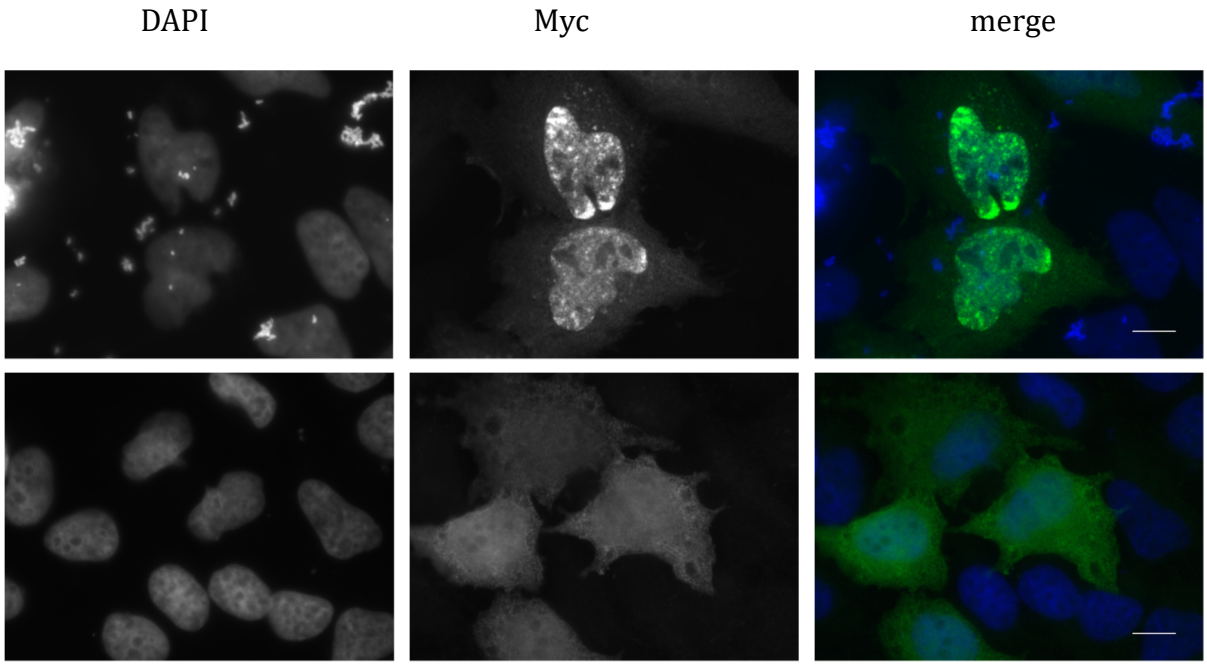


Figure 14 LtpK shows a diffuse cytoplasmic or nuclear localisation upon transfection
HeLa cells were transfected with pRK5Myc-LtpK. After 24h, cells were fixed and stained for immuno-fluorescence microscopy using specific antibodies. Representative of at least three independent experiments. Scale bar 10 μm.

2.7 LtpK does not co-localise with Histones upon ectopic expression in the nucleus

LtpK localises either in the cytoplasm of the cell or it appears in the nucleus, whereas nuclear localisation is rare and occurs only in ~ 5% of transfected cells. Other effectors were previously shown to interfere with histones in the nucleus. RomA/LegAS4 for example is a SET-domain effector which has been shown to modulate the host's epigenetic chromatin organisation (Rolando and Buchrieser, 2014, Rolando et al., 2013, Son et al., 2015). Therefore, it was decided to further investigate the nuclear localisation of LtpK.

HeLa cells were transfected with Myc-LtpK and localisation of LtpK with Histones H2, H3 or H4 was investigated via immunofluorescence. In those experiments, LtpK did not show a

nuclear localisation. No difference in the appreance of the different histones could be detected (Figure 15).

Only a small portion of LtpK locates to the nucleus upon transfection. Therefore, to enhance the localisation of LtpK in the nucleus, it was fused to a nuclear localisation sequence (NLS). The transfected cells were further stained for histones H2, H3 as well as H4 to investigate whether nuclear localised NLS-LtpK colocalises with one of these histones. Nuclear localised NLS-LtpK does not appear to co-localise with histone H2 or H3 (Figure 16).

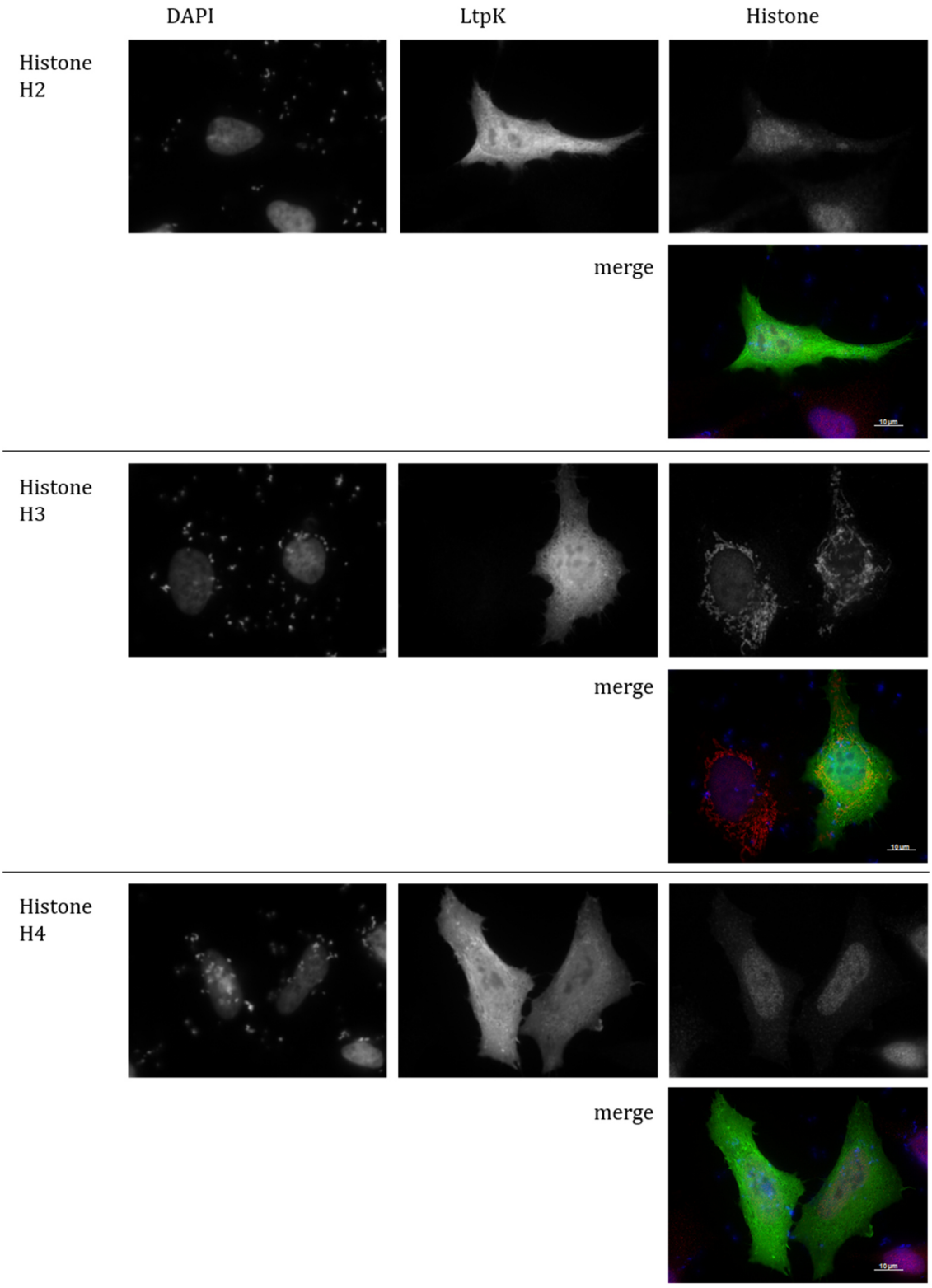


Figure 15 LtpK does not co-localise with Histone H2, H3 or H4 upon transfection

HeLa cells were transfected with pRK5Myc-LtpK. After 24h, cells were fixed and stained for immuno-fluorescence microscopy using specific antibodies against Myc as well the indicated Histones (Histone 2, 3 and 4). Representative of at least three independent experiments. Scale bar 10 µm.

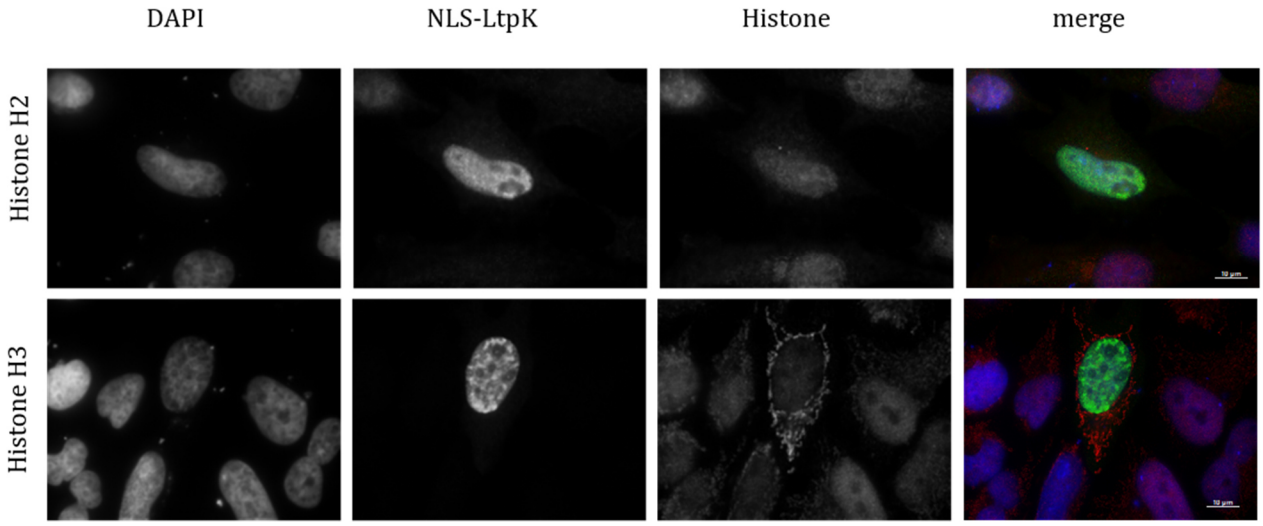


Figure 16 Nuclear localised NLS-LtpK does not co-localise with Histone 2 or Histone 3
HeLa cells were transfected with pRK5Myc-NLS-LtpK. After 24h, cells were fixed and stained for immuno-fluorescence microscopy using specific antibodies against Myc as well the indicated Histones (Histone 2 and 3). Representative of at least three independent experiments. Scale bar 10 µm.

2.8 Nuclear localised LtpK colocalises with ubiquitin upon ectopic expression

One of the hallmarks for LCV maturation is the accumulation of ubiquitinated proteins on the LCV. Ubiquitin was one of the proteins found in the pBio-pulldown in the pBio-LtpK sample (section 2.10) and the ubiquitin protein ligase E3 was found in the Y2H screen for LtpK (section 2.9). Although ubiquitin was not exclusively found for LtpK in the pBio-pulldown and the ubiquitin ligase could not be confirmed in the direct Y2H, it was decided to investigate the subcellular localisation of LtpK and ubiquitin. In order to investigate whether LtpK might play a role in ubiquitination, transfection experiments with LtpK with supsequent staining of ubiquitin were carried out.

HeLa cells were transfected with plasmids for expression of Myc-LtpK. After 24 hours, cells were fixed and stained for immunofluorenscence using specific antibodies against Myc and

ubiquitin. It was observed that when LtpK localises to compact structures in the nucleus, it does co-localise with ubiquitin (Figure 17).

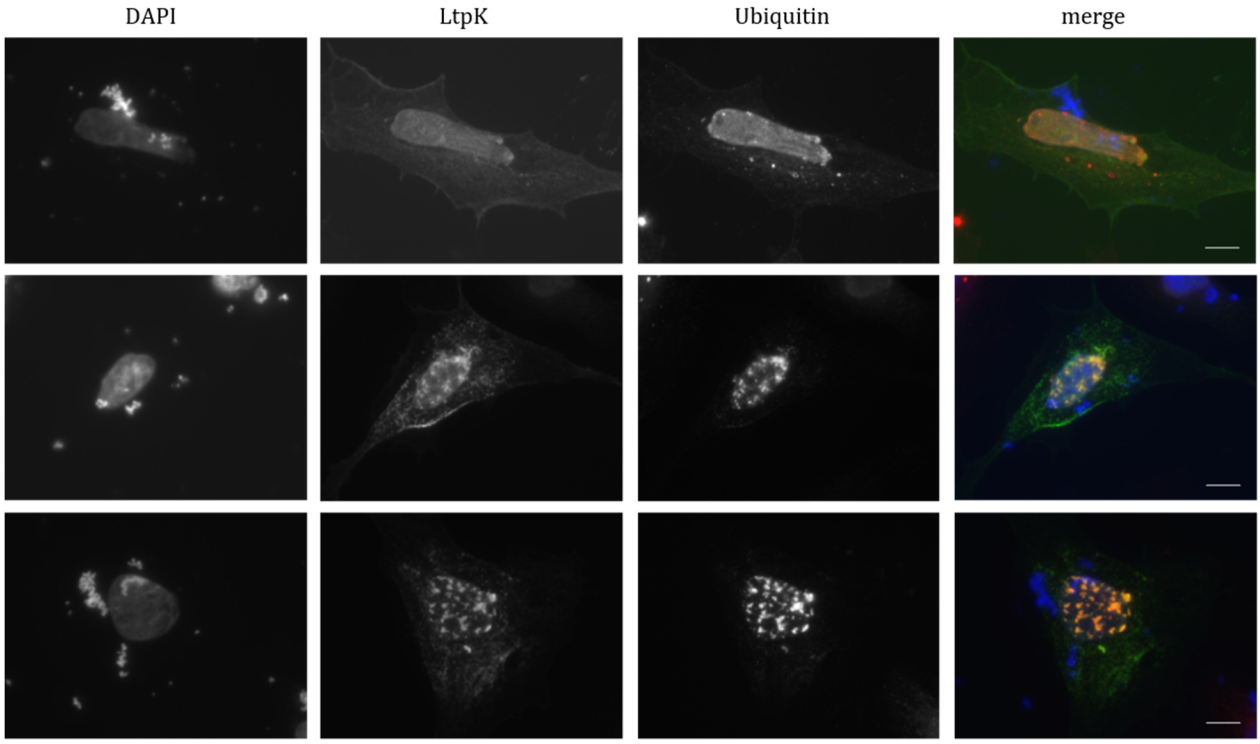


Figure 17 LtpK co-localises with ubiquitin upon transfection
HeLa cells were transfected with pRK5Myc-LtpK. After 24h, cells were fixed and stained for immuno-fluorescence microscopy using specific antibodies against Myc and ubiquitin. Representative of at least two independent experiments. Scale bar 10 μm.

2.9 A yeast two hybrid screen identified several proteins as potential interaction partners for LtpK

In order to find potential interaction partners of LtpK, two yeast-2-hybrid (Y2H) screens were performed using pGBKT7-LtpK (high expression) as well as pGBT9-LtpK (low expression) as bait and a pretransformed Matchmaker Clonetech HeLa cDNA library according to the manufacturer's protocol. Only four colonies grew on selective media for the first screen using pGBKT7-LtpK. The prey plasmid inserts were amplified from these colonies by PCR, the PCR product purified according to the PCR purification Kit protocol and sequenced. The protein coding sequences in the fragments were identified by BlastX search and described in Table 2.

Table 2 Potential interaction partners identified in the Y2H with pGBKT7-LtpK

Name of Gene	No of colonies	Description (Source: NCBI PUBMED)
SDHB: succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	2	Complex II of the respiratory chain, which is specifically involved in the oxidation of succinate
ASXL1: additional sex combs like 1	1	Gene is similar to the Drosophila additional sex combs gene, which encodes a chromatin-binding protein required for normal determination of segment identity in the developing embryo; member of the Polycomb group of proteins; thought to disrupt chromatin in localised areas, enhancing transcription of certain genes while repressing the transcription of other genes; functions as a ligand-dependent co-activator for retinoic acid receptor in cooperation with nuclear receptor coactivator 1
VBP1: von Hippel-Lindau binding protein 1	1	Interacts with the Von Hippel-Lindau protein to form an intracellular complex; ubiquitously expressed in tissue; located in the cell in the nucleus and cytoplasm; functions as a chaperone protein, is suspected that it may play a role in the transport of the Von Hippel-Lindau protein from the perinuclear granules to the nucleus or cytoplasm

In the second Y2H screen pGBT9-LtpK was used as bait, nine yeast colonies grew and the identified prey proteins are described in Table 3. None of the potential interactors from the first screen was found a second time.

Table 3 Potential interaction partners identified in the Y2H with pGBT9-LtpK

Name of Gene	No of colonies	Description (Source: NCBI PUBMED)
UBR7: ubiquitin protein ligase E3 component n-recognin 7	1	E3 ubiquitin-protein ligase which is a component of the N-end rule pathway. Recognizes and binds to proteins bearing specific N-terminal residues that are destabilizing according to the N-end rule, leading to their ubiquitination and subsequent degradation (By similarity)
ACBD3: acyl-CoA binding domain containing 3	1	Involved in the maintenance of Golgi structure by interacting with giantin, affecting protein transport between the endoplasmic reticulum and Golgi.
TMEM60: transmembrane protein 60	2	Uncharacterized transmembrane protein
L14: ribosomal protein L14	1	Ribosomal protein that is a component of the 60S subunit. The protein belongs to the L14E family of ribosomal proteins.
ATF6: activating transcription factor 6	1	A transcription factor that activates target genes for the unfolded protein response (UPR) during endoplasmic reticulum (ER) stress. Although it is a transcription factor, this protein is unusual in that it is synthesized as a transmembrane protein that is embedded in the ER. It functions as an ER stress sensor/transducer, and following ER stress-induced proteolysis,
APC: adenomatous polyposis coli	1 (no regrowth)	Tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway. It is also involved in other processes including cell migration and adhesion, transcriptional activation, and apoptosis.
DTWD1: DTW domain containing 1	1 (no regrowth)	Uncharacterized protein
Sec62: Homo sapiens SEC62 homolog (S. cerevisiae)	1 (no regrowth)	The Sec61 complex is the central component of the protein translocation apparatus of the endoplasmic reticulum (ER) membrane. The protein encoded by this gene and SEC63 protein are found to be associated with ribosome-free SEC61 complex.

2.9.1 Confirmation of interactions of LtpK by direct Y2H

In order to confirm the interactions of the proteins with LtpK, a direct Y2H was performed. For that, all the coding parts of the identified potential interactors were cloned in the pGADT7 yeast plasmid and co-transformed with pGBKT7- or pGBT9-LtpK. Both direct Y2Hs were plated on TDO (triple drop-out) and QDO (quadruple drop-out) selective media and growth restoration due to interaction monitored. Figure 18 shows the representative result for pGBKT7-LtpK. A summary of the results is shown in Table 4. As redox control/ mitochondria-associated proteins are common false positives in two-hybrid systems, the mitochondrial protein SDHB (succinate dehydrogenase) is very likely a false positive, which leaves VBP1, ASXL1 and ACBD3 as potential host targets for LtpK, wherein ACBD3 showed the strongest growth on the drop-out media.

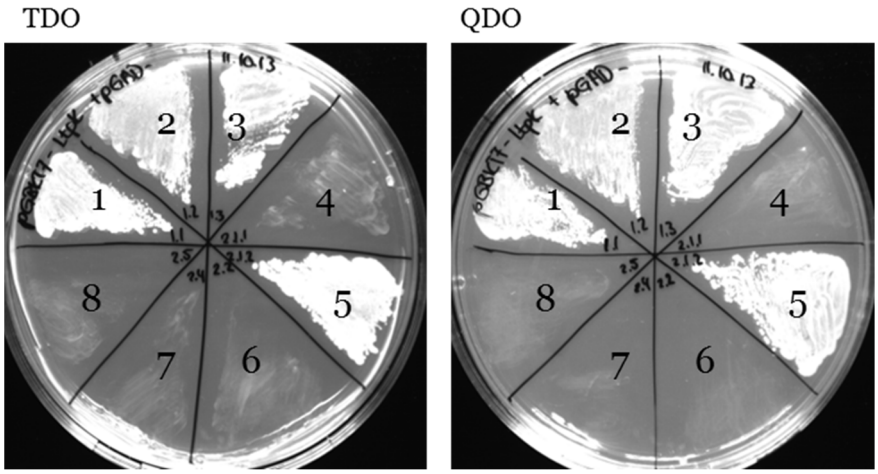


Figure 18 Direct Y2H of LtpK and potential interactors
S. cerevisiae AH109 was co-transformed with pGBKT7-LtpK and pGADT7 harbouring the putative interactor. Colonies were streaked on selective media TDO (triple drop out) and QDO (quadruple drop out). 1: SDHB, 2: VBP1, 3: ASXL1, 4: UBR7, 5: ACBD3, 6: TMEM60, 7: L14, 8: ATF6.

Table 4 Results of the direct Y2H assay between LtpK and potential interaction partners

pGADT7-	pGBKT7-LtpK		pGBT9-LtpK	
	TDO	QDO	TDO	QDO
SDHB	++	++	++	++
VBP1	++	++	+	+
ASXL1	++	++	+	++
UBR7	-	-	-	-
ACBD3	+++	+++	++	++
TMEM60	-	-	-	-
L14	-	-	-	-
ATF6	-	-	-	-

Symbols: - no growth, + slow growth, ++ medium growth, +++ fast growth

2.9.2 Subcellular localisation of LtpK and interactors

In order to further investigate whether LtpK interacts with those proteins in a cellular context, co-transfection experiments with plasmids for expression of HA-LtpK with Flag-ASXL1, Myc-VBP1 or Flag-ACBD3 were performed. For co-transfection of ACBD3 and LtpK, no co-localisation of LtpK with ACBD3 can be observed. ACBD3 localises to the Golgi and LtpK shows a diffuse cytoplasmic localisation. In co-transfection of LtpK with VBP1, the proteins do co-localise in the cytoplasm as well as diffusely in the nucleus. In co-transfection of LtpK with ASXL1, both proteins localise to compact structures in the nucleus and therefore a co-localisation can be observed between these two proteins too (Figure 19).

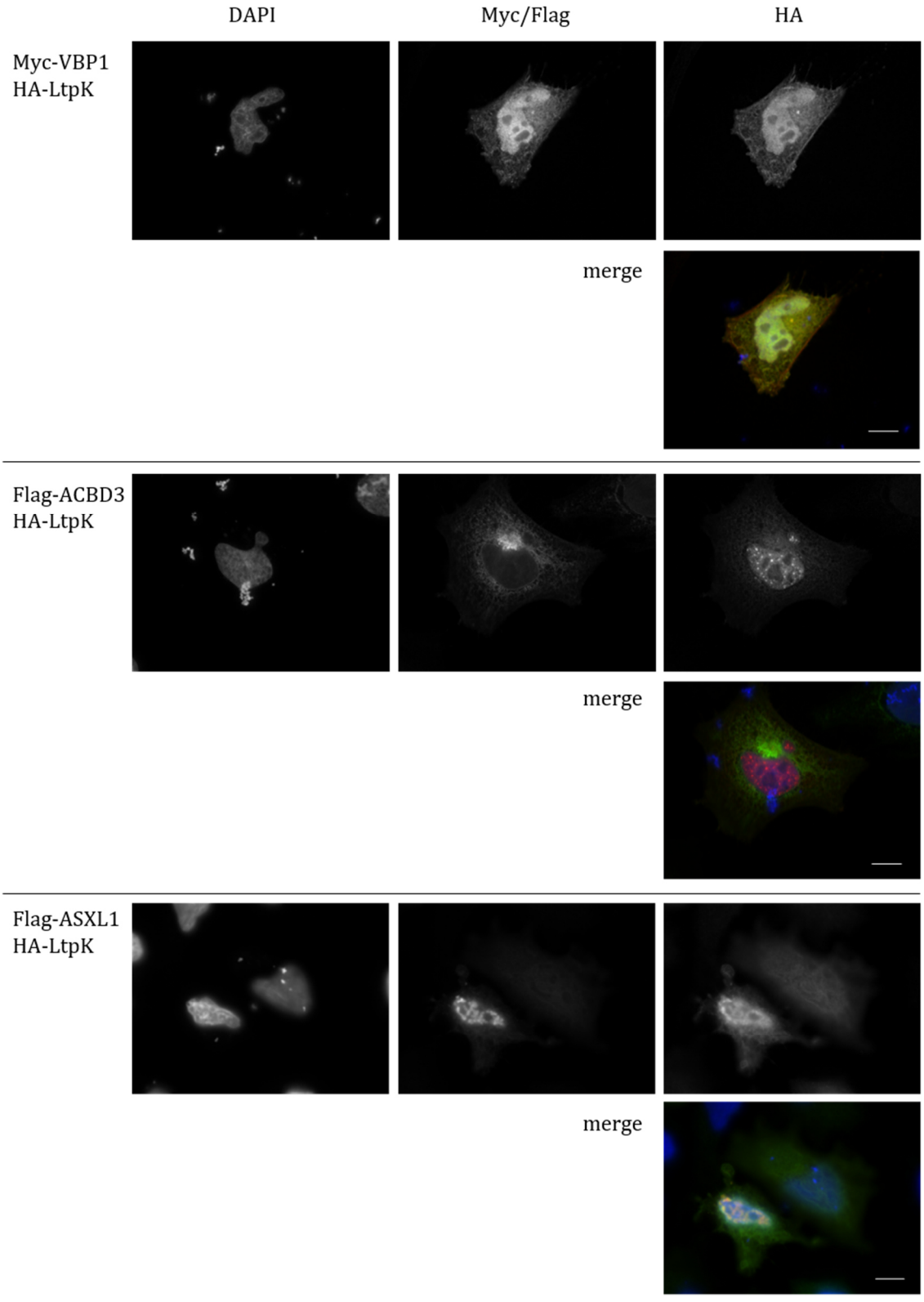


Figure 19 LtpK co-localises with ASXL1 and VBP1, but not ACBD3, upon transfection

HeLa cells were transfected with pRK5HA-LtpK and Flag-ASXL1, Myc-VBP1 or Flag-ACBD3. After 24h, cells were fixed and stained for immuno-fluorescence microscopy using specific antibodies against HA, Flag or Myc. Representative of at least two independent experiments. Scale bar 10 μm.

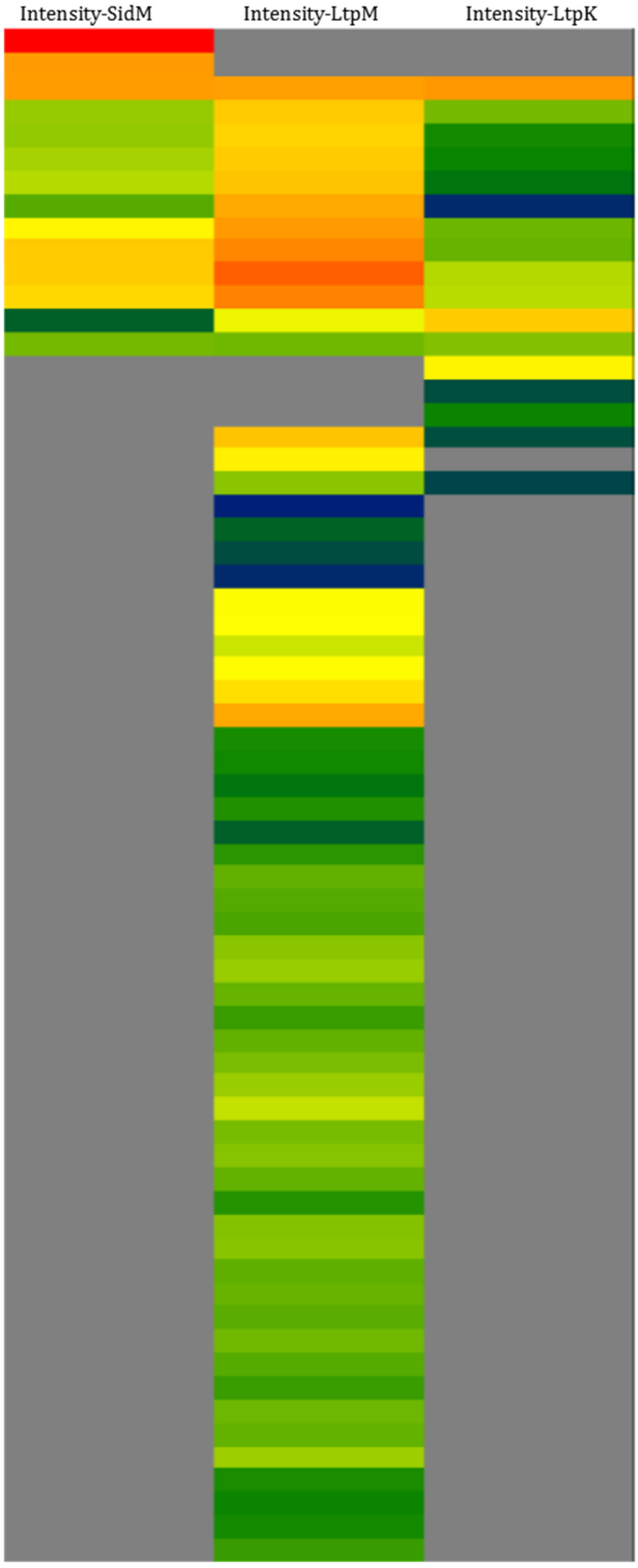
2.10 A pBio-pulldown with LtpK revealed other potential interaction partners

In order to validate and/or identify additional interaction partners of LtpK during infection, an alternative tandem-affinity purification which was developed in our research group was used (Mousnier et al., 2014, So et al., 2016). In this method, the effector of interest is fused to a biotinylation site and a hexahistidine-tag (referred to as Bio-tag). The bacteria expressing the tagged effector are used to infect a cell line (here A549) expressing the biotin ligase BirA. After infection and upon translocation of the effector in the cytoplasm of the host cell, the effector is biotinylated. Therefore, it is possible to purify translocated effectors in a tandem purification using a streptavidin pulldown and Ni-NTA affinity chromatography. Crosslinking the protein complexes before lysis of the cells was used to stabilize the interactions.

Following the tandem affinity purification, the samples were incubated with trypsin at 37°C overnight. On the following morning, samples are processed for mass spectrometry. The peptide mixtures are desalted using the Stage-Tip method (Broncel et 179 al., 2015) and dimethyl labelling was performed according to Boersema et al. (Boersema et al., 2009). The samples were light or heavy dimethyl labelled before they were vacuum dried and stored at -80°C. For Mass spectrometry, samples were resuspended in 20 µl 0.5% trifluoroacetic acid, 2% acetonitrile and transferred into LC-MS sample vials. Mass spectrometry and MS data processing was performed as described in (So et al., 2016). Briefly, data processing was done using MaxQuant (version 1.5.0.25) and identification of the peptides was done by matching MS/MS spectra with reference human and *Legionella pneumophila* (strain 130b or Paris) proteomes using Andromeda search engine (Cox 2011 J Proteome Res). Data were further analysed using Perseus (Version 1.5.0.9) and Heat maps generated using log2 light and heavy intensities.

This Bio-tag-pulldown for LtpK was performed several times using different crosslinking times and reagents, as well as lysis reagents and conditions in order to maximise the chance of recovering interaction partners.

Figure 20 shows an initial pBio-pulldown experiment using the effectors LtpK and LtpM as well the effector SidM as positive control in A549-Bir cells. PFA (1%) was used as crosslinking reagent and 1% Triton X100 for lysis. In this experiment, only SidM as the positive control could be purified with its interactor Rab1.



SidM
Ras-related protein Rab-1A
P00698
Putative elongation factor 1-alpha-like 3
Histone H1.4; H1.3; H1t; H1.1
Tubulin beta chain
Retinal dehydrogenase 1
Tubulin alpha-1C chain
Pyruvate kinase PKM
Annexin A2;Putative annexin A2-like protein
Actin, cytoplasmic
Aldo-keto reductase family 1 member B10
Alpha-1-antitrypsin;Short peptide from AAT
Heat shock 70 kDa protein
Alpha-1-acid glycoprotein 1
Desmoplakin
Junction plakoglobin
Vimentin
Cysteine and glycine-rich protein 1
Pyruvate carboxylase, mitochondrial
Clathrin heavy chain 1
ATP-citrate synthase
Heterogeneous nuclear ribonucleoprotein M
ATP-dependent RNA helicase DDX3X
Heterogeneous nuclear ribonucleoproteins A2/B1
Filamin-A
Zinc finger FYVE domain-containing protein 26
Plectin
Histone H2A
L-lactate dehydrogenase A chain
Tropomyosin alpha-4 chain
40S ribosomal protein S4
Protein S100-A11
RNA-binding motif protein
Myosin-9
Neuroblast differentiation-associated protein AHNAK
Heterogeneous nuclear ribonucleoprotein U
60S ribosomal protein L18
60S ribosomal protein L3
Adenosylhomocysteinase
40S ribosomal protein S3
Elongation factor 2
T-complex protein 1 subunit gamma
Heterogeneous nuclear ribonucleoproteins C1/C2
UDP-glucose 6-dehydrogenase
NAD(P)H dehydrogenase [quinone] 1
Alpha-enolase
Nucleolin
40S ribosomal protein S16
60S acidic ribosomal protein P2
Prelamin-A/C;Lamin-A/C
60S ribosomal protein L13
Alpha-actinin-4
40S ribosomal protein S2
60S ribosomal protein L10
Elongation factor 1-gamma
Tropomyosin alpha-3 chain
14-3-3 protein zeta
60S ribosomal protein L7a
Fascin
Polyadenylate-binding protein 1
Salivary acidic proline-rich phosphoprotein 1
Tropomyosin alpha-1 chain
Poly(rC)-binding protein 1
Non-POU domain-containing octamer-binding protein
P02789
40S ribosomal protein S13
Glucose-6-phosphate 1-dehydrogenase

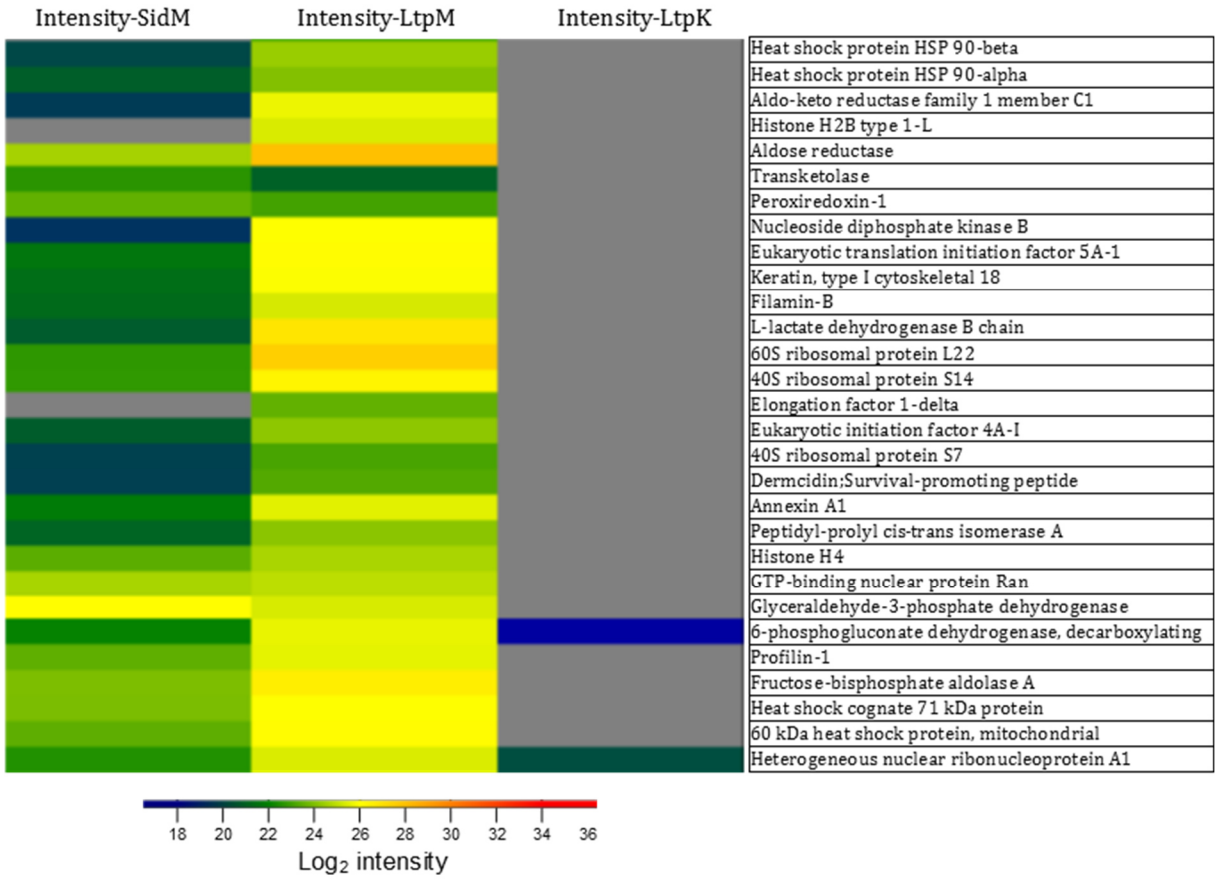
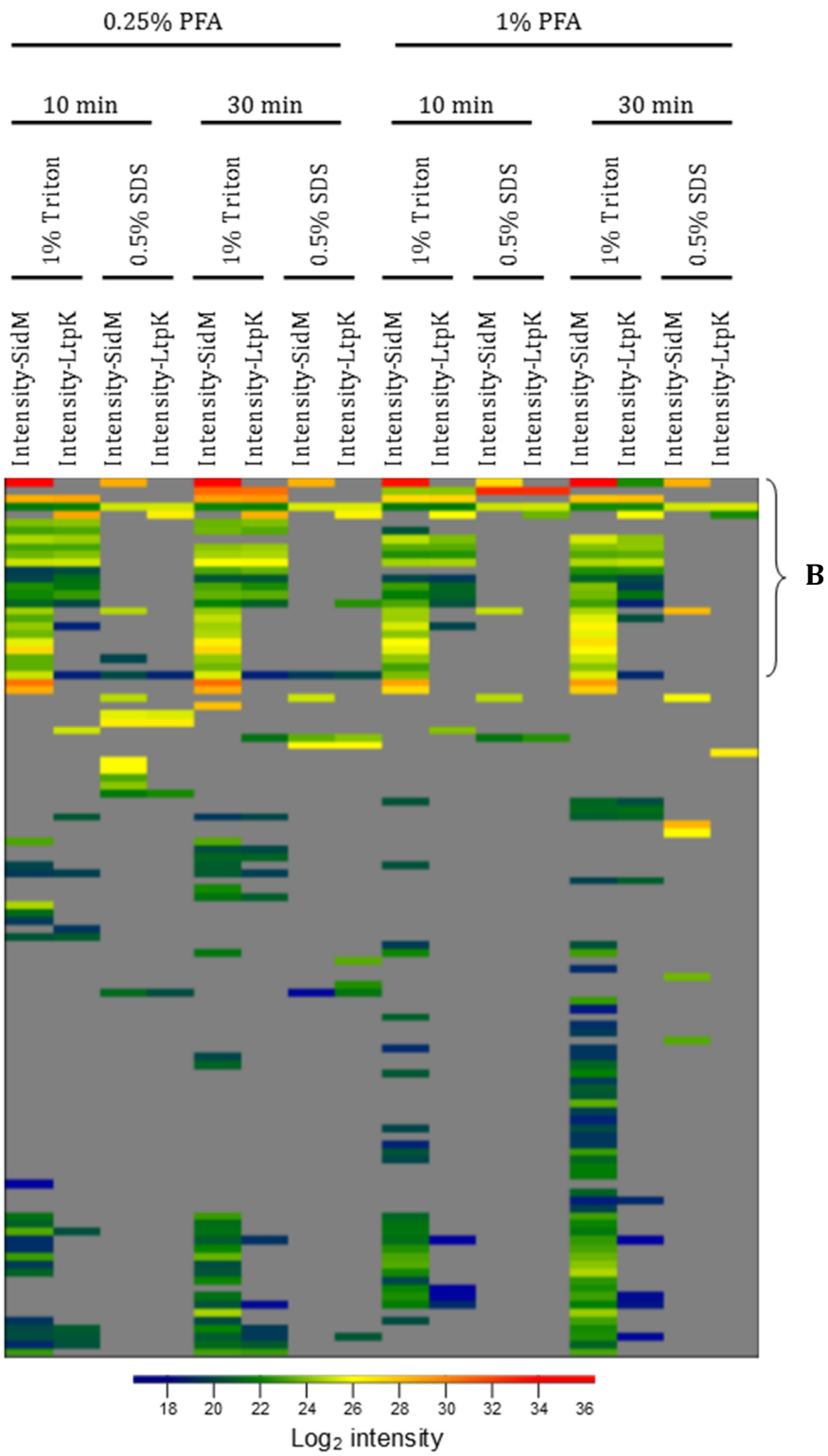


Figure 20 pBio-pulldown with SidM, LtpM and LtpK
A549-BirA cells were infected with *L. pneumophila* expressing His6-Bio-SidM, His6-Bio-LtpK or His6-Bio-LtpM. Effectors were purified with a tandem affinity (Ni2+ NTA and Neutravidin) purification (TAP). Each sample was crosslinked with 1% formaldehyde, lysed in Triton X-100, and subjected to LC/MS-MS analysis. Heat map showing intensities of all identified proteins across TAP. Proteins were ranked by enrichment factors.

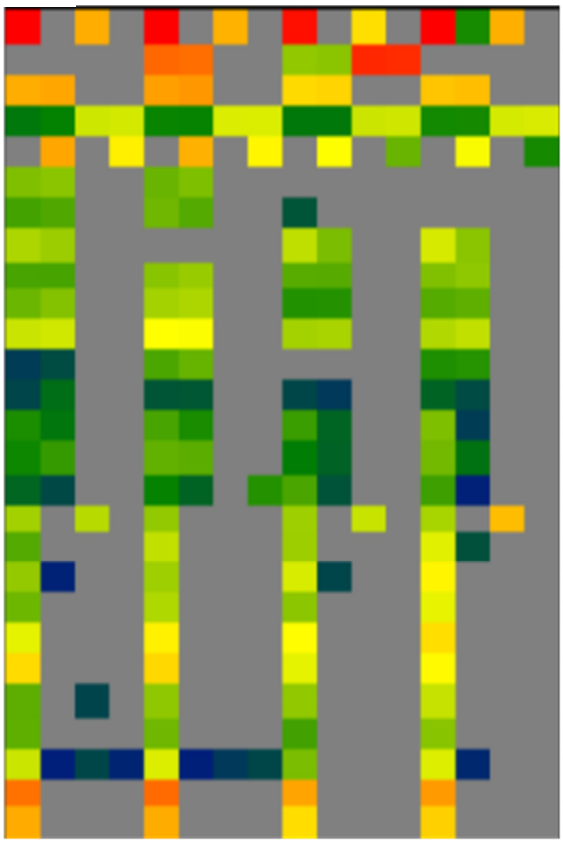
In order to find the right conditions to purify the effectors LtpK and LtpM, experiments testing different conditions were performed. Figure 21 A shows an experiment where different PFA concentrations (0.25% vs. 1%), different fixation times (10min vs. 30min) and different lysis conditions (1% triton X100 vs. 0.5% SDS) were tested and SidM as well as LtpK were used. It can be seen that more proteins could be purified in the samples lysed with 1% Triton X100 compared to 0.5% SDS. Moreover, fixation with 1% PFA showed the most proteins being purified compared to the other fixation variants, assuming that the protein complexes were stabilised effectively with 1 % PFA for 30 min. As a result, it was possible to purify SidM with the interactor Rab1a, which shows that the experiment was successful. LtpK is visible in the analysis too. However, no specific interactor for LtpK could be identified. A detailed list of the most abundant proteins is shown in Figure 21 B.

Although the positive control SidM usually worked in the pBio-pulldown experiments, the investigated effectors LtpK and LtpM were hardly seen in the analysis. The potential interacting partner of LtpK found in the Y2H screen ASXL1, ACBD3 and VBP1, could not be seen in the pBio pulldowns.

A


0.25% PFA
1% PFA
10 min
30 min
10 min
30 min
1% Triton
0.5% SDS
1% Triton
0.5% SDS
1% Triton
0.5% SDS
1% Triton
0.5% SDS
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
Intensity-SidM
Intensity-LtpK
B
18
20
22
24
26
28
30
32
34
36
Log2 intensity

B



SidM
K7EMR9
Pyruvate carboxylase, mitochondrial
Diacylglycerol kinase eta
LtpK
Acetyl-CoA carboxylase 1;Biotin carboxylase
Tubulin alpha-1C chain
Spermatogenesis-associated protein 7
60 kDa heat shock protein, mitochondrial
Methylcrotonoyl-CoA carboxylase subunit alpha
Elongation factor Tu, mitochondrial
Glutaminase kidney isoform, mitochondrial
Heat shock protein HSP 90-beta
Pyruvate kinase PKM;Pyruvate kinase
Heat shock cognate 71 kDa protein
Putative elongation factor 1-alpha-like 3
Zinc finger and BTB domain-containing protein 8A
Glyceraldehyde-3-phosphate dehydrogenase
Retinal dehydrogenase 1
lpw_31531
Annexin A2;Annexin;Putative annexin A2-like protein
Ras-related protein Rab-1B
Annexin A1
lpw_17241
Ubiquitin-60S ribosomal protein L40
Ras-related protein Rab-1A
Ras-related protein Rab-6B

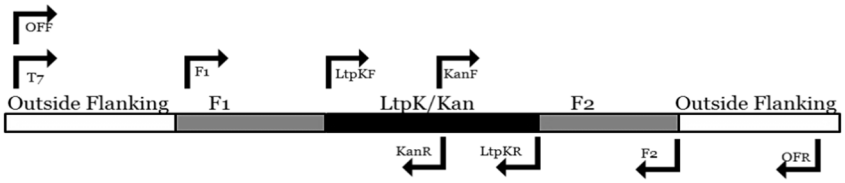
Figure 21 pBio-pulldown comparing different fixation and lysis methods
A549-BirA cells were infected with *L. pneumophila* expressing His6-Bio-SidM or His6-Bio-LtpK. Effectors were purified with a tandem affinity (Ni2+ NTA and Neutravidin) purification (TAP). Each sample was crosslinked with 0.25% or 1% formaldehyde for 10 min or 30 min. Cells were lysed with 1% Triton X-100 or 0.5% SDS. Samples were subjected to LC/MS-MS analysis. **A**, heat map showing intensities of all identified proteins across TAP. Proteins were ranked by enrichment factors. **B**, detailed list of enriched proteins.

2.11 Creation and characterisation of a *L. pneumophila ltpK* deletion mutant

To investigate the role of LtpK in infection, a *ΔltpK* deletion mutant was created. To delete *ltpk* from the *L. pneumophila* chromosome, the gene including flanking regions was amplified by PCR and ligated into pGEMT. The gene was removed by inverse PCR and a kanamycin (Kan) resistance cassette was ligated between the genomic regions flanking the gene in the chromosome. The kanamycin cassette including the flanking regions was amplified by PCR and the product incubated with *L. pneumophila* wild-type strain 130b to allow natural transformation and homologous recombination. To confirm the chromosomal deletion of the gene, genomic DNA (gDNA) was extracted from clones grown on selective kanamycin-containing-plates and several control PCRs were carried out.

The control PCRs as seen in Figure 22 showed that *ltpK* was successfully replaced with the kanamycin cassette in this clone. The *ltpK* gene cannot be detected any more (panel 3), but PCRs for the kanamycin cassette were positive (panel 4 and 5). Furthermore, the whole region was amplified by PCR and sequenced, which confirmed the replacement of *ltpK* with the kanamycin cassette and integrity of the flanking regions.

A



B

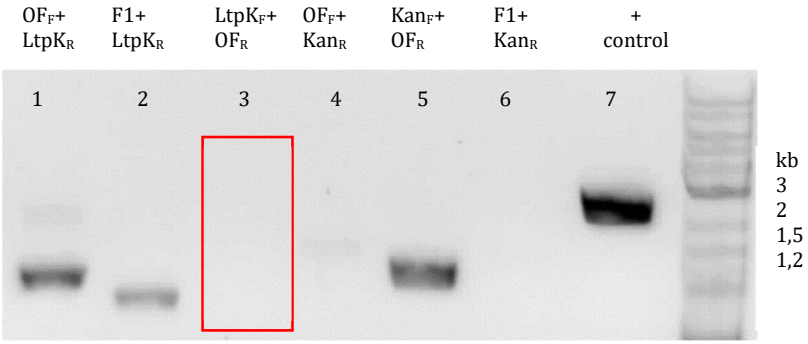


Figure 22 Confirmation of the chromosomal deletion of *ltpK* by PCR
(A) Schematic figure of the genomic region of *ltpK* and the primers used for the control PCRs. (B) Agarose gel of the control PCRs: To confirm the deletion of *ltpK* in the genome, gDNA was extracted and PCRs were carried out using a standard PCR program. Panel 1-6 were done using *ΔltpK* gDNA with different primer pairs as indicated; panel 7 is the positive control carried out with wild-type gDNA. Marker: 2-log DNA ladder.

2.11.1 Localisation of ubiquitin to the LCV is not altered in a *ΔltpK* deletion mutant compared to wildtype

Accumulation of ubiqutinated proteins is a hallmark of LCV maturation. As it was observed that nuclear localised LtpK co-localises with ubiquitin, this co-localisation was further investigated in order to find out whether LtpK plays a role in the accumulation of ubiquitinated proteins on the LCV. Therefore, infections with wildtype *L. pneumophila* or the *ltpK* deletion mutant were carried out and the ubiquitination pattern on the LCV was compared. A549 lung epithelial cells were infected and after 24 hours fixed and stained for immuno-fluorescence microscopy. In these experiments, the LCV stains positive for ubiquitin after infection with the wildtype as well as after infection with *ltpK* deletion mutant (Figure 23).

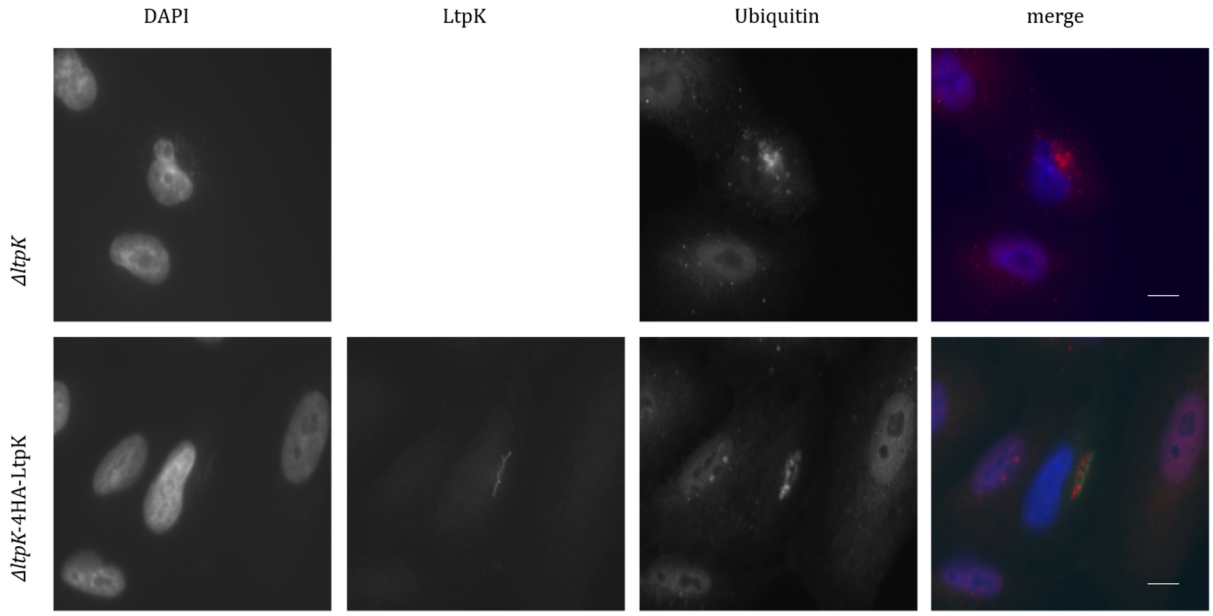


Figure 23 Localisation of ubiquitin to the LCV in a *ltpK* deletion mutant compared to wildtype
A549 cells were infected with wild-type *L. pneumophila* overexpressing LtpK fused to four HA tags (4xHA) or the *ltpK* deletion mutant. After 24h, cells were washed, fixed and stained for HA and ubiquitin using specific antibodies. Representative for two independent experiments. Scale bar 10 µm.

2.11.2 LtpK is not necessary for *L. pneumophila* replication in THP1 macrophages or *A. castllanii*

To investigate whether the deletion of LtpK has an effect on *L. pneumophila*, growth curves were performed by measurement of replication of fluorescent bacteria using the microplate reader. Figure 24 and Figure 25 show representative growth curves done by measuring fluorescence of the bacteria after infection. Across three repeats the *L. pneumophila ΔltpK* mutant did not show a significant difference in replication in THP1 macrophages or *A. castellanii* (Figure 24 and Figure 25).

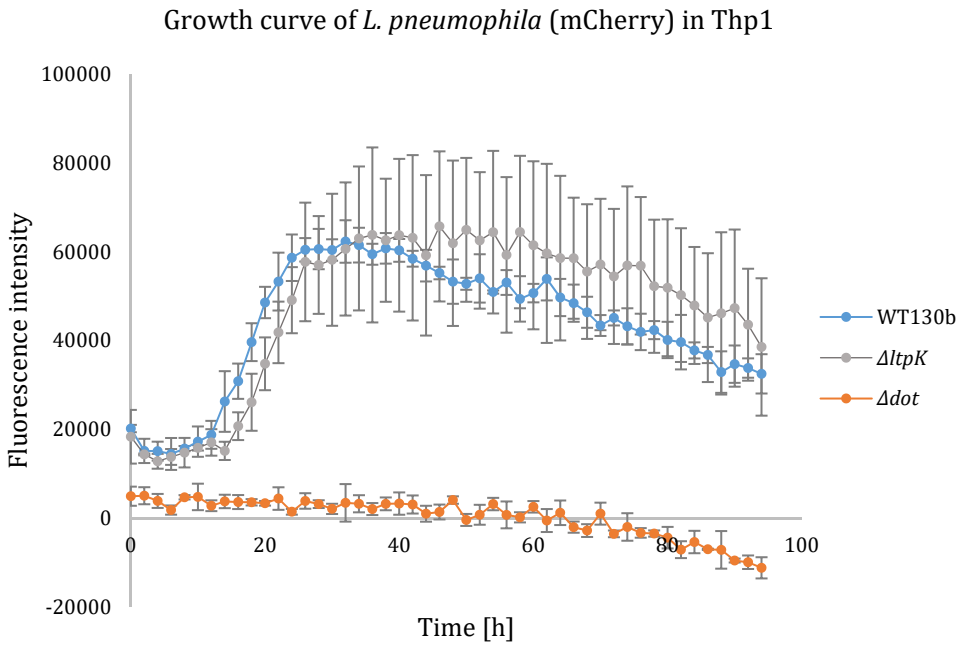


Figure 24 Growth curve of *L. pneumophila* WT and Δ*ltpK* expressing mCherry in Thp1 macrophages
ThP1 macrophages were infected with *L. pneumophila* WT, *ΔdotA* or *ΔltpK* expressing mCherry. Fluorescence was measured in a plate reader over 96 hours. Graphs are representative for at least three independent experiments.

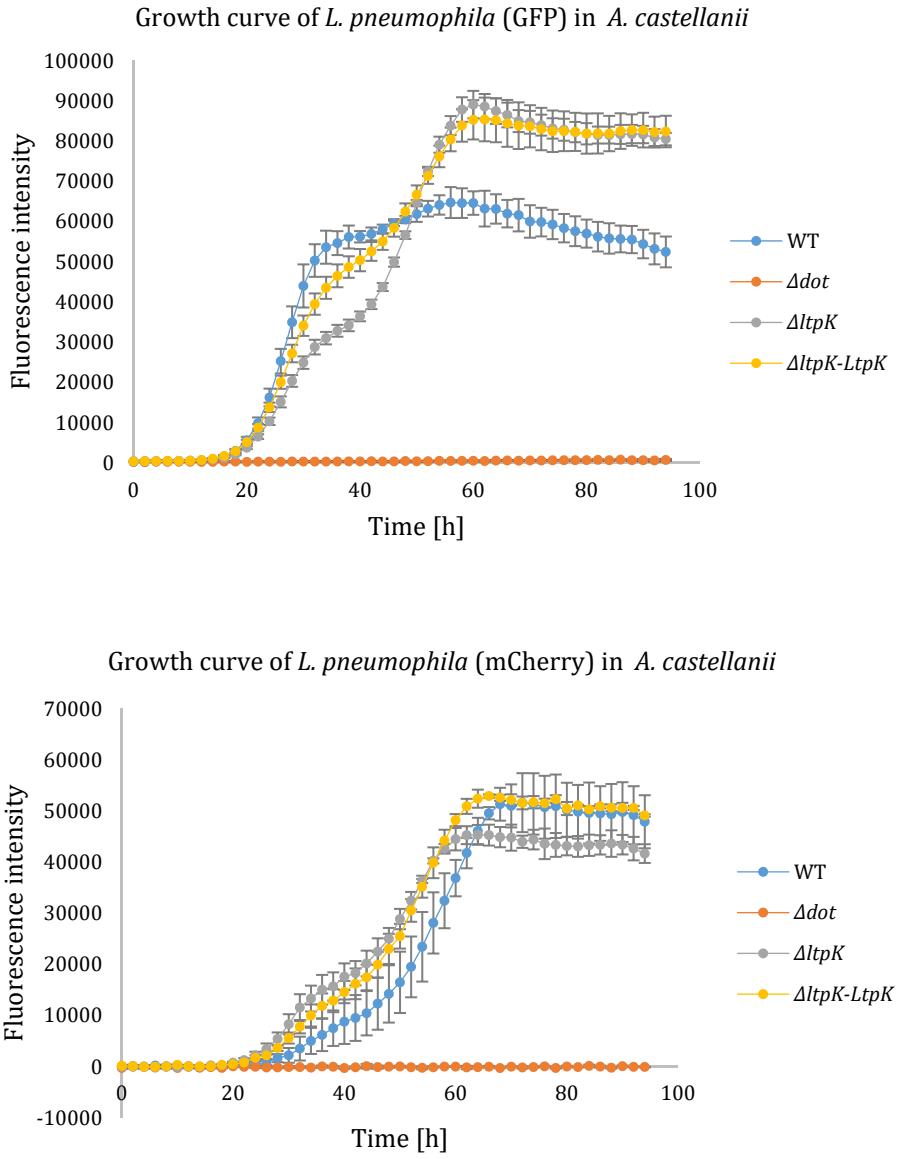


Figure 25 Growth curve of *L. pneumophila* WT, *ΔltpK* and *ΔltpK*-LtpK expressing GFP or mCherry in *A. castellanii*

A. castellanii was infected with *L. pneumophila* WT, *ΔdotA* or *ΔltpK* expressing GFP or mCherry. Fluorescence was measured in a plate reader over 96 hours. Graphs are representative for at least three independent experiments.

2.12 LtpK is dispensable for replication of *L. pneumophila* in mice

In vivo infections of AJ mice were performed with the *ΔltpK* deletion mutant in collaboration with Prof Hartland's research group (Melbourne, Australia). Pulmonary single infections of A/J mice with *L. pneumophila* 130b WT, the mutant *ΔltpK* strain or *ΔltpK* + p4HA-LtpK were carried out by intranasal inoculation. In the first experiment determination of bacterial CFU after 72 hours showed a significant attenuation for the *ΔltpK* strain compared to the WT and the complemented strains (Figure 26 A), however this could not be reproduced in a second experiment (Figure 26 B). When data from both experiments are combined the results show no significant difference (Figure 26 C).

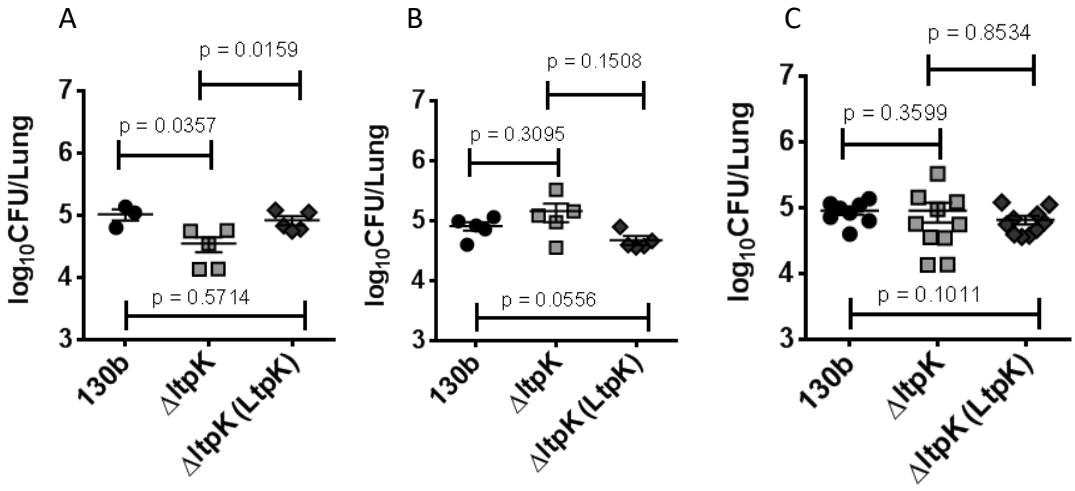


Figure 26 Virulence of *L pneumophila ΔltpK* in the lung of A/J mice
A/J mice were infected with the *L. pneumophila* 130b WT, *ΔltpK* deletion mutant or *ΔltpK* expressing 4HA-LtpK (*ΔltpK*-LtpK) by intratracheal inoculation. 72 h after infection mice were turned down, lungs homogenised and plated on CYE plates containing the appropriate antibiotics. CFUs were counted after three days. (Statistic: Mann-Whitney test, P<0,05) (A, B and C represent three independent experiments).

2.13 Discussion

Here we characterised the effector LtpK, which is homologue to the N-terminus of LtpD.

We find that the phosphorylation motif seems to be conserved between LtpD and LtpK. LtpD was previously shown to be serine- and tyrosine-phosphorylated upon transfection of A549 lung epithelial cells (Doctocal thesis Dr- Claire Harding, 2013). Here, we investigated whether LtpK is serine- and tyrosine-phosphorylated in A549 cells too. Moreover, phosphorylation of both effectors was investigated for the first time in the amoeba hosts *D. discoideum* and *A. castallanii.* The phosphorylation of LtpD in A549 cells could be confirmed and additionally it was shown that LtpD is also serine- and threonine phosphorylated in *D. discoideum*, but not in *A. castellanii*. However, only a small amount of LtpD could be purified after infection of *A. castellanii* and this might be below the detection limit. LtpK is serine- and tyrosine-phosphorylated in the amoeba hosts *A. castellanii* and *D. discoideum*. In contrast, in A549 cells LtpK is only detected for serine-phosphorylation. In A549-BirA cells however, bands for LtpK can be detected for serine- and tyrosine phosphorylation. As the test for total purification of LtpD and LtpK from A549-BirA cells was not successful, no clear conlusion can be drawn from these cells. This similar phosphorylation pattern of LtpD and LtpK is despite the fact that LtpK shows a very different cellular localisation upon transfection or infection, which suggests that LtpK interacts with different proteins. However, the role of the phosphorylation is not clear, as unfortunately no phenotype was discovered to assess the relevance of phosphorylation for the function of LtpK.

Notably, it was shown that the *L. pneumophila* Philadelphia homologue, Lpg2538, is required for bacterial replication in the absence of Bet5 during infection of *D. melanogaster* phagocytes, a protein involved in the tethering of ER-derived vesicles to target membranes (O'Connor et al., 2012). This indicates that LtpK rather plays a role in the cytoplasm/ ER interface for vesicle trafficking than in the nucleus. However, during this study, no growth defect with the *ltpK* deletion mutant compared to wildtype could be observed, the *ltpK*

mutant was able to replicate to the same numbers as the wildtype. The host cells tested in this study were Thp1 macrophages as well as the amoeba hosts *A. castellanii* and *D. discoideum*. O'Conner et al. used *D. melanogaster* cells in their study and a growth defect was observed when the host protein Bet5 was absent. In future experiments for LtpK, growth curves could be performed in Bet5 knock-out cells or amoeba cells lacking the homologues protein to Bet5.

The other possibility is that LtpK has dual roles early in acting on proteins which direct pathways similar to Bet5-dependent signalling and in the nucleus at very late stages of infection. We performed different assays to identify targets which could give us further information about the role of LtpK.

In transfection experiments, it was shown that LtpK co-localises with ubiquitin when localised to the nucleus. Ubiquitination is one of the post-translational modifications (PTMs) of proteins. Ubiquitination can have very diverse effects on the fate of a protein, ranging from proteasomal degradation, changing cellular location, to modulating the activity or protein interactions (Glickman and Ciechanover, 2002, Mukhopadhyay and Riezman, 2007, Pickart and Eddins, 2004, Schnell and Hicke, 2003).

The host ubiquitin pathway is hijacked by many bacterial pathogens which use their virulence factors to establish successful infections (Zhou and Zhu, 2015). It is known that ubiquitinated proteins accumulate during infection (Dorer et al., 2006) and that *L. pneumophila* modulates the host's ubiquitination machinery to its advantage. *L. pneumophila* mimics eukaryotic E3 ubiquitin ligases, the effector LubX (*Legionella* U-box protein) for example contains two domains that have a remarkable similarity to the U-box, which is a domain found in eukaryotic E3 ubiquitin ligases (Kubori et al., 2008). The SidE effector family of *L. pneumophila* was shown to ubiquitinate multiple Rabs, small GTPases, and thereby catalysing ubiquitination by using ADP-ribosylation without the need for the E1 and E2 enzymes (Qiu et al., 2016, Berk and Hochstrasser, 2016).

2.13.1 Characterisation of putative cellular interacting proteins of LtpK

In the Y2H screen with LtpK, several different proteins were identified as putative interaction partners for LtpK. The first, SDHB, is a member of the respiratory chain in mitochondria. In a compendium of two-hybrid system hits, redox control/mitochondria-associated proteins are common false positives (Serebriiskii et al., 2000), which makes it likely that SDHB is not a true interaction partner of LtpK.

Another putative interaction partner for LtpK identified via the Y2H screen is von Hippel-Lindau binding protein 1 (VBP1). VBP1 localises to the nucleus and the cytoplasm of the cell and is ubiquitously expressed. As the name says, that protein interacts with von Hippel-Lindau protein (VHL), which itself is a tumour suppressor involved in ubiquitinylation and degradation of hypoxia inducible factor (HIF). Also, VHL elongates and stabilizes microtubules and stabilizes the spindle during mitosis. It was previously shown that other pathogens also produce proteins which interact with VBP1. One example is HBx of Hepatitis B Virus, which was shown to interact with VBP1 to induce NFκB production and cell proliferation (Kim, 2007). Also, HIV1-IN (integrase) was shown to interact via VBP1 with the VBP1-VHL complex, which leads to degradation of HIV1-In, leading to expression of further virus proteins (Mousnier et al., 2007). Interestingly, silencing of expression of the *Drosophila* homologue of VBP1 was shown to attenuate growth of *L. pneumophila* in fly phagocytes (Dorer et al., 2006). The underlying mechanism or its relevance in human infection are still unknown, however this might indicate that manipulation of VBP1 by effectors might be beneficial for *Legionella*. Nevertheless, the interaction of LtpK with VBP1 has to also be treated with caution, as VBP1 acts as a chaperone and might have rather promiscuous protein binding specificity. Recently, the VBP1 appeared as a hit in the research group in a Y2H screen several times for another protein, indicating that it might be a false positive in Y2H screens. LtpK and VBP1 have the same diffuse subcellular localisation, which makes it hard to draw a conclusion whether the proteins actually co-localise. Further experiments

elucidating the interaction of LtpK and VBP1 in infection are required to validate if VBP-1 is the physiological target of LtpK.

The second confirmed potential interaction partner identified in the Y2H assay was ASXL1. ASXL1 is involved in both transcriptional activation and transcriptional repression in a context-dependent manner (Katoh, 2013). It is a member of protein complexes involved in the epigenetic regulation of gene expression, interacts with various transcription activators and repressors (e.g. BAP1 deubiquitinase leading to gene repression by deubiquitinylation of histone H2A).

Acyl-coenzyme A binding domain containing 3 protein (ACBD3, also known as PAP7, GOLPH1, GOCAP1) was found in the Y2H screen as another potential interacting partner for LtpK. It is a Golgi protein involved in the maintenance of Golgi apparatus structure. ACBD3 plays a central role in several cellular signalling cascades after its release from the Golgi apparatus. It is shown to be involved in cAMP-dependent steroidogenesis, apoptosis or iron homeostasis. Acyl-CoA-binding protein (ACBP) homologues were identified in all four eukaryotic kingdoms, Animalia, Plantae, Fungi and Protista, and eleven eubacterial species (Burton et al., 2005). In context of the evolution and selection of *L. pneumophila* effector proteins in amoeba, ACBD3 could represent a conserved target of cross-host relevance.

It was shown that viral proteins interact with ACBD3 to recruit phosphatidylinositol 4-kinase class III beta (PI4KIIIβ/PI4KB), a factor required for viral replication. In particular, it was shown that the 3A protein of picornaviruses interacts with ACBD3 to recruit PI4K (Greninger et al., 2012, Greninger et al., 2013, Sasaki et al., 2012, Teoule et al., 2013).

L. pneumophila is also known to subvert host PI metabolism very efficiently. Several effectors are known to bind to different phosphoinositides or directly modify the PI pattern on the LCV. For example, SidF is a PI 3-phosphatase that hydrolyzes *in vitro* phagosomal/endosomal PtdIns(3,4)P2 (and also PtdIns(3,4,5)P3), eventually yielding PtdIns(4)P (Hsu et al., 2012). Another PI 3-phosphatase is SidP, which hydrolyses *in vitro* PtdIns(3)*P* as well as

PtdIns(3,5)P2 (Toulabi et al., 2013). Also, *L. pneumophila* produces effectors that recruit PI-modifying enzymes. The Arf1 GEF RalF and the Rab1 GEF SidM could modulate the LCV PI pattern by activating small host GTPases and subsequent recruitment of the PI 4-kinase (PI4K) IIIβ (Godi et al., 1999) or the PI 5-phosphatase OCRL1, respectively (Lichter-Konecki et al., 2006, Hyvola et al., 2006). It was shown that OCRL1 which dephosphorylates PI(4,5)P2, yielding PI4P is recruited to the LCV (Weber et al., 2009a). The bacterium may benefit from the enrichment of PI4P on the LCV because it mimics the cis-Golgi compartment, leading to a better recipient site for the fusion of ER-derived vesicles.

However, ACBD3 is thought to be a Golgi protein and predominantly localizes to Golgi structures. It is only released from the Golgi during apoptosis. As a potential PI4K recruiting enzyme, it would be expected that it would be recruited to the LCV in infection. However, it was not found in the proteome of the LCV (Hoffmann et al., 2013). LtpK on the other hand cannot be seen on the LCV neither, it shows diffuse cellular localization in transfection and a diffuse and nuclear localization in infection. However, LtpK might localize to the LCV or the Golgi in amounts not detectable by immunofluorescence. Although ACBD3 has a putative nuclear localization sequence and nine predicted DNA binding sites, it could not be detected via immunostaining in the nucleus yet (Liu et al., 2006).

Further research focused on validating the cellular interaction partners for LtpK using a tandem affinity purification approach which allows determination of effector interactomes from infected cells and define the role of the interaction during infection of cells with *L. pneumophila*. Using the pBio-pulldown, it is possible to purify protein complexes from cells. This is particularly interesting for research of effectors of pathogenic bacteria, as the effector can be purified from infected cells in complex with the proteins it interacts with during infection, the interactome. This method does not only focus to find a cellular target of the effector, but can instead also identify other bacterial proteins and other effectors the effector of interest might interact with.

Using the approach for LtpK, it was possible to purify it from infected cells. Unfortunately, none of the putative interacting proteins which were found in the yeast-2-hybrid screen could be confirmed. Unfornately, we could not consistently identify a target in all approaches or clearly demonstrate the relevance of these interaction during infections.

2.13.2 Characterisation of the nuclear localisation of LtpK

LtpK has a diffuse cytoplasmic or nuclear localisation during transfection and infection. In very late stages of infection (after 24 hours), it was also enriched in the nucleus. This might be due to disintegration of the nuclear envelope in the dying cells and passive diffusion of LtpK to the nucleus. In transfection, some LtpK was also found in the nucleus but enrichment was not prominent. We did not find an effect or co-localisation with histone H2a, H3 or H4. Transciption was found to be modulated during *L.pneumophila* infection by the effector RomA/LegAS4. RomA/LegAS4 methylates Histone H3 on position K14, which interferes with acetylation on K14 and thereby prevents transcription, or methylates Histone H3 on position K4 activating rDNA transcription (Li et al., 2013b, Rolando et al., 2013). As LtpK was not seen to colocalise with Histones, it might have another effect upon its localisation in the nucleus.

Several other *L. pneumophila* effectors were found to modulate and modify the cellular transcription and translation machinery. As many *L. pneumophila* effectors contain motifs characteristic for eukaryotic organisms (Gomez-Valero et al., 2011, Gomez-Valero et al., 2014), an effector was identified to harbour a eukaryotic-like SET domain (Suppressor of variegation-Enhancer of zeste-Trithorax). Methyltransferases harbour a SET domain to transfer a methyl-group on Lysine residues of Histones (Dillon et al., 2005). Methylation and also acetylation of histones changes the epigenetic code and is a mechanism to regulate gene expression (Ruthenburg et al., 2007, Tchurikov, 2005, Jenuwein and Allis, 2001). This effector RomA/LegAS4 translocated to the nucleus of infected cells, where is modified the methylation status of Histone H3 and therefore changes transcription in the host cell. The effector RomA was found and characterised in the *L. pneumophila* Paris strain and LegAS4 in

the Philadelphia strain, they have 96% sequence identity. RomA methylates H3K14 genome wide and inhibits gene expression (Rolando et al., 2013). Methylation on H3K14 competes with acetylation of the same residue, which is a marker for gene activation. LegAS4 however methylates H3K9 which leads to an increase of rDNA transcription (Li et al., 2013b).

Other SET-domain proteins evolved in pathogenic bacteria and also viruses to modulate the host cell's epigenetic code and therefore to enhance intracellular survival of the pathogen (Nwasike et al., 2016). For example, the viral SET protein vSET methylates host chromatin on H3K27 to promote the replication of the chlorella virus *Paramecium bursaria* PBCV-1 (Mujtaba et al., 2008). Another example is the *Bacillus* protein BaSET, which regulates growth of the bacterium as well as the host's inflammatory response by specifically trimethylating the host's histone H1 rather than the histone proteins H2A, H2B, H3, and H4, and thereby epigenetically repressing NF-κB activation in infected macrophages (Mujtaba et al., 2013). *Chlamydia pneumoniae* is another bacterium which uses a similar mechanism. In this pathogen, the SET-like protein cpnSET methylates histone-like proteins Hc1 and Hc2 for a morphological switch (Murata et al., 2007). In *Chlamydia trachomatis,* the nuclear effector protein (NUE) was shown to preferentially methylate host cell chromatin at histones H2B, H3, and to a lesser extent H4, but not H2A, in an *in vitro* methylation assay (Pennini et al., 2010).

All those nuclear effector proteins of pathogens are shown to manipulate the chromatin epigenetic code via their SET domain. However, LtpK does not have a SET domain and was shown to not co-localise with histones in the nucleus. The role of LtpK upon its localisation in the nucleus remains unknown.

Chapter 3

Characterisation of the effector LtpM

3. Characterisation of the effector LtpM

3.1 Domain organisation of the effector LtpM

The effector LtpM is present in the *L. pneumophila* Paris strain as well as in recently sequenced environmental and outbreak isolates. It shares sequence similarity in its C-terminal domain with the C-terminal domain of the effector LtpD (for more details see section 3.2), however, has no other similarities or homologies to LtpD in the N-terminal domain. Bioinformatic analysis of LtpM reveal two more features in the N-terminus of LtpM. An overview of the domain organisation of LtpM is shown in Figure 27. Firstly, LtpM has two Ankyrin-repeats (AA 365-394 and 407-437). Ankyrin repeats are one of the most widely existing protein motifs and are the most common protein-protein interaction motif found in nature (Li et al., 2006, Al-Khodor et al., 2010). It was long believed that Ankyrin-repeats in microbial proteins were acquired from eukaryotes through horizontal gene transfer. However, Ankyrin-repeats were also found in the genome of archaea and free living bacteria, which suggests that this motif evolved several times in different species or it has a more ancient origin (Al-Khodor et al., 2010). Ankyrin repeats appear to be conserved only in structure, but not in function, as they are found in a large number of functionally very diverse proteins (Bork, 1993, Mosavi et al., 2004). As domains with Ankyrin repeats are known to mediate protein-protein interactions, that domain might be necessary for LtpM to interact with another protein.

In addition to the Ankyrin-repeat, structural homology prediction indicated a low, partial homology of the first 145 AA of LtpM to the glycosyl transferase domain of the Photorabdus insect toxin PaToc, with a DxN motive at AA 140-142. For more details on this domain, see section 3.17.

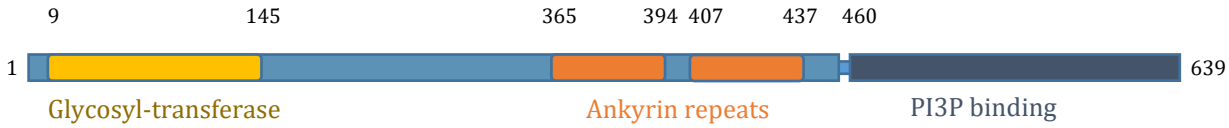


Figure 27 Domain organisation of the effector LtpM
The C-terminal domain of LtpM is homologues to the PI3P-binding of LtpD and is therefore characterised for its PI3P binding ability. LtpM has two Ankyrin-repeats at AA 365-437 in the middle of the protein and before the PI3P binding domain. Moreover, it is a glycosyltransferase domain in the N-terminus, AA 1-145, with the characteristic DxN motive at AA 140-142.

3.2 LtpM has sequence homology in its C-terminal domain to the effector LtpD

The C-terminus of LtpM shares 68.3% identity with the C -terminus of the previously characterised effector LtpD (LtpDC). Figure 28 shows an alignment of these domains (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/).

This is interesting because the C-terminal domain of LtpD was characterised to bind to PI3P-rich cellular compartments and to PI3P in a PIP-array. Therefore, it was decided to investigate whether the effector LtpM has a similar PI3P-binding preference and a new PI3P-binding domain could be characterised.

```
LtpM455-639       1 SDEVIQPSSKDERGFTPEQIMVIKVAMQEALSSMGTSHFVSGRDDKKESL     50
                    ||||:|.||..|..|||:|..:|..|||:||:||||||.|||||||::.|
LtpD496-679       1 SDEVMQSSSNREHSFTPQQSTIIIEAMQKALTSMGTSRFVSGRDDKEKLL     50

LtpM455-639      51 VQCKSAFENAPNGTERHEALRNFVTAASTPRQGLLNFLPAAYGETRSAIA    100
                    ||.|||||||.||.||||||||||.|||||||:|||.|||||:||||.|
LtpD496-679      51 VQFKSAFENATNGQERHEALRNFVTTASTPRQGVLNFFPAAYGKTRSATA    100

LtpM455-639     101 FFEHIKKQDSSLFDALKPMREDKGPEVARQSNSSVETEATKPVARAREIM    150
                    |:||:|.||.||.:.||.|..:||.:|..|||||.||:||.|..:||.||
LtpD496-679     101 FYEHVKNQDPSLVEVLKTMDGEKGRKVVAQSNSSDETKATMPADQARGIM    150

LtpM455-639     151 LHYKLAMQAERRGELKTDNDSVDSQNLCGTRLK    183
                    .|:|.|:|.||..||||.||||||||.|.||
LtpD496-679     151 QHFKRAIQVERGSELKTGNDSVDSQNLCDTGLK    183
```

```
Aligned_sequences: 2
1: LtpM455-639
2: LtpD496-679
Matrix: EBLOSUM62
Gap_penalty: 14
Extend_penalty: 4

Length: 183
Identity:     125/183 (68.3%)
Similarity:   143/183 (78.1%)
Gaps:           0/183 ( 0.0%)
Score: 611
```

Figure 28 Homology of the C-termini of the effectors LtpD and LtpM
The C-termini of the effectors LtpD and LtpM were aligned using the emboss-matcher and sequence homology and similarity was calculated by an algorithm based on Bill Pearson's lalign application, version 2.0u4 (Feb. 1996).

3.3 LtpM is a translocated effector

To confirm LtpM is ideed translocated by the Dot/Icm tranlocation system of *L. pneumophila*, a translocation assay was performed. This was done in the same way as for LtpK described in section 2.3. LtpD was used as a positive control, while the *L. pneumophila* protein FAB was used as negative control. Neither of the tested proteins was translocated by the *ΔdotA* mutant. No increase of the translocation ratio was observed for infection with WT bacteria expressing TEM-FAB showing that no unspecific release of the TEM1-tagged protein occurs. For LtpM a strong increase in the translocation ratio was observed, showing that LtpM, like LtpK, is a translocated effector (Figure 29).

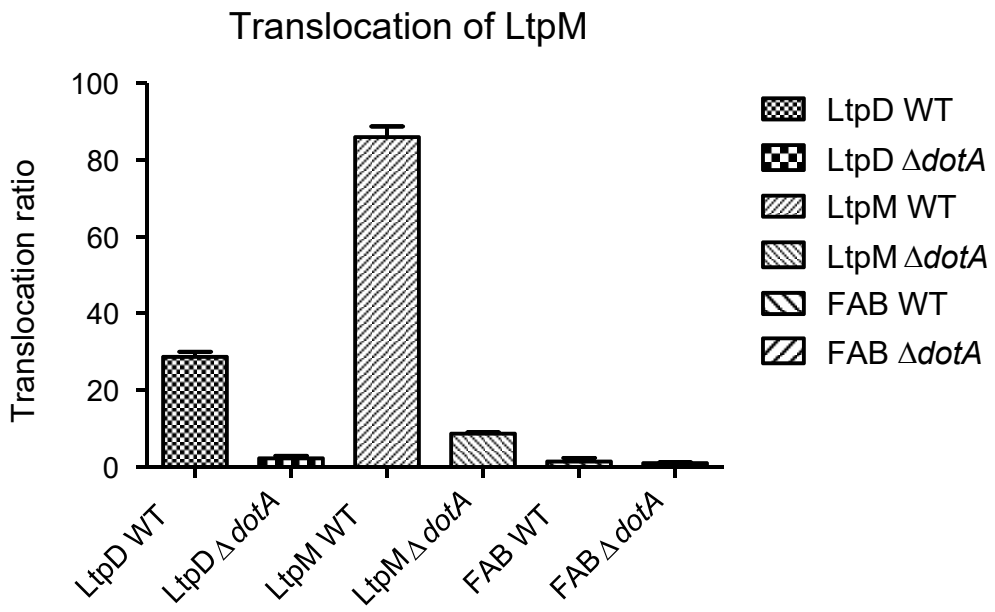


Figure 29 Translocation of LtpM into RAW264.7 macrophages
RAW264.7 macrophages were infected with *L. pneumophila* WT and Δ*dotA* overexpressing the confirmed effector LtpD, the putative effector LtpM as well as the protein FAB fused to β-lactamase. Translocation was measured calculating the ration of cleaved (520nm) to uncleaved (447nm) substrate CCF2/AM. Performed in triplicates; Error bars represent +/- SD; Graph representative for three independent experiments.

3.4 LtpM binds to PI3P via its C-terminal domain

3.4.1 LtpM co-localises with PI3P markers in transfection

The homology to LtpD suggests that LtpM might also bind to PI3P with its C-terminal domain. To test this hypothesis, full length LtpM, the C-terminal domain and the N-terminal domain were co-transfected with the PI3P marker SetA (Jank et al., 2013) in HeLa cells. This showed a strong co-localization of the full length LtpM and the C-terminal domain with the PI3P binding domain of SetA, whereas the N-terminal domain showed a diffuse cytoplasmic localisation (Figure 30). This indicates that the C-terminal domain of LtpM is responsible for the subcellular localisation and targets LtpM to the vesicular structures.

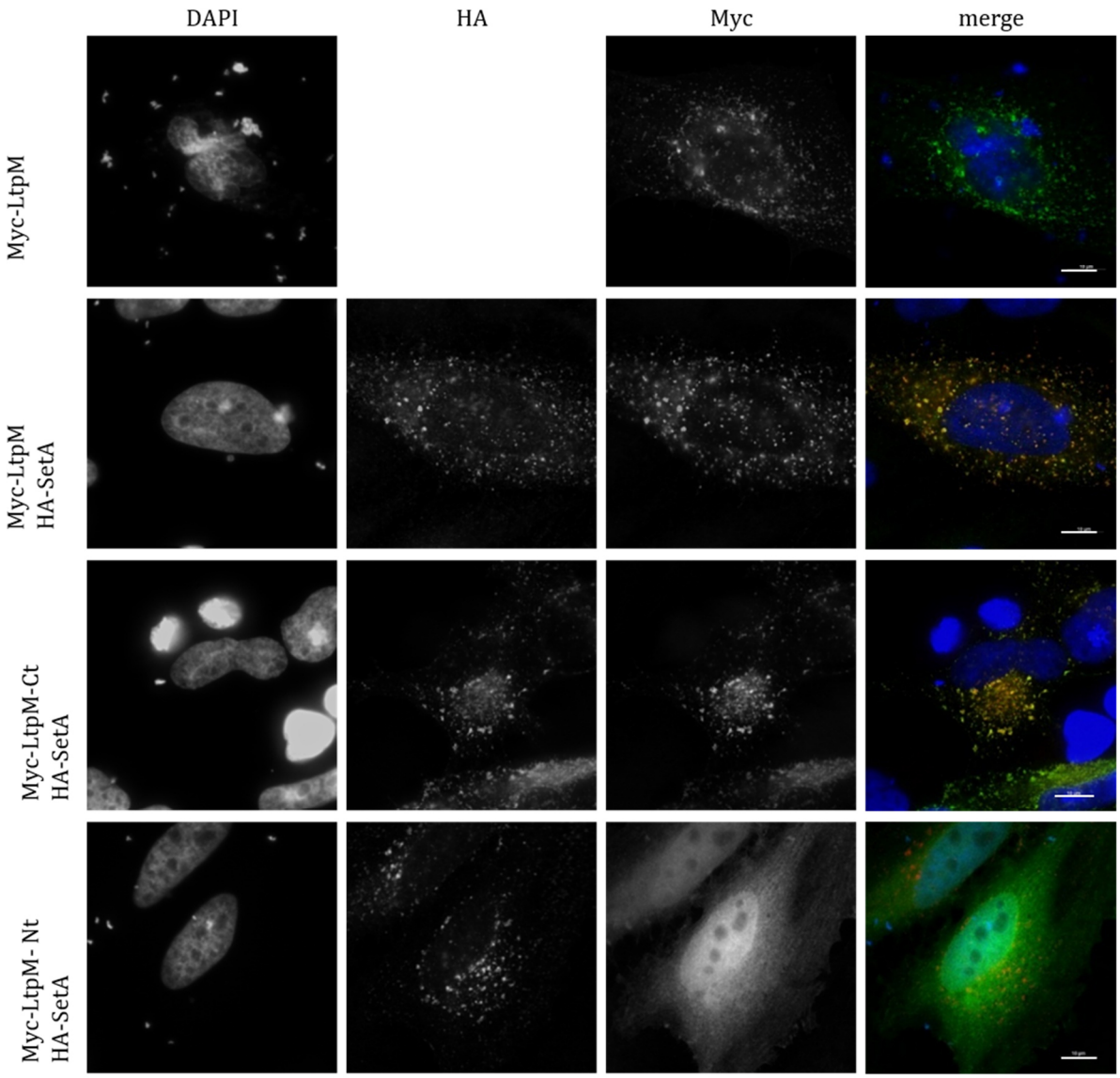


Figure 30 LtpM co-localizes with PI3P markers upon transfection
HeLa cells were co-transfected with full length Myc-LtpM, the C-terminus (Myc-LtpM Ct) of LtpM or the N-terminus (Myc-LtpM Nt). Constructs were co-transfected with the PI3P binding domain of SetA. Scale bar 10 µm.

3.4.2 Binding of LtpM to PI3P in a PIP-array

Direct binding and PI specificity can be tested *in vitro* using a PIP array. Therefore, the full length LtpM or the C-terminal domain were cloned in the pGEX-vector, which allows expression of a GST fusion protein in *E. coli* BL21*. Expression tests were performed at 37°C, 30°C and 18°C at different IPTG concentrations. The highest amount of soluble proteins could be obtained at 18°C with induction using 1mM IPTG (data not shown). 1 L cultures of *E. coli* BL21* were incubated under optimal induction conditions described above in order to purify the proteins. Proteins were purified using Glutathione Sepharose 4B. The different fractions of the purification process were collected and analysed by SDS-PAGE. Results are shown in Figure 31 A. Proteins were dialysed over night with PBS at 4° and purity and stability analysed by SDS-PAGE (Figure 31 B).

Compared to the full length LtpM, the C-terminal domain was better expressed and could be purified more readily. Therefore, the PIP-array was performed with the C-terminus of LtpM only. GST was used as negative control, whilst the positive control was a PI4P binding protein provided with the PIP-array kit. The result of the PIP-array can be seen in Figure 31 C. The positive and negative controls show some background binding to different PIs. However, the signal obtained for the C-terminus of LtpM binding to PI3P is much stronger than the signal of any of the controls, indicating that it binds PI3P.

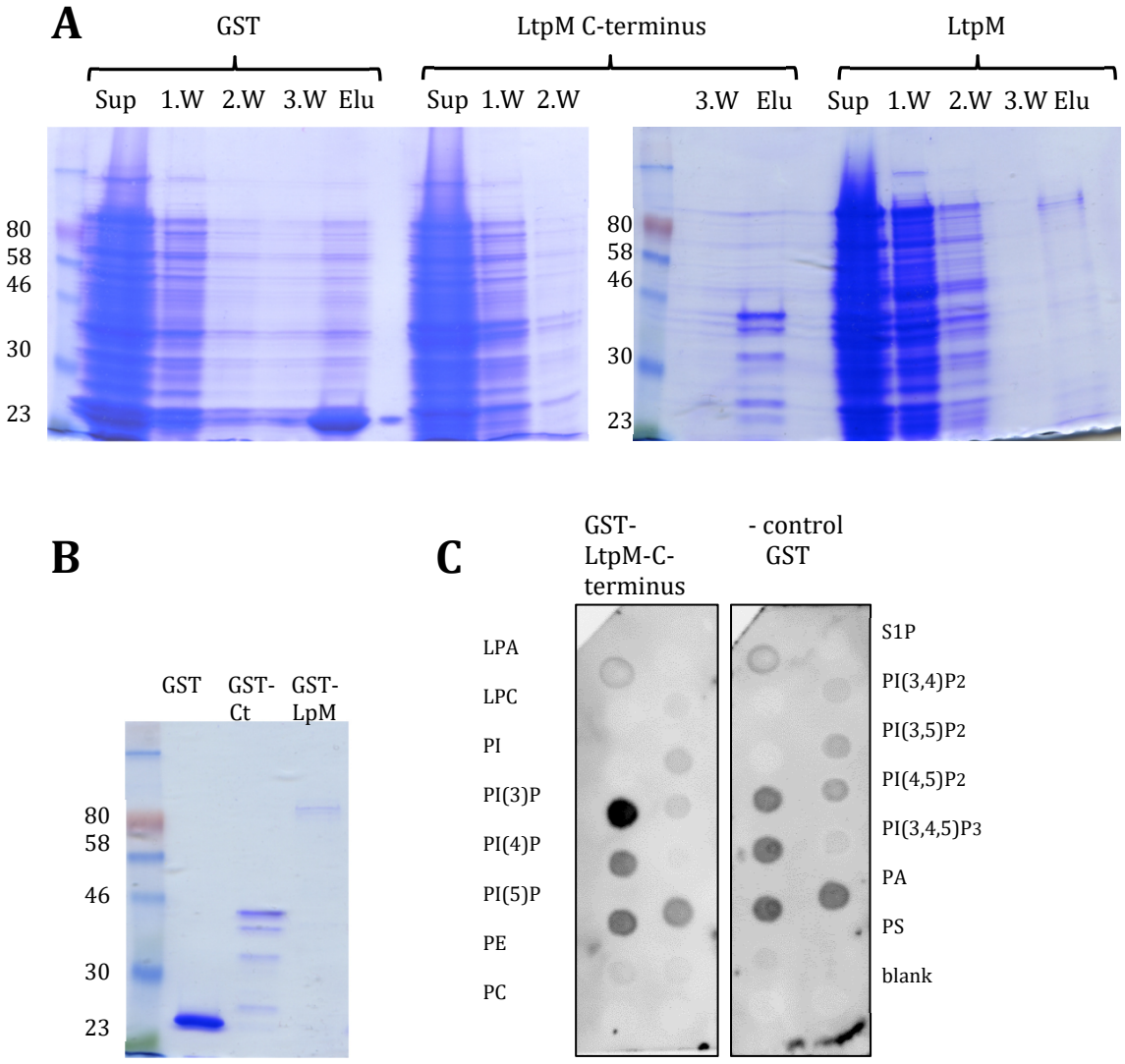


Figure 31 LtpM full length and C-terminus purification and PIP-array
GST-tagged full length LtpM, C-terminus (Ct) or GST alone were produced in *E.coli* BL21* and purified using Gluthatione Sepharose 4B. The different fractions were analysed via SDS-PAGE. GST: 25 kDa; GST-C-terminus: 45 kDa; GST-LtpM: 97 kDa (B) SDS-PAGE analysis of the purified proteins after dialysis. (C) PIP-array performed with the LtpM C-terminus as well a positive control (PI4P binding protein) and negative control (GST). (W)=Wash; (Elu) = Elution.

3.5 Inhibition of PI3K does not abolish vesicular localisation of LtpM

In order to provide further evidence that LtpM binds to PI3P, cells were treated with Wortmannin, a covalent inhibitor of phosphoinositide 3-kinases (PI3K) to disrupt PI3P generation in the cell. After the treatment with Wortmannin, the vesicular localisation of LtpM is clearly reduced, but not completely abolished. Especially in experiments with the full length LtpM there are still vesicles detectable in the cell, indicating that LtpM localizes to these vesicles not just by its binding to PI3P but possibly by interacting with proteins (Figure 32). In contrast the C-terminus of LtpM showed a more diffuse localisation after the treatment with Wortmannin.

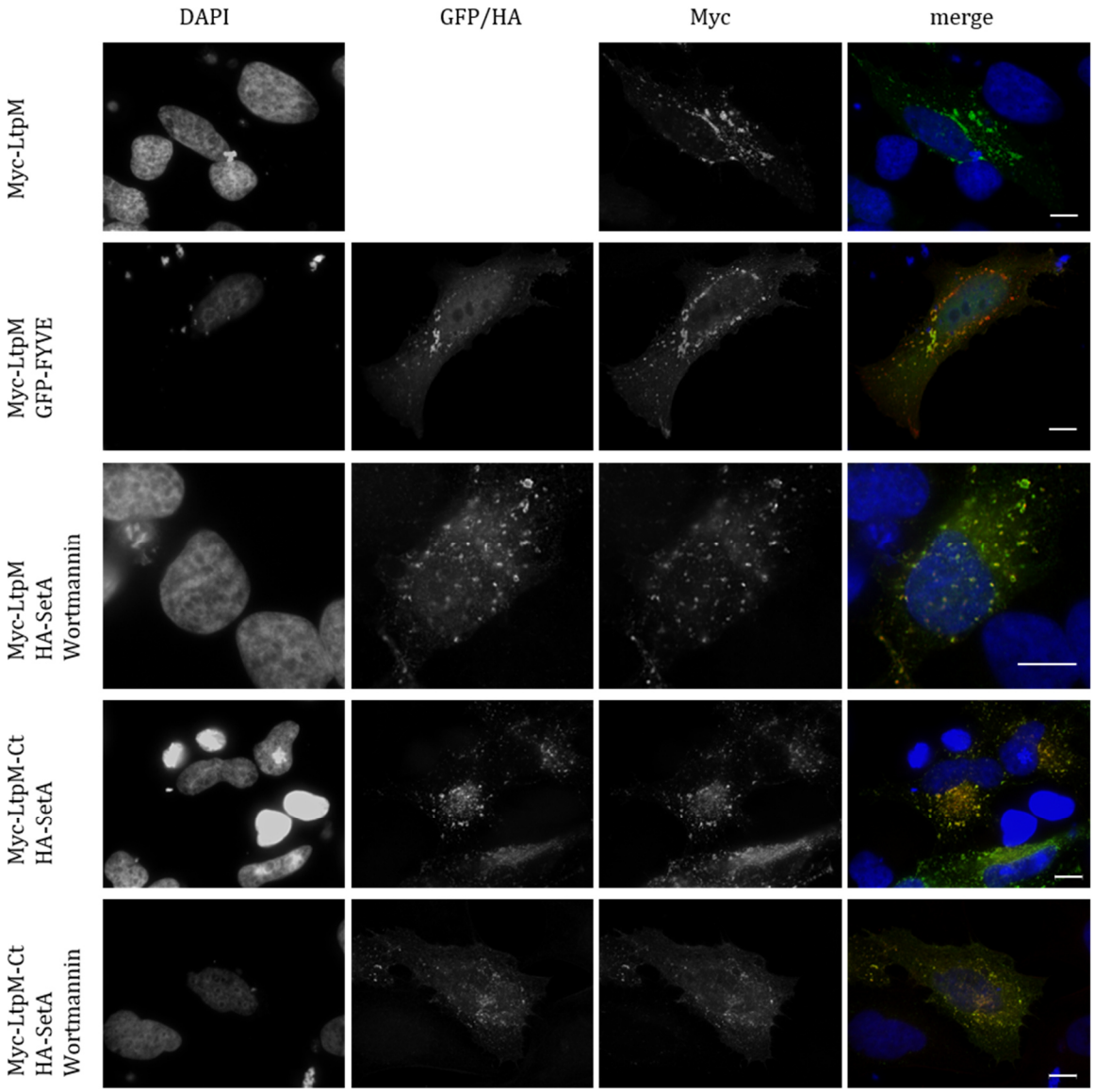


Figure 32 LtpM co-localizes with PI3P markers upon transfection
HeLa cells were co-transfected with full length Myc-LtpM, the C-terminal domain (Myc-LtpM-Ct) or the N-terminal domain of LtpM (Myc-LtpM-Nt) and the PI3P binding domain of SetA. To disrupt PI3P, cells were treated with 100 nM Wortmannin for 6 hours prior to fixation and subsequent staining. Scale bar 10 µm.

3.6 LtpM co-localises with markers of the endo-lysosomal pathway upon ectopic expression

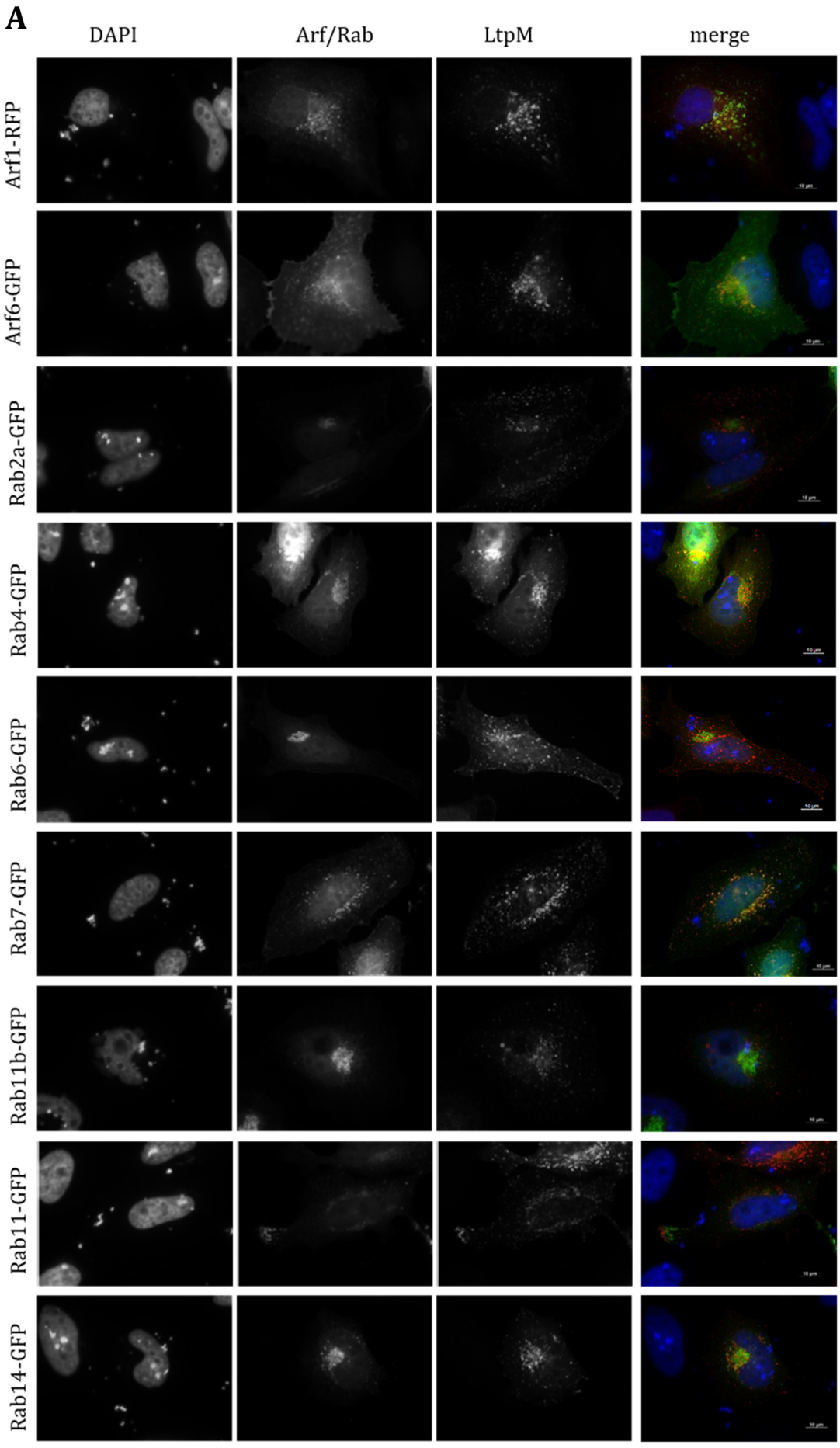
PI3P is a marker for endosomal membranes. To further assess to which specific subcompartment LtpM localises and whether LtpM has any effects on the endocytic pathway, co-transfections were carried out. Myc-LtpM was either co-transfected with markers of endosomal compartments fused to GFP or transfected cells were stained with specific antibodies. LtpM co-localises with some markers of the endocytic pathway, but a redistribution of those markers could not be observed when compared to cells which were not transfected with LtpM (Figure 33). No effects on other organelles, eg Golgi or ER, were observed.

Table 5 shows a summary of the tested endo-lysosomal markers and the results.

Table 5 Overview of the co-localisation of LtpM with tested endo-lysosomal markers

LtpM	Compartment	**Co-localisation?**
Arf1	Golgi	-
Arf6	Endocytosis	-/+
Rab2	Golgi	-
Rab4	endosomes, recycling	-/+
Rab6	Golgi to ER	-
Rab7	early endosomes	++
Rab11b	Golgi	-
Rab11	recycling endosomes	-/+
Rab14	TGN and early endosomal	-/+

Symbols: - no co-localisation, +/- occasional co-localisation, ++ clear co-localisation


A
DAPI
Arf/Rab
LtpM
merge
Arf1-RFP
Arf6-GFP
Rab2a-GFP
Rab4-GFP
Rab6-GFP
Rab7-GFP
Rab11b-GFP
Rab11-GFP
Rab14-GFP

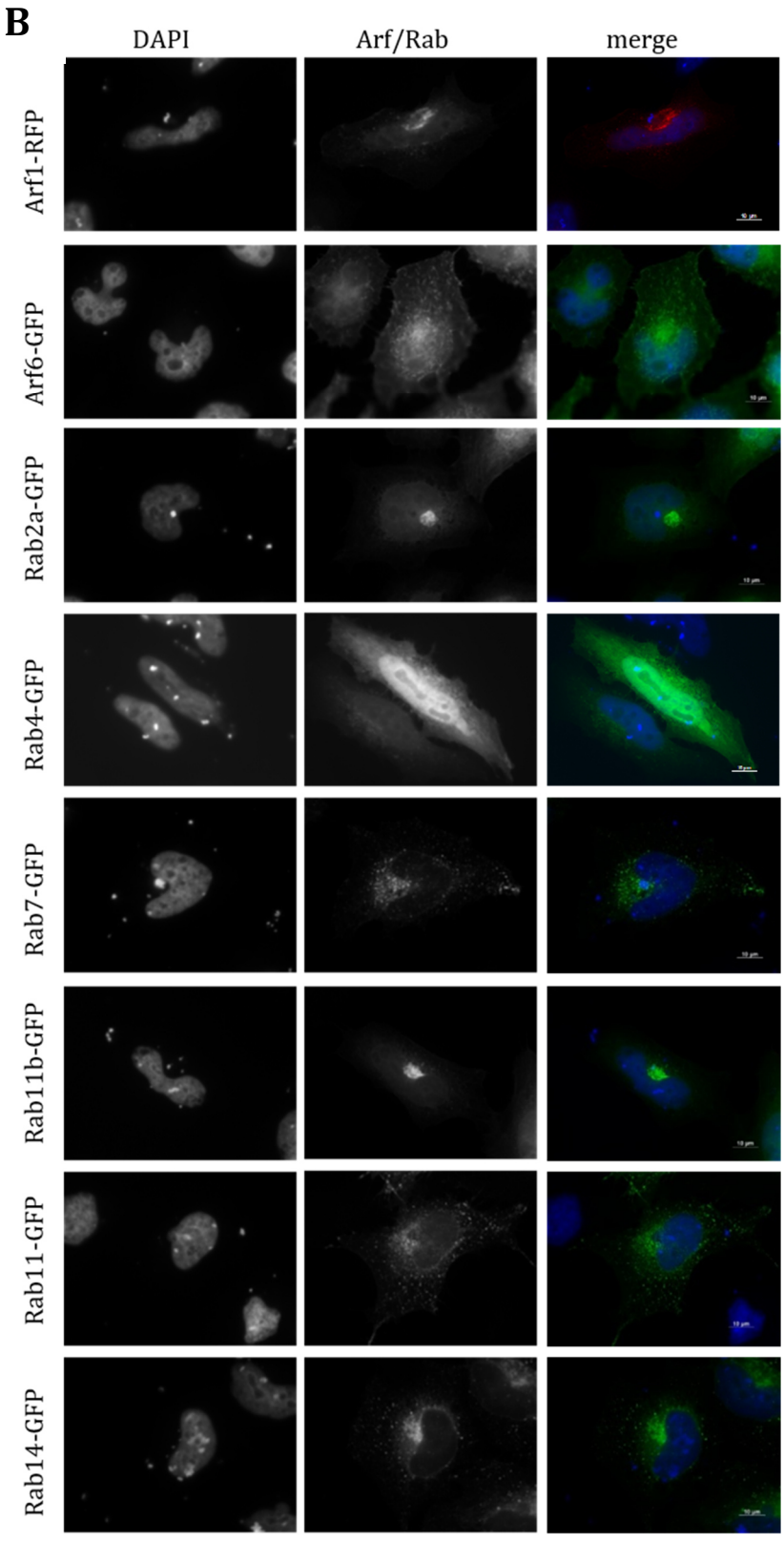


Figure 33 Co-transfection of LtpM with cellular trafficking markers
HeLa cells were co-transfected with HA-LtpM and the indicated cellular trafficking marker Arf or Rab fused to GFP or RFP (A) or for comparison cells were only transfected with the cellular trafficking marker (B). Cells were fixed and stained for immunofluorescence microscopy using specific antibodies against HA. Scale bar 10 µm.

3.7 LtpM is dispensable for intracellular growth of *L. pneumophila* in different host cells

The gene encoding for *ltpM* is found in the *L. pneumophila* Paris strain but not in other prototypical *L. pneumophila* strains such as 130b or Philadelphia. The gene was replaced in the Paris strain with a kanamycin resistance cassette, using the same methodology as described for the *ltpK* mutant.

Intracellular growth of *L. pneumophila* Δ*ltpM* was analysed using a fluorescence plate reader assay. THP1 macrophages, *D. discoideum* or *A. castellanii* were infected with *L. pneumophila* Paris WT, *ΔltpM* deletion mutant or the *Δdot* strain expressing GFP or mCherry (MOI 1 or 10). Replication was measured in the plate reader as increase of fluorescence over time. Figure 35 shows that LtpM does not have a role in intracellular replication in THP1 macrophages, *D. discoideum* or *A. castellanii*.

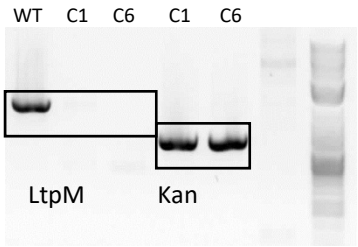
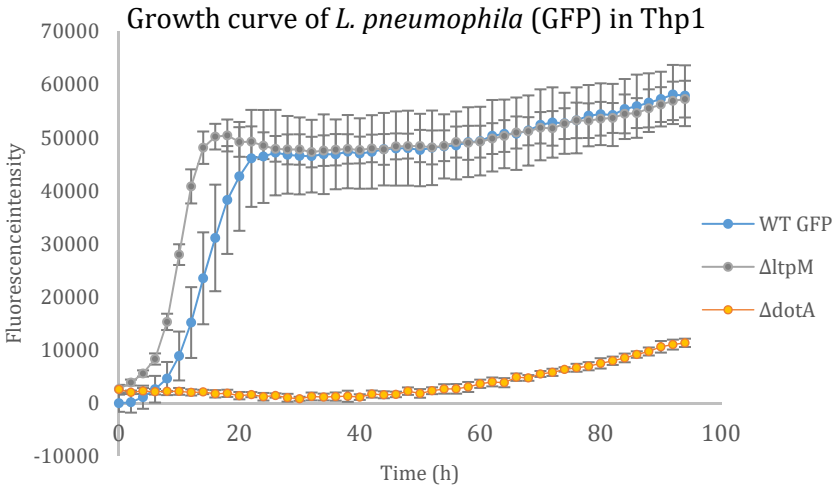
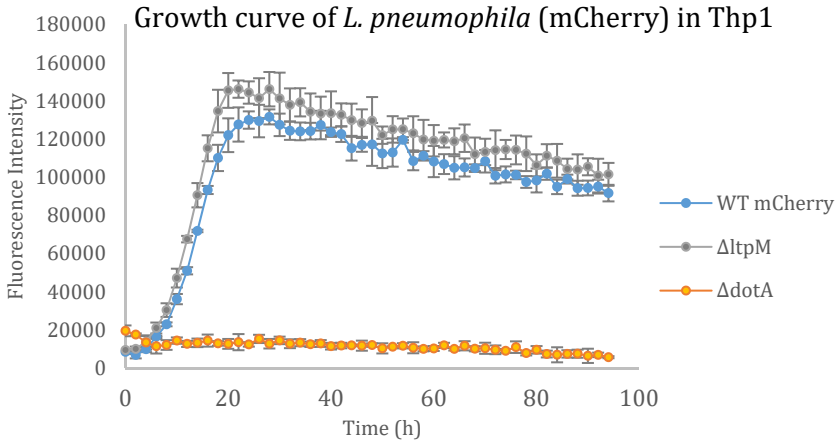


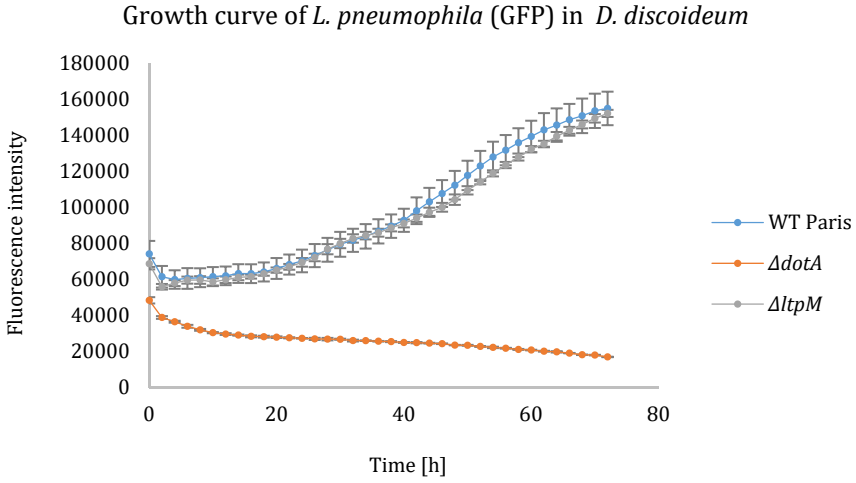
Figure 34 Replacement of the *ltpM* gene with a kanamycin resistance cassette
PCRs were carried out using a standard PCR program. Two clones, C1 and C6, were tested for deletion of *ltpM* and insertion of the kanamycin cassette. WT Paris gDNA was used as positive control for LtpM. Marker: 2-log DNA ladder.

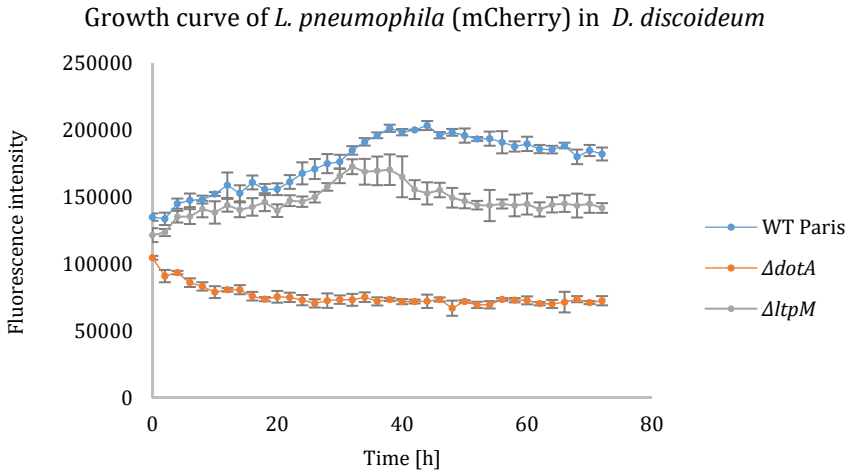
A


Growth curve of *L. pneumophila* (GFP) in Thp1
Fluorescenceintensity
70000
60000
50000
40000
30000
20000
10000
0
-10000
0
20
40
60
80
100
Time (h)
WT GFP
ΔltpM
ΔdotA

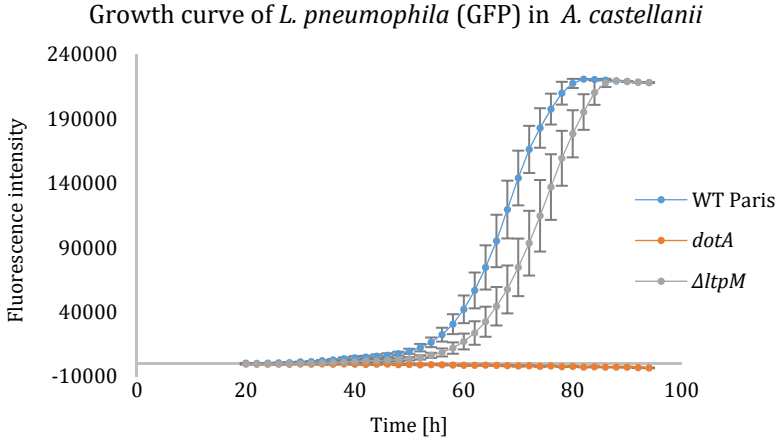

Growth curve of *L. pneumophila* (mCherry) in Thp1
Fluorescence Intensity
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
20
40
60
80
100
Time (h)
WT mCherry
ΔltpM
ΔdotA

B


Growth curve of *L. pneumophila* (GFP) in *D. discoideum*
Fluorescence intensity
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
20
40
60
80
Time [h]
WT Paris
ΔdotA
ΔltpM



C



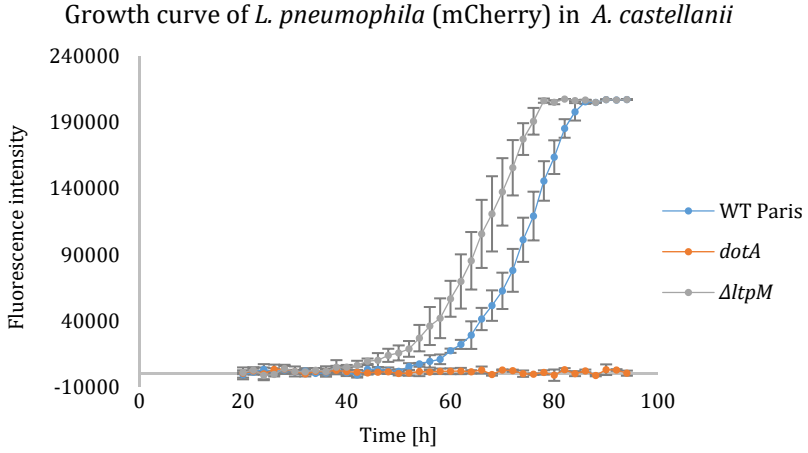


Figure 35 Intracellular growth of the *L. pneumophila ΔltpM* mutant in different hosts

ThP1 macrophages (A), *D. discoideum* (B) or *A. castellanii* (C) were infected with *L. pneumophila* WT, *ΔdotA* or *ΔltpM* expressing GFP or mCherry. Fluorescence was measured in a plate reader over 96 hours. Graphs are representative for at least three independent experiments.

3.8 Deletion of LtpM does not affect localisation of Rab5 during infection

As LtpM localises to the LCV and early endosomes via its C-terminal PI3P binding domain and *L. pneumophila* is known to harbour many effectors modulating the endo-lysosomal pathway, it was investigated whether LtpM might have a role in one of those pathways too. The *L. pneumophila ΔltpM* deletion mutant does not show a growth defect in the tested cell lines. However, it is very common that the deletion of a single effector does not have an effect on the intracellular growth of *L. pneumophila*. It was therefore investigated further whether LtpM affects the endo-lysosomal pathway by comparing the *ΔltpM* deletion mutant to the wildtype strain by staining for specific markers during infection.

A549 lung epithelial cells expressing Rab5-GFP were infected with *L. pneumophila* expressing 4HA-LtpM as well as the Δ*ltpM* deletion mutant. 6 and 24 hours after infection, cells where washed, fixed and stained for the indicated cellular markers. This way, it was investigated whether the cellular marker Rab5 might distribute differently when cells are infected with the wildtype in comparison with the *ΔltpM* deletion mutant. Rab5 shows the same distribution in the wildtype *L. pneumophila* strain as in the *ΔltpM* deletion mutant, indicating that LtpM is not necessary for the localisation or distribution of Rab5 during *L. pneumophila* infection.

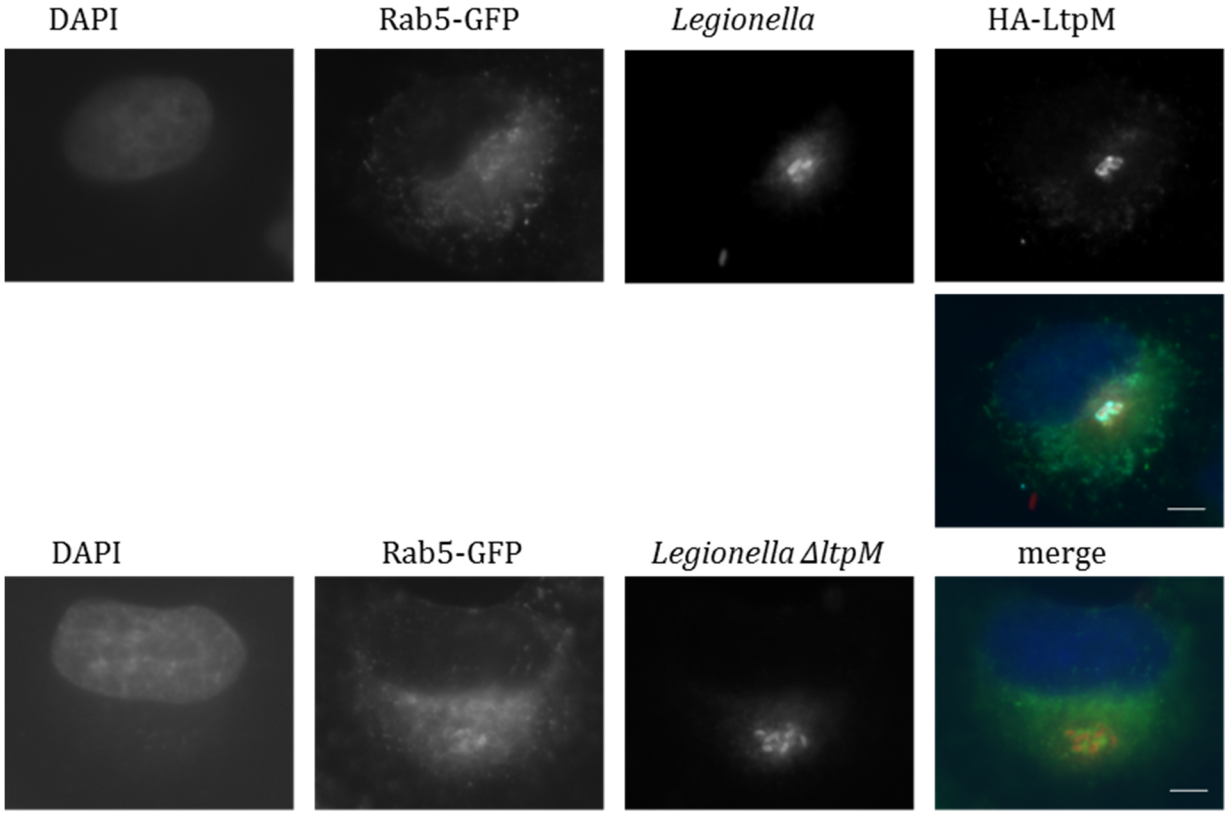


Figure 36 Comparison of the distribution of Rab5 during infection with the wildtype or the *ΔltpM* strain

A549 lung epithelial cells stably expressing Rab5-GFP were infected with either the *L. pneumophila* wildtype strain or the *ΔltpM* deletion mutant. 6 hours after infection cells were washed, fixed and stained for fluorescence microscopy using specific antibodies against *Legionella* LPS and HA. Scale bar 5 µm.

3.9 Localisation of ubiquitin to the LCV does not differ in a *ΔltpM* deletion mutant compared to wildtype

LtpK was shown to co-localise with ubiquitin upon transfection and subsequently it was investigated whether accumulation of ubiquitin on the LCV differs in a *ltpK* deletion mutant. In parallel, the same experiment was performed for LtpM to investigate whether LtpM might have a function in that process.

A549 lung epithelial cells were infected with the *ltpM* deletion mutant or wildtype *L. pneumophila* and the ubiquitination pattern on the LCV was compared. After 24 hours, infected cells were fixed and stained for immuno-fluorescence microscopy. In these experiments, the LCV stains positive for ubiquitin after infection with the wildtype as well as after infection with *ltpM* deletion mutant (Figure 37).

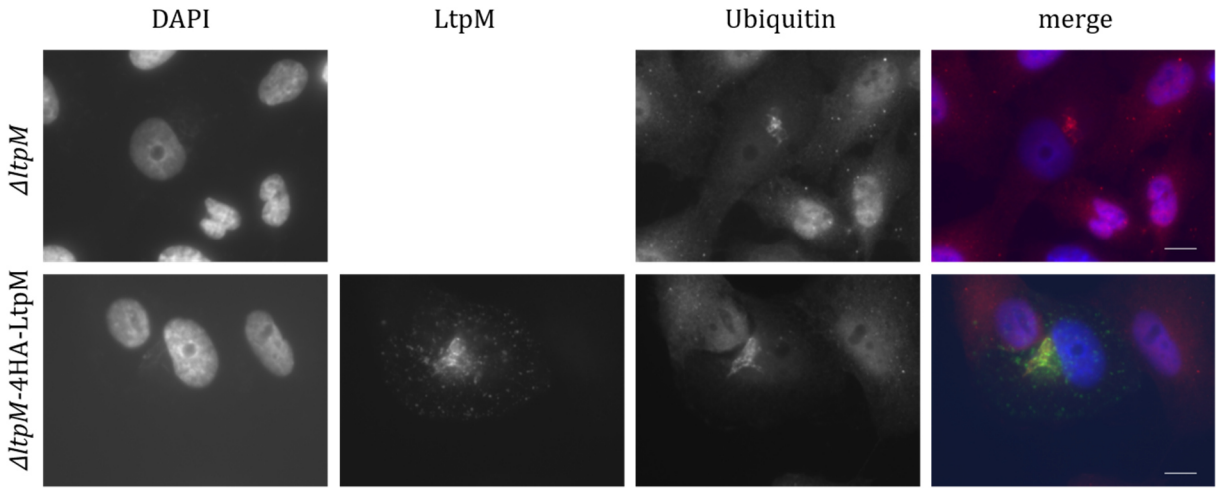


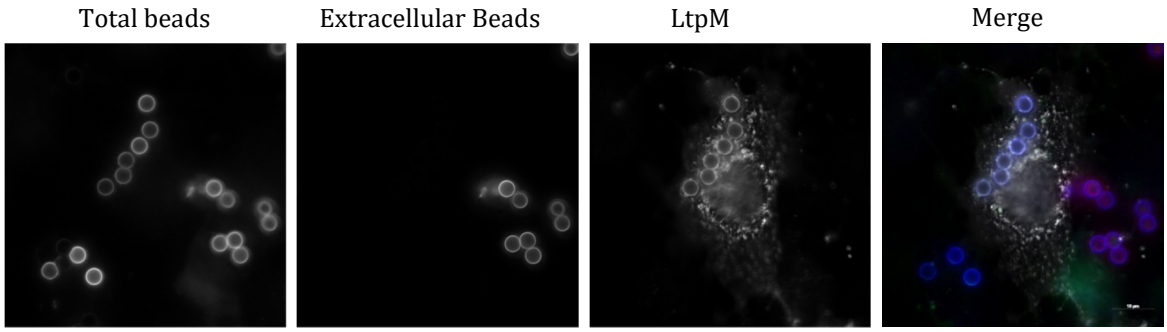
Figure 37 Localisation of ubiquitin to the LCV in a *ltpM* deletion mutant compared to wildtype
A549 cells were infected with wild-type *L. pneumophila* overexpressing LtpM fused to four HA tags (4xHA) or the *ltpM* deletion mutant. After 24h, cells were washed, fixed and stained for *Legionella*-LPS, Myc and ubiquitin using specific antibodies. Representative for two independent experiments. Scale bar 10 µm.

3.10 LtpM does not interfere with phagocytosis

Via binding to PI3P, LtpM localises to the LCV but also to early endosomes. To further clarify the role of LtpM on early endosomes, a phagocytosis assay was used to investigate whether LtpM interferes with phagocytosis or vesicle transport of the phagocytosed particles. LtpM and the FcγRIIa were ectopically expressed in Cos-7 cells and phagocytosis assessed using opsonised agarose beads. Control cells were transfected with pFcγRIIa and empty pRK5myc vector. After 18 hours, transfected cells were challenged with opsonised agarose beads for 90 minutes, then fixed and stained differentially with specific antibodies against myc, external beads and total number of beads (Figure 38 A). To determine the efficiency of phagocytosis, external and total number of beads were counted for each cell.

Notably, the beads after uptake were surrounded by LtpM-positive membranes; however, there was no difference in the efficiency of phagocytosis between the LtpM-transfected cells and the cells transfected with the empty pRK5myc vector (not shown) (Figure 38 B).

A



B

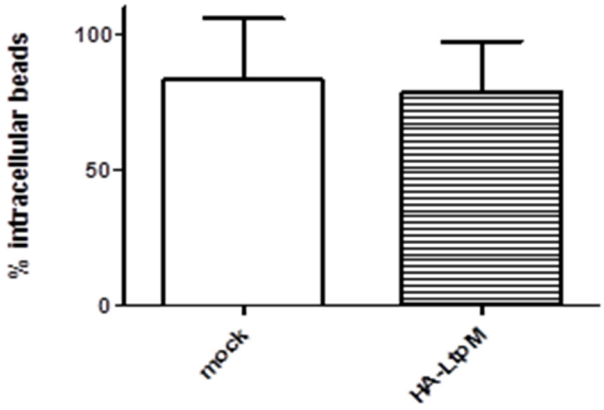


Figure 38 Phagycotosis is not affected in LtpM-transfected cells
Cos-7 cells were co-transfected with plasmids encoding FcγRIIa and Myc-tagged LtpM or empty vector. 18 hours after transfection, cells were challenged with IgG-opsonised agarose beads for 90min. Cells were stained with anti-myc antibody (blue) and RBC were differentially stained to reveal external (cyan) and total (red) beads (**A**). For both and internal and external beads were analysed to determine the percentage of phagocytosis (**B**). Quantification of two independent experiments and representative images are shown. Results are the mean ± SD.

3.11 Mutation of a potential PI-phosphatase motif in LtpM does not affect localisation of PI3P and PI4P markers

LtpM harbours a motif similar to the so called CX5R motif, which is found in different PI-phosphatases. These phosphatases are characterised via two motifs, the first characteristic motif is FN(V/F)G(V/I)NE and the second, more important one is CKS(G/A)KDRT. Both motifs can be found in the sequence of LtpM, although they are both not completely conserved. The first is **FN**DA**GV**T and the second is **CKSA**FENA, conserved residues are bold. The LtpM data strongly indicates that LtpM binds and localises to PI3P-containing membranes, however the LCV contains mainly PI4P at later time points of infection. In order to test if LtpM affects PI4P distribution, cells were transfected with the PI4P marker SidC and either with control constructs LtpD or SetA or with LtpM. As a control, LtpD or SetA were co-transfected with SidC. In these cells SidC localises almost exclusively to the Golgi apparatus, which is rich in PI4P, whereas LtpD and SetA show a vesicular staining as previously shown (Harding et al., 2013). SidC does not colocalise with LtpD or SetA. As another control, LtpM was co-transfected with SetA, both proteins bind to PI3P and colocalize in vesicular structures as previously shown. However, in some cells it appeared that when LtpM and SidC are co-transfected, some SidC seems to lose its clear defined localisation at the Golgi apparatus and appears more diffuse cytoplasmic (Figure 39). SidC gets partly redistributed in the cell and co-localises with LtpM to some extent. To further investigate this effect, the CX5R motif of LtpM was mutated and in further transfection experiments characterised.

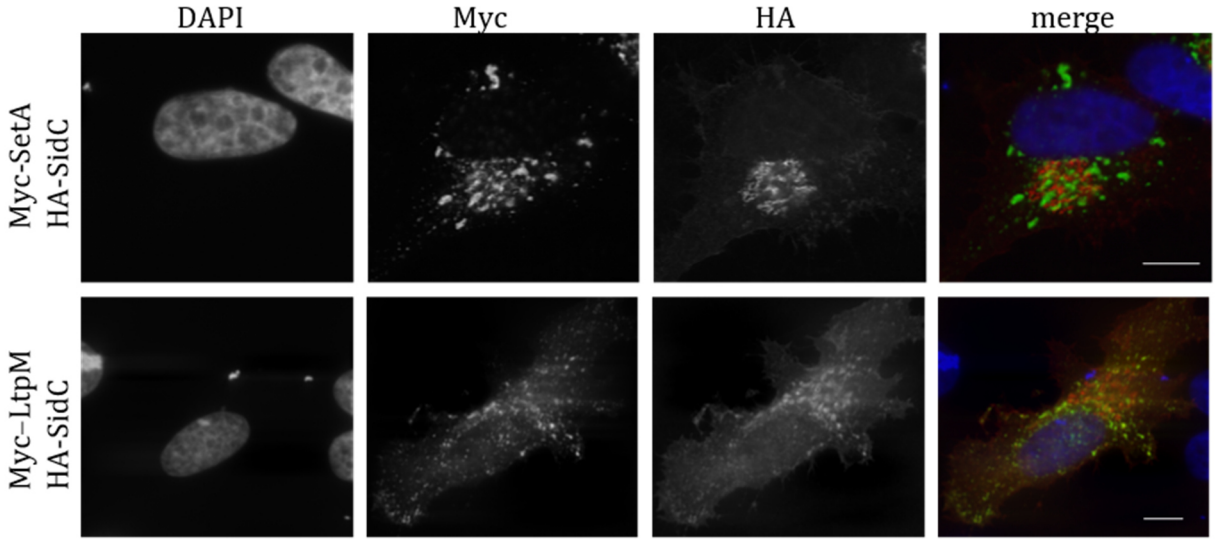


Figure 39 LtpM seems to influence SidC localisation upon co-transfection
HeLa cells were co-transfected with HA-SidC and Myc-SetA or Myc-LtpM and HA-SidC. 24 hours after transfection, cells were fixed and immunostained for the different proteins. Two lower panels represent different cells of the the same transfection. Scale bar 10 µm.

Wildtype LtpM or $LtpM_{C508A}$ were co-transfected with the PI3P marker SetA or the PI4P marker SidC. From the co-transfections of wildtype LtpM or $LtpM_{C508A}$ with SetA it can be seen that both the wild type and mutant LtpM co-localise with PI3P, the mutation of C508A does not change localisation of LtpM upon transfection (Figure 40 A). As shown in Figure 40 B, SidC localises predominantly to the Golgi, whether transfected with wildtype LtpM or $LtpM_{C508A}$. The diffuse localisation of SidC occurred sporadically, but did not correlate to co-transfection with LtpM.

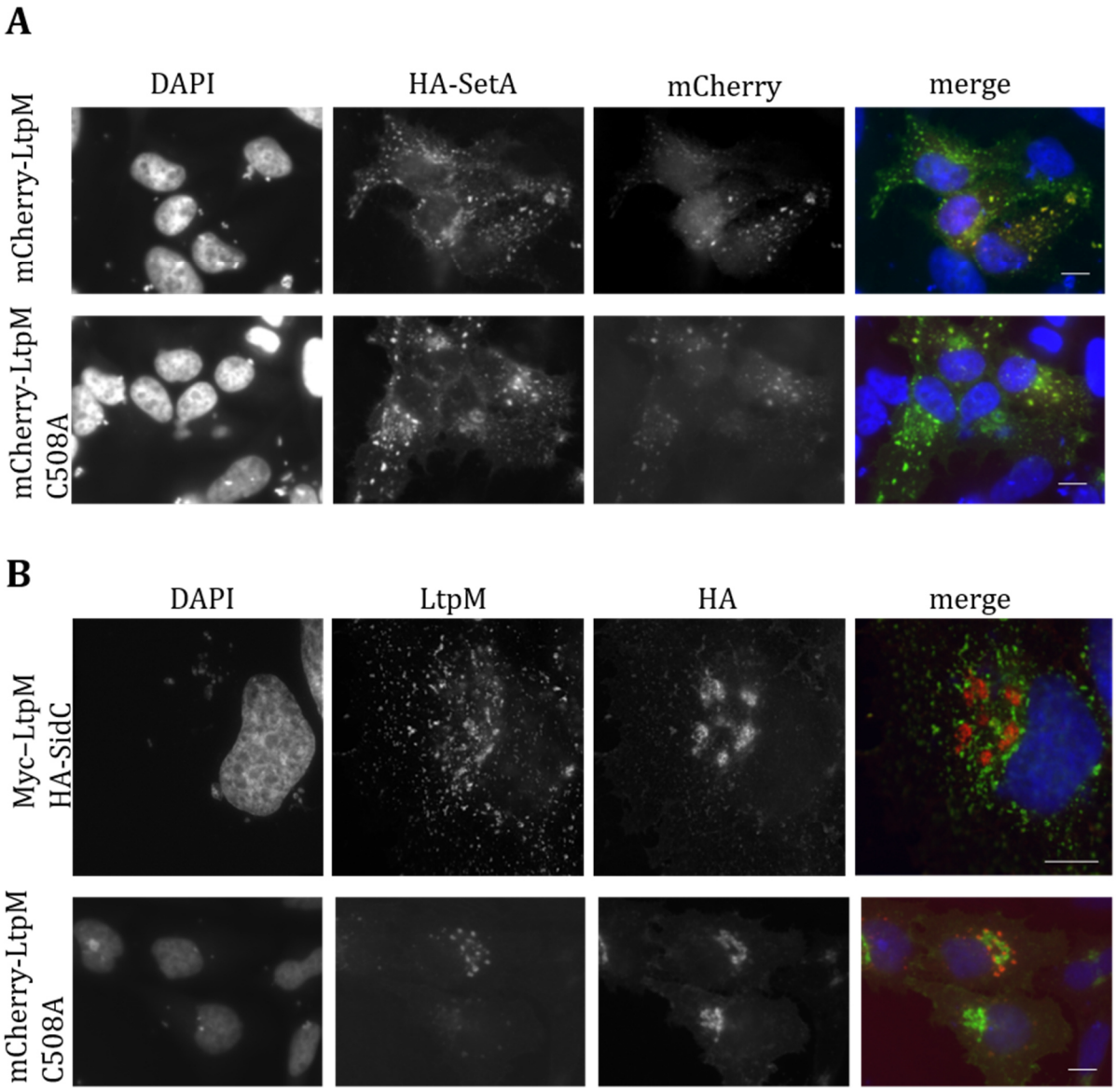


Figure 40 LtpM does not influence SidC localisation upon co-transfection
HeLa cells were co-transfected with SetA and LtpM or LtpM C508A (A) and SidC with LtpM or LtpM C508A (B). 24 hours after transfection, cells were washed, fixed and stained for fluorescence microscopy using specific antibodies. Scale bar 10 µm.

3.12 Localisation of LtpM to the LCV and early endosomes during infection of lung epithelial cells

The localisation of LtpM was investigated by immunofluorescence staining after infection. A549 lung epithelial cells were infected with *L. pneumophila* overexpressing 4HA-LtpM (MOI=40). Cells were fixed and stained after 6 hours and after 24 hours (data not shown) with HA- and *Legionella* LPS-antibodies. LtpM localises to the LCV, but is also located at small vesicular structures distributed throughout the cell (Figure 41).

To determine the nature of the small vesicles to which LtpM localises after 24h of infection, cells were infected with *L. pneumophila* overexpressing 4HA-LtpM and then stained for vesicular endosomal marker proteins. During infection LCV localised LtpM did not co-localise with any marker, but the vesicular portion of LtpM observed after 24 hours of infection co-localized/ partially co-localised with EEA1 (Figure 42). Similar results were obtained after transfection of either the full length or the C-terminus of LtpM. Combined infection and transfection data indicate that LtpM might be involved in the manipulation of early endosomal trafficking. It should be noted that in transfection LtpM partially co-localises with β-Cop, a marker for the secretory pathway between ER and Golgi (data not shown). As LCV biogenesis strongly depends on the interception of the secretory pathway, this might indicate that LCV localised LtpM influences this pathway.

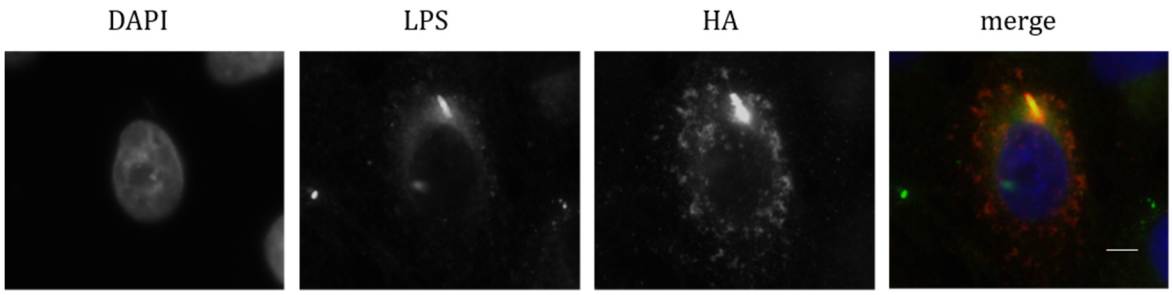


Figure 41 LtpM localises to the LCV and to vesicular structures 6 h post infection
A549 cells were infected with wild-type *L. pneumophila* overexpressing LtpM fused to four HA tags (4xHA). After 6 h, cells were washed, fixed and stained for *Legionella*-LPS and HA using specific antibodies. Representative for three independent experiments. Scale bar 5 µm.

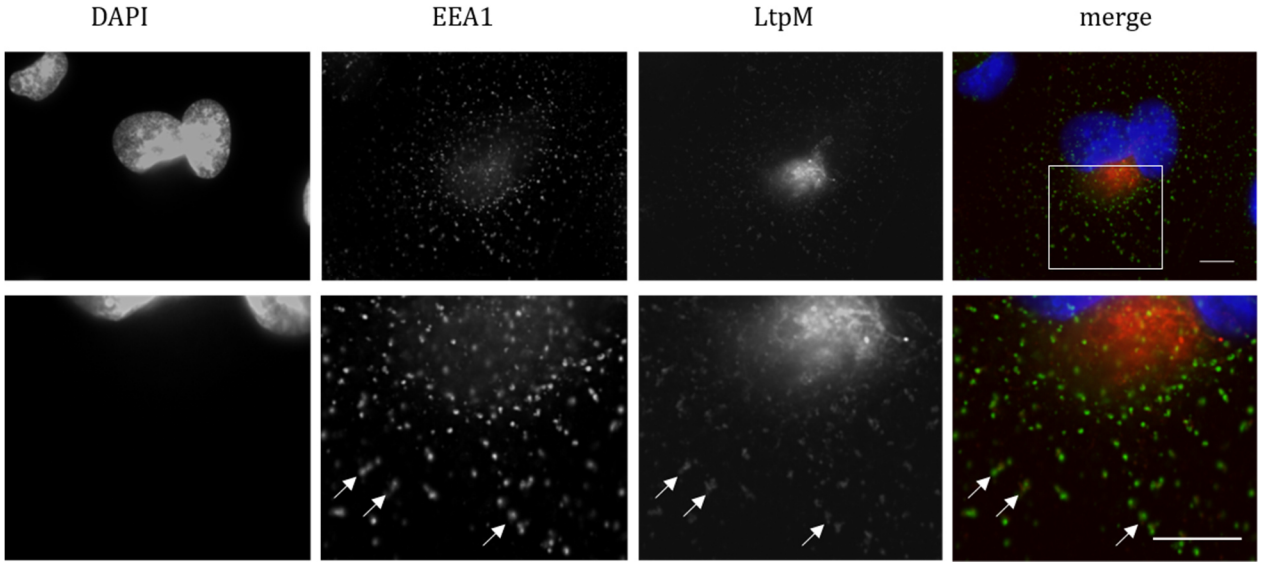


Figure 42 LtpM colocalizes with endosomal markers in infection
A549 cells were infected with *L. pneumophila* overexpressing HA-LtpM. After 24 h cells were fixed and immunostained for HA-LtpM and the early endosomal marker protein EEA1. Scale bar 10 µm.

3.13 LtpM localises to the LCV in different host cells

As LtpM was shown to localise to the LCV in lung epithelial cells, it was further investigated where it localises in other host cells and whether it has the same localisation in amoeba hosts. *A. castellanii* and *D. discoideum* were used in the experiments as amoeba hosts, as well as Raw 264.7 macrophages to test another mammalian cell line. *A. castallanii, D. discoideum* and the Raw 264.7 macrophages were infected with *L. pneumophila* expressing 4HA-LtpM at a MOI of 1. 24 hours after infection, cells were fixed and stained with antibodies against *Legionella* LPS and HA as well as DAPI to visualize the nucleus. For the Raw 264.7 macrophages, the experiment was carried out for 6 hours, after which the cells were fixed and stained as previously described. At these time points, LtpM is found on the LCV in all tested cell lines, which agrees with the previous results with the A549 lung epithelial cells (Figure 43).

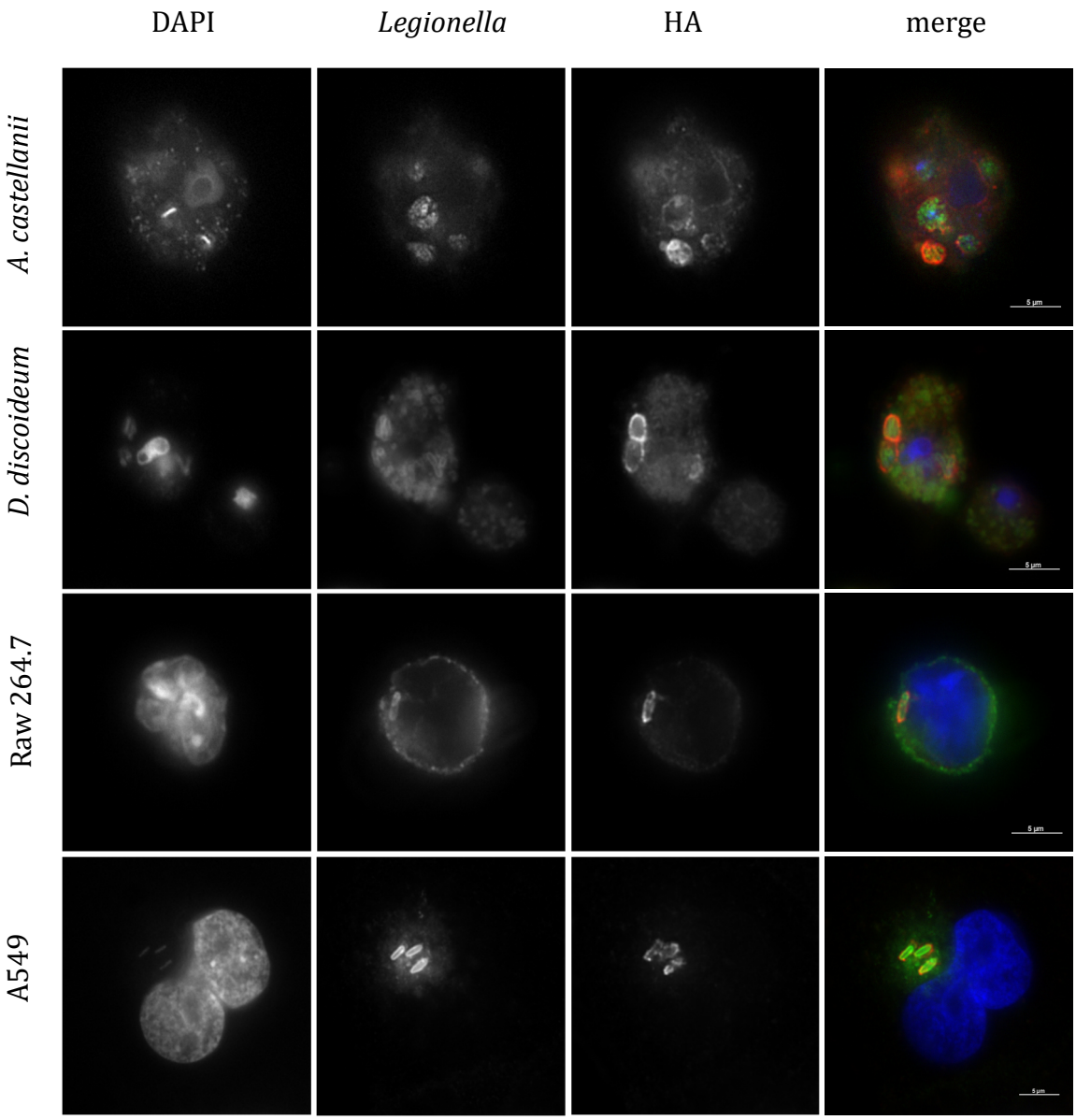


Figure 43 Localisation of LtpM to the LCV in different host cells
Different cells were infected with *L. pneumophila* overexpressing HA-LtpM; *Dictyostelium discoideum, Acanthamoeba castellanii* and Raw 264.7 macrophages MOI:1; A549 lung epithelial cells MOI: 25. In the amoeba *Dictyostelium discoideum* and *Acanthamoeba castellanii*, 4HA-LtpM is detectable on the LCV after 18 hours of infection. In Raw 264.7 macrophages as well as A549 lung epithelial cells, LtpM localises to the LCV after 6 hours of infection (scale bar 5 µm).

3.14 A yeast two hybrid screen identified several proteins as potential interaction partners for LtpM

In order to find potential interaction partners of LtpM, a Y2H screen was performed as for LtpK, using pGBT9-LtpM as bait and a pre-transformed Matchmaker Clonetech HeLa cDNA library as described for LtpK, but instead of plating on QDO, it was plated on the less stringent medium TDO instead. Only eleven colonies were recovered. Putative interaction partners for LtpM identified in this screen are listed in Table 6 . However, further research using a direct Y2H assay could not confirm the potential interaction of LtpM with any of those proteins, the directly transformed yeast cells were not able to grow on the selective SD-agar plates.

Table 6 Potential cellular interaction partners for LtpM identified by Y2H

Name of gene	No of colonies	Description (Source: NCBI PUBMED)
helicase-like transcription factor (HLTF)	1	Member of the SWI/SNF family. Members of this family have helicase and ATPase activities and are thought to regulate transcription of certain genes by altering the chromatin structure around those genes; contains a RING finger DNA binding motif.
poly (ADP-ribose) polymerase 1 (PARP1)	2	Chromatin-associated enzyme, poly(ADP-ribosyl)transferase, which modifies various nuclear proteins by poly(ADP-ribosyl)ation; modification is dependent on DNA and is involved in regulation of various important cellular processes such as differentiation, proliferation, tumor transformation and in the regulation of the molecular events involved in the recovery from DNA damage.
Sjogren syndrome antigen B (autoantigen La) (SSB)	2	involved in diverse aspects of RNA metabolism, including binding and protecting poly(U) termini of nascent RNA polymerase III transcripts from exonuclease digestion, processing 5' and 3' ends of pre-tRNA precursors, acting as an RNA chaperone, and binding viral RNAs associated with hepatitis C virus.
Ras suppressor	1	involved in the Ras signal transduction pathway,

protein 1 (RSU1)		growth inhibition, and nerve-growth factor induced differentiation processes, as determined in mouse and human cell line studies. In mouse, the encoded protein was initially isolated based on its ability to inhibit v-Ras transformation.
microtubule interacting and transport, domain containing 1 (MITD1)	1	Required for efficient abscission at the end of cytokinesis, together with components of the ESCRT-III complex
phosphatidylinositol 4-kinase type 2 beta (PI4K2B)	1	Phosphatidylinositol 4-kinases (PI4Ks) phosphorylate phosphatidylinositol to generate phosphatidylinositol 4-phosphate (PI4P), an immediate precursor of several important signalling and scaffolding molecules. PIP itself may also have direct functional and structural roles. PI4K2B is a primarily cytosolic PI4K that is recruited to membranes, where it stimulates phosphatidylinositol 4,5-bisphosphate synthesis
glypican 5 (GPC5)	1	Cell surface heparan sulfate proteoglycans are composed of a membrane-associated protein core substituted with a variable number of heparan sulfate chains. Members of the glypican-related integral membrane proteoglycan family (GRIPS) contain a core protein anchored to the cytoplasmic membrane via a glycosyl phosphatidylinositol linkage. These proteins may play a role in the control of cell division and growth regulation.
centrosomal protein 135kDa (CEP135)	1	Centrosomal protein, which acts as a scaffolding protein during early centriole biogenesis, and is also required for centriole-centriole cohesion during interphase. Mutations in this gene are associated with autosomal recessive primary microcephaly-8
serine/threonine kinase 38 like	1	Involved in the regulation of structural processes in differentiating and mature neuronal cells

3.15 LtpM could not be expressed in *S. cerevisiae* AH109 for a yeast two hybrid screen

As shown in section 3.14, Yeast-2-hybrid screens were performed with LtpM in order to identify cellular proteins LtpM might interact with. In order to identify interaction partners, different yeast expression constructs of LtpM and its domains were constructed and transformed into yeast. This yielded very few colonies for the full-length LtpM and N-terminal domain, but not C-terminal domain when the high expression level vector pGBKT7 were used. Preliminary experiments were performed to ensure LtpM is expressed and is not toxic for the yeast cells. Growths curves were performed overnight with pGBKT7-LtpM-N-terminus and pGBKT7-LtpM-C-terminus as well as the low expression plasimds pGBT9-LtpM-N-terminus and pGBT9-LtpM-C-terminus and no toxicity was observed (Figure 44 A and B). However, when the expression of the C-terminus, the N-terminus as well as full length LtpM was controlled by Western-blot analysis, only the C-terminus of LtpM was detectable. No expression of the N-terminus of the full-length protein could be detected (Figure 44 C). As yeast cells are difficult to lyse and usually only pores are formed in the cell wall, it is common that large proteins remain inside the cells and therefore cannot be detected via Western-blot. Therefore, at this stage it was decided to continue with the Y2H screen.

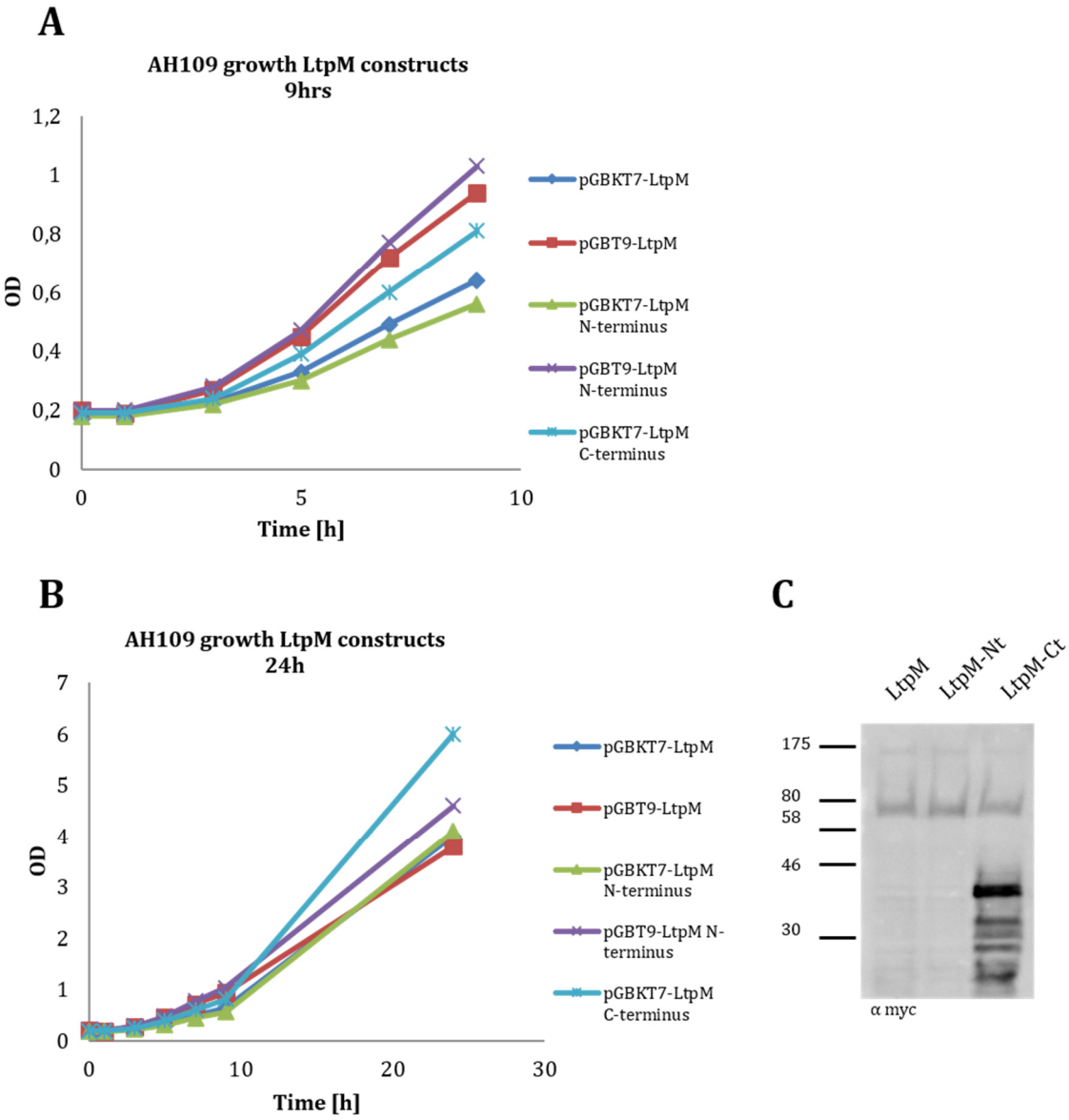


Figure 44 Control experiments for Y2H screen with LtpM
After transformation a yeast AH109 with the yeast expression plasmids harbouring the LtpM constructs, a growth curve was performed and OD600 nm of the cultures measured for the first 9 hours (A) and after 24 hours (B). Yeast was lysed and expression tested of myc-LtpM, myc-LtpM-N-terminus (LtpM-Nt) and myc-LtpM-C-terminus (LtpM-Ct) via Western-Blot (C). Exected sizes: LtpM 72kDa + 18 kDa tag= 90 kDa; LtpM-Nt 52 kDa + 18 kDa tag = 70 kDa; LtpM-Ct 20 kDa + tag 18 kDa = 38 kDa.

3.16 LtpM inhibits growth of *S. cerevisiae* BY4712

Because the preliminary experiments for the Y2H screen indicated that LtpM might be toxic for *S. cerevisiae* AH109, it was decided to investigate this further by completing a specific yeast toxicity assay. In this experiment, LtpM and LtpK, which was used a control, were cloned into the yeast expression vector pYES under control of a glucose-repressed/ galactose inducible promoter and transformed into the strain BY4712. Transformed cells were grown on selective SD agar plates to control for the presence of the plasmid before culturing them in parallel in either galactose or glucose containing SD. Growth was measured over 48 hours at 30°C in the plate reader by measuring OD600 nm. In the medium containing glucose, all transformed strains grew at the same rate. However, when cultured in galactose containing medium which induces the expression of the proteins, the strain transformed with pYES-LtpM grew significantly less well compared to the strains harbouring pYES-LtpK or pYES empty (Figure 45).

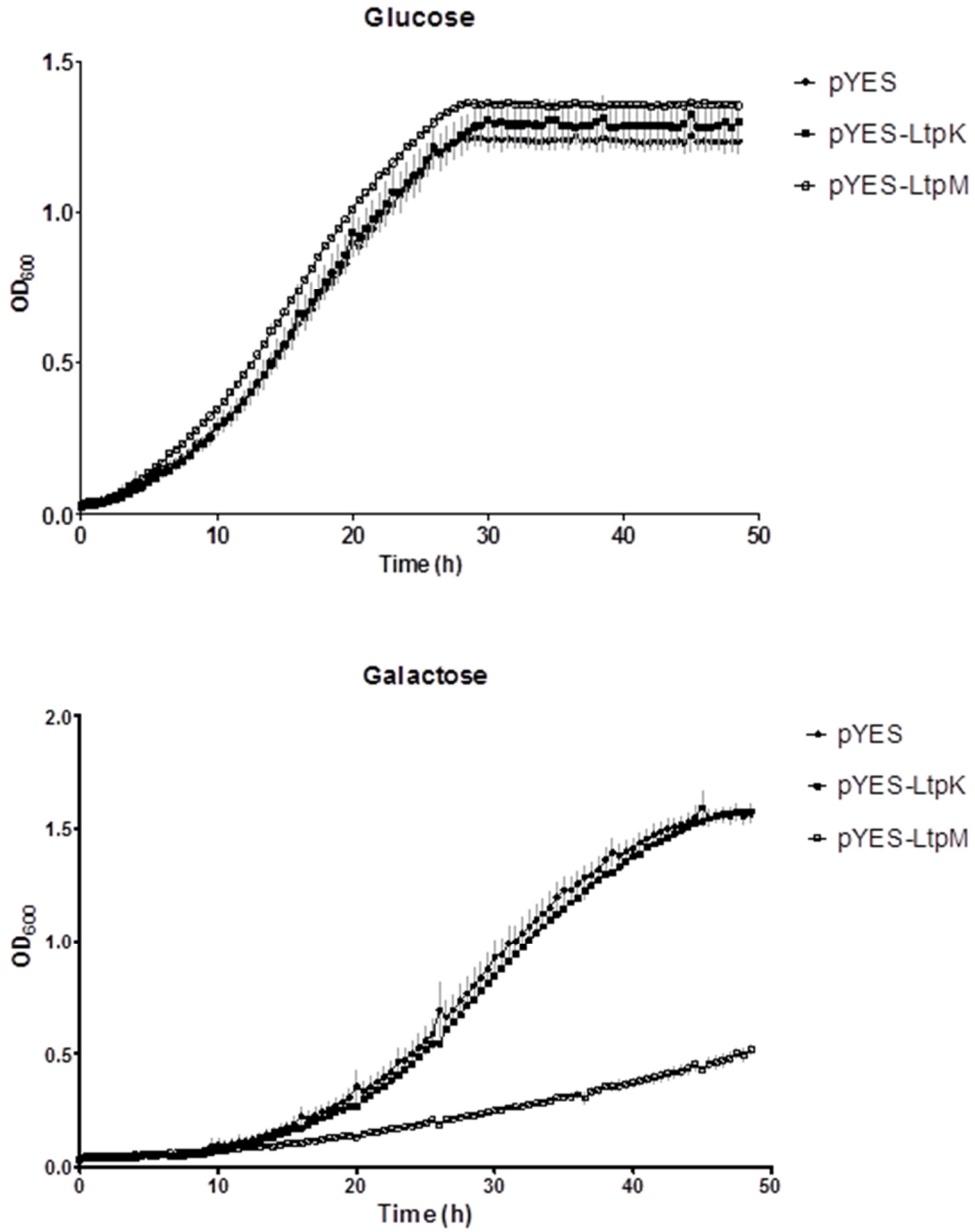


Figure 45 Yeast growth curve of pYES-LtpK and pYES-LtpM
Growth of yeast BY4712 expressing LtpM or LtpK from a galactose-inducible promoter on glucose- or galactose-medium. Yeast strains harbouring an empty vector (pYES) were used as control. Growth was measured in a plate reader measuring OD600 over 48 hours.

3.17 LtpM has a glycosyltransferase domain

The observed toxicity of LtpM towards yeast promted us to reassess the bioinformatics prediction data for any, even weak, similarities to enzymes and known toxins. Phyre2 structural homology prediction showed a similiarity with very low confidence of the first 145 residues of LtpM to the glycosyltransferase domain of PaTox, an insecticidal toxin from *Photorhabdus asymbiotica* (The Phyre2 web portal for protein modeling, prediction and analysis (Kelley et al., 2015).

It showed 75% coverage (with 68.3% confidence and 21% identity) in the secondary structure in the first 145 AA of the protein (Figure 46). The activity of PaTox as other classical glycosyltransferases was shown to depend on a DxD motif, which LtpM also harbours in similar position as a DxN motif.

Although the secondary structures of LtpM and the known glycosyltransferase PaTox show 75% coverage, alignment of the primary amino acid sequence does not show this similarity. Sequence alignment of LtpM with known glycosyltransferases like PaTox from *P. asymbiotica*, Toxin A and B from *C. difficile,* SetA and Lgt1, 2 and 3 from *L. pneumophila* shows that although harbouring some conserved residues like the D of the DxD motif, the primary amino acid sequence of LtpM differs from the other known glycosyltransferases (Figure 47).

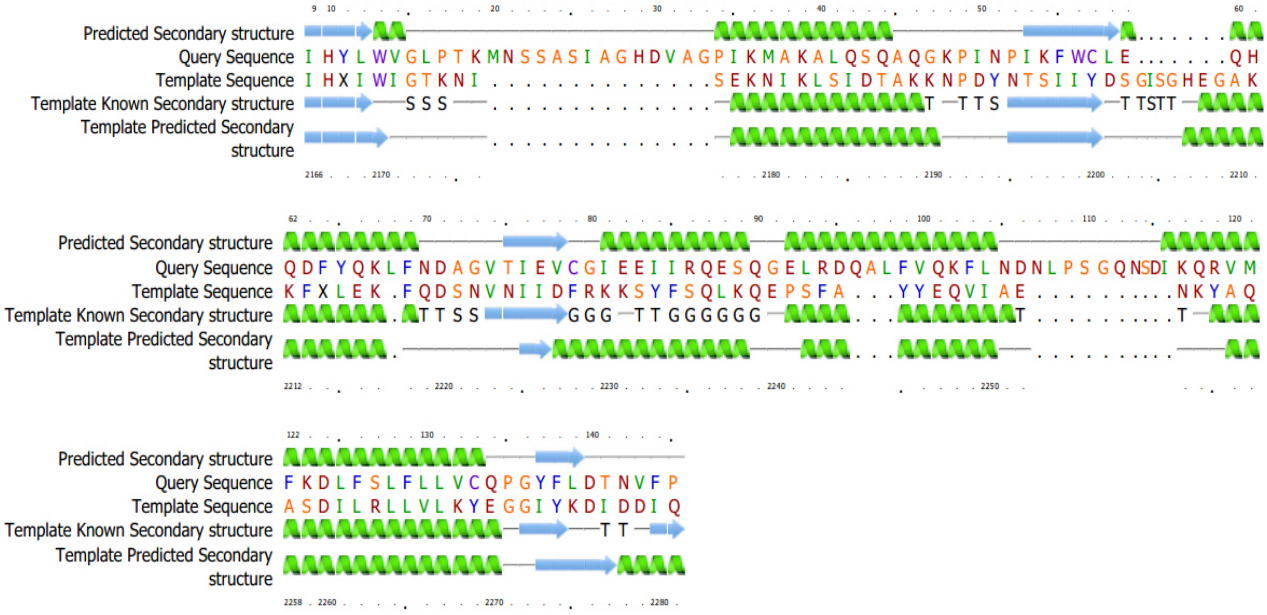


Figure 46 Predicted secondary sequence alignment of LtpM and glycosyltransferase domain of PaTox

Secondary structure prediction of LtpM (query sequence) using the Phyre2 server and alignment with the secondary structure of the glycosyltransferase PaTox resulted in 75% coverage with with 68.3% confidence and 21% identity in the first 145 amino acids (AA) of LtpM.



Figure 47 Amino acid sequence alignment of LtpM with known glycosyltransferases

The predicted glycosyltransferase domain of LtpM was aligned with the known glycosyltransferases PaTox (*P. asymbiotica*), Toxin A and B (*C. difficile*) and the *L. pneumophila* glycosyltransferases SetA, Lgt1, 2 and 3.

3.18 Toxicity of LtpM in yeast depends on its glycosyltransferase motif

LtpM was found to be toxic in yeast and has a glycosyltransferase domain. As the effector SetA was previously characterised with very similar features, it was investigated whether the toxicity in yeast depends on the activity of the glycosyltransferase motif DxN. Therefore, the DxN motif was mutated to NxN (LtpM D140N), which was previously shown to inactivate the glycosyltransferase activity of PaTox and other glycosyltransferases like SetA (Jank *et al*., 2013). LtpM D140N was cloned into the yeast expression vector pYES and tested in the yeast toxicity assay. Moreover, different truncated versions of LtpM were cloned into the pYES vector to further identify the minimal domain in LtpM which is necessary for the toxicity in yeast (Figure 48).

This revealed that the full-length wildtype version of LtpM is toxic for yeast, whereas LtpM D140N mutant does not inhibit the growth of yeast. The truncated versions of the protein do not inhibit growth neither. Only LtpM 1-271 shows an intermediate phenotype, but does not inhibit growth of yeast as strongly as the wildtype. This is shown in growth curves using the plate reader (Figure 49 A) as well as spotted on plates (Figure 48 B).

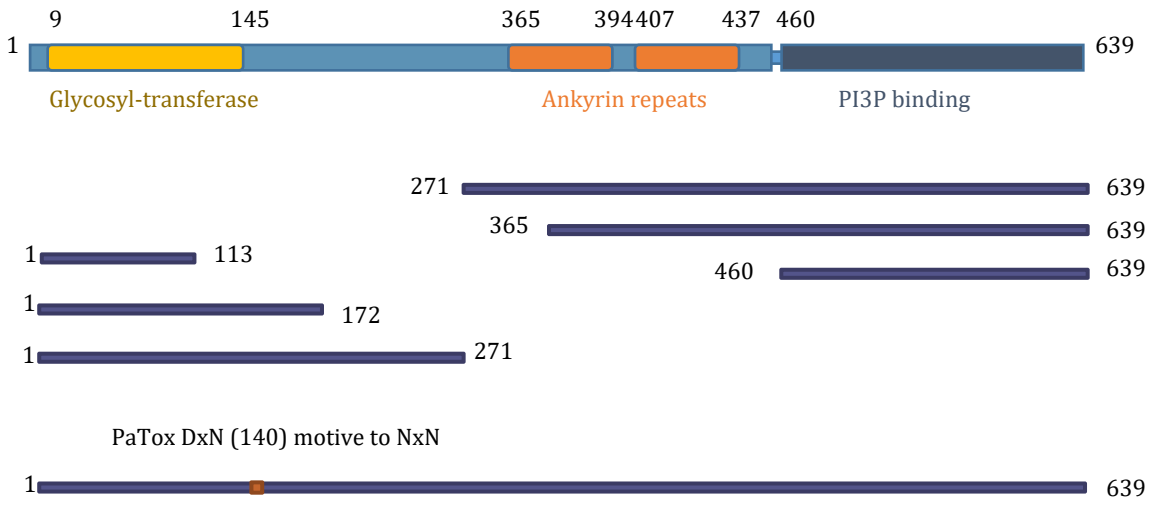
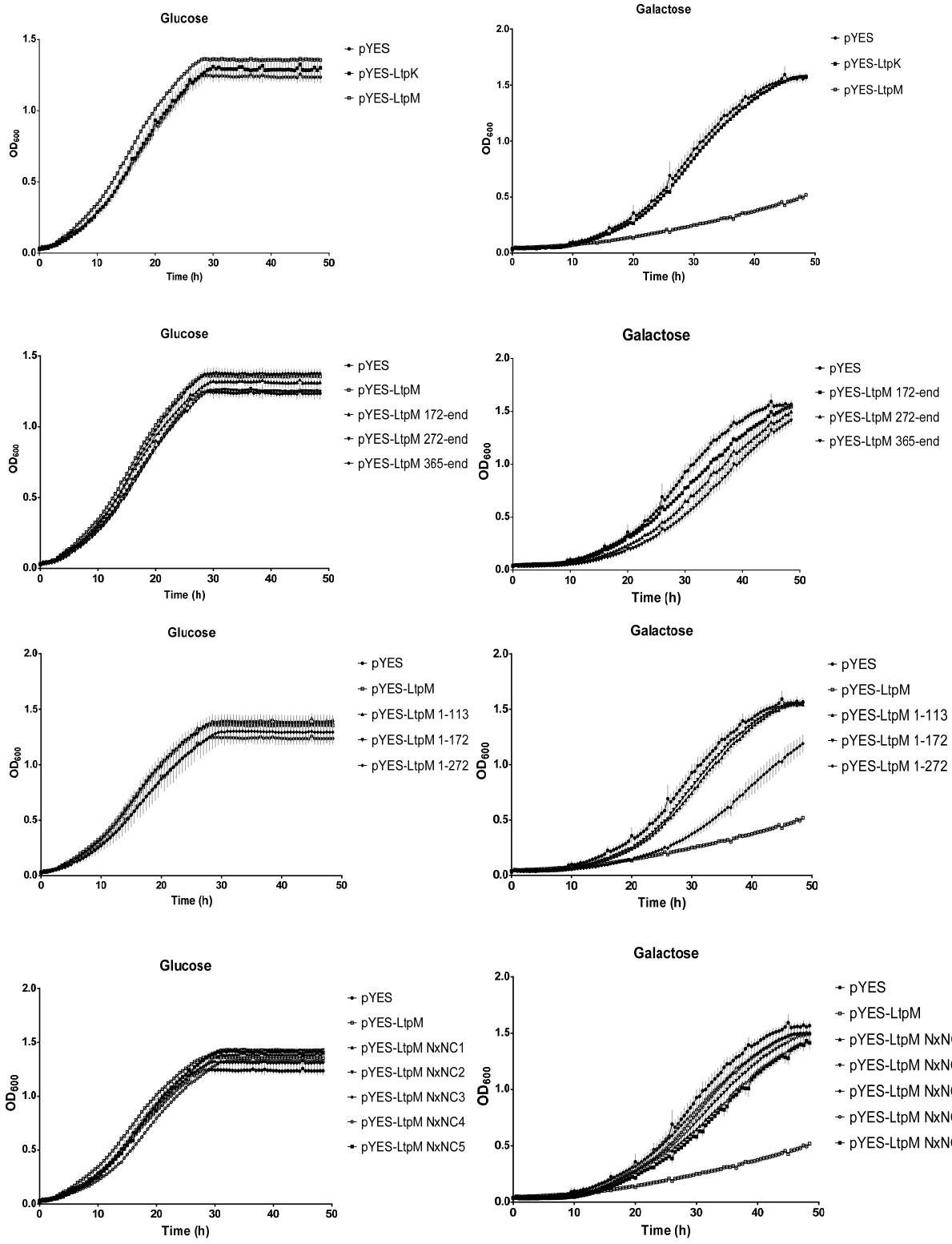


Figure 48 Truncated versions of LtpM used in the yeast toxicity assay
To characterise the minimal domain responsible for the yeast toxicity of LtpM, different truncated versions of LtpM as well a LtpMD140N point mutant were cloned in the yeast expression vector pYES.

A


Glucose
Galactose
pYES
pYES-LtpK
pYES-LtpM
pYES-LtpM 172-end
pYES-LtpM 272-end
pYES-LtpM 365-end
pYES-LtpM 1-113
pYES-LtpM 1-172
pYES-LtpM 1-272
pYES-LtpM NxNC1
pYES-LtpM NxNC2
pYES-LtpM NxNC3
pYES-LtpM NxNC4
pYES-LtpM NxNC5
OD600
Time (h)

B

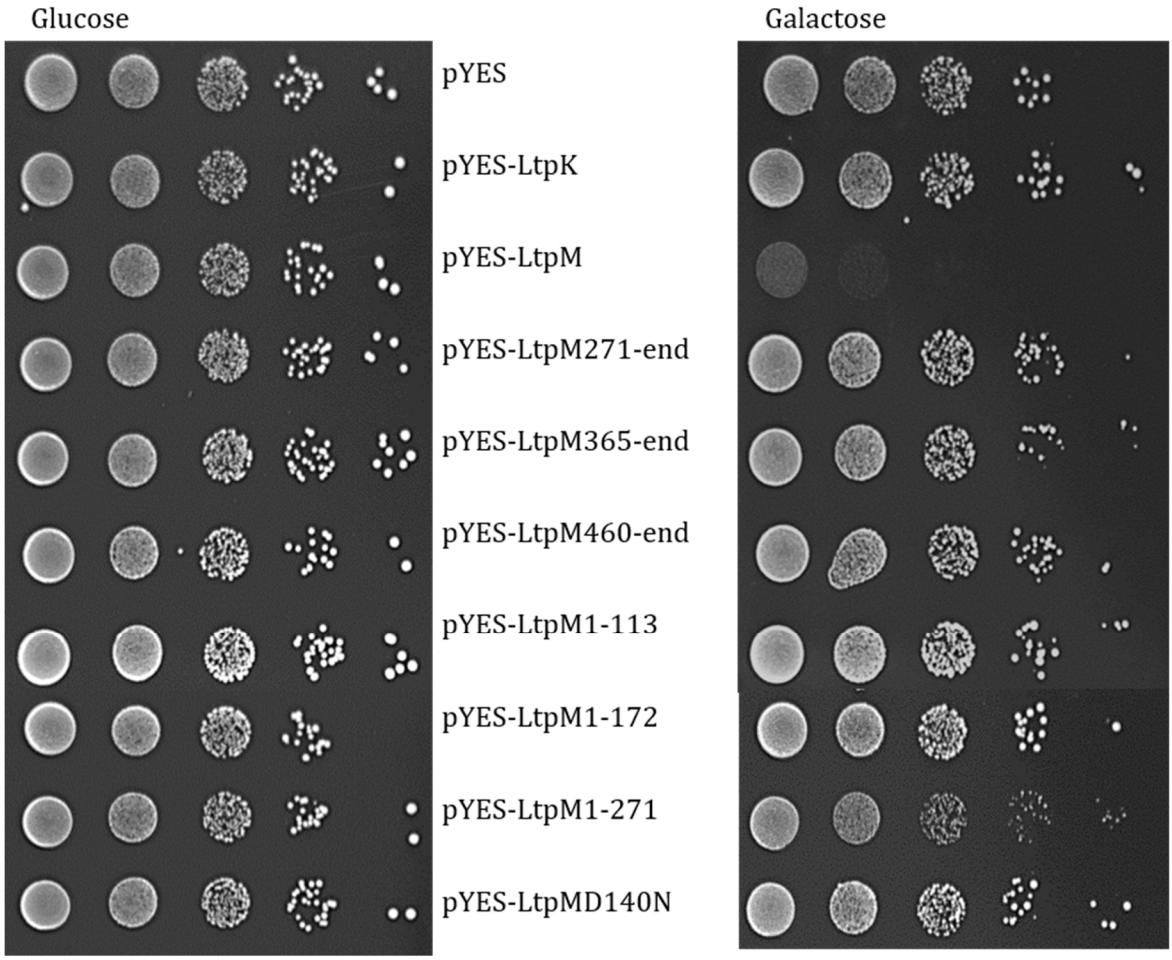


Figure 49 Toxicity of LtpM in yeast depends on the DxN glycosyltransferase motif
Truncated versions of LtpM and LtpM D140N were transformed in yeast under the Galactose inducible promoter in the expression vector pYES. Yeast harbouring the empty vector or the effector LtpK were used as controls. Growth was measured with glucose or galactose by (**A**) a plate reader measuring OD600 nm over 48 hours or by (**B**) plating 5-fold serial dilutions and incubation for 4 days at 30°C.

3.19 LtpM uses UPD-glucose as donor substrate and glucosylates itself

The glycosyltransferase activity was kindly investigated by our collaborators Prof. Aktories and Dr. Jank at the University of Freiburg.

UDP-sugars are mainly used by glycosyltransferases as activated sugar donor substrates in glycosylation reactions. Moreover, glycohydrolase activity is used by many glycosyltransferases to cleave donor sugar substrates even in the absence of their acceptor substrate, which was also found for glucosyltransferases SetA (Jank et al., 2013). To identify the donor substrate of LtpM, various radiolabelled UDP-[14C]sugars were used and the hydrolase activity of LtpM analysed using thin layer chromatography. Results show that LtpM, like SetA, uses UDP-glucose (UDP-Glc) as donor substrate (Figure 49 A). A point mutation of the DxN motif to AxN abolished the hydrolase activity of LtpM, but the addition of 10 mM EDTA did not (Figure 49 B). Moreover, an auto-glucosylation of LtpM was observed when UDP-[14C]glucose was used as a donor substrate (Figure 49 C). This auto-glucosylation was abolished in the point mutant LtpM AxN, but was not inhibited by the addition of EDTA (Figure 49 D).

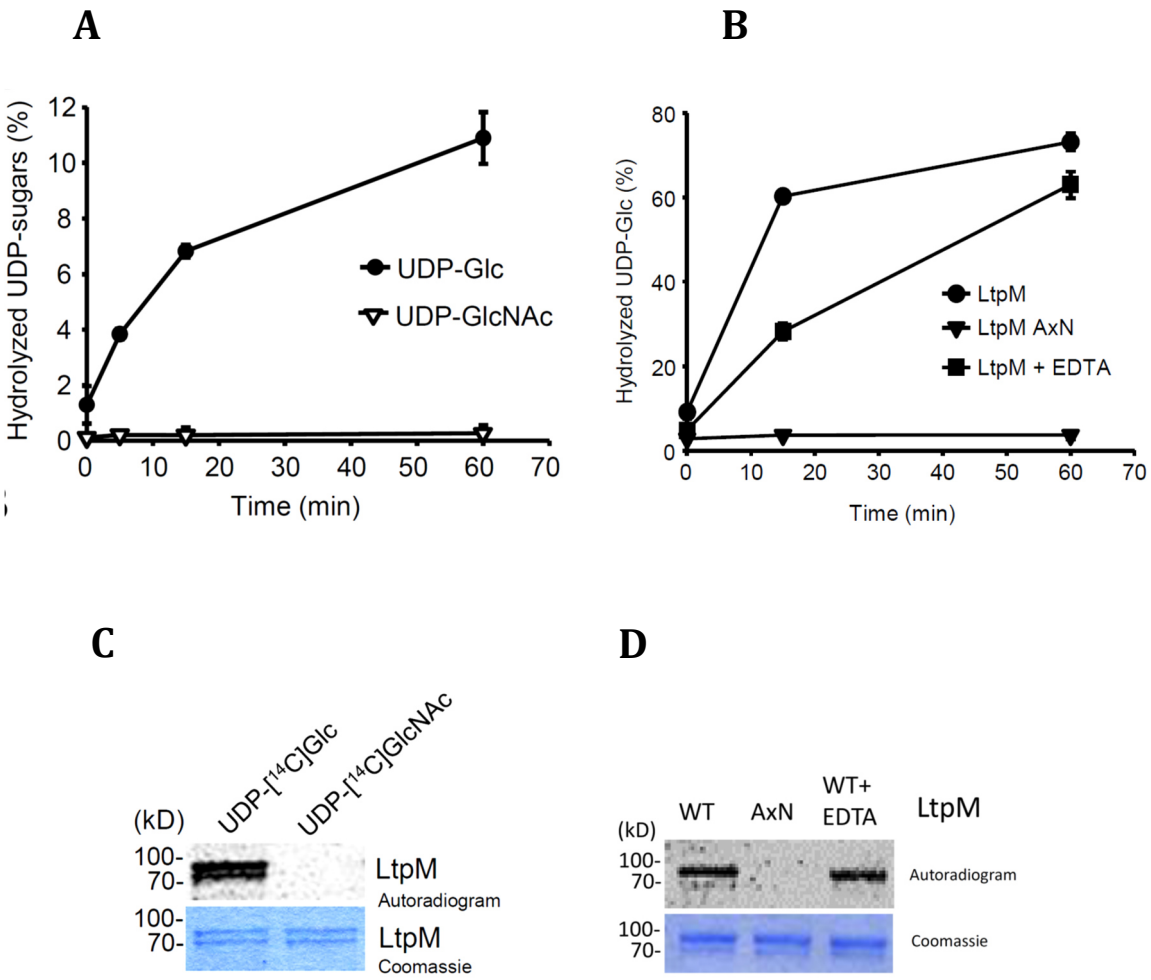


Figure 50 LtpM hydrolyses UDP-glucose and demonstrates autoglucosylating activity

A time course of UDP-sugar hydrolase activity using LtpM (1µM) in a buffer containing 50 mM Hepes, 1mM MnCl2, 2mMMgCl2 was performed at 30°C. The reaction components were separated by PEI-thin layer chromatography. Products were detected by autoradiography and reveal that LtpM uses UDP-glucose (UDP-Glc) but not UDP-N-acetylglucosamine (**A**). Enzymatic hydrolysis of UDP-[14C]glucose (10 mM) was tested with LtpM, LtpM AxN point mutant and LtpM + EDTA (each 1µM) at 30°C for 60 min. Products were separated at indicated timepoints by thin layer chromatography and analysed by autoradiography. The addition of EDTA does not inhibit the hydrolysis of UDP-glucose by LtpM (**B**). Auto-glucosylation of LtpM is shown with UDP-[14C]Glc but not UDP-[14C]GlcNAc. Radiolabelled proteins were separated by SDS-PAGE and visualized using autoradiography or Coomassie as control (**C**). Only wildtype LtpM, but not the point mutant LtpM AxN auto-glucosylates itself. EDTA does not inhibit auto-glucosylation (**D**).

3.20 Discussion

3.20.1 Characterisation of the binding of LtpM to PI3P

The *L. pneumophila* effector LtpM localises to vesicular structures during transfection of HeLa cells and to the LCV and vesicular structures during infection of A549 cells. To further investigate the cellular localisation of LtpM, it was co-transfected with the PI3P-binding domains FYVE and SetA as well as co-stained in infection with the early endosome marker EEA1. It was shown that it co-localises with FYVE (Fab-1, YGL023, Vps27, and EEA1) in transfection experiments as well as with EEA1 during infection. As EEA1 contains a FYVE domain (Stenmark and Aasland, 1999), it has been well characterized as a PI3P binding protein itself (Simonsen et al., 1998, Stenmark et al., 1996, Christoforidis et al., 1999). As PI3P is predominately present on early endosomes, lysosomes and autophagosomes in a cell, these results indicate that LtpM might bind to intracellular compartments via PI3P. In previous studies it was shown that LtpD contains a PI3P-binding domain and localises to the LCV (Harding et al., 2013a) and so do other PI3P binding effectors of *L. pneumophila*, like SetA, LidA and LpnE (Neunuebel et al., 2012, Weber et al., 2009a, Jank et al., 2012). This gives an indication that PI3P is present on the LCV and an important determinant for membrane targeting *Legionella* effectors. In fact, it was shown that PI3P is present on the LCV during early time points of infection, but wild-type LCVs gradually loose this phosphatidyl-inositol within 2 hours (Weber et al., 2013). To further investigate the subcellular localisation of LtpM and characterise the PI3P binding, cells were treated with the phosphoinositide-3-kinase (PI3K)-inhibitor Wortmannin. By inhibiting PI3K, PI3P should be depleted in Wortmannin treated cells. The localisation of LtpM, the C-terminal domain of LtpM and the PI3P-binding domain of SetA was monitored. Interestingly, after treatment with Wortmannin, LtpM still localises to the same vesicular structures as in the control cells, and these vesicles were also positive for the PI3P-binding domain of SetA, indicating the PI3P was not depleted in those cells. The same effect was recently found for the effector CvpB of

Coxiella burnetii (Martinez et al., 2016). In this study, it is shown that the effector CvpB localises to PI3P-rich compartments, also after treatment with an PI3K-inhibitor. An increase of PI3P levels in infected cells was observed, which is due to the presence of CvpB, to support the biogenesis of the replicative compartment. It was shown that CvpB does not possess PI3-kinase activity itself, but uses its C-terminal domain to interfere with the recruitment of the PI5-kinase PIKfyve at PI3P-positive membranes. CvpB thereby perturbs the function of the PI5-kinase, either by an unidentified catalytic activity or steric, and preserves PI3P at the *Coxiella* containing vacuole (CCV). A similar mechanism might be possible for LtpM.

As PI3P is present on early endosomes and also on the LCV, LtpM might bind to these compartments due to a PI3P binding domain. As it is a minimal domain in the C-terminus of LtpD which enables binding to PI3P (LtpD472-626) and the C-termini are 68% homologous, it is likely that the C-terminus of LtpM contains a PI3P binding domain as well. Therefore, a PIP array was performed with the C-terminal domain of LtpM, which showed a strong binding to PI3P. However, as the C-terminus as well as the positive and negative control showed substantial background binding on the PIP-arrays. The array was repeated in the meantime and showed a clear binding of LtpM to PI3P.

Many intracellular bacterial species target PI metabolism to ensure their survival and replication (Hilbi, 2006, Weber et al., 2009b, Pizarro-Cerda and Cossart, 2004) and in fact as discussed above viruses also depend on PI metabolism during replication and therefore recruit PI-metabolising enzymes.

3.20.2 Characterisation of a putative phosphatase motif in LtpM

After co-transfection of LtpM with the PI4P marker SidC, in some cells it could be observed that SidC loses its defined localisation at the Golgi. This observation as well as a phosphatase motif in the sequence of LtpM led to the hypothesis that LtpM might modulate the PI-metabolism in a cell.

A class of phosphatases is characterized by specific motifs in their sequence. The first characteristic motif is FN(V/F)G(V/I)NE and the second, more important one is CKS(G/A)KDRT, mainly referred to as CX5R, the cysteine is thought to be the catalytic residue (Norris et al., 1997). The effector SidF was previously identified via this CX5R motif (Hsu et al., 2012). In the sequence of LtpM both motives can be found, although they are both not completely conserved. The first is **FN**DA**GV**T and the second is **CKSA**FENA, conserved residues are bold.

A recent study suggests a role for an inositol phosphate phosphatase in the virulence of *Salmonella* infections. SopB, a protein secreted by *Salmonella dublin*, was shown to have sequence similarity to the 4-phosphatase active site motif CX5R. The *L. pneumophila* effector SidF was identified due to the CX5R motif and is a PI 3-phosphatase that hydrolyses *in vitro* phagosomal/endosomal PtdIns(3,4)*P*2.

The CX5R motif is not only indicative of PI phosphatase, but can be found in other phosphatases, e.g. tyrosine phosphatase of *Yersinia* (Zhang et al., 1994). The cysteine within the Cys-Xaa5-Arg motif functions as the nucleophile for catalysis, and cysteine modifying reagents are potent inhibitors of this class of phosphatases (Zhou et al., 1994). SidF and other phosphatases share a structurally conserved core which contains four parallel β strands and one α helix (Hsu et al., 2012). The catalytic CX5R motif is located on a coil between the C-terminus of one of the β strands and the first turn of the helix. Sequence-based structural prediction does not indicate strong homology of LtpM with SidF or other phosphatases or any other protein, which suggests that LtpM might have a new fold. Future structural studies as well as site-directed mutagenesis of conserved residues will be needed to clarify the relevance of the putative motif of LtpM for its function.

To study this hypothesis, a point-mutation was introduced in the CX5R motif of LtpM, in which the Cysteine-508 of the catalytic centre was replaced with Alanine (Norris et al., 1997). Co-localisations of LtpM or LtpM-C508A with SetA or SidC were investigated by co-

transfection. High levels of co-localisation were seen between LtpM wild type or LtpM-C508A with SetA. In contrast, in experiments with SidC neither LtpM or LtpM-C508A showed any co-localisation. As LtpM was shown previously to bind to PI3P-rich compartments, this result was expected, but shows that the point mutation at position 508 does not alter the subcellular localisation of LtpM. The result showed that the LtpM does not seem to have an effect on SidC localisation, SidC showed a similar cellular distribution when co-transfected with either LtpM or LtpM-C508A and the diffuse distribution of SidC occurred only sporadically.

To determine whether LtpM phosphatase activity a malachite green assay or Pi ColorLock gold kit could be used with LtpM and the LtpM-C508A point mutant (Hsu et al., 2012). In case free phosphates are released it can be measured and quantified by spectrophotometry, as the solution turns green in case of the malachite green assay (Maehama and Dixon, 1999) or changes colour in case of the PiColorLock™ phosphate detection reagent (Innova Biosciences). This would give an indication which PIPs, if any, LtpM could catalyse.

3.20.3 Glycosyltransferase activity of LtpM

The glycosyltransferase domain in the first 145 AA of LtpM was first identified by a bioinformatics domain analysis using the PHYRE2 server. Although only showing 21% identity in the sequence, LtpM was further analysed for glycosyltransferase activity. Our and the data of our collaborators Prof Aktories and Dr Jank at the University of Freiburg, Germany, clearly show that LtpM seems to be a glycosyltransferase.

Several glycosyltransferases were previously found in the genome of *L. pneumophila* by Prof Aktories and colleagues (Belyi and Aktories, 2010, Belyi et al., 2013, Belyi et al., 2011, Belyi et al., 2006, Belyi et al., 2009, Belyi et al., 2008, Lu et al., 2010). *Legionella* glycosyltransferase 1 (Lgt1) was identified in a screen for *Legionella* virulence factors with UDP-glycosyltransferase activity. Lgt1 harbours the DxD motif characteristic for type A

glycosyltransferases and shares the catalytic core residue of the clostridial large glucosylating toxins (Belyi et al., 2003, Belyi et al., 2006). In type A glycosyltransferases, the DXD-motif is crucial for divalent cation binding and is located upstream of a short hydrophobic patch. It was shown that only mutation of the first aspartic acid lead to a reduction in enzyme activity, demonstrating its fundamental importance for the function of type A glycosyltransferases (Hurtado-Guerrero et al., 2010). In LtpM, the DxD-morif is only partially conserved as a DxN motif. This supports the theory that the first aspartic acid is essential for the function and enzymatic activity of glycosyltransferases. However, the non-conserved motif might also indicate that LtpM works via a different catalytic mechanism than other glycosyltransferases.

Moreover, the crystal structure of Lgt1 was resolved and it shows high structural homology to the glycosyltransferase domain of TcdB (Lu et al., 2010). The Lgt-family of glycosyltransferase was further investigated and 13 Lgt1-like genes were found in the genomes of the *L. pneumophila* strains. They are grouped in the three sub-families Lgt1, Lgt2 and Lgt3. All the 13 Lgts are substrates of the Dot/Icm secretion system which leads to the conclusion that they play a role for the pathogenicity of *L. pneumophila* and might target a host protein for glycosylation. The different *L. pneumophila* strains harbour a mixture of combinations of the Lgts, where the Philadelphia-1 strain harbours Lgt1, Lgt2 and Lgt3, other strains only Lgt1 and Lgt3 (Belyi et al., 2011). The eukaryotic elongation factor 1A (eEF1A) was identified as the substrate of Lgt1, which is glycosylated in infected macrophages by Lgt1 (Tzivelekidis et al., 2011) and which seems to be a substrate for all other Lgts, including the Lgt2 and Lgt3 families, as they all glycosylate eEF1A at Serine-53. The elongation factor eEF1A helps to deliver aminoacyl-tRNAs to the A-site of the ribosome and therefore plays an important role in the process of protein synthesis (Marintchev and Wagner, 2004). The glycosylation of eEF1A by the Lgts might inactivate this protein and therefore inhibit eukaryotic protein synthesis, which was investigated in an *in vitro* cell-free translation assay (Belyi et al., 2008). Moreover, the glycosylation of eEF1A, inhibition of

protein synthesis and cell death could also be seen after delivery of Lgt protein into mammalian cells (Belyi et al., 2008, Belyi et al., 2012). It will be interesting to test if LtpM also targets translation or has different host cell targets.

Another glycosyltransferase in *L. pneumophila* is SetA. SetA harbours the GT-characteristic DXD-motif at residues D134SD136. In SetA, the mutation of both aspartic acids to alanines diminishes the toxic phenotype in yeast. SetA also shows an altered trafficking phenotype in transformed yeast and localises to endosomes upon ectopic expression, the hypothesis was raised that SetA might inactivate factor of the endosomal protein sorting machinery via glycosylation (Jank *et al*., 2013).

Only the full-length wildtype LtpM inhibits growth of yeast in the toxicity assay. All of the various truncated versions of LtpM were not able to inhibit the yeast growth to the same extent as that of wildtype LtpM, although they did include the full glycosyltransferase domain. Only $LtpM_{1\text{-}271}$ showed an intermediate phenotype and was able to partially inhibit the growth of yeast. This indicates that the other domains of LtpM are necessary for the full function of the protein.

3.20.4 Similar domain structure of LtpM and SetA

Our domain analysis revealed a PI3P binding domain in the C-terminus and a glycosyltransferase domain in the N-terminus of LtpM. This is a very similar structure as the effector SetA, which however does not have Ankyrin-repeat domains and classical motif.

SetA and LtpM have similar sizes and molecular weights: SetA consists of 644 AA and a weight of 73 kDa, while LtpM consists of 639 AA and a weight of 72 kDa. SetA is present in all sequenced pathogenic *L. pneumophila* strains. However, with more *L. pneumophila* genomes becoming available, LtpM is not found solely in the Paris strain anymore, but in environmental and outbreak isolates.

In a PIP array, the C-terminal domain of SetA was shown to bind to PI3P (Jank *et al.,* 2013). Furthermore, the C-terminal domain was shown to be responsible to guide SetA to vesicular structures in the host cell which were identified as early endosomes. The same was shown for LtpM in this study. Upon ectopic expression, LtpM localises to vesicular structures which were identified as early endosomes by co-staining with the early endosomal marker EEA1. A PIP-array confirmed the hypothesis that the C-terminal domain of LtpM binds to PI3P.

Like LtpM, SetA is toxic for yeast cells. However, SetA is also toxic in mammalian cells upon ectopic expression, which was not observed for LtpM. The toxic effect for mammalian cells was found after microinjection of SetA into embryonic bovine lung (EBL) cells, which were then imaged over 8 hours. However, to investigate the localisation of SetA in mammalian cells, HeLa cells were used and recombinant SetA ectopically expressed. In the first 6 hours, there was no toxic effect on the HeLa cells, which leaves the possibility that LtpM might have a toxic effect in another mammalian cell line, and it might have a toxic effect to a lower extent compared to SetA.

Taken together, although LtpM and SetA hardly share any sequence homology, the two effectors harbour a very similar domain structure, cellular localisation and enzymatic activity.

3.20.5 Future perspectives for the characterisation of LtpM

No cellular target or interaction partner for LtpM could be found during the course of this study. It was attempted to find a target via Y2H screens and also via pBio-pulldown experiments. The Y2H screen was not successful as LtpM turned out to be toxic for yeast cells. In the pBio-pulldown, it was not possible to purify LtpM from infected cells and identify it in the mass-spectrometry analysis. However, as the enzymatic function of LtpM was discovered, this opens possibilities for other methods targeted to this activity. As it was found that LtpM has a glycosyltransferase domain with mono-O-glycosyltransferase activity,

the glycosylation status of LtpM transfected cells compared to mock transfected cells could be investigated. With commercially available kits it might be possible to pull glycosylated proteins out of cells and enriching proteins with that PTM. By comparing mock- with a LtpM-transfected cells and further mass-spectrometry analysis, it might be possible to see a difference and identify a protein which is only glycosylated in the LtpM-transfected cells (Hennrich and Gavin, 2015, Patrie et al., 2013), followed by glycopeptide analysis to identify the glycosylation sited of the protein (Desaire, 2013).

Finding a cellular target for LtpM by Y2H was not successful because of the toxicity of LtpM for yeast cells. As the domain responsible for this toxicity was identified and the LtpM D140N point mutant is no longer toxic, the Y2H screen can now be repeated with the catalytically inactive mutant. Moreover, as the C-terminal domain of LtpM is likely solely important for the localisation to PI3P-rich membranes, the N-terminus of LtpM 1-460 with the point mutation can be used for the Y2H screen.

In order to further characterise the toxicity of LtpM in yeast cells, alternative truncated versions of the protein as well as other point mutants can be tested. Only the full length wildtype protein showed full toxicity in the assay. A truncated version of LtpM harbouring the glycosyltransferase domain LtpM 1-271 showed an intermediate phenotype, but was not sufficient to inhibit growth of yeast as strongly as the wildtype full-length LtpM. At the same time, a single point mutation inactivates the enzymatic activity and the toxic effect. To further characterise this, longer truncated versions of LtpM could be tested to investigate exactly which domains are necessary for a toxicity comparable to the wildtype. One interesting question to answer is the role of the Ankyrin-repeats for toxicity. This could be investigated by testing the whole N-terminal domain LtpM 1-460 harbouring the glycosyltransferase domain as well as the Ankyrin-repeats. Additionally, the deletion of the Ankyrin-repeats from the wildtype LtpM would give insight into the role for the Ankyrin-repeats and whether they play a role for the toxic effect in yeast. Also, other directed point

mutations could be introduced in the sequence of LtpM to gain more insight in the important residues. Structural characterisation of LtpM should give insight in the catalytic mechanism, eg how substrate activation and transfer works without a classical DxD motif.

Chapter 4
General Discussion

4. General Discussion, Conclusions and Perspectives

This study focuses on the characterization of the *L. pneumophila* effector proteins LtpK and LtpM, which share domains with the effector LtpD.

A translocation assay was performed to determine if the homologues LtpD, LtpK, LtpM and Lpp0303, are translocated by *L. pneumophila* in a T4SS dependent manner. Also, the subcellular localisation of the effectors in infection was investigated by immuno-fluorescence microscopy. Whereas none of these assays could validate Lpp0303 as effector, LtpK and LtpM could be detected robustly in host cells. Lpp0303 was proposed to be an effector candidate, based on the homology of its N-terminal domain with the effector LtpD. However, as the T4SS translocation signal is encoded in the C-terminus, one could speculate that Lpp0303 and LtpD might be derived from a common non-effector ancestor and only in case of LtpD evolution or replacement of the C-terminal extension has generated an effector. In conclusion, the protein Lpp0303 does not seem to be translocated and further research was therefore solely focussed on LtpK and LtpM.

L. pneumophila harbours a very large effector arsenal, in fact with more than 300 it has the highest number of effectors found so far in any pathogenic bacteria, which were identified by translocation assays using fusion proteins as well as by bioinformatics (Burstein et al., 2016, Burstein et al., 2009, Zhu et al., 2011, Segal, 2013). In the well characterised Philadelphia strain, this is approximately 10% of the entire proteome (Chien et al., 2004, Ensminger, 2016). It is a question of interest how *L. pneumophila* developed such a large arsenal of effectors and why it requires so many, and further how it is able to maintain them. An essential fact when studying the effectors of *L. pneumophila* and their role in pathogenicity is that they are almost always dispensable for growth in macrophages. At the same time, translocation of the effectors is essential, as a translocation mutant is not able to survive and

replicate in macrophages (Segal et al., 1998, Vogel et al., 1998, Berger and Isberg, 1993). A study showed that even the deletion of 31% of the effectors did not have a drastic impact on replication of *L. pneumophila* in mouse macrophages (O'Connor et al., 2011). In fact, only two effectors where shown to be essential for pathogenesis so far. One of which is the iron transporter IroT/MavN (Isaac et al., 2015, Portier et al., 2015) and the other one is SdhA (Laguna et al., 2006), which is required for maintaining the integrity of the LCV during infection and intracellular replication (Creasey and Isberg, 2012, Harding et al., 2013b, Ge et al., 2012). It is a common model that this is due to the functional redundancy of the effectors, several of which would have similar roles during infection so that deletion does not cause a drawback for survival of the bacterium.

Another model that goes hand in hand with functional redundancy is that *L. pneumophila* maintains this big arsenal of effectors because of the constant circulation through many different host cells in the environment. The specific selective pressure of each host has made *L. pneumophila* a generalist, which can survive and replicate in a range of different cells with no host-specific specialisation (O'Connor et al., 2011, O'Connor et al., 2012, Ensminger, 2016, Ensminger et al., 2012, Faulkner et al., 2008). Therefore, the deletion of an individual effector would not cause a defect in most of the hosts and even the deletion of a bigger cluster of effector causes only host-specific effects (O'Connor et al., 2011).

This complicates the study of effector function using genetics in *L. pneumophila*. It was also the case for the deletion mutants for LtpK and LtpM in this study which did not show growth defects in the tested host cells, which were THP1 macrophages, *D. discoideum* as well as *A. castellanii*. With that phenotype, LtpK and LtpM join most other investigated *L. pneumophila* effectors, even those for which an enzymatic function was identified.

To add another level of complication in studying the effectors of *L. pneumophila*, it was found that some of the effectors do not modulate host proteins, but instead regulate other effectors. The fact that in *L. pneumophila* effectors exist which regulate other effectors is another

explanation for the high number of effectors. An effector of an effector was termed a metaeffector by Kubori, Nagai and their colleagues (Kubori et al., 2010). One of those metaeffectors was identified by the same group, the E3 ubiquitin ligase LubX which ubiqitinates the effector SidH and therefore targets it for degradation (Kubori et al., 2008, Kubori et al., 2010, Quaile et al., 2015). Another example of a metaeffector in *L. pneumophila* is SidJ, which seems to regulate the appearance of the SidE-family of effectors on the vacuole (Havey and Roy, 2015, Jeong et al., 2015).

As no cellular protein could be positively identified as a target for LtpK and neither for LtpM in the methods used, there is the possibility that they act on another effector.

Not regulating the other effector directly, but regulating its effect in the cell is the function of yet another class of effectors. Pairs of effectors have been identified in which one removes or inactivates the action of the other on the cellular target. Therefore, they are not classified as metaeffectors, but act on a similar level of regulation. One of those pairs is Lem3 and AnkX. Lem3 removes a phosphocholine group on Rab1, which was added to Rab1 before by AnkX (Mukherjee et al., 2011, Tan et al., 2011). Another example is the effector pair DrrA/SidM and SidD. Rab1 is ampylated by DrrA/SidM, whereas SidD de-ampylates Rab1 later in infection (Brombacher et al., 2009, Neunuebel et al., 2011, Tan and Luo, 2011, Hardiman and Roy, 2014).

Bearing in mind how many different hosts and baits *L. pneumophila* has in the environment, this complicates the study of the molecular mechanisms and role of the effectors in a laboratory setting in a traditional way. It is not only a question of finding the right host cell for the experiment, but moreover also using the appropriate methods to investigate the function of the effector. One question of interest when studying effectors is the cellular target or interaction partner of the effector. Traditionally, one way to find a cellular target is a Yeast-Two-Hybrid (Y2H) screen, in which a cDNA library constructed from HeLa cells is used as “prey” protein, whereas the effector of interest is used as “bait” protein. This method is

applied to identify protein-protein interactions and is also successfully used for bacterial effector proteins. When the Y2H was used for LtpK and LtpM, only a few proteins were identified as potential interaction partners and unfortunately, these could not be confirmed in other methods. There are several potential reasons why the Y2H screens in this study were not successful. As discussed above, it is possible that one effector acts on another one, in which case the search for a cellular target would not be successful. A second potential reason is that the effector of interest has a specific target in another host cell which is highly different to the counterpart in HeLa cells. A Y2H screen using a cDNA library of a natural host of *L. pneumophila*, e.g. *A. castellanii*, could be performed and might solve this limitation. For LtpM, the reason for the unsuccessful Y2H became clear when testing it in the yeast toxicity assay: LtpM is toxic for yeast due to its glycosyltransferase activity. It is necessary to mutate the DxN motif responsible for the toxicity before repeating a Y2H screen with LtpM.

However, new methods are being developed which help gain further insight into the broad pathogenic mechanisms of *L. pneumophila* infections.

One of those methods is the pBio-pulldown, a tandem purification of effectors from infected cells, which was also used in this study. The benefit of this method is the pulldown of the effector during infection, so while it is interacting with its target protein. If that target protein is another effector, it is possible to identify it with this method. Also, by purifying the effector from the infected cell, it is possible to identify whole protein complexes and therefore gain a clearer understanding of the processes during infection. By varying time points of the pulldown after infection, it is moreover a good method to glean insights into the different processes on a time scale during infection and therefore be able to investigate what happens at which stage of infection.

This approach was also applied to the effectors LtpK and LtpM during this study. There are many variables during the protocol of the method which need to be established for the different effectors individually. This was done for LtpK and LtpM by varying the number of

plates, fixation times, fixation methods, lysis detergents and also tandem- versus single-step-pulldown. It was possible to purify LtpK from infected cells using this method, however, no clear pattern of a interactome could be identified. The proteins identified in the same fraction as LtpK were different in each experiment.

It was not possible to purify LtpM in the pBio-pulldown. We attempted to optimize the protocol for LtpM by trying different variables, however, LtpM could not be identified in mass-spectrometry analysis. Although different fixation and lysis conditions were applied including with or without sonication, it might be due to the binding of LtpM to membranes. As LtpM has a PI3P binding domain, it is possible that this binding to the PI3P-rich compartments in the cell fixates LtpM to the membranes, which might prevent it from being released and instead remains in the insoluble fraction following the lysis step.

Establishing new methods will help to further investigate and understand the processes of *L. pneumophila* pathogenesis in general, and the function of its effectors in particular. Moreover, the constant improvement of bioinformatics, the analysis and discovery of protein domains and motifs, as well as the modelling of protein structures, gives first indications for the functions, localisation or potential interacting proteins.

In essence, bioinformatics and experimental data of this study strongly suggest that functional domains have been swapped between the effectors LtpD, LtpK, and LtpM, generating new effectors. Future work on the function of LtpK and LtpD will reveal how far the proteins functionally diverged after the fusion event. The data for Lpp0303 also illustrates that swapping events might sometimes be unsuccessful because a translocation signal gets lost resulting in “dead effectors”. Taken together, this study not only uncovers a new glycosyltransferase family but also provides evidence that domain swapping seems to be a common, important mechanism for the evolution of the T4SS effector repertoire and virulence in *Legionella* species.

Chapter 5

Material and Methods

5. Material and Methods

5.1 Cells and strains

5.1.1 Prokaryotic cell lines

Table 7 Prokaryotic cell lines used in this study

Bacterial Strain	Genotype/ Characteristic	Reference
***E. coli* BL21* (DE3)**	F- *ompT hsdS*B (rB-mB-) *gal dcm* (DE3)	Novagen, Darmstadt
***E. coli* Top10**	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-	Invitrogen
***L. pneumophila* 130b ATCC BAA-74**	O1, clinical isolate	Newton et al, 2008
***L. pneumophila* 130b *dotA*::Kan**	dotA disrupted with KanamycinR gene	Dr Hartland, University of Melbourne
***L. pneumophila* 130b *ltpK*::Kan**	*lptK* gene replaced with a Kan^r cassete	This study
***L. pneumophila* Paris**	Worldwide epidemic strain	(Cazalet et al., 2004)
***L. pneumophila* Paris *ltpM*::Kan**	*lptM* gene replaced with a Kan^r cassete	This study
***L. pneumophila* Paris *dotA*::Kan**	dotA gene replaced with a Kan^r cassete	

5.1.2 Eukaryotic cell lines

Table 8 Eukaryotic cell lines used in this study

Cell line	Cell type	Origin
HeLa (ATCC® CCL-2™)	epithelial cells from adenocarcinoma	Human
A549 (ATCC® CCL-185™)	alveolar basal epithelial cells from lung adenocarcinoma	Human
THP1 (ATCC® TIB202™)	monocytes from acute monocytic leukemia	Human
RAW 264.7	macrophage; Abelson murine leukemia virus induced tumor	Mouse
***S. cerevisiae* AH109**	ATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ, MEL1) and Y187 (MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-, URA3::GAL1UAS-GAL1TATA-lacZ, MEL1)	Clontech
***Dictyostelium discoideum* AX2-214**	Axenic growth in HL5; clone214 of the axenic isolate AX2	Dictybase.org
Acanthamoeba castellanii		Kindly provided by Prof. C. Buchrieser

5.2 Media

5.2.1 Liquid Media and Agar Plates for prokaryotic cell lines

Table 9 Liquid Media and Agar Plates for prokaryotic cell lines

Culture Medium/ Supplement	
Luria Bertani (LB) agar plates	
LB liquid medium	
AYE agar plates	AYE base with addition of 1.5 g/l activated charcoal powder and 1.5 g/l agar
AYE liquid medium	10 g/l ACES, 10 g/l yeast extract (Merk), 1 g/l α-ketoglutarate, pH 6.9, supplemented with 6 µM $Fe(NO_3)_3$ and 30 µM L-cysteine after autoclaving

5.2.2 Liquid Media for eukaryotic cell lines

Table 10 Media and buffers used for growth of *Dictyostelium discoideum*

Media/ buffers for *Dictyostelium discoideum*	
HL5 1L	5 g proteose peptone 5 g thiotone E peptone 10 g glucose 5 g yeast extract 0.35 g $Na_2HPO_4*7H_2O$ 0.35 g KH_2PO_4
Lo-Flo 1L Filter sterilize	1 ml 1000x FM salts 1 (see below) 1 ml 1000x FM salts 2 (see below) 0.1 ml 10,000x FM trace elements (see below) 11 g glucose·$1H_20$ 5ml 1M K_2HPO_4 5 g casein peptone (not vitamin-free)
1000x FM salts 1	NH4Cl 500 mM MgCl2 200 mM CaCl2 10 mM
1000x FM salts 2	FeCl3 50 mM
10,000x FM trace elements 100 ml pH 6.5	4.84 g Na_2-EDTA·$2H_20$ 2.3 g $ZnS0_4$·H_20 1.11 g H_3BO_4 0.51 g $MnCl_2$·$4H_20$ 0.17 g $CoCl_2$·$6H_20$ 0.15 g $CuSO_4$·$5H_20$ 0.10 g $(NH_4)_6Mo_7O_{24}$·$4H_20$
Electroporation Buffer (EB) pH 6.1	Sucrose (50 mM) KH_2PO_4 (10 mM)
Electroporation Healing Solution	$MgCl_2x6H_2O$ (0,1 M) $CaCl_2x2H_2O$ (0,1 M)
Sörensen Phosphate-Buffer (SorC) (Malchow et al., 1972) pH 6	Na_2HPO_4 (2 mM) KH_2PO_4 (15 mM) $CaCl_2$ x $2H_2O$ (50 mM)

Table 11 Media and buffers used for growth of *Acanthamoeba castellanii*

Media/ buffers for *Acanthamoeba castellanii*	
ATCC Medium: 712 PYG with Additives pH 6.5	2% bactotryptone, 0.1% yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3

Table 12 Media and buffers used for growth of *S. cerevisiae*

Media for *S. cerevisiae*	Company	Catalogue number
YPD rich Yeast Medium	1% yeast extract [Difco, 10215203], 2% peptone [Difco, 211705], and 2% glucose	
SD selective Yeast Medium	0.67% yeast nitrogen base [Difco, 211940], 2% glucose, supplemented with the appropriate amino acids and vitamins.	

Table 13 Culture Media and Supplement for mammalian cell lines

Culture Medium/ Supplement Mammalian cells	Company	Catalogue number
DMEM Low Glucose	Sigma	D5546
RPMI-1640 Medium With L-glutamine 6X500ML	Sigma	R8758-6X500ML
Opti-MEM® Reduced Serum Medium, GlutaMAX™	Life technologies - Invitrogen	51985-034
GlutaMAX™-I Supplement, 200 mM 20 × 100 ml	Invitrogen/Gibco	35050-087
Non-essential amino acid solution	Sigma	M7145-100ML
EDTA cell culture	Sigma	E8008
Trypsin	Sigma	T4674
Fetal Bovine Serum	Gibco	10270-106
YPD rich Yeast Medium	1% yeast extract [Difco, 10215203], 2% peptone [Difco, 211705], and 2% glucose	
SD selective Yeast Medium	0.67% yeast nitrogen base [Difco, 211940], 2% glucose, supplemented with the appropriate amino acids and vitamins.	
ATCC 712 PYG with Additives	2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$, 2.5 mM NaH_2PO_3, 2.5 mM K_2HPO_3	

Table 14 Dimethyl labelling buffers for pBio-pulldown MS labelling

Dimethyl labelling buffers	
PB 7.5	2ml of 50mM Na_2HPO_4 7ml of 50mM NaH_2PO_4
Light labelling buffer (100ul per sample)	90% of PB 7.5

	5% of 4% CH_2O in water 5% of 0.6M $NaBH_3CN$ in water
Medium labelling buffer (100ul per sample)	90% of PB 7.5 5% of 4% CD_2O in water 5% of 0.6M $NaBH_3CN$ in water
Heavy labelling buffer (100ul per sample)	90% of PB 7.5 5% of 4% $C_{13}D_2O$ in water 5% of 0.6M $NaBD_3CN$ in water

5.3 Antibiotics

Table 15 Antibiotics used in this study

Antiobiotic	Stock concentration
Ampicillin	100 µg/ml
Chloramphenicol	30 µg/ml
Kanamycin	50 µg/ml
Gentamycin	100 µg/ml
Geneticin (G418 Sulfate)	50mg/mL
Antibiotics were added to media at the following final concentrations for *L. pneumophila*	**For *E. coli***
kanamycin, 25 µg/ml	100 µg/ml
chloramphenicol, 6 µg/ml	12.5 µg/ml
ampicillin, 100 µg/ml	100 µg/ml

5.4 Antibodies

5.4.1 Primary Antibodies

Table 16 Primary antibodies used in this study

Antibody	Mono-/polyclonal	Origin	Company/ Cat no	Dilution
HA.11, clone 16B12	Monoclonal	Mouse	Cambridge Bioscience MMs-101P-1000	1:1000
HA-TRITC, Clone HA-7	Monoclonal	Mouse	Sigma H9037	1:200
Myc Tag, clone 4A6	Monoclonal	Mouse	Millipore 05-724	1:200
***Legionella* serogroup 1**	Monoclonal	Mouse	Virostat 6026	1:900
Legionella pneumophila	Polyclonal	Rabbit	Affinity BioReagents PA1-7227	1:900
IMPA1 (H-85)	Polyclonal	Rabbit	Santa Cruz Biotech sc-99227	1:500
EEA1	Monoclonal	Mouse	BD Transduction Laboratories™ 610457	1:500
Transferrin Receptor	Polyclonal	Rabbit	Millipore CBL47	1:200
Giantin	Polyclonal	Rabbit	Abcam ab24586	1:500
beta-COP	Monoclonal	Mouse	Sigma G6160-.2ML	1:400
Flag M2	Monoclonal	Mouse	Sigma F1804	1:500
FLAG® M2-FITC	Monoclonal	Mouse	Sigma F4049	1:100
Myc- FITC Clone 9E10	Monoclonal	Mouse	Sigma F2047-100	1:250
Rac 2 (C-11)	Polyclonal	Rabbit	Santa Cruz sc-96	1:500
Sec22b/Sec22L1/ERS-24	Monoclonal	Mouse	Sigma WH0009554M1-100UG	1:500

Bovine Serum Albumin	Monoclonal	Mouse	Sigma B2091	1:100
Chicken anti-c-myc	Monoclonal	Chicken	Bethyl A190-103A	1:200
Histone H2A	Polyclonal	Rabbit	ThermoFisher 720148	1:200
Histone 3	Polyclonal	Rabbit	Abcam ab1791	1:400
Histone 4	Polyclonal	Rabbit	Abcam ab7311	1:200
GST	Monoclonal	Mouse		1:2000
Mono-Poly-Ubiquitin			Nr 140	1:1000

5.4.2 Secondary Antibodies

Table 17 Secondary antibodies used in this study

Antibody	Fluorophore	Origin	Company/ Cat no	Dilution
Mouse IgG (H+L)	Alexa488	Donkey	Jackson Immunoresearch 715-545-150-JIR	1:200
Mouse IgG (H+L)	RRX	Donkey	Jackson Immunoresearch 715-295-150	1:500
Rabbit IgG (H+L)	Alexa488	Donkey	Jackson Immunoresearch 711-485-152	
Rabbit IgG (H+L)	RRX	Donkey	Jackson Immunoresearch 711-295-152	
Mouse IgG (H+L)	AMCA (Coumarin)	Donkey	Jackson Immunoresearch 715-155-150	1:50
Chicken IgY (H+L)	Alexa647	Goat	Jackson Immunoresearch 103-605-155	1:100
Mouse IgG (Fc fragment)	HRP (POD)	Goat	Jackson Immunoresearch 115-035-008	1:10000
Rabbit IgG (Fc fragment)	HRP (POD)	Goat	Jackson Immunoresearch 111-035-008	1:50 000

5.5 Plasmids

Table 18 Plasmids cloned and used in this study

Name	Resistance	Characteristic	Reference
pXDC61	CM	empty plasmid	de Felipe et al., 2008)
pMMB207c-HA4	CM	empty plasmid	(Dolezal et al., 2012)
pGBKT7	Kan	empty plasmid	Clontech
pMMB207c-Bio	CM	empty plasmid	Dr. A. Mousnier
pRK5myc	Amp	empty plasmid	Clontech
pGEX	Amp	empty plasmid	GE Healthcare
pQE-HS2	Amp	empty plasmid	Qiagen
pGBT9	Amp	empty plasmid	Clontech
pCMV-RFP-LtpD		(Dr C. Harding)	Dr C. Harding
pRK5myc-LtpD Δ153	Amp	LtpD Δ153 with N-terminal Myc-tag; mammalian expression	Dr C. Harding
pRK5myc-SidC	Amp	SidC with N-terminal Myc-tag; mammalian expression	Dr C. Harding
pGBT9-LtpD	Kan	LtpD with N-terminal GAL4 DNA binding domain; for Y2H	Dr C. Harding
pGAD-IMPA1	Amp	IMPA1 with N-terminal GAL4 activation domain	Dr C. Harding
pGBKT7-LtpD last 54AA	Kan	last 54 AA of LtpD with N-terminal GAL4 DNA binding domain; for Y2H	Dr C. Harding
pRK5myc-LtpD	Amp	LtpD with N-terminal Myc-tag; mammalian expression	Dr C. Harding

pGBT9-LtpDcterm	Kan	LtpD C-terminus with N-terminal GAL4 DNA binding domain; for Y2H	Dr C. Harding
pGEM-LtpKF1F2	Amp	LtpK with flanking regions; for chromosomal deletion of LtpK	This study
pRK5myc-SetA PI3P binding site	Amp	PI3P binding site of SetA with N-terminal Myc-tag; mammalian expression	This study
pRK5HA-SetA PI3P binding site	Amp	PI3P binding site of SetA with N-terminal HA-tag; mammalian expression	This study
pMMB207c-4xHA-SetA PI3P bs	CM	PI3P binding site of SetA with N-terminal HA-tag; expression in *Legionella*	This study
pEGFP-2xFYVE		PI3P marker FYVE fused to GFP; mammalian expression	Dr C. Harding
pRK5myc-0303 full length	Amp	Lpp0303 with N-terminal Myc-tag; mammalian expression	This study
pRK5myc-LtpD cterm	Amp	LtpD C-terminus with N-terminal Myc-tag; mammalian expression	Dr C. Harding
pRK5myc-0356	Amp	Lpp0356 with N-terminal Myc-tag; mammalian expression	Dr C. Harding
pRK5myc-0303 cterm	Amp	Lpp0303 N-terminus with C-terminal Myc-tag; mammalian expression	This study
pXDC61-LtpK	CM	LtpK with N-terminal TEM-Tag; expression in *Legionella*	Dr C. Harding
pRK5HA-GFP	Amp	GFP with N-terminal HA-tag; mammalian expression	Dr C. Harding
pGBKT7-0356 cterm	Kan	Lpp0356 with N-terminal GAL4 DNA binding domain	This study
pGEX-0356 cterm	Amp	Lpp0356 N-terminus with C-terminal GST-tag; for expression in *E.coli*	This study
pGBKT7-LtpD last 54AA	Kan	last 54 AA of LtpD with N-terminal GAL4 DNA binding domain; for Y2H	Dr C. Harding
pMMB207c Bio-LtpK	CM	LtpK with N-terminal Biotin-tag; expression in *Legionella*	This study
pMMB207c-Bio-LtpD	CM	LtpD with N-terminal Biotin-tag; expression in *Legionella*	Dr C. Harding
pGBKT7-LtpK	Kan	LtpK with N-terminal GAL4 DNA binding domain; for Y2H	This study
pMMB207c-4xHA-0303 full length	CM	Lpp0303 with N-terminal HA-tag; expression in *Legionella*	This study
pMMB207c-4xHA-LtpK	CM	LtpK with N-terminal HA-tag; expression in *Legionella*	This study
pGEM-KanF1F2 C1 (LtpK KO)	Amp+ Kan	Kanamycin cassette with LtpK flanking regions; for chromosomal deletion of LtpK	This study
pMMB207c-4xHA -SidC	CM	SidC with N-terminal HA-tag; expression in *Legionella*	Dr C. Harding
pRK5myc-0356 cterm	Amp	Lpp0356 N-terminus with N-terminal Myc-tag; mammalian expression	This study
pXDC61-0303 full length	CM	Lpp0303 with N-terminal TEM-Tag; expression in *Legionella*	This study
pGEX-0356 full length	Amp	Lpp0356 with N-terminal GST-tag; expression in *E.coli*	This study
pXDC61-0303cterm	CM	Lpp0303 C-terminus with N-terminal TEM-Tag; expression in *Legionella*	This study
pMMB207c-Bio-0356	CM	Lpp0356 with N-terminal Biotin-tag; expression in *Legionella*	This study
pQE His-Strep-LtpK Paris	Amp	LtpK *L. pneumophila* strain Paris with His and Strep tag; expression in *E.coli*	This study
pQE His-Strep- LtpK 130b	Amp	LtpK *L. pneumophila* strain 130b with His and Strep tag; expression in *E.coli*	This study
pMMB207c-Bio-0303	CM	Lpp0303 with N-terminal Biotin-tag; expression in *Legionella*	This study
pMMB4207c-4xHA-SidC cterm PI4P binding	CM	PI4P binding site of SidC with N-terminal HA-tag; expression in *Legionella*	This study
pRK5-HA-SidC	Amp	SidC with N-terminal HA-tag; mammalian expression	This study
pGBT9-LtpK	Amp	LtpK with N-terminal GAL4 DNA binding domain; for Y2H	Dr C. Harding
pXDC50-mCherry	CM	mCherry expression in *Legionella*	X.Charpentier

pXDC31-GFP	CM	GFP for expression in *Legionella*	X.Charpentier
pGEMt-F1LtpMF2	Amp	LtpM with flanking regions; for chromosomal deletion of LtpM	This study
pGBT9-LtpM full length	Amp	LtpM with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGEX-LtpM full length	Amp	LtpM with C-terminal GST-tag; expression in *E.coli*	This study
pGEMt-F1KanF2 LtpM KO	Amp+ Kan	Kanamycin cassette with LtpM flanking regions; for chromosomal deletion of LtpM	This study
pGBKT7-LtpM full length	Kan	LtpM with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGBT9-LtpM N-terminus	Amp	LtpM with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGBKT7-LtpM N-terminus	Kan	LtpM with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGAD-SDHB	Amp	SDHB with N-terminal GAL4 activation domain	This study
pGAD-VBP1	Amp	VBP1 with N-terminal GAL4 activation domain	This study
pGAD-ASXL1	Amp	ASXL1 with N-terminal GAL4 activation domain	This study
pGAD-SDHB-2	Amp	SDHB.2 with N-terminal GAL4 activation domain	This study
pGAD-UBR7	Amp	UBR7 with N-terminal GAL4 activation domain	This study
pGAD-ACBD3	Amp	ACBD3 with N-terminal GAL4 activation domain	This study
pGAD-TMEM60	Amp	TMEM60 with N-terminal GAL4 activation domain	This study
pGAD-TMEM60-2	Amp	with N-terminal GAL4 activation domain	This study
pGAD-L14	Amp	L14 with N-terminal GAL4 activation domain	This study
pGAD-ATF6	Amp	ATF6 with N-terminal GAL4 activation domain	This study
pRK5myc-LtpK	Amp	LtpK with N-terminal Myc-tag; mammalian expression	Dr C. Harding
pXDC61-LtpK	CM	LtpK with N-terminal TEM-Tag; expression in *Legionella*	Dr C. Harding
pXDC61-LtpM	CM	LtpM with N-terminal TEM-Tag; expression in *Legionella*	Dr C. Harding
pET28 empty	Kan	empty plasmid	
pGEX-LtpD 472-626	Amp	expression in *E.coli*	Dr C. Harding
pGBKT7-LtpM N-terminus -	Kan	LtpM shorter N-terminus with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGBT9-LtpM N-terminus -	Amp	LtpM shorter N-terminus with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGBKT7-LtpM C-terminus +	Kan	LtpM longer C-terminus with N-terminal GAL4 DNA binding domain; for Y2H	This study
pGBT9-LtpM C-terminus +	Amp	LtpM longer C-terminus with N-terminal GAL4 DNA binding domain; for Y2H	This study
pET28-C-Strep empty	Kan	empty plasmid; expression in *E.coli*	
pRK5-HA-LtpK	Amp	LtpK with N-terminal HA-tag; mammalian expression	This study
pRK5-HA-LtpM	Amp	LtpM with N-terminal HA-tag; mammalian expression	This study
pET28-C-Strep LtpM	Kan	LtpM C-terminal Strep-tag, N-terminal His-tag; expression in *E.coli*	This study
pET28-C-Strep LtpK	Kan	LtpK C-terminal Strep-tag, N-terminal His-tag; expression in *E.coli*	This study
pET28-LtpK	Kan	LtpK C-terminal His-tag, N-terminal His-tag; expression in *E.coli*	This study
pCS2-myc6-VBP1	Amp	VBP1 with 6 Myc-tags; mammalian expression	Mousnier et al., 2007
pcDNA3-2xflag-mASXL1	Amp	with 3 N-terminal flag-tags; mammalian expression	Lee et al., 2010
pCI-neo3xflag-ACBD3	Amp	full length ACBD3 with 3 N-terminal flag-tags; mammalian expression	Téoulé et al., 2013
pDest27-GST-ACBD3	Amp	full length ACBD3 with N-terminal GST-tag; mammalian expression	Téoulé et al., 2013
pGFP		mammalian expression	
pmCherry		mammalian expression	

pRK5HA-LtpM N-terminus	Amp	mammalian expression	This study
pRK5myc-LtpM N-terminus	Amp	mammalian expression	This study
pGFP-LtpM		mammalian expression	This study
pmCherry-LtpM		mammalian expression	This study
pRK5HA-LtpM C508A	Amp	mammalian expression	This study
pRK5myc-LtpM C508A	Amp	mammalian expression	This study
p4HA-LtpM C508A	CM	expression in *Legionella*	This study
P2xflag-ASXL1		mammalian expression	
pRK5myc-LtpK new cloned	Amp	mammalian expression	This study
pDM1203 empty		*Dictyostelium* exoression	
pGADt7-HLTF (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-La/SS-B protein (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-EIF3A (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-PARP1 (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-RSU1 (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-MITD1 (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-PI4K2B (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-GPC5 (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-serine/threonine kinase 38 like (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGADt7-NASP (Y2H hit)	Amp	with N-terminal GAL4 activation domain Yeast expression	This study
pGFP-LtpM C508A		mammalian expression	This study
pmCherry-LtpM C508A		mammalian expression	This study
pGADt7-VBP1 full length	Amp	Yeast expression	This study
pYES empty	Amp	Yeast expression	
pTX-GFP-BirA		*Dictyostelium* expression	This study
pMX-IP-HA-LtpM		mammalian expression	This study
pGFP-4HA-LtpK	CM	expression in *Legionella*	This study
pmCherry-4HA-LtpK	CM	expression in *Legionella*	This study
pGFP-4HA-LtpM	CM	expression in *Legionella*	This study
pmCherry-4HA	CM	expression in *Legionella*	This study
pYES-LtpK	Amp	Yeast expression	This study
pYES-LtpM	Amp	Yeast expression	This study
pRK5-Bio-LtpK-myc	Amp	mammalian expression	This study
pRK5-Bio-LtpK-NLS-myc	Amp	mammalian expression	This study
pMX-LtpK		mammalian expression	This study
p4HA-GFP	CM	expression in *Legionella*	This study
pTX-LtpK		*Dictyostelium* expression	This study
pGFP-4HA-LtpK S318A	CM	expression in *Legionella*	This study
pGFP-4HA-LtpK FF314AA	CM	expression in *Legionella*	This study
p4HA-LtpK FF314AA	CM	expression in *Legionella*	This study
p4HA-LtpK S318A	CM	expression in *Legionella*	This study
pYES-LtpM 271-end	Amp	Yeast expression	This study
pYES-LtpM 365-end	Amp	Yeast expression	This study

pYES-LtpM 460-end	Amp	Yeast expression	This study
pYES-LtpM1-113	Amp	Yeast expression	This study
pYES-LtpM1-172	Amp	Yeast expression	This study
pYES-LtpM1-271	Amp	Yeast expression	This study
pRK5HA-LtpM 271-end	Amp	mammalian expression	This study
pRK5HA-LtpM 365-end	Amp	mammalian expression	This study
pRK5HA-LtpM 460-end	Amp	mammalian expression	This study
pRK5HA-LtpM NxN D140N	Amp	mammalian expression	This study
pRK5HA-LtpM DxD N142D	Amp	mammalian expression	This study
pRK5myc-LtpM DxD N142D	Amp	mammalian expression	This study
p4HA-LtpJ	CM	expression in *Legionella*	E. So, unpublished
pTX-GFP		*Dictyostelium* expression	
pEGFP-BirA		mammalian expression	

5.6 Oligonucleotides: Primers

Table 19 Oligonucleotides used in this study

Name	Sequence
CM001 Lpp0303 Kpn1 FW	cactgaggtacccaaagcaaatctgttcgtaaggaacg
CM002 Lpp0303 Kpn1 RV	tactagggtaccttatatttccttccccgatgcgccc
CM003 Lpp0303 BamH1 FW	agtcgaggatcccaaagcaaatctgttcgtaaggaacg
CM004 Lpp0303 Pst1 RV	tagcatctgcagttatatttccttccccgatgcgccc
CM005 LtpK EcoR1 pGBKT7 FW	aattgaattcaaaacaaaatatgaaataagtcaagataaa
CM006 LtpK Xba1 RV	gtcagtctagattaacgaagaattaatctatcagc
CM007 Lpp0303 342-end BamH1 FW	agtcgaggatcccgttcacctaaacctagtgttcaattg
CM008 Lpp0303 432-end Xma RV	tagcatgagctcttatatttccttccccgatgcgccc
CM009 Lpp0356 455-end BamH1 FW	atgctaggatccagcgatgaggtgattcaaccc
CM010 Lpp0356 455-end EcoR1 RV	gtacgaattctctaatttttttaacctagtgccacataaa
CM011 0245 last 54AA Xma1 FW	cactaacccgggatcaaattcatcagacgaaacg
CM012 Lpp0356 455-end BamH1 FW	tgcataggatccacagcgatgaggtgattcaaccc
CM013 LtpK Xho1 RV	gatacctcgagacgaagaattaatctatcagcatcc
CM014 Lpp0303 N-term RV Pst1	gcatctgcagttacgcctcatcctgcatgcgttttgcagc
CM015 Lpp0303 HindIII RV	tagcataagcttttatatttccttccccgatgcgccc
CM016 SidC RV XbaI	ggctatctagactatttctttataactcccgtgtac
CM017 LtpK outside flanking FW	cctgggaggagtttaccgagaaacagtgtcttttagcgg
CM018 LtpK outside flanking RV	ggtcatgggggaagacagggtattgtttgcctcc
CM019 0356 N-term Pst1 RV	cgtaacctgcagagggttgaatcacctcatcgct
CM020 0356 FW EcoR1	cgtaggaattcgacaacgatcaactgtcagtta
CM021 LtpM Flanking1 FW	gggcagattactagatcaactgtg
CM022 LtpM Flanking2 RV	cccttattgaaattattaagcttcaggg
CM023 LtpM KO Flanking1 RV BamH1	atcagggatccccatgataatctccaaatgattcatac
CM024 LtpM KO Flanking2 FW PstI	actgc**ctgcag**tatgtggcactaggttaaaaaattagg
CM025 LtpM KO Flanking2 FW BamH1	atcgggatcctatgtggcactaggttaaaaaattagg
CM026 LtpM outside Flanking1 FW	gcggaagttctatgggctag
CM027 LtpM outside Flanking2 RV	cccaggcaactgcaatatataaaatg
CM028 LtpM FW BamH1 +2	cgtaggatccctgacaacgatcaactgtcagtta

CM029 LtpM N-term Pst1 RV stop	cgtaacctgcagattagggttgaatcacctcatcgct
CM030 LtpM RV Pst	cgtaacctgcagctaatttttttaacctagtgccac
CM031 LtpM outside Flanking2 RV	cattttatatattgcagttgcctggg
CM032 LtpM Arf-GAP Cterm FW	atcgtgaattcgctgatggaacattacttcatcat
CM033 LtpM N-term minus Arf-GAP RV Pst1	agcactgcagttatccatcagcaatattaacaatata
CM034 LtpK Xho1 RV with stop	gatacctcgagttaacgaagaattaatctatcagcatcc
CM035 LtpM RV Not1 no stop	cagtgcggccgcatttttttaacctagtgccacata
CM036 LtpM C508A FW	ggtgcgttttcaaatgctgatttggcttgaacaagagattccttttttatc
CM037 LtpM C508A RV	gataaaaaggaatctcttgttcaagccaaatcagcatttgaaaacgcacc
CM038 LtpM Nterm RV XbaI	cgtaactctagaattagggttgaatcacctcatcgct
CM039 GFP-BirA FW Kpn1	acgtggtaccgccaccatggtgagcaagggc
CM040 GFP-BirA RV BamH1	acgaggatccttatttttctgcactacgcaggg
CM041 GFP-BirA FW BamH1	atgcggatccgccaccatggtgagcaagggc
CM042 GFP-BirA RV SpeI	acgaactagtttatttttctgcactacgcaggg
CM041 SidC PIP4 dom FW Kpn1	gtcataggtaccaaatattcctccaagccattattgg
CM042 SidC RV BamH1	ggctaggatccctatttctttataactcccgtgtac
CM045 ACBD3 FW NdeI	ctgatcatatggcggcggtgctgaacgca
CM046 ACBD3 RV EcoRI	atcaggaattcttatctagtataatagactc
CM047 VBP1 FW NdeI	gtcagcatatggcggccgttaaggacag
CM048 VBP1 RV EcoR1	atcaggaattcttatgctttgttcttgg
CM049 ASXL1 Mus Musculus FW NdeI	ctgacatatgaaggacaaacagaagaggaag
CM050 ASXL1 Mus Musculus RV EcoRI	gtcagaattcttatctcaccacaaggcacaa
CM051 ACBD3 FW EcoR1	ctaggaattcgcggcggtgctgaacgca
CM052 ACBD3 RV SacI	atcaggagctcttatctagtataatagactctgtagtag
CM053 ASXL1 Mus Musculus RV Sac1	gactgagctcttatctcaccacaaggcacaa
CM054 pRK5 universal FW BamH1	actgaggatccatcgattgaattggccaccatg
CM055 LtpM RV Not1	gtactgatgcggccgcctaatttttttaacctagtgccacataaa
CM056 GFP-BirA FW pDM1203 overhang	ataaaaaataaaaatcagatctagtactaggccaccatggtgagcaagggcgaggagctg
CM057 GFP-BirA RV pDM1203 overhang	aatttatttatttaactagtactagatctgttatttttctgcactacgcagggatatttc
CM058 pRK5 universal FW PacI	ctgatcttaattaaatcgattgaattggccaccatg
CM059 RBS-GFP pXDC31 FW EcoRI	ctagcgaattcctctagatttaagaaggagatata
CM060 GFP pXDC31 RV EcoRI	ctacggaattcttatttgtatagttcatccatgccat
CM061 RBS-mCherry pXDC50 FW EcoRI	ctagtgaattctttaagaaggagatatacatatg
CM062 mCherry pXDC50 RV EcoRI	tcagtgaattcttatttgtacagctcatccatgcca
CM063 LtpK RV KpnI	gtcagggtaccttaacgaagaattaatctatcagc
CM064 LtpM RV KpnI	cgtaacggtaccctaatttttttaacctagtgccac
CM065 LtpK FF314AA	gagaaaggagaaaaggaatatgcagccctcccatcaccacaag
CM066 LtpK FF314AA RV	cttgtggtgatgggagggctgcatattccttttctcctttctc
CM067 LtpK S318A FW	ggaatatttttttctcccagctccacaagtattacg
CM068 LtpK S318A RV	cgtaatacttgtggagctgggagaaaaaaatattcc
CM069 pRK5 universal XhoI FW	cagtcctcgagatcgattgaattggccaccatg
CM070 LtpM Nterm 1-113 RV Xba1	catacgtctagaattttgtcctgaaggaaggttatc
CM071 LtpM Nterm 1-271 RV Xba1	ctacgtctagatggtgcgagagacttcctgctgaa
CM072 LtpM Nterm 1-365 RV Xba1	gatcatctagaaatattaacaatataagaaggatgtga
CM073 LtpM Cterm 460-end FW Xba1	catacgtctagaagggttgaatcacctcatcgct
CM074 LtpM Cterm 113-end FW BamH1	cagtcggatccgataaccttccttcaggacaaaat
CM075 LtpM Cterm 271-end FW BamH1	cagtcggatccttcagcaggaagtctctcgcacca
CM076 LtpM Cterm 365-end FW BamH1	cagtcggatcctcacatccttcttatattgttaatatt
CM077 LtpM Cterm 460-end FW BamH1	cagtcggatccagcgatgaggtgattcaaccc
CM078 LtpM Nterm 1-172 RV Xba1	catacgtctagaaaagtcatttgatttctgaaatcc

CM079 LtpM D140N FW	ccaggatatttccttaacactaatgtatttccggcaaca
CM080 LtpM D140N RV	tgttgccggaaatacattagtgttaaggaaatatcctgg
CM081 LtpM FKD122AKA FW	aagcaaagagtgatggccaaagctttattttcattgttc
CM082 LtpM FKD122AKA RV	taagaacaatgaaaataaagctttggccatcactctttg
CM083 pRK5 universal SacI FW	cagtcgagctcatcgattgaattggccaccatg
CM084 LtpM Nterm with stop 1-113 RV Xba1	catacgtctagattaattttgtcctgaaggaaggttatc
CM085 LtpM Nterm with stop 1-172 RV Xba1	catacgtctagattaaaagtcatttgatttctgaaatcc
CM086 LtpM Nterm with stop 1-271 RV Xba1	ctacgtctagattatggtgcgagagacttcctgctgaa
CM087 LtpM Nterm with stop 1-365 RV Xba1	gatcatctagattaaatattaacaatataagaaggatgtga
CM088 LtpM Nterm 1-113 RV Not1	tcgatgctgacgccggcgattttgtcctgaaggaaggtt
CM089 LtpM Nterm 1-271 RV Not1	tcgatgctgacgccggcgtggtgcgagagacttcctgct
CM090 LtpM N142D FW	ggatatttccttgacactgatgtatttccggcaacagat
CM091 LtpM N142 RV	atctgttgccggaaatacatcagtgtcaaggaaatatcc
T7	taatacgactcactataggg
SP6	atttaggtgacactatag
CH02 leg246 pGTB9 RV	agctgctgcagctaaatctttaaaccagtgtcgc
CH09 leg2716 pXDC61 FW.	cggtccgggtaccaaaacaaaatatgaaataagtcaag
CH39 lpp0356 pXDC61 FW	gattagggtaccgacaacgatcaactgtcag
CH51 2716 mutant 1 FW	catccaatcagttaaccagtgaaaggtcttgag
CH52 2716 mutant 1 RV	gtatgaattctcataaaaaaccctggctgcagcttg
CH53 2716 mutant 2 FW	gtatgcgaattcgaaagccattttatctcatgaaaatg
CH54 2716 mutant 2 RV	cgaagaattaatctatcagcatccgcttc
CH121 SidC PIP4 dom pRK5 FW BamH1	gtcataggatccaaatattcctccaagccattattgg
CH40 lpp-356 pXDC61 RV	cgtaacggatccctaatttttttaacctagtgccac
CH61 356 pRK5 FW	ggatacggatccatggacaacgatcaactgtcag
CH62 356 pRK5 RV	cgtaacctgcagctaatttttttaacctagtgccac
CH46 lpp-356 pXDC61 RV	cgtaactctagactaatttttttaacctagtgccac
GNS465 SetA PIPD KpnI FW	cactgaggtaccgtaaatccaacggctcaagaat
GNS466 SetA PIPD XbaI RV	tcgcatctagattatattcttaaaccatgattgttatcgt
GNS467 SetA PIPD BamHI FW	cactgaggatccgtaaatccaacggctcaagaat
GNS468 SetA PIPD EcoRI RV	tcgcagaattcttatattcttaaaccatgattgttatcgt

5.7 Kits

Table 20 Laboratory Kits used in this study

Name of Kit	Company	Catalogue number
Miniprep kit	Peqlab	12-6942-02
DNeasy Tissue Kit	Qiagen	69504
RNeasy Mini Kit (50)	Qiagen	74104
QiaQuick PCRpurification kit (250)	Qiagen	28106
QIAquick Gel Extraction Kit (250)	Qiagen	28706
QIAGEN OneStep RT-PCR Kit (100)	Qiagen	210212
HiPerFect Transfection Reagent	Qiagen	301705
Nucleofector® Kit V	Lonza	VCA-1003
KOD Hot Start DNA Polymerase	Novagen	71086-3
Quickchange II site directed mutagenesis Kit	Agilent	200524
Fast SYBR® Green Master Mix 5-Pack	Invitrogen	4385617

5.8 Cell culture methods for prokaryotic cell lines

5.8.1 Culture of *Escherichia coli*

E. coli cultures were either grown in liquid Luria-Bertani (LB) medium shaking 200rpm at 37°C or static on LB plates at 37°C, containing the appropriate antibiotics.

5.8.2 Transformation of chemically competent *Escherichia coli*

Chemically competent *E. coli* TOP10 were thawed on ice. 50 µl were added to the ligation mix and incubated on ice for 30 min. A heat shock followed at 42°C for 45 s, bacteria were immediately transferred back on ice for a further incubation for 3-5 min. Prewarmed LB liquid medium was added and bacteria were incubated shaking at 37°C for 1 h before plating on LB agar plates containing the appropriate antibiotic.

5.8.3 Culture of *Legionella pneumophila*

L. pneumophila cultures were either grown static on charcoal-yeast extract (CYE) plates supplemented with 6 µM $Fe(NO_3)_3$ and 30 µM L-Cysteine for 3-4 days at 37 ˚C then inoculated into ACES yeast extract (AYE) plates at 37°C or in liquid AYE medium supplemented with FeNO3 and L-Cysteine shaking at 37°C, containing the appropriate antibiotics.

5.8.4 Transformation of chemically competent *Legionella pneumophila*

Competent *L. pneumophila* were thawed on ice. 50 µl of cells were combined with 100-200 ng of plasmid DNA in a 1.5 ml Eppendorf tube and transferred in a precooled 2 mm cuvette (Biorad). The cuvette was placed in the electroporator (Biorad Micropulser) and pulsed U = 2.5 kV. Immediately prewarmed 450 µL AYE (without antibiotic) were added and cells were transferred into a sterile 1.5 mL tube. For 5 h – 6 h at 37°C cells were incubated shaking

before plating on AYE plates containing the appropriate antibiotic. Colonies could be seen after 3-4 days 37°C.

5.8.5 Chromosomal deletion in *Legionella pneumophila*

To delete a gene, it was first replaced between its flanking regions (400-1000 bp) with an antibiotic resistance cassette via cloning. The PCR amplified region was incubated in a high concentration (5 PCR reactions) with *L. pneumophila* in a liquid culture at 37°C and 30°C for several days and plated each day on Kan-plates. To confirm the chromosomal deletion of the gene, colonies grown on Kan-plates were either picked and screened by PCR, directly or gDNA was extracted. Extraction of genomic DNA from *L. pneumophila* was performed using the Qiagen DNeasy Tissue Kit following the user's manual. Screening was done via colony screening PCR.

5.9 Cell culture methods for eukaryotic cell lines

5.9.1 Culture of HeLa and A549 cell lines

The epithelial cell lines HeLa and A549 were cultured in the same way in 75 cm cell culture flasks with DMEM medium (DMEM low glucose, 10% heat inactivated (hi) FCS, 1% GlutaMaxx, 1% non-essential amino acids). When cells were 80-100% confluent, they were washed with PBS and then incubated with 5 ml Trypsin/EDTA at 37°C until cell dispersed. Trypsin was quenched by adding 5 ml culture medium. Cells were centrifuged (300 g, 3 min), resuspended in fresh medium and seeded in the desired dilution, usually 1:10.

5.9.2 Culture of RAW 264.7

RAW 264.7 macrophages were cultured in 75 cm cell culture flasks with RPMI medium (RPMI/10% hiFCS, 1% GlutaMaxx) in a water saturated 95% air/ 5% CO_2 atmosphere. When

a confluence of 60-80% was reached, cells were detached using a cell scraper and seeded in the desired dilution, usually 1:10.

5.9.3 Culture of THP-1

THP-1 cells were grown in suspension in RPMI medium (RPMI/10% hiFCS, 1% GlutaMaxx). When cells reached a density of 8x10^5/ml, cells were centrifuged (300 g, 3 min) and resuspended in fresh culture medium at a density of 2-3x10^5. Cells were passaged every 3-4 days. Monocytic differentiation was induced by PMA (phorbol 12-myristate 13-acetate) in a concentration 1:20000 (80 nM) when seeding the cells.

5.9.4 Culture of *Saccharomyces cerevisiae*

S. cerevisiae strains were cultured according to their genotypes either with rich YPD medium (YPD agar plates or YPD liquid medium) or with selective SD medium (SD agar plates or SD liquid medium; SD/–Ade, –His, –Leu, –Trp, in different combination according to the genotype) following the instructions of the Clontech user manual to perform a Yeast 2 Hybrid screening. The cells were cultured at 30°C in a shaking incubator in liquid medium or static on agar plates. Cultures can be stored on agar plates at 4°C for up to one month.

5.9.5 Axenic growth of *Dictyostelium discoideum* attached on surfaces

Inoculate *D. discoideum* cells from frozen storage stocks into fresh HL5 growth medium (see chapter 5.2.2) Grow cells either in plastic Petri dishes (~10 ml medium) or in cell culture flasks (75 cm^2: 10-12 ml, 150 cm^2: 15 ml) at 21-23°C. Change medium frequently (every 2-3 days). For detachment of cells, firmly tapping the flasks on a flat surface and transfer aliquot to a new flask. Subculture the cells as soon as they reach stationary density.

5.9.6 Axenic growth of *Acanthamoeba castellanii* attached on surfaces

Inoculate *A. castellanii* cells from frozen storage stocks into fresh ATCC medium 712 PYG with additives (see chapter 5.2.2). Grow cells either in plastic Petri dishes (~10 ml medium) or in cell culture flasks (75 cm^2: 10-12 ml, 150 cm^2: 15 ml) at 21-23°C. Change medium frequently (every 2-3 days). For detachment of cells, firmly tapping the flasks on a flat surface and transfer aliquot to a new flask. Subculture the cells as soon as they reach stationary density.

5.10 Transfection of HeLa cells

Cells were seeded in 24 well plates in a density of 1x10^5 per well one day before transfection, so that they should be 50-60% confluent. 500 ml of medium was removed before transfection. Transfection reagents were prepared as indicated in the Genejuice (Novagen) manual (0.75 µl GeneJuice to 20 µl OptiMEM serum-free medium/transfection), mixed by vortexing and incubated for 5 min at RT. 0.25 µg DNA per transfection were added to 20.75 µl Genejuice:OptiMEM and mixed gently. For co-transfections, 0.125 µg of each plasmid were used. GeneJuice reagent/DNA mixture was incubated at room temperature for 15 min. The entire volume (20.75 µL plus volume DNA) of GeneJuice reagent/DNA mixture was added drop-wise to cells in complete growth medium. Cells were incubated at 37° C (5% CO_2). 24 h post transfection (or later if necessary) cells were stained for microscopy.

5.11 Transfection of THP-1 cells

Transfection of THP-1 cells with siRNA was performed using the Amaxa® Cell Line Nucleofector® Kit V following the manufacturer protocol.

5.12 Transformation of Yeast

On the day before the transformation, a large single colony of S. cerevisiae AH109 in inoculated in 10 ml YPD and incubated at 30°C, 200 rpm overnight. On the following morning, a subculture is started from the overnight culture at OD600 0.2 (100 ml for 10 transformations) and incubated at 30°C, 200 rpm until OD600 is at 0.4-0.6. The cells are harvested by centrifugation at 3000 rpm for 5 min at RT. In the meantime, Hering sperm DNA is thawed and boiled for 5 min at 100°C. The cells pellet is washed with 10 ml sterile water per 50 ml culture and again centrifuged. The cell pellet is then resuspended in 1 ml 100 mM LiAc and transferred in an Eppendorf tube. Cells are pelleted by centrifugation at 13000 rpm for 15 s and the supernatant removed. Then, the pellet is resuspended in the 500 µl 100 mM LiAc and aliquoted in 50 µl samples in fresh sterile Eppendorf tubes before centrifuging and removing the supernatant again. The yeast cells are now prepared for the transformation mixture, which is added to the pelleted cells in the following order: 240 µL 50% (w/v) PEG3350, 36 µL 1M LiAc, 25 µL Hering Sperm DNA (heat-denatured), 50 µL water, plasmid DNA (~ 1 µg). The tubes and then vortexed to resuspend the cells and incubated at 30°C for 30 min. After the incubation, the heat shock follows at 42°C for 20-25 min in a water bath. The cells are pelleted by centrifugation at 13000 rpm for 15 s and resuspended in 200 µl sterile water. 50 µl of the freshly transformed yeast cells are plated on the appropriate SD drop out agar plates and incubated at 30°C for 3-4 days until colonies are visible.

5.13 Yeast 2 Hybrid

Yeast 2 Hybrid screens were performed for the indicated constructs mating with a pretransformed HeLa library from Clontech, following the user manual by Clontech. The investigated effectors LtpK or LtpM were cloned into the plasmids pGBKT7/pGBT9 and transformed into the yeast strain AH109. Transformants were mated with the library

following the Clontech manual and the mated culture was plated on QDO (quadruple drop out: SD/–Ade, –His, –Leu, –Trp) or TDO (triple drop out: SD/–Ade or –His, –Leu, –Trp) plates to select for presence of both plasmids as well as protein interactions. Colonies are re-streaked on QDO and the library insert amplified by PCR using AD-LD primers from Clontech (5' CTATTCGATGATGAAGATACCCCACCAAACCC 3' and 5' AATCGTAGATACTGAAAAA CCCCGCAAGTTCACT 3') and send for sequencing. The Sequences were then identified by BlastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) searching the *Homo sapiens* library. To confirm protein interactions, the library sequences were cloned in pGADT7 and directly transformed in AH109 with the appropriate plasmid containing the effector LtpK or LtpM, as well the empty plasmid as negative control.

5.14 Yeast growth curves

Freshly transformed yeast was taken from plate (SD -Ura) and resuspended in SD with no added sugar source. The OD600 was measured and diluted to OD600 0.1 in SD + Glucose and SD + Galactose. 200 ul/well was added in a 96 well plate (white, clear bottom) in triplicates and plate reader assay started, at 30° for 96h measurement of OD600 every 30 min.

Serial dilutions were prepared in SD in a 96well plate and spotted on SD agar (-Ura) + Glucose/Galactose and incubated at 30° C for four days.

5.15 Methods for Molecular Biology

5.15.1 Cloning

Cloning was performed following general protocols for molecular biology. PCR was done using the KOD polymerase Hot Start kit by Novagen. PCR product got purified using the PCR purification kit by Qiagen. Purified PCR product as well as plasmid was digested with the appropriate restriction enzymes (NEB) at 37°C for 2-4 h. After another purification step the

PCR product was ligated into the vector using a vector:product ration of 1:5 with T4 ligase for 1 h at RT or overnight at 4°C.

5.15.2 Transformation of chemically competent *E. coli* via heat shock

E. coli cells are thawed on ice carefully (50 µL). 1 µl Plasmid-DNA or 10 - 30µL of the ligationmix is added to the competent cells and incubated on ice for 30 min. Then the heat shock is performed for 45s at 42°C, before immediately placing the cells back on ice for 5 min. 450 µL of fresh, pre-warmed sterile LB medium is added to the cells and they are incubated for 1h shaking at 200 rpm at 37°C. 50 µl of the freshly transformed bacteria as well as the remaining 450 µl are plated on LB plates with appropriate antibiotics and incubated overnight at 37°C in a static incubator.

5.15.3 Agarose-Gel Electrophoresis

Separation of DNA fragments for analytical and preparative purposes was done by agarose gel-electrophoresis. 1% agarose gels were used. To visualize DNA, 1:10000 SYBR® Safe was added to the gel. A 2-log DNA standard (NEB) was used in every electrophoresis to determine the fragment sizes. Samples were mixed with loading buffer and applied to the slots. Electrophoresis was performed in 1x TAE Buffer at 120 V for 20 min.

5.15.4 Plasmid preparation

Plasmid preparations were performed using the Peqlab Miniprep Kit following the user manual. 4-10 mL of LB containing the appropriate antibiotic were inoculated with colony from plate or from a frozen stock and incubated at 37°C, overnight, shaking at 200 rpm. The plasmid DNA is isolated using a mini- or midi-preparation kit (Peqlab Miniprep Kit, refer to manual), and eluted DNA in 50 µl H20 per column. DNA concentration of 2 µl eluate is measured using the Nanodrop and 20 ul of a concentration between 20 and 100 ng/ul is send for sequencing.

5.15.5 Preparation of competent *L. pneumophila*

On the first day, 10% glycerol is cooled over night at 4°C and a 10 mL AYE overnight culture of *L. pneumophila* is set up (plus kan25 for *ΔdotA* or mutant strains), let not grow to stationary phase. Bacteria are inoculated to OD600 0.1 as it is done for infections, and are grown for 18 hours. On the next morning, 100 mL of AYE is pre-warmed in disposable, single use plastic Erlenmeyer flask to 37°C. Meanwhile, the 10% glycerol is cooled down on ice. The subculture is inoculated with the overnight culture to a starting OD600 of 0.1 and incubated shaking for 5 to 6 hours at 37°C until OD600 reaches 0.3 – 0.5. Then, the cells are cooled on ice and kept at 4°C for the rest of the procedure. The cell suspension is transferred in 50 mL Falcon tubes and spun down (4000 x *g*, 10 min, 4°C), supernatant is removed completely. Cells are resuspended in 35 mL ice cold 10% sterile glycerol and incubated on ice for 10 min. This cold glycerol wash (Step 8-10) is repeated two more times using 10 mL 10% Glycerol to resuspend. Cells are spun down (4000 x *g*, 10 min, 4°C), supernatant removed and the pellet resuspended in 8 mL 10% ice cold glycerol, then in the next step in 750 µl 10% ice cold glycerol. 50 µL of the freshly prepared competent cells are aliquoted to Eppendorf tubes and frozen at -80°C.

5.15.6 Extraction of mRNA from eukaryotic cells

mRNA extractions from eukaryotic cells were performed using the RNeasy Mini Kit (Qiagen) following the company's instructions. Concentrations of extracted RNA were measured using the Nanodrop spectrophotometer.

5.15.7 Reverse-Transcriptase PCR (RT-PCR)

1-2 µg of RNA were used as template in the beginning of the reaction. DNA was digested using DNase by Promega. RT-PCR to rewrite extracted mRNA in cDNA was performed using the QIAGEN OneStep RT-PCR Kit following the manufacturer's protocol.

5.15.8 Quantitative Real-Time PCR (q-RT-PCR)

1 µl of cDNA from the RT-PCR reaction was used as template. Quantitative Real-Time PCR was performed using the Fast SYBR® Green Master Mix 5-Pack (Invitrogen) following the manufacturer's manual.

5.16 Methods for protein biochemistry

5.16.1 SDS-PAGE

For SDS-Polyacrylamide gel-electrophoresis equipment from BioRad was used. Separation gels contained 10-12% polyacrylamide. Protein samples were mixed with 2x or 4x SDS sample buffer, boiled for 10 min at 90°C and added to the gel slots. Protein separation was performed for 1.5-2h at 120 V.

5.16.2 Western Blot analysis

For detection of proteins with specific antibodies, a wet western blot analysis was done after the separation via SDS-PAGE. The transfer was performed at 300 mA for 1.5 h. To avoid unspecific binding of the antibodies the membrane was blocked with 5% milk for 1 h at RT or overnight at 4°C. The primary antibody was diluted in blocking buffer as indicated and added to the membrane. After incubation for at least 1 h the membrane was washed three times for 10 min with PBS-T and incubated for 1-1.5 h with the appropriate secondary HRP-conjugated antibody diluted in PBS as indicated. After three additional washing steps with PBS-T the membrane was incubated for 1 min with EZ-ECL according to the manufactures manual and developed using a chemo luminescence imager.

5.16.3 Purification of GST-tagged proteins

For the protein purification of GST-tagged proteins for the PIP-array, 1L cultures of *E. coli* BL21* transformed with the particular plasmid were prepared, spinned down and the pallet was frozen down until further purification. The cells were lysed in 10 ml ice cold PBS plus 1 tablet "Roche protease inhibitor mix" per 10 mL using the EmulsiFlex and spinned down after lysis. Supernatants were incubated with 200 µL Glutathione Sepharose 4B (GE Healthcare, 17-0756-01), which was washed with PBS twice and resuspended in PBS to obtain a 50% slurry. Samples were incubated rotating for at least 30 min before centrifuges (5min, 500g) and washed three times with PBS. Flow through and washes were kept for analysis. The protein was eluted in 100 µL elution buffer (50 mM TrisHCl, 10 mM reduced Glutathione pH 8.0, prepare fresh) for 10 min at RT using gentle agitation. Samples were centrifuged again (5min, 500 x *g*) and elutions collected in fresh tubes. Fractions were analysed by SDS-PAGE and/or Western Blot.

5.17 Infection Methods

5.17.1 Infection of eukaryotic cell lines with *Legionella pneumophila*

Legionella strains are streaked out on CYE-agar plates four days prior to the experiment. For immuno-staining of infections, 1x10^5 A549 were seeded per well on coverslips in a 24 well plate one day before infection. *L. pneumophila* cultures were grown for 21 hours in AYE medium containing the appropriate antibiotic and if necessary induced with 1mM IPTG 1 h before infection. Cells were infected with MOI 40 and centrifuged (600 x *g*, 5 min, RT) to synchronize infection. Infected cells were incubated in growth medium containing 1 mM IPTG and 6 ug/ mL chloramphenicol at 37°C, 5% CO_2 for the desired times, usually 6h and 24h.

5.17.2 Infection of *Dictyostelium discoideum* with *Legionella pneumophila*

Legionella strains are streaked out on CYE-agar plates four days prior to the experiment. The *Dictyostelium* cells are subcultured and feeded two days before the experiment. On the day before the experiment a *Legionella* overnight culture is set up in AYE containing the appropriate antibiotics, at OD 0.1 in 3 ml medium (21h before infection). *Dictyostelium* cells are prepared either on the day before the experiment or at least two hours before infection. The cells are resuspended in HL5 and diluted to reach a concentration of 5x10^5/ml. Sterile glass coverslips are placed in each well of the 24-well plate. *Dictyostelium* is seeded at a concentration of 2.5x10^5/well (0.5 ml/well) and are allowed to settle down over night (cells replicate about once over night), and medium changed to LoFlo medium before infection. Amoeba cells survive in LoFlo medium, but do not replicate. If seeded two hours before the experiment, cells are seeded at 4x10^5 cells/well in LoFlo medium. *Legionella* dilutions are prepared and the OD600 of the overnight cultures is measured. The cultures are diluted in LoFlo for the infection and the volumes for a the appropriate MOIs are calculated. Cells are infected with different MOIs, ranging from 0.1 to 50, and centrifuged (600 x *g*, 5 min, RT) to synchronize infection. Infected cells were incubated in growth medium containing 1 mM IPTG and 6 ug/ mL chloramphenicol at 22°C for the desired times. For fixation and staining, the general immunofluorescence staining protocol is followed.

5.17.3 Infection of *Acanthamoeba castellanii* with *Legionella pneumophila*

Legionella strains are streaked out on CYE-agar plates four days prior to the experiment. The *A. castellanii* cells are subcultured and feeded two days before the experiment. On the day before the experiment a *Legionella* overnight culture is set up in AYE containing the appropriate antibiotics, at OD 0.1 in 3 ml medium (21h before infection). *A. castellanii* cells are prepared either on the day before the experiment or at least two hours before infection. The cells are resuspended in PYG 712 medium and diluted to reach a concentration of 1x10^5/ml. Sterile glass coverslips are placed in each well of the 24-well plate. *A. castellanii*

is seeded at a concentration of 0.5x10^5/well (0.5 ml/well) and are allowed to settle down over night (cells replicate about once over night), and medium changed to LoFlo medium before infection. If seeded two hours before the experiment, cells are seeded at 1x10^5 cells/well in LoFlo medium. *Legionella* dilutions are prepared and the OD600 of the overnight cultures is measured. The cultures are diluted in LoFlo for the infection and the volumes for a the appropriate MOIs are calculated. Cells are infected with different MOIs, ranging from 0.1 to 50, and centrifuged (600 x *g*, 5 min, RT) to synchronize infection. Infected cells were incubated in growth medium containing 1 mM IPTG and 6 ug/ mL chloramphenicol at 22°C for the desired times. For fixation and staining, the general immunofluorescence staining protocol is followed.

5.17.4 Translocation Assay

RAW264.7 macrophages were seeded ($7.5x10^4$ cells/well in a black 96 well plate with translucent bottom, 200 µl per well) three days before the assay and incubated at 37°C, 5% CO_2. On day of assay, macrophages were washed with warm 150 µL RPMI (Glutamax, 10% FCS). After infection (MOI 40) cells were centrifuged (600 x *g*, 5 min, RT) to synchronize infection. After 1h at 37°C, 5% CO_2, RPMI was replaced with 100 µl of HBSS (20mM HEPES, 3 mM Probenecid, pH 7.4) and 20 µl of 6x CCF2/AM solution was added (freshly prepared with the CCF2/AM loading kit; CCF2/AM final concentration, 1 µM; Invitrogen). Cells were incubated at RT for 1h 45 min protected from light. Cells were washed four times (twice with 200 µL, once with 150 µL and once with 100 µL) with HBSS (20mM HEPES, 3 mM Probenecid, pH 7.4). Fluorescence was measured using the Fluostar Optima reader.

5.17.5 Purification of His-tagged biotinylated effector proteins after infection

A 90% confluent monolayer of A549 cell line, stably expressing GFP-BirA and grown in 10 cm^2 tissue culture dishes, was infected at MOI 15 for 24h with post-exponential growth phase *L. pneumophila* 130b WT carrying the pICC plasmid coding for the His_6-Bio-tagged

effector proteins. The cell media was supplemented with 6 µg/ml chloramphenicol, 1 mM IPTG and 4 µM D-Biotin throughout the infection, and was replaced after 2h of infection. At the end of infection, cells were washed 2 times in PBS and, if required, protein complexes were cross-linked with 1% formaldehyde (PFA) for 30 min at 37°C or RT. Cross-linking was quenched with 125 mM Glycine for at least 5min at room temperature. Cells were then washed 3 times with PBS and dry dishes can be stored at -80°C. Purification of protein complexes was performed on ice in denaturing conditions. Cells were incubated in 1 ml RIPA lysis buffer with protease inhibitor per 10 cm dish for 30 min and then harvested by scraping into an Eppendorf tube. Depending on the experiment, lysis with or without sonication was compared. Lysates were cleared by centrifugation for 20min at 17,000 x *g* and supernatants were incubated 1h with the His-resin, Ni-NTA Agarose (Qiagen), on a rotating wheel at 15 rpm at 4°C. 60 µl of Ni-NTA agarose per sample was prepared beforehand by washing it twice in lysis buffer (centrifugation for 1 min at 1000 x *g*). The columns were then washed 5 times with wash buffer (1% Triton X-100, 20mM imidazole; pH 8) (1 min spin at 1000 x *g*, 4°C). The supernatant after the first centrifugation step is kept in a fresh Eppendorf tube as the His-flow through fraction. The purified His_6-tagged proteins were then eluted with 200 µl elution buffer for 10 min at RT with gentle agitation (PBS, 1% Triton X-100, 250mM imidazole buffer). After centrifuging the supernatant is transferred into a clean Eppendorf tube and another 200 µl ice cold elution buffer is added and the step repeated. The two elutions are combined and an aliquot for the Western Blot analysis is taken. For the control experiment, the different fractions were analysed by SDS-PAGE and Western Blot after this point. For the purification of complexes, another purification step followed. The Neutravidin resin (50ul suspension per sample) is prepared by washing 2x PBS 1% Triton X-100. The His-elution is centrifuged to ensure no His-resin is taken over to the next step, almost the whole supernatant is taken and incubated with the equilibrated Neutravidin-resin for 1 hour at RT on a rotating wheel. After one hour, samples are centrifuged and the supernatant kept to analyse by Western Blot. The resin is transferred to a low-bind Eppendorf tube and

washed 4 times with 1ml with ice cold PBS 1% Triton X-100. (1min, 1000 x *g* spins) and 4 times with 1ml with AMBIC. After the last washing step, 50 µl AMBIC are left with the resin and 5 µl trypsin (20µg/100µl) is added. The samples are incubated with the trypsin at 37°C overnight in a shaker at 1000 rpm. On the following day, samples are processed for mass spectrometry by stage tipping.

The supernatants, which contain tryptic peptides, are collected by addition of 1 x 80 µl AMBIC and 1 x 80 µl 0.1% formic acid and 10 min vortex shaking followed by 2 min of 3000 x *g* centrifugation. The peptide mixtures are desalted using the Stage-Tip method (Broncel et al., 2015) and dimethyl labelling was performed according to Boersema et al. (Boersema et al., 2009). The samples were light or heavy dimethyl labelled. Briefly, peptide mixtures were loaded onto sorbent (SDB-XC poly(styrenedivinylbenzene) copolymer, from 3 M) and desalted with 150 µl water. 100 µl of dimethyl labelling solution (Table 14) were passed over 30mins through the Stage-Tip. The membrane was washed with 150 µl water and peptides eluted using 79% acetonitrile. Samples were vacuum dried and stored at -80°C until usage. Samples were resuspended in 20 µl 0.5% trifluoroacetic acid, 2% acetonitrile and transferred into LC-MS sample vials.

Mass spectrometry and MS data processing was performed as described in (So et al., 2016).

Briefly, data processing was done using MaxQuant (Cox 2008 Nat. Biotechnol.) (version 1.5.0.25) and identification of the peptides was done by matching MS/MS spectra with reference human (Uniprot, downloaded on 19/01/2015) and *Legionella pneumophila* (strain 130b or Paris) proteomes using Andromeda search engine (Cox 2011 J Proteome Res). Fixed modifications were not set. In silico digestion of the reference proteomes was done using the trypsin/P setting, cleavage was allowed after arginine/lysine residues but only if it is not followed by a proline. Light (+28Da) and heavy (+32Da) dimethyl labelled lysines and N-termini were used for quantification by a build-in algorithm in MaxQuant (Cox 2008 Nat. Biotechnol). A maximum of two missed cleavages was allowed. The false discovery rate was

set to 0.01 for peptides, proteins and sites. All other parameters were used as pre-set from the software.

Data were further analysed using Perseus (Version 1.5.0.9). Reverse and identified by site hits were removed. Proteins identified with at least 1 unique and 1 razor peptide were included for further analysis. Light and heavy intensities were logarithmized (log2). A unique peptide threshold was not applied in order to identify as many potential interacting proteins as possible and not exclude them prematurely. Heat maps were generated using log2 light and heavy intensities with imputed values removed.

5.17.6 Immunoprecipitation of *L. pneumophila* effectors for analysis of phosphorylation

1.5 x 10^7 A549 cells were infected with WT *L. pneumophila* 130b expressing 4HA-LtpK or 4HA-LtpD at an MOI of 15. After two hours, infected cells were washed twice with D-PBS. 24 hours after infection, cells were washed twice with D-PBS before lysis in 1 ml ice-cold RIPA-buffer with protease inhibitor cocktail (Sigma). Cells were scraped and the lysate was then centrifuged (13000 rpm, 20 min) to remove cellular debris and the supernatant applied to pre-equilibrated mouse anti-HA agarose (Sigma) beads and incubated together on a wheel for 2 h at 4 ˚C. The beads were then washed 6 times in RIPA buffer and the protein eluted using HA-peptide following the manufactures instructions. Laemmeli buffer was added to eluted protein and the proteins separated and transferred using blocking buffer (10 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 2% BSA,) instead of PBS at all points.

5.17.7 Intracellular growth curve with *Legionella pneumophila* in THP-1 by plating

Three days before infection, THP-1 monocytes were seeded and differentiation was induced with 80nM PMA (5x10^5/well in 24 well plate). *L. pneumophila* strains were cultured for 21 h in liquid AYE medium containing the appropriate antibiotics. Medium of THP-1 cells was

replaced with fresh RPMI containing 1mM IPTG and the appropriate antibiotic before infecting with MOI 1. Bacteria were centrifuged on cells (600g, 5 min, RT) to synchronize infection and incubated 1h at 37°C, 5% CO_2. To kill extracellular bacteria, RPMI was replaced with RPMI containing Gentamycin (100 µg/ml) as well as IPTG and the resistance antibiotic. After 1 h Gentamycin treatment, medium was again replaced to the RPMI/IPTG/antibiotic and incubated for different time points. In order to stop infection at different time points, supernatant was collected in 1.5 ml Eppendorf tubes and 200 µl 0,05% digitonin was added to the cells for 5 min. 300 µl of prewarmed AYE liquid medium was added, resuspended several times and added to the supernatant to combine intra- and extracellular bacteria. Serial dilutions were made in PBS and plated on AYE agar plates containing the selective antibiotic. CFUs were counted three days after plating.

5.17.8 Intracellular growth curve with *Legionella pneumophila* in THP-1 using the plate reader

L. pneumophila strains expressing either GFP or mCherry were streaked out four days before infection and cultured for 21 h before infection in liquid AYE medium containing the appropriate antibiotics as well as IPTG. THP-1 monocytes were differentiated with 80nM PMA and seeded in a black 96-well plate with translucent bottom two days before infection ($8.5x10^4$ cells/well). On day of infection, medium of THP1s is changed to 100 µL RPMI without phenolred (1% Glutamax, 10% FBS, 1mM IPTG, cam6). THP1s are infected with MOI 1 or 10, infections are synchronized by centrifugation (5 min, 600g). After one hour of infection, RPMI medium is replaced with RPMI containing 100 µg/mL Gentamicin to kill extracellular bacteria. After one more hour of infection, cells are washed three times with D-PBS before RPMI (with supplements, without Gentamicin) is added back to the wells and the measurement in the plate reader is started. The plate reader and CO_2 unit have to be set at 37°C and equilibrated before the measurement.

5.17.9 Intracellular growth curve with *Legionella pneumophila* in *D. discoideum* or *A. castellanii* using the plate reader

L. pneumophila strains expressing either GFP or mCherry were streaked out four days before infection and cultured for 21 h before infection in liquid AYE medium containing the appropriate antibiotics as well as IPTG. The amoeba hosts *D. discoideum* and *A. castellanii* are seeded in a black 96-well plate with translucent bottom four hours before the infection in LoFlo medium (1mM IPTG, cam6) ($8.5x10^4$ cells/well). *Legionella* dilutions are prepared and the OD600 of the overnight cultures is measured. The cultures are diluted in LoFlo for the infection and the volumes for a the appropriate MOIs are calculated. For a 96 hour growth curve, the amoeba hosts are infected with MOI 0.1, 1 or 10, infections are synchronized by centrifugation (5 min, 600g). Measurement in the plate reader is started.

5.17.10 *In vivo* infection of A/J mice

All animal work took place at the University of Melbourne and the University of Melbourne Animals Ethics Committee approved mouse procedures. The virulence of *L. pneumophila* 130b, the Δ*ltpK* mutant and complemented strain were examined as described previously (Newton et al., 2008). Briefly, 6 to 8 week old male or female A/J mice were anesthetized and inoculated intranasally with approximately 2.5 x 10^6 CFU of each *L. pneumophila* strain under investigation. 72 hours following inoculation, mice were killed and their lung tissue isolated. Tissue was homogenized and complete host cell lysis was achieved by incubation in 0.1% saponin for 15 min at 37 ˚C. Serial dilutions of the homogenate were plated onto both plain and antibiotic selective CYE agar plates to determine the number of viable bacteria and the ratio of wild-type to mutant bacteria colonizing the lung.

5.18 Immunofluorescence staining and Microscopy

After infections, transfection or a similar assay for microscopy, samples were processed in the following way: Cells were washed three times with PBS before fixing with PFA (3.2%) for 20 min at RT. PFA was removed and washed out three times with PBS and once with 20 mM NH4Cl in PBS for 10 min at RT. For permeabilization 0.1% Triton-X100 was added for 5 min and washed with PBS afterwards. Blocking was performed with 2% BSA for at least 1h. For incubation with the antibodies, drops of 30 µl (antibody in blocking buffer in desired dilution) were added on the top of coverslips and incubated for at least 45 min. Three washing steps with between first and second antibodies were performed. After antibody incubations, cover slips were rinsed 3 times in large volumes of PBS and once with distilled H_2O before mounting them upside down in 5 µl Pro-long anti-fade Gold on glass slides.

5.19 Phagocytosis assay in Cos7 cells

5 x104 Cos7 cells/well are seeded on 13mm glass coverslips in a 24-well plate (10% FCS-DMEM + L-Glutamine). On the next day, the cells are transfected using GeneJuice (Merck) following the manufacturer's instructions. Briefly 97ul Optimem Media are mixed with 3ul transfection reagent and incubated at RT for 5min. A total of 1ug plasmid DNA is added and incubated for 15 min. 500 ul of fresh medium is added to each well (10% FCS-DMEM + L-Glutamine) and 50 ul of transfection mixture / well. Cells are incubated at 37°C 5% CO2 for ~18 h. On the next day, cells are washed 2X with PBS and incubated with Serum Free-DMEM for 2h.

For the IgG opsonisation of beads, labelling buffer is used (Labelling buffer: 20 mM MES, 8 mM HEPES (40 mL H2O, 1 ml 1M MES, 400 ul 1M HEPES)). 10ul of beads slurry per well is used and washed in labelling buffer. Beads are spun down at 13500 rpm for 3 mins and resuspended in 1 mL labelling buffer. For the labelling, 10 uL Antibody Mouse anti-BSA is

used and incubated with the beads at RT with rotation for 1 hour. After that, the beads are washed again, spun down at 13500 rpm and resuspended in 1 mL labelling buffer. The beads get spun down again and resuspend in SF-DMEM. To challenge the cells with opsonised beads (in SF-DMEM with Hepes 10mM), add the beads and spin at 500 g for 5 mins. Place the cells at 4°C for 15 min to allow binding. The bead suspension is removed after that and 500 ul of fresh serum containing media is added. The cells are incubated at 37 °C 5% CO2 for 90min. After that, cells are washed 1X cold PBS on ice (tissue culture PBS).

For the staining, the general immunofluorescence staining protocol is followed.

The beads are coated with an antibody Mouse anti-BSA. For external bead staining incubate coverslips for 7 mins on ice with Ab Donkey anti-mouse RRX (Red) 250 ul/cvs 1:500 in 0.2% BSA/PBS. Then cells are washed 1X with cold PBS. After that, cells are fixed with PFA 4% 25 min at room temperature and then washed 3X PBS (Samples can be left overnight at 4°C at this stage).

The samples are incubated with 50 mM NH4Cl PBS for ~15 min and permeabilized with Triton X100 0.2% in PBS for 2min. Immediately after that, the samples are washed 3X with PBS and incubated with 0.2% BSA PBS for 15min. Samples are incubated with 25 ul/cvs for 45 mins in 0.2% BSA/PBS Ab 1:50 Donkey anti-mouse AMCA (for internal beads staining) and 1:200 Chicken anti-myc primary (for effector staining). Samples are washed 3X with PBS. Samples are incubated in 0.2% BSA/PBS for 5-10 minutes. For secondary antibody staining, samples are incubated with 25 ul/cvs for 45 mins in 0.2% BSA/PBS containing 1:100 Goat anti-chicken Alexa 647 and then washed 3X with PBS. Coverslips are mounted on slides with Antifade Gold and dried overnight, then stored at 4°C.

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Appendices

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Figure 3 Transmission electron microscopy of endothelial cells infected with *L. pneumophila*

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