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Characterisation of the *Legionella pneumophila* Effector RidL

Dissertation

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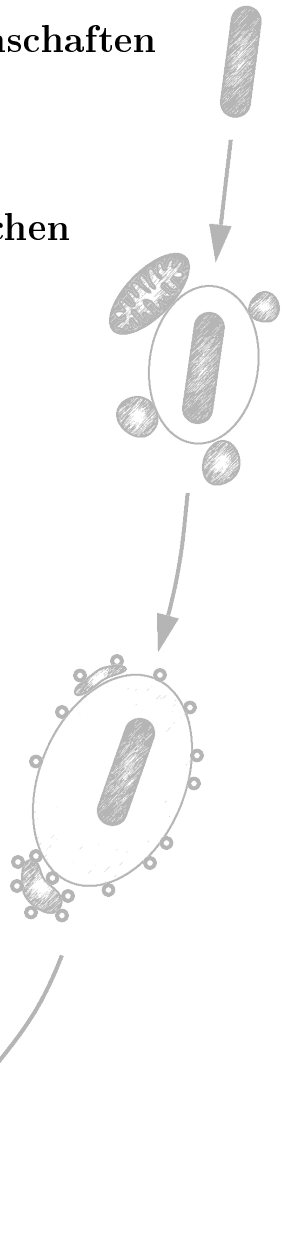

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Contents

List of Abbreviations

AIDS	acquired immunodeficiency syndrome
Amp	Ampicillin
AYE	ACES yeast extract
BAR	Bin-Amphiphysin-Rvs
Cam	Chloramphenicol
CDMPR	cation-dependent mannose 6-phosphate receptor
CFU	colony forming units
CIMPR	cation-independent mannose 6-phosphate receptor
CNX	calnexin
CTx	cholera toxin
CTxB	cholera toxin subunit B
CYE	charcoal yeast extract
DAG	diacylglycerol
Dot	defective organelle trafficking
EE	early endosome
EHEC	enterohemorrhagic *Escherichia coli*
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
GAP	GTPase-activating protein
GARP	Golgi-associated retrograde protein
Gb3	globotriaosylceramide
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GEF	guanine nucleotide exchange factor
GGA	Golgi-localised, γ-adaptin ear-containing, ARF-binding protein
GLC	Golgi-like compartment
GM1	glycolipid monosialotetrahexosylganglioside
HIV	human immunodeficiency virus
HPV	human papillomavirus
HRPO	horseradish peroxidase
HVS	herpesvirus saimiri

Icm	intracellular multiplication
IL-2	Interleukin-2
IPTG	isopropyl-1-thio-β-D-galactopyranoside
Kan	Kanamycin
Lck	Lymphocyte-specific protein tyrosine kinase
LCV	*Legionella*-containing vacuole
LE	late endosome
LPS	lipopolysaccharide
Lqs	*Legionella* quorum sensing
LYS	lysosome
MHC	major histocompatibility complex
MOI	multiplicity of infection
MPR	mannose 6-phosphate receptor
Neo	Neomycin
OD_{600}	optical density at 600 nm
PBS	phosphate-buffered saline
PC	phosphocholine
PEx	*Pseudomonas aeruginosa* exotoxin A
PFA	paraformaldehyde
PI	phosphoinositide
PI3K	phosphatidylinositol 3-kinase
PI4KIIIβ	phosphatidylinositol 4-kinase IIIβ
PKC	protein kinase C
PKD	protein kinase D
PM	plasma membrane
PtdIns	phosphatidylinositol
PX	phox
R	resistant
RAB6IP1	Rab6-interacting protein-1
RAB6IP2	Rab6-interacting protein-2
RBS	ribosomal binding site
RE	recycling endosome
RidL	retromer interactor decorating LCVs

SCV	*Salmonella*-containing vacuole
SIF	*Salmonella*-induced filament
SKIP	SifA- and kinesin-interacting protein
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors
SNX	sorting nexin
SPI-1	*Salmonella* pathogenicity island 1
SPI-2	*Salmonella* pathogenicity island 2
STx	Shiga toxin
STxA	Shiga toxin subunit A
STxB	Shiga toxin subunit B
STx1	Shiga-like toxin 1
STx2	Shiga-like toxin 2
SVAT	spacious vacuole-associated tubule
SV40	Simian virus 40
TBS	Tris-buffered saline
TCR	T-cell receptor
TEN	tubular endosomal network
TfR	transferrin receptor
Tip	tyrosine kinase-interacting protein
TIP47	tail-interacting protein of 47 kDa
TGN	*trans*-Golgi network
t-SNARE	target-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors
T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4SS	type IV secretion system
T5SS	type V secretion system
Vps	vacuolar protein sorting
v-SNARE	vesicle-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors

List of Publications

Work described in this thesis has been published previously:

Finsel, I.*, Ragaz, C.*, Hoffmann, C., Harrison C., Weber S., van Rahden, V., Johannes, L., Hilbi, H. (2013), The *Legionella* effector RidL inhibits retrograde trafficking to promote intracellular replication. *Cell Host & Microbe* Jul 17;14(1):38-50.

Urwyler, S., Finsel, I., Ragaz C, Hilbi, H. (2010), Isolation of *Legionella*-containing vacuoles by immuno-magnetic separation. *Curr Protoc Cell Biol* Mar, Chapter 3: Unit 3.34.1-3.34.14

Hoffmann, C., Finsel, I., Hilbi, H. (2012), Purification of pathogen vacuoles from *Legionella*-infected phagocytes. *J Vis Exp* Jun 19;(64);e4118

Hoffmann, C., Finsel, I., Hilbi, H. (2013), Pathogen vacuole purification from *Legionella*-infected amoeba and macrophages. *Methods Mol Biol* 954: 309-21.

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Other publications during the time of dissertation:

Hilbi, H., Weber, S., Finsel, I. (2011), Anchors for effectors: Subversion of phosphoinositide lipids by *Legionella*. *Front Microbiol* 2: 91

Hoffmann, C., Finsel, I., Otto, A., Pfaffinger, G., Rothmeier, E., Hecker, M., Becher, D., Hilbi, H. (2013), Functional analysis of novel Rab GTPases identified in the proteome of purified *Legionella*-containing vacuoles from macrophages. *Cell Microbiol* Dec 26; doi: 10.1111/cmi.12256. [Epub ahead of print]

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Summary

The Gram-negative bacterium *Legionella pneumophila* naturally parasitises environmental amoebae, but is also able to infect human alveolar macrophages in a mechanistically similar manner. This can result in the mild "Pontiac fever", a flu-like illness, or a potentially lethal pneumonia termed "Legionnaires' disease". Crucial for establishing an intracellular replication niche is the Icm/Dot type IV secretion system (T4SS), which translocates approximately 300 different "effector" proteins into the host cell. These substrates enhance uptake efficiency into phagocytes and direct formation of a replication-permissive compartment, called the *Legionella*-containing vacuole (LCV), and ultimately the egress of the bacteria. Some of the effectors interfere with small GTPases, phosphoinositide metabolism or the ubiquitination machinery, and modulate host cell signalling and vesicle trafficking.

We developed a method to isolate intact LCVs by using immuno-magnetic separation with an LCV-specific antibody followed by density gradient centrifugation. Proteomic analysis of the purified phagosomes together with findings of previous studies showed, that the vacuoles harbour markers of the endosomal network, associate with mitochondria, early secretory vesicles and the endoplasmic reticulum, but avoid fusion with lysosomes.

Our investigations of the novel *L. pneumophila* effector RidL revealed that the LCV also communicates with the retrograde vesicle trafficking pathway of infected cells. This pathway recycles amongst others acid-hydrolase receptors, such as the cation-independent mannose 6-phosphate receptor (CIMPR), from the tubular endosomal network back to the *trans*-Golgi. This transport requires the multiprotein "retromer" complex, which consists of two major subunits: the heterotrimeric cargo-selective subcomplex comprising the proteins Vps26, Vps29 and Vps35 and the membrane-deforming heterodimeric subcomplex composed of any combination of the phosphoinositide (PI)-binding sorting nexins SNX1 or SNX2 plus SNX5 or SNX6.

Pull-down experiments with lysates of RAW 264.7 macrophages or *D. discoideum* amoebae revealed Vps26, Vps29 and Vps35 to be retained by the then uncharacterised protein RidL, which represented an intriguing, novel effector interaction. Like most T4SS substrate mutants, *L. pneumophila* lacking *ridL* showed no phenotype for growth in liquid AYE medium and uptake into phagocytes compared to wild-type bacteria. However, intracellular replication was strongly impaired for the mutant strain in several host cell lines. RidL is preferentially expressed in the late post-exponential growth phase and translocated in an T4SS-dependent manner at early time-points of the infection, suggesting a role shortly after the uptake of the bacteria. The effector exhibited a

bipolar localisation on the LCV membrane, but upon overexpression the protein covered the entire vacuole. Interestingly, RidL bound the lipid phosphatidylinositol 3-phosphate (PtdIns(3)P), a known eukaryotic endosomal membrane anchor, and also specifically bound to the retromer subunit Vps29. Although the protein had no effect on the acquisition of Vps26, Vps29 and Vps35, the percentage of LCVs positive for the retrograde cargo receptors CIMPR or sortilin was reduced in presence of RidL, suggesting interference with the retrograde transport pathway. Furthermore, significantly less SNX1- and SNX2-positive LCVs were detected in cells infected with wild-type *L. pneumophila* compared to the *ridL* mutant strain. Moreover, RidL competed with SNX1 for binding at PtdIns(3)P-positive membranes. To directly examine the influence of RidL on retrograde trafficking, the retromer-dependent transport of cholera and Shiga toxin inside cells was analysed in macrophages infected with wild-type or Δ*ridL L. pneumophila*, and in HeLa cells ectopically producing RidL, respectively. In both cases, the trafficking was inhibited by RidL, and for cholera toxin the transport was arrested at the endosomal stage. In line with these findings, siRNA knockdown experiments revealed that a functional retrograde pathway restricted intracellular growth of *L. pneumophila*.

Taken together, we postulate that RidL (Retromer interactor decorating LCVs) inhibits retrograde trafficking at endosomes by binding to the retromer subunit Vps26 and/or by competition with sorting nexins, thus promoting intracellular replication of *L. pneumophila*.

Collectively, the results obtained in this thesis shed light on the host factor composition of LCVs and provide mechanistic insights into a novel *L. pneumophila* effector protein.

Zusammenfassung

Das Gram-negative Bakterium *Legionella pneumophila* parasitiert normalerweise Umweltamöben, kann aber auch humane Alveolarmakrophagen in mechanistisch ähnlicher Weise infizieren. Dies kann zu dem leichten "Pontiac"-Fieber, einer grippeähnlichen Krankheit, oder zu einer potentiell tödlichen Pneumonie, "Legionärskrankheit" genannt, führen. Entscheidend für die intrazelluläre Replikation ist das Icm/Dot Typ IV Sekretionssystem (T4SS), welches schätzungsweise 300 verschiedene "Effektor"-Proteine in die Wirtszelle transloziert. Diese erhöhen die Aufnahmeeffizienz in Phagozyten und steuern die Bildung eines replikationspermissiven Kompartiments, der sogenannten Legionellen-enthaltenden Vakuole (LCV), und schlussendlich die Freisetzung der Bakterien. Einige der Effektoren wirken auf kleine GTPasen, den Phosphoinositidmetabolismus oder den Ubiquitinylierungsapparat und modulieren Signalwege und den Vesikeltransport der Wirtszelle.

Wir haben eine Methode entwickelt, intakte LCVs durch immuno-magnetische Auftrennung mit Hilfe eines LCV-spezifischen Antikörpers und anschließender Dichtegradientenzentrifugation zu isolieren. Die Proteomanalyse der aufgereinigten Phagosomen zeigten zusammen mit Ergebnissen früherer Studien, dass die Vakuolen Marker des endosomalen Netzwerkes tragen und sich mit Mitochondrien, frühen sekretorischen Vesikeln und dem endoplasmatischen Retikulum assoziieren, aber eine Fusion mit Lysosomen verhindern.

Unsere Untersuchungen des neuartigen *L. pneumophila* Effektors RidL deckten auf, dass die LCV zusätzlich auch mit dem retrograden Vesikeltransport von infizierten Zellen interagiert. Dieser Transportweg führt unter anderem Rezeptoren von sauren Hydrolasen, wie den kationenunabhängigen Mannose-6-Phosphat-Rezeptor (CIMPR), vom tubulären endosomalen Netzwerk zum *trans*-Golgi zurück. Dieser Rücktransport benötigt den Multiproteinkomplex "Retromer", der aus zwei Hauptuntereinheiten besteht: dem heterotrimären, frachtselektiven Unterkomplex, der die Proteine Vps26, Vps29 und Vps35 umfasst, und dem membrandeformierenden, heterodimären Unterkomplex bestehend aus einer beliebigen Kombination der Phosphoinositid-bindenden Sorting Nexine SNX1 oder SNX2 plus SNX5 oder SNX6.

Co-Immmunopräzipitationsexperimente mit Lysaten von RAW 264.7 Makrophagen oder *D. discoideum* Amöben offenbarten, dass Vps26, Vps29 und Vps35 von dem damals uncharakterisierten Protein RidL gebunden wurden, was eine neuartige Effektorinteraktion darstellte. Wie die meisten Effektormutanten zeigte auch *L. pneumophila*, dem *ridL* fehlte, verglichen mit dem Wildtyp keinen Phänotyp für Wachstum in flüssigem AYE-Medium oder Aufnahme in Phagozyten. Allerdings war die intrazel-

luläre Replikation für den Mutantenstamm in mehreren Zelllinien stark beeinträchtigt. RidL wird bevorzugt in der späten post-exponentiellen Wachstumsphase exprimiert und zu einem frühen Zeitpunkt der Infektion T4SS-abhängig transloziert, was eine Funktion kurz nach Aufnahme der Bakterien nahelegt. Der Effektor lokalisierte bipolar auf der LCV-Membran, bedeckte aber bei Überexpression die gesamte Vakuole. Interessanterweise band RidL an das Lipid Phosphatidylinositol-3-Phosphat (PtdIns(3)P), einem bekannten eukaryotischen, endosomalen Membrananker, und außerdem band es spezifisch an die Retromeruntreinheit Vps26. Obwohl das Protein keinen Effekt auf die LCV-Lokalisation von Vps26, Vps29 und Vps35 hatte, war der Anteil an LCVs, die positiv für die retrograden Frachtrezeptoren CIMPR und Sortilin waren, in Anwesenheit von RidL niedriger, was auf eine Störung im retrograden Transportweg hindeutete. Weiterhin wurden signifikant weniger SNX1- und SNX2-positive LCVs in Zellen ermittelt, die, im Vergleich zu *ridL*-Mutanten, mit Wildtyp *L. pneumophila* infiziert wurden. Überdies konkurrierte RidL mit SNX1 um die Bindung an PtdIns(3)P-positive Membranen. Um den Einfluss von RidL auf den retrograden Transportweg direkt zu prüfen, wurde die retromerabhängige Beförderung von Cholera- und Shigatoxin in Zellen untersucht. Dazu wurden Makrophagen mit Wildtyp oder Δ*ridL L. pneumophila* infiziert, oder der Toxintransport in HeLa-Zellen untersucht, die RidL ektopisch produzieren. In beiden Fällen wurde der retrograde Transport durch RidL unterbrochen und, wie für Choleratoxin gezeigt, auf der endosomalen Stufe gestoppt. Folgerichtig zeigten RNAi-Interferenzexperimente, dass ein funktionierender retrograder Lieferweg intrazelluläres Wachstum von *L. pneumophila* einschränkt.

Wir postulieren daher, dass RidL den retrograden Transportweg durch Bindung an die Retromeruntereinheit Vps26 und/oder durch Konkurrieren mit Sorting Nexinen an den Endosomen inhibiert und auf diese Weise intrazelluläre Replikation von *L. pneumophila* fördert.

Zusammengefasst tragen die Resultate dieser Doktorarbeit zu einem umfassenderen Verständnis der LCV-Zusammensetzung bei, sowie zu einem mechanistischen Verständnis eines neuartigen *L. pneumophila* Effektors.

1 Introduction

1.1 *Legionella pneumophila*: Pathogen & Model Organism

1.1.1 Natural Habitats and Epidemiological Relevance

At a convention of the American Legion in Philadelphia in 1976, a thus far unidentified lung disease sickened over 200 people, killing every sixth. This severe pneumonia was termed "Legionnaires' disease" caused by the soon identified *Legionella pneumophila* (Fraser *et al.*, 1977; McDade *et al.*, 1977). The Gram-negative bacterium naturally replicates in free-living protozoa, making *L. pneumophila* a primarily waterborne parasite (Rowbotham, 1980). However, the main portion of the bacteria is not free-living but associated with complex preexisting aquatic biofilms formed e.g. by species such as *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* (Mampel *et al.*, 2006; Murga *et al.*, 2001; Rogers *et al.*, 1994). Several species of amoebae have been shown to support intracellular replication of *L. pneumophila* and are thus used as model systems, including *Acanthamoeba castellanii*, *Hartmanella vermiformis* or *Dictyostelium discoideum* (Fields, 1996; Hägele *et al.*, 2000; Solomon *et al.*, 2000). In contrast, extracellular growth of the fastidious bacteria could only be demonstrated with special laboratory media (Feeley *et al.*, 1979).

Co-evolution of *Legionella* and its natural hosts has been proposed to select for virulence features, which are also required for survival in other cell types (Brüggemann *et al.*, 2006; Greub and Raoult, 2004), including human alveolar macrophages, the cells affected during Legionnaires' disease (Nash *et al.*, 1984). The relatively recent emergence of this illness has two major causes: Firstly, inhalation of contaminated aerosols is facilitated by anthropogenic water-supplies, e.g. cooling towers, showers, whirlpools or spas (Fields, 1996). Secondly, as an opportunistic pathogen, *L. pneumophila* benefits from rising numbers of immuno-compromised people as a result of demographic change and increased survival chance after high-risk surgeries (Länger and Kreipe, 2011; Marston *et al.*, 1994). Infection is not necessarily fatal, but may lead to the flu-like "Pontiac fever" instead of the Legionnaires' pneumonia (Glick *et al.*, 1978).

L. pneumophila is the best studied species of the family *Legionellaceae*, which consists of the single genus *Legionella* and whose members are described as obligate aerobic, mono-flagellated γ-proteobacteria (Benson *et al.*, 1998; Fry *et al.*, 1991). There are several different subtypes of the species, e.g. the original isolate from the first outbreak "Philadelphia 1" or the France-endemic strain "Paris", which are responsible for the vast majority of legionellosis cases (Victor *et al.*, 2002).

L. pneumophila utilises an impressive array of different mechanisms and translocated factors (**Section 1.1.2**) to ensure intracellular growth in its host cells. After uptake (**Section 1.1.3**), the bacteria evade lysosome fusion (**Section 1.1.4**) and form an ER-like, replication-permissive vacuole, the *Legionella*-containing vacuole (LCV) (**Section 1.1.5**). At the end of this process the pathogens are released from the cell (**Section 1.1.6**) and can begin a new infection cycle (Hubber and Roy, 2010).

Identification and analyses of the factors facilitating host cell subversion contribute not only to our comprehension of the pathogenicity of the bacterium, but also allow insights into eukaryotic cell signalling and trafficking turning *Legionella pneumophila* into a valuable model organism.

1.1.2 The Icm/Dot T4SS

One of the key components determining *L. pneumophila* virulence is its Icm/Dot (intracellular multiplication/defective organelle trafficking) type IV secretion system (T4SS). This translocation machinery is ancestrally related to systems conjugating genetic material between bacteria (Christie, 2001). Although *L. pneumophila* retained the complex's ability to exchange plasmids with other prokaryotes, the main function has become the transfer of virulence factors, so called "effector" proteins, directly into the host cell (Burns, 2003; Vogel *et al.*, 1998).

The T4SS consists of twenty-seven proteins forming two large subcomplexes (Vincent *et al.*, 2006, 2012) that span the inner and outer bacterial membrane enabling the penetration of the eukaryotic plasma membrane for effector delivery (Burns, 2003). The transmembrane subcomplex comprises many proteins including the inner membrane proteins IcmG (DotF) and IcmE (DotG) and two outer membrane lipoproteins DotC and DotD, which recruit the pore forming complex IcmK (DotH) (Vincent *et al.*, 2006). The second subcomplex, the DotL type IV coupling protein complex, is found at the inner membrane and consists of DotL (IcmO), DotM (IcmP), DotN (IcmJ), IcmS and IcmW (Vincent *et al.*, 2006). The proteins interact with substrates prior to their translocation, coupling them to the secretion system (Vincent *et al.*, 2012). The best

studied examples are the so called chaperone-like "type IV adaptors" IcmS and IcmW, whose initial binding is required for the delivery of several effectors (Cambronne and Roy, 2007; Ninio *et al.*, 2005).

Numerous studies on *L. pneumophila* focused on the identification of effector proteins. Techniques applied for this purpose include the investigation of a C-terminal secretion signal (Kubori *et al.*, 2008; Nagai *et al.*, 2005), interaction with T4SS components (Ninio *et al.*, 2005), presence of coregulators (Altman and Segal, 2008; Zusman *et al.*, 2007, 2008), interaction with host components (Murata *et al.*, 2006), genetic screenings in yeast (de Felipe *et al.*, 2008; Heidtman *et al.*, 2009; Shohdy *et al.*, 2005) and *in silico* methods like screens for genes with eukaryotic-like domains (Brüggemann *et al.*, 2006; Cazalet *et al.*, 2004; de Felipe *et al.*, 2005; Pan *et al.*, 2008) or a machine learning approach (Burstein *et al.*, 2009). Verification of secretion was accomplished by various translocation assays and/or use of specific antibodies (de Felipe *et al.*, 2008; Luo and Isberg, 2004; Nagai *et al.*, 2005).

Currently, approximately 300 different effectors have been identified. It is hypothesised that this number is unusually high due to the host diversity *L. pneumophila* encounters in its natural environment, resulting in functional redundancy of effectors and considerably complicating scientific analyses (O'Connor *et al.*, 2011).

In addition to the T4SS, *L. pneumophila* also possesses an Lss type I secretion system (T1SS), an Lsp type II secretion system (T2SS), another type IV secretion system called Lvh (Lvh T4SS) and a type V secretion system (T5SS) only found in the Paris strain (De Buck *et al.*, 2007). Little is known about the T1SS and T5SS, but the T2SS and the Lvh T4SS seem to be important for infection of mammalian lungs or for infections by *L. pneumophila* grown at lower temperatures, respectively (Ridenour *et al.*, 2003; Rossier *et al.*, 2004). Some T2SS and several T4SS substrates are expanded upon in the following sections and an overview of a complete intracellular infection cycle is depicted in **Figure 1.1**.

1.1.3 Entry into Host Cells

L. pneumophila can actively stimulate actin-mediated, macropinocytotic internalisation into professional phagocytes dependent on its Icm/Dot T4SS, e.g. bacteria defective for this system were taken up less efficiently by amoeba and HL-60 cells compared to wild-type bacteria (Hilbi *et al.*, 2001; Lu and Clarke, 2005; Watarai *et al.*, 2001). Additionally, Wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, blocks phagocytosis of an *icm/dot* mutant, but not of the wild-type strain, suggesting different uptake mechanisms (Khelef *et al.*, 2001).

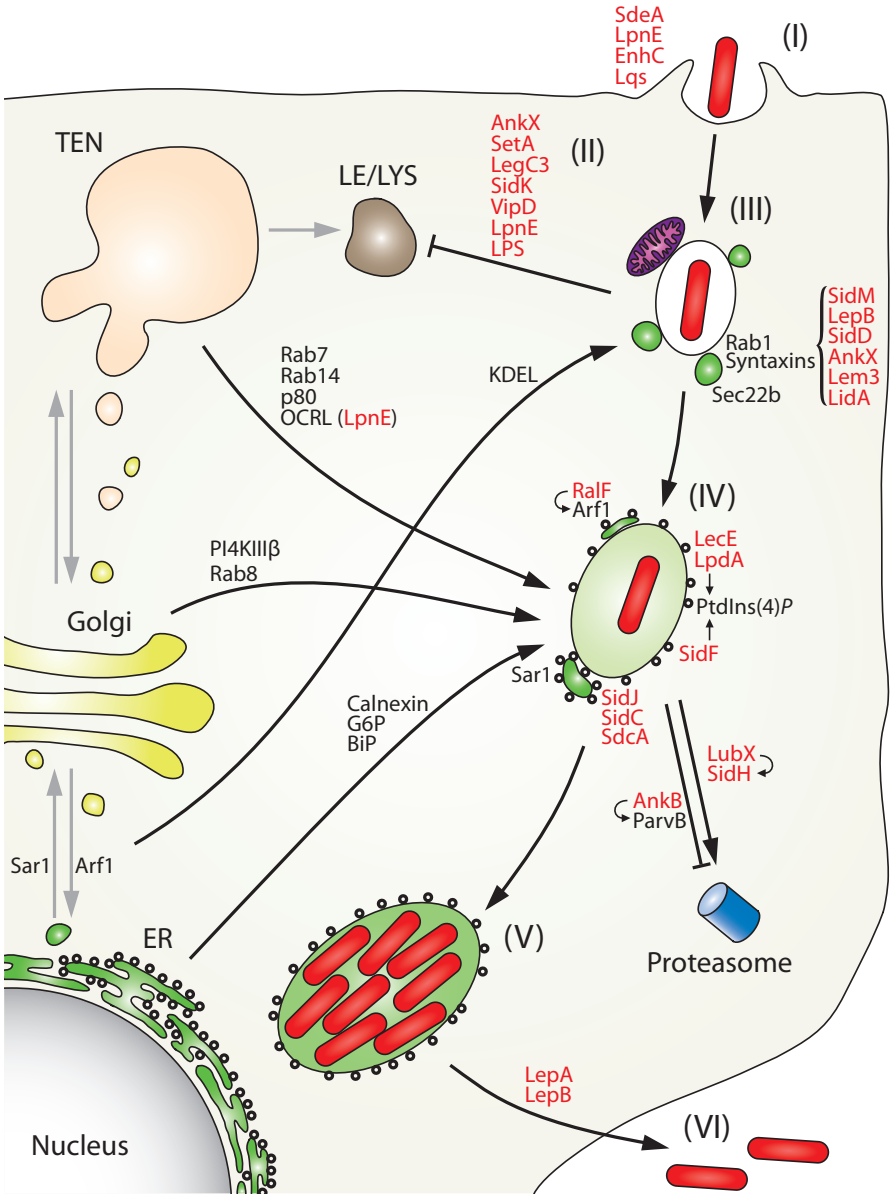

Figure 1.1: Intracellular infection cycle of *L. pneumophila*. The bacteria promote their own uptake into cells with the Icm/Dot T4SS (I; **Section 1.1.3**), communicate extensively with endosomes (TEN = tubular endosomal network) and immediately block fusion with lysosomes (LYS) (II; **Section 1.1.4**). Merging with ER exit site vesicles (III) precedes the association of the LCV with rough ER-derived vesicles and interactions with the proteasome (IV), enabling *L. pneumophila* to replicate (V; **Section 1.1.5**). Ultimately, the bacteria escape their host cell (VI; **Section 1.1.6**). Virulence factors of *L. pneumophila* are shown in red.

One of the relevant effectors for internalisation is SdeA (LaiA), which has been shown to be important for adhesion and uptake, although its mode of action has not yet been determined (Bardill *et al.*, 2005; Chang *et al.*, 2005). T4SS-independent virulence factors, like LpnE or the mostly periplasmic EnhC, also play a role in efficient internalisation, as these proteins were shown to enhance invasiveness by yet unknown mechanisms (Liu *et al.*, 2008; Newton *et al.*, 2006) (**Figure 1.1**). Furthermore, cell-to-cell signalling via small molecules, produced and detected by the Lqs (*Legionella* quorum sensing) system, contributes to efficient uptake and is also important for intracellular replication (Kessler *et al.*, 2013; Tiaden and Hilbi, 2012). Interestingly, although the pathogen encounters only phagocytic host cells in its natural habitat, the filamentous morphotype of *L. pneumophila* has been reported to promote entry into non-phagocytic lung epithelial cells (Prashar *et al.*, 2012).

1.1.4 Evasion of Phagolysosome Fusion

After uptake *L. pneumophila* resides in its LCV and immediately arrests the "default" phagolysosome fusion in an Icm/Dot T4SS-dependent manner to avoid degradation (Roy *et al.*, 1998). Several T4SS effectors have been identified to contribute to this important step, e.g. AnkX, which interferes with microtubule-dependent vesicular trafficking, but does not alter the microtubule network itself (Pan *et al.*, 2008). It has been shown that the effector phosphocholinates the GTPase Rab35, a regulator in the endocytic pathway, hereby altering its activity (Mukherjee *et al.*, 2011). Furthermore, glycosylation, another post-translational modification, performed by the effector SetA, is proposed to influence a currently unknown component of the endocytic trafficking machinery (Heidtman *et al.*, 2009) (**Figure 1.1**). Additionally, the translocated factor LegC3 interferes with the lysosomal route supposedly by inhibiting fusion of endosomes and lysosomes, thus protecting the bacteria from degradation (Bennett *et al.*, 2013; de Felipe *et al.*, 2008). Acidification of the phagosome, a prerequisite for bacterial lysis, is inhibited by the T4SS substrate SidK, which specifically targets VatA, a key component of the host multi-subunit V-ATPase, which is responsible for establishing a proton gradient (Horwitz and Maxfield, 1984; Xu *et al.*, 2010). Further evidence that communication with the endocytic pathway is not completely inhibited, is that the late endosomal/lysosomal GTPase Rab7 is present on LCV membranes as well as other endosomal markers like Rab14 or the copper transporter p80 (Urwyler *et al.*, 2009). Finally, the phospholipase VipD binds to the activated GTPases Rab5 and Rab22, thus preventing their interaction with downstream target proteins and consequently blocking endosomal trafficking (Ku *et al.*, 2012; Shohdy *et al.*, 2005).

However, not all bacterial factors contributing to lysosomal evasion rely on the Icm/Dot secretion system, e.g. the aforementioned LpnE, although its translocation mechanism remains undiscovered (Newton *et al.*, 2007). *L. pneumophila* can also release membrane vesicles containing lipopolysaccharide (LPS) into the phagosome, thereby inhibiting lysosomal fusion (Fernandez-Moreira *et al.*, 2006).

1.1.5 Establishing a Replication-permissive Vacuole

While avoiding lysosomal degradation, *L. pneumophila* simultanously begins modifying the LCV to a replication-permissive compartment resembling the endoplasmic reticulum (ER) (Horwitz, 1983; Tilney *et al.*, 2001). The remodelling process takes several hours and includes two timed and distinct steps. Firstly, mitochondria are found to associate with the pathogen vacuole shortly after uptake into the host cell (Horwitz, 1983). Additionally, the LCV fuses with smooth ER exit site vesicles, shown by colocalisation of the ER-Golgi cycling signal peptide KDEL (Kagan and Roy, 2002).

However, a few hours after infection the frequency of mitochondria and ER exit site vesicle association on the pathogen vacuole decreases, whereas ribosome appearance increases (Horwitz, 1983; Swanson and Isberg, 1995). The observation of ribosomes on LCVs coincides with colocalisation of ER specific proteins, like the enzyme glucose 6-phosphatase or the chaperones calnexin or BiP (Kagan and Roy, 2002; Robinson and Roy, 2006; Swanson and Isberg, 1995). This suggests that the ribosomes originate not from the cytoplasmic pool, but are originally ER associated, indicating a fusion of the LCV with rough ER membranes (**Figure 1.1**). Initial interaction with the ER exit site vesicles is assumed to prepare the LCV for ER association, resulting in initiation of bacterial replication 4-6 h post infection with a bacterial doubling time of about 2h (Horwitz and Silverstein, 1980; Tilney *et al.*, 2001).

The process of LCV formation has to be coordinated spatiotemporally. One of the factors determining site-specific spatial organisation are phosphoinositides (PIs), differentially phosphorylated derivatives of phosphatidylinositol (PtdIns). Among others, these lipids contribute to organelle identity and regulate membrane trafficking by providing binding sites to host regulatory proteins (Di Paolo and De Camilli, 2006). However, PIs are also exploited by *L. pneumophila* proteins as binding targets for effectors (Hilbi *et al.*, 2011). The LCV membranes, for instance, harbour PtdIns(4)*P*s, a PI normally associated with the secretory pathway. In addition to providing a binding target, enrichment in PtdIns(4)*P* may contribute to a resemblance of the phagosome to a *cis*-Golgi network, facilitating acquistion of ER vesicles (Weber *et al.*, 2006).

It has been reported that host enzymes are involved in PI modulation on LCVs

and therefore effector binding. The most prominent examples are the *D. discoideum* PtdIns(4,5)P_2 5-phosphatase Dd5P4 and its mammalian homologue OCRL, which binds the effector LpnE, and the host PtdIns 4-kinase IIIβ (PI4KIIIβ), which might contribute to PtdIns(4)P levels on LCVs (Brombacher *et al.*, 2009; Weber *et al.*, 2009). Also of note and consistent with its role in the lysosomal pathway, inhibition of the PI metabolising PI3Ks increases intracellular replication of *L. pneumophila* and survival of a T4SS defective mutant (Weber *et al.*, 2006).

Furthermore, the two LCV-localising T4SS substrates LecE and LpdA have been shown to increase the level of diacylglycerol (DAG) on the vacuole. This leads to recruitment of protein kinase C (PKC) and protein kinase D (PKD) and subsequent activation of PKD by PKC. This in turn facilitates the binding of PI4KIIIβ generating PtdIns(4)P (Viner *et al.*, 2012). Additionally, through the recently identified effector SidF, a phosphatidylinositol polyphosphate 3-phosphatase, *L. pneumophila* can directly enrich the LCVs in PtdIns(4)P (Hsu *et al.*, 2012).

Normally, in host cells the localisation of the different PI compounds is often controlled by lipid kinases/phosphatases recruited by small GTPases of the Rab family. GTPases are key regulators of vesicle transport and can switch between a GDP-bound, inactive conformation and a GTP-bound, active conformation, which interacts with downstream target proteins (Stenmark, 2009). Guanine nucleotide exchange factors (GEFs) catalyse incorporation of GTP, whereas GTPase-activating proteins (GAPs) prompt hydrolysis of bound GTP to GDP thus inactivating the GTPase. GDP dissociation inhibitors (GDIs) prevent the release of GDP, thereby stabilising the inactive state, and are in turn themselves targeted by GDI displacement factors (GDFs).

A well-studied example of the interference of GTPase activity by *L. pneumophila* effectors is Rab1, which mediates ER-Golgi transport and fusion and can be detected on LCVs minutes after uptake (Kagan *et al.*, 2004) (**Figure 1.2**). The GTPase is recruited by the PtdIns(4)P-binding Icm/Dot substrate SidM (DrrA), which is a Rab GEF as well as a GDF, consequently activating Rab1 (Brombacher *et al.*, 2009; Machner and Isberg, 2006, 2007; Murata *et al.*, 2006; Schoebel *et al.*, 2009). Inactivation in turn is caused by the effector LepB, a Rab GAP, resulting in Rab1 dissociation from LCV membranes (Gazdag *et al.*, 2013; Ingmundson *et al.*, 2007). However, SidM can also covalently attach an AMP to Rab1, subsequently blocking the interaction with LepB and prolonging GTPase activation (Muller *et al.*, 2010). The effector SidD is responsible for de-AMPylation, thus reverting Rab1 into its original state (Neunuebel *et al.*, 2011; Tan *et al.*, 2011). Recently, it has been shown that the T4SS substrate AnkX also modifies Rab1 by phosphocholination, similar to Rab35 as mentioned in **Section 1.1.4**, thus modulating the secretory pathway (Mukherjee *et al.*, 2011). Again, there

is an antagonistic process, de-phosphocholination, which is catalysed by the effector Lem3 (Tan *et al.*, 2011). These post-translational modifications have also been shown to inhibit GDI re-association with Rab GTPases after their spontaneous dissociation. AnkX is even able to modify inactive Rab1, thus enabling the incorporation of the GDP-bound version into membranes, where the de-phosphocholination step by Lem3 is required to enable Rab1 activation by a GEF (Mukherjee *et al.*, 2011; Oesterlin *et al.*, 2012). Additionally, the PtdIns(3)*P*-binding T4SS substrate LidA interacts with activated Rab1, thereby arresting the GTPase in its active state and thus supporting the GEF activity of SidM (Brombacher *et al.*, 2009; Machner and Isberg, 2006; Neunuebel *et al.*, 2012). The requirement of Rab1 for the transport of ER vesicles to LCVs may provide a rationale for the bacterial efforts of manipulating this GTPase, whose overall function is crucial for intracellular replication (Kagan *et al.*, 2004).

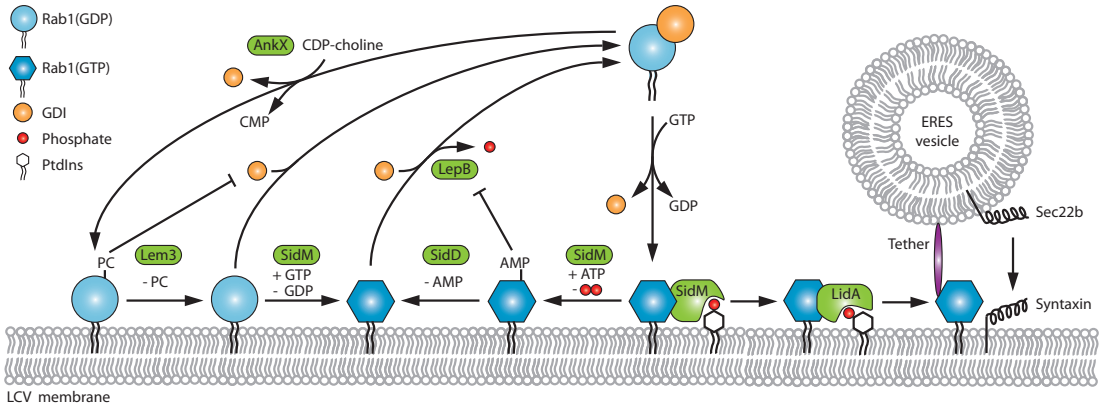

Figure 1.2: Regulation of Rab1 by *L. pneumophila* effectors on LCVs. Rab1 can be activated and recruited to the LCV by the PtdIns(4)*P*-binding effector SidM. Dissociation of the GTPase is facilitated by LepB and can be inhibited by SidM-catalysed AMPylation, a modification which itself can be reversed by SidD. AnkX covalently attaches a phosphocholine (PC) to active or inactive (depicted here) Rab1, hereby preventing its re-association with the GDI after spontanous dissociation. Lem3 mediates the required de-phosphocholination restoring GEF accessibilty and enabling Rab activation. The active GTPase can also be bound by the PtdIns(3)*P*-binding LidA and interacts with tethering factors for ER exit site vesicle docking.

In the current model for the recruitment of ER vesicles, active Rab1 stimulates the tethering of ER-derived vesicles with the LCV, resulting in membrane fusion. This is mediated by a noncanonical interaction of the ER exit site vesicle membrane protein Sec22b and plasma membrane originating syntaxins localising to the LCV (Derré and Isberg, 2004; Kagan *et al.*, 2004). These proteins are so-called soluble *N*-ethylmaleimide-

sensitive factor attachment protein receptors (SNAREs) and merge membranes by forming a tight complex among each other and thus between the opposing compartments. The noncanonical pairing between the plasma membrane and the phagosome contributes to the establishment of a replication permissive vacuole (Arasaki *et al.*, 2012). Interestingly, sequence analysis revealed some *L. pneumophila* proteins to have SNARE-like domain organisations themselves, e.g. the above mentioned IcmG (DotF) or LegC3, which are both able to block membrane fusion (Bennett *et al.*, 2013; Paumet *et al.*, 2009).

The second stage of the biphasic LCV maturation, namely the association with ER membranes, involves the GTPase Arf1, which mediates host cargo transport between the *cis*-Golgi and the ER by directing vesicle coat assembly (Donaldson and Jackson, 2011). RalF, the first identified *L. pneumophila* T4SS effector, is an Arf1 GEF and required for the recruitment of the GTPase to the LCV (Nagai *et al.*, 2002). Arf1 seems to be specifically necessary for the fusion process with the pathogen vacuole, whereas Sar1, also a GTPase in the secretory pathway, is required for the initial tethering of membranes (Robinson and Roy, 2006). Infection of cells expressing a dominant negative form of Arf1, reduction of Arf1 levels by chemical inhibitors or siRNA knockdown inhibited intracellular growth of *L. pneumophila* (Dorer *et al.*, 2006; Kagan and Roy, 2002). Interestingly, Arf1 is also a known recruiting factor for PI4KIIIβ and might thus contribute to establishing a PtdIns(4)P-positive LCV (Godi *et al.*, 1999). In line with the findings above, bacteria lacking an effector called SidJ show a delayed acquisition of ER proteins and also decreased replication rates (Liu and Luo, 2007).

These data may suggest that the nutrient supply required for intracellular multiplication of *L. pneumophila* is only provided after the fusion with ER membranes. Contradictory to this reasoning are studies regarding the T4SS substrate SidC, which localises to LCVs by binding PtdIns(4)P (Weber *et al.*, 2006). Deletion of *sidC* and its paralogue *sdcA* led to a strong decrease in recruitment of ER vesicles to LCVs, but had unexpectedly no apparent effect on intracellular replication (Luo and Isberg, 2004; Ragaz *et al.*, 2008).

Another method for temporal coordination of LCV formation has been reported for the effectors LubX and SidH, exploiting ubiquitin mediated degradation by the host cell proteasome (Voges *et al.*, 1999). SidH, which is present within cells early after infection, is polyubiquitinated by the U-box-type E3 ubiquitin ligase LubX, whose cytoplasmic levels reach their maximum at a later timepoint (Kubori *et al.*, 2008, 2010; Luo and Isberg, 2004). This ubiquitination marks SidH for proteasomal degradation, clearing it from the cell and thus making LubX its negative temporal regulator termed a "meta-effector".

AnkB is another effector interfering with proteosomal machinery. It has been shown to reduce the levels of ubiquitination of ParvB which normally mark the protein for proteolysis by competing with eukaryotic E3 ligases (Lomma *et al.*, 2010). Abundancy of ParvB, which links integrins and associated proteins with signal trafficking directing actin cytoskeletal dynamics and cell survival, seems to be beneficial for *L. pneumophila*, as replication was reduced for *ankB* mutant bacteria or in ParvB knockdown cells (Al-Khodor *et al.*, 2008; Lomma *et al.*, 2010; Sepulveda and Wu, 2006).

Only after the conversion of the LCV from a plasma membrane-derived vacuole to a compartment resembling the ER and reversion of the initial block of acidification, do the bacteria begin intracellular replication (Sturgill-Koszycki and Swanson, 2000; Tilney *et al.*, 2001).

1.1.6 Release from Host Cells

Having successfully completed intracellular growth, *L. pneumophila* exits the host cells. One mechanism is nonlytic egress, i.e. fusion of the LCV with the host plasma membrane. The effectors LepA and LepB, which show SNARE-like motifs, have been reported to be involved in this process in some hosts, although the underlying mechanism is unclear (Chen *et al.*, 2004). In contrast, lytic egress of bacteria mediated by pore formation has been discussed as another possibility, reporting the *icmT* gene to be crucial, which is not surprising considering its requirement for translocation of effectors. (Alli *et al.*, 2000; Molmeret *et al.*, 2002). Finally, even bacterial escape from the LCV to the cytosol prior to release has been documented (Molmeret *et al.*, 2004). It has been speculated that the employed egress method of *L. pneumophila* depends on the infected cell.

1.2 Retrograde Vesicle Trafficking

1.2.1 Retrograde Trafficking and Retromer Function

L. pneumophila has been shown to communicate with the secretory as well as the endocytic pathway, exhibiting extensive interference with host cellular trafficking (see sections above and (Hilbi and Haas, 2012)). Remarkably, LCVs have also been shown to accumulate OCRL, a PtdIns(4,5)P_2 5-phosphatase implicated in the retrograde retrieval of receptors from endosomes to the *trans*-Golgi network (TGN) (Choudhury *et al.*, 2005;

Weber *et al.*, 2009). These transmembrane proteins include e.g. the iron transporter DMT1-II (Tabuchi *et al.*, 2010), the hydrolase-binding sortilin (Canuel *et al.*, 2008) as well as the cation-dependent and cation-independent mannose 6-phosphate receptors CDMPR and CIMPR, respectively (Ghosh *et al.*, 2003). The MPRs' primary function is well characterised, especially the CIMPR's, which is the binding and subsequent delivery of newly synthesised, mannose 6-phosphate-tagged acid hydrolase precursors from the TGN to endosomes, where the enzymes are processed for use in the endolysosomal pathway. This anterograde transport is enabled by the Arf1-dependent activation of a Golgi-localised, γ-adaptin ear-containing, ARF-binding (GGA) protein through the cytoplasmic acidic cluster-dileucine signal of CIMPR, resulting in clathrin coat assembly and vesicle formation (Misra *et al.*, 2002; Mullins and Bonifacino, 2001; Shiba *et al.*, 2002).

However, to avoid degradation in lysosomes the cargo receptors have to cycle back to the TGN, a step mediated by retrograde vesicle trafficking and the "retromer complex" (Arighi *et al.*, 2004; Seaman, 2004). Its core components are the membrane-deforming subcomplex, which induces/stabilises membrane tubulation, and the cargo-selective subcomplex, which binds the target receptor (Cullen and Korswagen, 2011; Seaman, 2012).

1.2.2 Retromer Core Components

The membrane-deforming subcomplex, first identified in *Saccharomyces cerevisiae*, consists of the two sorting nexins (SNXs) Vps5p and Vps17p (Vacuolar protein sorting) (Seaman *et al.*, 1998). Due to gene duplication, two homologues of Vps5p, SNX1 and SNX2, and two homologues of Vps17p, SNX5 and SNX6, exist in mammals requiring one protein of each group for dimerisation (Haft *et al.*, 1998; Horazdovsky *et al.*, 1997; Wassmer *et al.*, 2007). For instance in mice, absence of SNX1 can be compensated by SNX2 and vice versa, whereas lack of both genes is fatal (Schwarz *et al.*, 2002). It is currently not fully understood which functions or whether at all specific functions are accomplished by different SNX combinations. SNX proteins contain a C-terminal Bin-Amphiphysin-Rvs (BAR) domain, a dimerisation motif which forms a rigid bent structure allowing the sensing of membrane curvature (Peter *et al.*, 2004). Interestingly, as their concentration increases, SNX proteins are able to bend the membranes themselves, resulting in higher ordered helical arrays and thus tubule formation (Frost *et al.*, 2008, 2009; Roux *et al.*, 2010).

The cargo-selective subcomplex comprises the proteins Vps26, Vps29 and Vps35

in mammals (Cullen and Korswagen, 2011). Vps26 and Vps29 bind independently at the distal ends of the largest subunit, Vps35, a horseshoe-shaped, α-helical solenoid, resulting in a stable, but flexible, stick-shaped trimer (Hierro *et al.*, 2007; Norwood *et al.*, 2011). The association of the heterotrimer with cargo proteins is accomplished by Vps35, recognising several tripeptide sorting motifs, e.g. YLL for DMT1-II (Tabuchi *et al.*, 2010), FLV for sortilin or WLM for CIMPR (Seaman, 2007). However, recent studies have also revealed the possibility that Vps26 or SNX proteins bind to cargo receptors or that the cargo recognition or the membrane-deforming retromer subcomplex might be dispensable (Fjorback *et al.*, 2012; Harterink *et al.*, 2011; Nisar *et al.*, 2010).

1.2.3 Recruitment of the Retromer

Recruitment of the two subcomplexes is mechanistically independent, as RNAi knockdown of the cargo-selective subcomplex had no effect on the SNX-dimer association to membranes (**Figure 1.3**) (Arighi *et al.*, 2004; Seaman, 2004). Although it has been reported that depletion of SNX1 and SNX2 reduced the cargo-selective subcomplex's localisation to membranes, this supposition is meanwhile disputed due to the very weak nature of interactions (Rojas *et al.*, 2007; Seaman, 2012; Swarbrick *et al.*, 2011).

Membrane association of sorting nexins is mediated by its phox homology (PX) domain, which is capable of binding PIs (Cozier *et al.*, 2002). SNX1 and SNX2 interact with PtdIns(3)P or PtdIns(3,5)P_2, early and late endosomal markers, respectively, whereas SNX5 preferentially binds to PtdIns(4,5)P_2, mostly found in plasma membranes and to to a lesser extent at the Golgi, while SNX6 shows highest affinity for PtdIns(4)P (Cozier *et al.*, 2002; Koharudin *et al.*, 2009; Niu *et al.*, 2013). However, PI-binding specifity of SNX5 and SNX6 becomes relevant only at later stages of retrograde trafficking and their membrane recruitment is mediated by dimerisation with SNX1 or SNX2 (Koharudin *et al.*, 2009; Niu *et al.*, 2013). Levels of PtdIns(3)P and thus SNX recruitment depend amongst other factors on the activity of PI kinases and phosphatases, e.g. the PI3K Vps34, which itself has been shown to be regulated by Vps30/Beclin1 and the GTPase Rab5 (Burda *et al.*, 2002; Rojas *et al.*, 2008; Ruck *et al.*, 2011).

Recruitment of the cargo-selective subcomplex to endosomal membranes on the other hand is dependent on the active Rab7 GTPase and its regulators (Rojas *et al.*, 2008). For instance, depletion of the protein CNL5, implicated in a lysosomal storage disorder called Batten disease, leads to a decrease of GTP-bound Rab7 and subsequently lysosomal degradation of sortilin and CIMPR (Mamo *et al.*, 2012). Another regulator interfering with Rab7 activity is the Rab GAP TBC1D5 which directly binds Vps29

and causes Rab7 to dissociate from the membrane, thus negatively regulating the subcomplex's recruitment (Harbour *et al.*, 2010; Seaman *et al.*, 2009).

To sum up, the prerequisite for active Rab5 (regulates the PtdIns(3)P-level for SNX binding) as well as Rab7 (recruits Vps-trimer) determines the spatiotemporal site of retromer acitivity at the transition of early to late endosomes, where also the PI switch from PtdIns(3)P to PtdIns(3,5)P_2 occurs (van Weering *et al.*, 2012).

Interestingly, the coat protein clathrin has also been reported to play a role in the early stages of the retrograde pathway. Clathrin and some clathrin-associated factors, e.g. Hrs, epsinR and RME-8, are discussed to locally accumulate cargo on endosomes preceeding and facilitating retromer recruitment (Borner *et al.*, 2006; McGough and Cullen, 2011; Popoff *et al.*, 2007, 2009). However, it is currently not clear whether there is also a membrane curvature induced by clathrin prior to retromer recruitment or not (Johannes and Popoff, 2008; McGough and Cullen, 2012).

1.2.4 Recycling of Cargo

Once the retromer has been assembled at the endosome, several additional interactors catalyse correct tubule and vesicle formation (**Figure 1.3**). For instance, the recycling of MPRs from late endosomes requires the GTPase Rab9 and its effectors p40 and TIP47 (tail-interacting protein of 47 kDa), a cargo adaptor which interacts with the cargo receptors during vesicle formation (**Figure 1.4**) (Carroll *et al.*, 2001; Diaz and Pfeffer, 1998; Diaz *et al.*, 1997; Lombardi *et al.*, 1993).

Furthermore, similar to other vesicle transport pathways, the cytoskeleton is implicated in retrograde trafficking. SNX5 and SNX6 have been shown to bind p150$^{\text{Glued}}$, a subunit of dynactin, which is an interactor of the microtubule motor dynein thus indicating factors promoting the transport to the Golgi (Hong *et al.*, 2009; Wassmer *et al.*, 2009). Lack of p150$^{\text{Glued}}$ leads to extended tubules, suggesting also a role in fission from endosomes possibly by applying longitudinal force (Wassmer *et al.*, 2009).

Moreover, involvement of actin has been shown by recruitment of the WASH complex to endosomes via the cargo-selective subcomplex (Gomez and Billadeau, 2009; Harbour *et al.*, 2010). WASH promotes actin nucleation and consists of several subunits including Fam21, which directly binds to Vps35. Similar to p150$^{\text{Glued}}$, depletion of functional WASH leads to abnormal elongation of retromer-tubules, indicating again perturbation of vesicular scission. This may be due to the absence of a required actin polymerisation burst mediated by WASH to separate vesicle and donor membranes or/and lack of a required interaction of the complex with the scission factor dynamin-2 (De-

rivery *et al.*, 2009). Both processes, microtubule-dependent pulling and actin-dependent pushing of tubules, seem to be necessary for successful retrograde trafficking. It is also possible that the actin network stabilises membrane microdomains that constrict and enrich cargo proteins (Puthenveedu *et al.*, 2010). Alternatively or additionally, the actin-stabilised microdomains could alter local lipid composition or facilitate regulation of nearby signalling.

Moreover, EHD3 and its paralogue EHD1, which bind to the retromer Vps heterotrimer and stabilises the SNX-induced tubules, are required for efficient cargo transport (Gokool *et al.*, 2007; Naslavsky *et al.*, 2009). It is also possible that EHD1, sharing similarities with dynamin GTPases, but possessing ATPase acitivity, contributes to tubule scission (Daumke *et al.*, 2007).

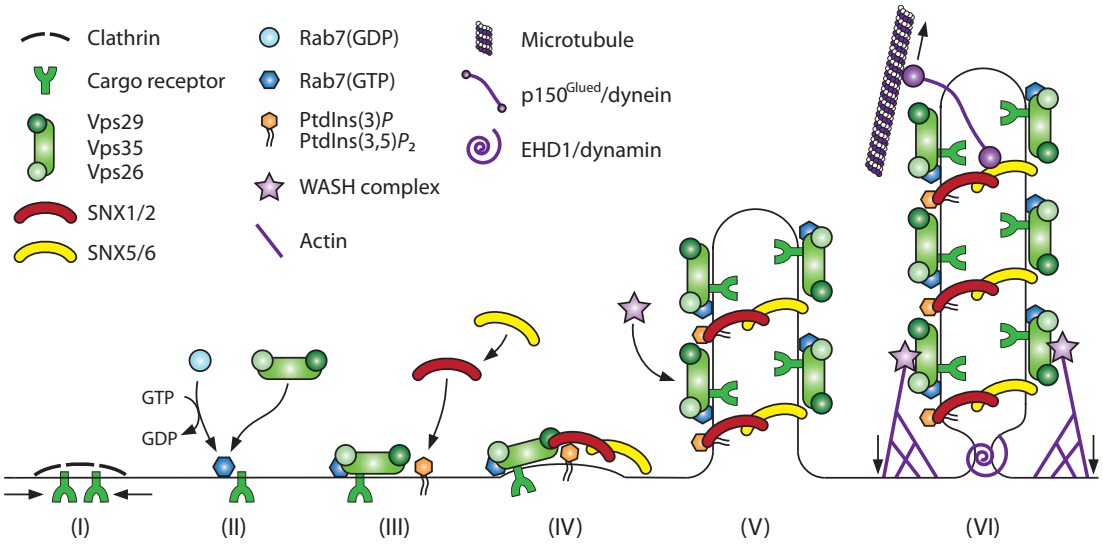

Figure 1.3: Recruitment of the retromer and vesicle formation. Firstly, cargo receptors are locally acumulated by clathrin (I) and the cargo-selective subcomlex is recruited to the endosomal membrane by active Rab7 (II). SNX1/2 binds to resident PtdIns(3)P or PtdIns(3,5)P_2 enabling the formation of a heterodimer with SNX5/6 (III), which induces membrane curvature (IV). Further remodelling results in tubules that associate with the WASH complex (V), which is required for an actin polymerisation burst. This actin-dependent pushing of the nascent vesicle together with binding of SNX5/6 to p150^{Glued}, a protein of the microtubule motor complex, which pulls the tubule away from its originating membrane, and additional scission factors, like EHD1 and dynamin, enable the separation of the cargo receptor-loaded vesicle from endosomes (VI). Adapted from Cullen and Korswagen (2011).

There is currently some dissent whether retrieval of Wntless, the cargo receptor for Wnt, which creates extracellular morphogenic gradients, is an example of a novel,

distinct retrograde pathway (compare (Cullen and Korswagen, 2011) versus (Seaman, 2012)). This hypothesis arose after studies had shown that transport of Wntless is dependent on SNX3, which possesses no BAR, but a PX domain with strong specificity for PtdIns(3)P. The sorting nexin is essential for correct localisation of the Vps-trimer and replaces the usual membrane-deforming subcomplex (Harterink *et al.*, 2011; Jong and Lemmon, 2001; Zhang *et al.*, 2011). On the other hand, other investigations indeed indicated a role for SNX1 in this retrograde pathway, thereby contradicting the above hypothesis (Shi *et al.*, 2009).

After retrograde vesicles have been transported along microtubules, they dock and fuse with the TGN. GCC185, a TGN-resident protein, has been shown to be crucial for vesicle recycling of Rab9-positive vesicles from the endosomes, suggesting its function at the docking step, as it is a member of the Golgin family of putative tethers (Reddy *et al.*, 2006). Furthermore, suppression of the tethering GARP (Golgi-associated retrograde protein) complex also interferes with retromer-dependent transport (Pérez-Victoria *et al.*, 2008). Additionally, the requirement of the Rab6-interacting protein-1 (RAB6IP1), which has been shown to bind SNX1, implicates the TGN resident GTPase Rab6 in docking (Wassmer *et al.*, 2009). Dissociation of the motor protein complex from the retromer is induced by binding of SNX6 to Golgi-localised PtdIns(4)P, hereby interrupting the interaction with $p150^{Glued}$ (Niu *et al.*, 2013). A similar mechanism may be true for SNX5, which binds to TGN-resident PtdIns(4,5)P_2 (Koharudin *et al.*, 2009).

Ultimately, fusion of the membranes itself is driven by formation of a tight complex of SNAREs, which are localised on the transport vesicles (v-SNAREs) as well as on the target membrane (t-SNAREs) (Jahn and Scheller, 2006). For instance, successful retrieval of MPRs requires a complex of the t-SNAREs syntaxin 10, syntaxin 16 and Vti1a and the v-SNARE VAMP3 (Ganley *et al.*, 2008).

In summary, the retromer core components are recruited independently to the endosomal membrane and together with additional cofactors mediate tubulation and scission of a cargo-enriched vesicle, which is transported along microtubules back to the TGN.

1.2.5 Toxins Exploit Retrograde Trafficking

A number of viruses and toxins exploit retrograde trafficking for transport from the plasma membrane to the ER, e.g. the Simian virus 40 (SV40), the plant toxin ricin, *Pseudomonas aeruginosa* exotoxin A (PEx), cholera toxin (CTx) and toxins belonging to the Shiga toxin family, possibly the best studied and most often used molecular tools

for investigating retromer-dependent transport (Spooner *et al.*, 2006). This group includes Shiga toxin (STx) secreted by *Shigella dysenteriae*, the causative agent of the severe dysentery "shigellosis", and the Shiga-like toxins 1 (STx1; 99% identical to STx) and 2 (STx2; 56% identical to STx) from enterohemorrhagic *Escherichia coli* (EHEC) strains (Bergan *et al.*, 2012; Cherla *et al.*, 2003).

The toxins are members of the AB_5 family of protein toxins, consisting of an enzymatically active subunit A (STxA) and a nontoxic homopentamer B (STxB) interacting with the glycosphingolipid globotriaosylceramide (Gb3) on cell membranes (Fraser *et al.*, 1994; Jacewicz *et al.*, 1986). After binding the toxin is internalised by a clathrin-dependent or a clathrin-independent mechanism, in which case the STxB itself induces tubular membrane invaginations (**Figure 1.4**) (Johannes and Römer, 2009; Römer *et al.*, 2007; Sandvig and van Deurs, 2005). After endocytosis STx is directly transported from the early endosomes (EE) to the TGN (Mallard *et al.*, 1998). This route is different from retrieval of CIMPR, which is Rab9-dependent and originates from late endosomes (LE) (Lombardi *et al.*, 1993) (**Section 1.2.4**). Furthermore, reduction of retrograde STx transport upon overexpression of the recycling endosome (RE)-resident Rab11a suggests that also this compartment is part of the trafficking route (Wilcke *et al.*, 2000). However, a high number of components and their role in retrograde transport are shared between the EE/RE and LE pathways, most importantly the requirement of the complete retromer complex (Bujny *et al.*, 2007; Popoff *et al.*, 2007; Utskarpen *et al.*, 2007). Other required factors are for instance clathrin and its associated factors, EpsinR and RME-8, the vesicle constrictors dynamin and the regulators EHD1 and EHD3 (Gokool *et al.*, 2007; Lauvrak *et al.*, 2004; Naslavsky *et al.*, 2009; Saint-Pol *et al.*, 2004; Shi *et al.*, 2009).

A role in docking has been attributed to the Golgi-localised Rab6A' and RAB6IP2 (Mallard *et al.*, 2002; Monier *et al.*, 2002). Additionally, the tethering factor Golgin-97 may be involved in the initial docking process (Lu *et al.*, 2004). Like for MPRs, SNAREs are required for ultimate membrane fusion at the TGN and in the case of STx the complex consists of the t-SNAREs syntaxin 6, syntaxin 16 and Vti1a and the v-SNAREs VAMP3 or VAMP4 (Mallard *et al.*, 2002). A second SNARE complex comprised of the t-SNAREs syntaxin 5, GS28 and Ykt6, and the v-SNARE GS15 has also been suggested to be involved in STx trafficking from endosomes to the TGN (Tai *et al.*, 2004). The reason for the requirement of another SNARE complex has not been elucidated yet.

Once at the TGN, STx travels dependent on Rab6, but independent of the coat protein complex COP-I, through the Golgi and finally to the ER, with the details of the pathways being largely unknown (Girod *et al.*, 1999; McKenzie *et al.*, 2009; White

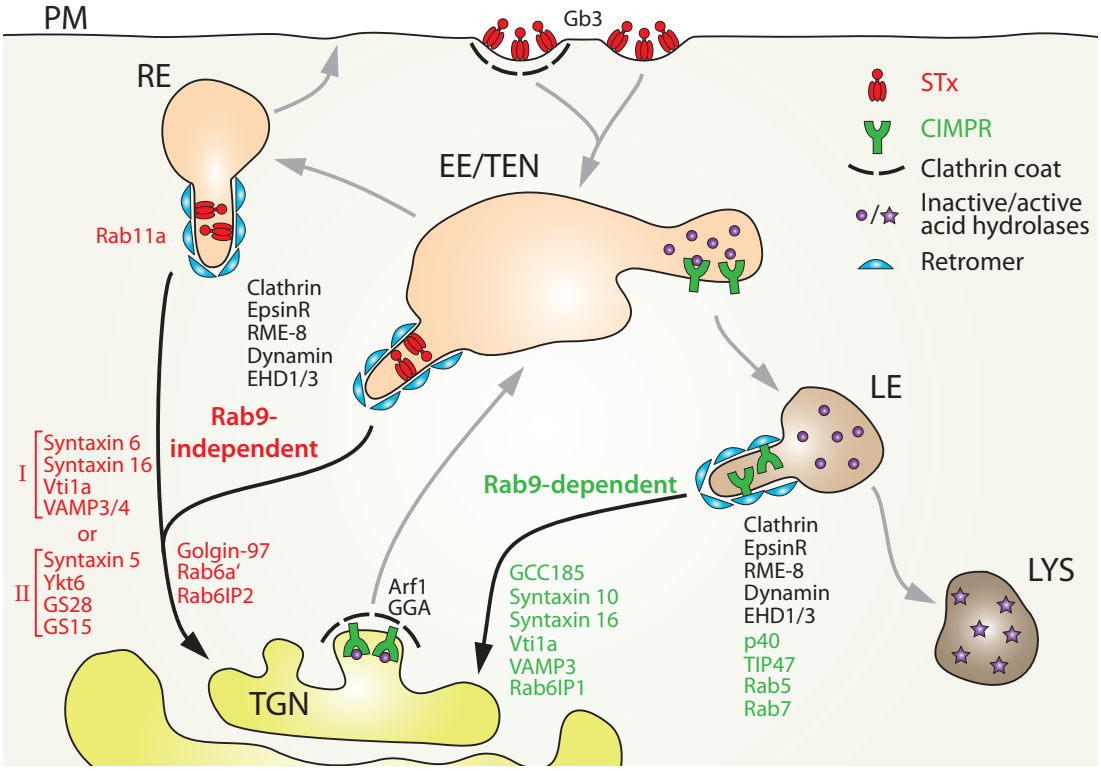

Figure 1.4: Retrograde trafficking routes. STx (specifically associated factors shown in red) and CIMPR (specifically associated factors shown in green) have different retrieval pathways, but share the retrograde core components, like the retromer. STx binds Gb3 at the plasma membrane (PM) and is internalised in a clathrin-dependent or -independent way, in which case the STxB itself forms tubular membrane invaginations. Once at the EE/TEN or RE, the toxin is transported in a retromer dependent, but Rab9-independent manner to the TGN, involving various tethering factors and two alternate sets of SNARE complexes (I, II). In contrast, loaded CIMPR is transported from the TGN to the TEN to deliver acid hydrolase precursor proteins, a process requiring Arf1, GGA and clathrin. The empty receptor is then recycled from LEs in a Rab9-dependent pathway, which also utilises specific tethering and fusion factors at the TGN.

et al., 1999). After reaching the ER, the toxin is cleaved by furin and the released and activated STxA can interact with resident chaperones (Garred *et al.*, 1995). These proteins target the subunit to the translocon channel of the ER-associated degradation (ERAD) apparatus, which enables the delivery, termed retro-translocation, of the subunit into the cytosol (Yu and Haslam, 2005). The toxicity of STxA derives from its RNA *N*-glycosidase activity, which removes an adenine base from the 28S ribosomal RNA and thus practically stops protein biosynthesis (Endo *et al.*, 1988; Saxena *et al.*, 1989). This ultimately leads to apoptosis of the cell caused by a ribotoxic stress response (Tesh, 2010).

Like STx, the CTx from *Vibrio cholerae* is transported in a retrograde manner through the cell to the cytosol, since it also lacks the ability to disrupt cellular membranes (Spooner *et al.*, 2006). CTx is an AB_5-toxin as well, but binds to the surface glycosphingolipid monosialotetrahexosylganglioside (GM1), triggering uptake dependent on cell type either through caveolin-coated vesicles, clathrin-coated vesicles or by the so-called Arf6 endocytic pathway (Holmgren *et al.*, 1973a,b; Sánchez and Holmgren, 2011). Whereas transport from endososmes to the TGN resembles trafficking of STx (Nichols *et al.*, 2001), CTx is delivered directly from there to the ER without passage through the Golgi cisternae (Feng *et al.*, 2004; Nichols *et al.*, 2001). ER resident proteins cleave and activate the toxin and the A subunit is subsequently translocated into the cytosol by the ERAD machinery (Tsai and Rapoport, 2002; Tsai *et al.*, 2001). There, the catalytic subunit transfers an ADP-ribosyl onto the heterotrimeric G protein Gs-α component of adenylyl cyclase, thus constitutively activating it. This leads to elevated cAMP levels resulting in loss of water and electrolytes, which is manifested as the typical severe diarrhoea associated with cholera (Moss and Vaughan, 1977).

1.3 Aims of the Thesis

With currently approximately 300 known translocated effector proteins *L. pneumophila* has a wide array of tools to manipulate its host cells. Identification and characterisation of these effectors is vital for the comprehension of *Legionella* pathogenesis. Moreover, a deeper understanding of the interference performed by these effectors also shed light onto basic cellular processes and pathways of eukaryotic host cells, making *L. pneumophila* an excellent model organism. Unfortunately many effector proteins exhibit functional redundancy making analyses considerably more difficult.

The aim of this thesis is the characterisation of the *L. pneumophila* effector protein RidL (Lpg2311, Ceg28), which has previously been shown to be a substrate of the Icm/Dot T4SS (Huang *et al.*, 2011; Zhu *et al.*, 2011; Zusman *et al.*, 2007). This is done by various approaches, with a special focus on the effector's impact on retrograde trafficking. The sequence of *ridL* is analysed regarding conservation and homologies and its expression, protein production pattern and translocation is documented. Also the spatiotemporal localisation of RidL after infection is analysed, utilising amongst others a newly developed method for the isolation of LCVs. The influence of RidL on uptake and intracellular replication is shown by different techniques. Finally, the function of RidL in host cells is analysed by investigating protein-protein and protein-lipid inter-

actions, its impact on LCV composition and changes in host cell pathways, especially concentrating on the retrograde trafficking route.

2 Materials and Methods

2.1 Materials

2.1.1 Laboratory Equipment

Item	Model	Manufacturer
Autoclave	STERIMAQUET	MAQUET (Rastatt)
Autoclave	Varioklav classic	H+P (Oberschleißheim)
Benchtop centrifuge	5417R	Eppendorf (Hamburg)
Cell homogeniser		Isobiotec (Heidelberg)
Centrifuge	3-30K	Sigma (St. Louis)
Centrifuge	5810	Eppendorf (Hamburg)
Centrifuge	Megafuge 1.0R	Thermo (Waltham)
CO_2 incubator	Heraeus HeraCell 240	Thermo (Waltham)
Colony counter	Countermat flash	IUL (Barcelona)
Confocal microscope	Leica TCS SP5	Leica (Mannheim)
Culture microscope	Primo Vert	Zeiss (Oberkochen)
Diaphragm vacuum pump	MZ 2C	Vacuubrand (Wertheim)
Electrophoresis chamber	Mini-Protean 3	Bio-Rad (Munich)
Electrophoresis chamber	Mini-Subcell GT	Bio-Rad (Munich)
Electrophoresis chamber	Subcell GT	Bio-Rad (Munich)
Electroporation device	GenePulser XCell	Bio-Rad (Munich)
French press	SIM AMINCO	Spectronic (New York)

Gel imaging system	ChemiDoc MP System	Bio-Rad (Munich)
Gel imaging system	GelDoc EQ	Bio-Rad (Munich)
Hot plate magnetic stirrer	RCT basic	IKA (Staufen)
Ice maker	AF30	Scotsman (Vernon Hills)
Incubation cabinet	Certomat BS-1	Sartorius (Goettingen)
Incubation cabinet	Oribital shaker, Forma	Thermo (Waltham)
Incubator	Heraeus BR6000	Thermo (Waltham)
Incubator	Heraeus Function Line	Thermo (Waltham)
Incubator	IPP500	Memmert (Schwabach)
Magnetic separator	MACS Multistand	Miltenyi Biotec (Bergisch-Gladbach)
Medical film processor	FPM-100A	Fuijifilm, EU (Düsseldorf)
Mixer	Vortex-Genie 2	IKA (Staufen)
Nucleofection device	Amaxa Nucleofector I	Lonza (Basel)
pH-meter	Level 1	inoLab (Weilheim)
Pipettes	Pipetman	Gilson (Middleton)
Pipettor	Pipetus	Hirschmann (Eberstadt)
Power supply	PAC100	Bio-Rad (Munich)
Precision balance	BP61-S	Sartorius (Goettingen)
Precision balance	PG2002-S	Mettler-Toledo (Greifensee)
Protein transfer device	MAXI-Semi-Dry-Blotter	Roth (Karlsruhe)
Rocking platform shaker	Duomax 1030	Heidolph (Schwabach)
Rocking platform shaker	Mini MR-1	Biosan (Riga)
Rolling mixer	RM5-35s 1732	Fröbel (Lindau)
Spectrophotometer	Helios Epsilon	Thermo (Waltham)
Spectrophotometer	NanoDrop ND-1000	PeqLab (Erlangen)
Superspeed centrifuge	Sorvall RC-5B	DuPont (Wilmington)
Suspension mixer	CMV	Fröbel (Lindau)

Thermal cycler	T3	Biometra (Goettingen)
Thermal mixer	Thermomixer comfort	Eppendorf (Hamburg)
UV-transilluminator		Bachofer (Reutlingen)
Water bath	Wasserbad 1005	GFL (Burgwedel)

Standard laboratory equipmentwas used in addition to the equipment listed above.

2.1.2 Strains and Plasmids

Strain/Plasmid	Relevant Properties	Reference
Escherichia coli		
BL21(DE3)		Novagen
TOP10		Invitrogen
Legionella pneumophila		
CR06	JR32 *ridL*::Kan^{R} (Δ*ridL*)	(Finsel *et al.*, 2013b)
GS3011	JR32 *icmT3011*::Kan^{R} (Δ*icmT*)	(Segal and Shuman, 1998)
JR32	Virulent serogroup 1 strain Philadelphia	(Sadosky *et al.*, 1993)
Dictyostelium discoideum		
Ax3/pCaln-GFP	P_{act15}, G418^{R}	(Müller-Taubenberger *et al.*, 2001)
Ax3/pCR110	DdVps10-GFP	(Finsel *et al.*, 2013b)
Ax3/pCR111	DdVps26-GFP	(Finsel *et al.*, 2013b)
Ax3/pCR112	DdVps29-GFP	(Finsel *et al.*, 2013b)
Ax3/pCR113	DdVps35-GFP	(Finsel *et al.*, 2013b)
Ax3/pSU12	GFP-Rab8a	(Urwyler *et al.*, 2009)
Ax3/pSU22	GFP-Rab1a	(Urwyler *et al.*, 2009)
Ax3/pSU23	GFP-Rab7a	(Urwyler *et al.*, 2009)
Ax3/pSU31	GFP-Rab14	(Urwyler *et al.*, 2009)
Ax3/pSW111	Dd5P4$_{1\text{-}132}$-GFP	(Weber *et al.*, 2009)

A549	Human alveolar basal epithelial cells	gift from U. Greber (Zurich)
HeLa	Human cervix adenocarcinoma cells	gift from U. Greber (Zurich)
RAW 264.7	Murine macrophage cell line	ATCC TIB-71
Acanthamoeba castellanii		ATCC 30234

Plasmids

pCR2	pGEX-4T-1-*sidC*	(Weber *et al.*, 2006)
pCR5	pET28a(+)-*ridL*	(Finsel *et al.*, 2013b)
pCR6	pGEX-4T-1-*ridL*	(Finsel *et al.*, 2013b)
pCR33	*Legionella* expr. vector, Δ*mobA*, RBS, M45-$(Gly)_5$, Cam^R, (= pMMB207-C-RBS-M45)	(Weber *et al.*, 2006)
pCR34	pMMB207-C-RBS-M45-*sidC*	(Weber *et al.*, 2006)
pCR38	pMMB207-C-RBS-M45-*ridL*	(Finsel *et al.*, 2013b)
pCR76	pMMB207-C-P_{tac}-RBS-*gfp*-RBS-MCS	(Finsel *et al.*, 2013b)
pCR77	pMMB207-C-P_{tac}-RBS-*dsred*-RBS-MCS	(Finsel *et al.*, 2013b)
pCR79	pMMB207-C-P_{tac}-RBS-*gfp*-RBS-MCS-*sidC*	(Finsel *et al.*, 2013b)
pCR80	pMMB207-C-P_{tac}-RBS-*dsred*-RBS-MCS-*sidC*	(Finsel *et al.*, 2013b)
pCR93	pEGFP-N1-*ridL* (RidL-GFP)	(Finsel *et al.*, 2013b)
pCR94	pEGFP-C1-*ridL* (GFP-RidL)	(Finsel *et al.*, 2013b)
pCR95	pcDNA3.1(+)-*ridL*	(Finsel *et al.*, 2013b)
pCR96	pDXA-HC-*ridL-gfp*	(Finsel *et al.*, 2013b)
pCR110	pDXA-HC-*DdVps10-gfp*	(Finsel *et al.*, 2013b)
pCR111	pDXA-HC-*DdVps26-gfp*	(Finsel *et al.*, 2013b)
pCR112	pDXA-HC-*DdVps29-gfp*	(Finsel *et al.*, 2013b)

pCR113	pDXA-HC-*DdVps35-gfp*	(Finsel *et al.*, 2013b)
pCR115	pGEX-4T-1-*DdVps26*	(Finsel *et al.*, 2013b)
pCR116	pGEX-4T-1-*DdVps29*	(Finsel *et al.*, 2013b)
pcDNA3.1(+)	Mammalian expr. vector, PCMV, Neo^R, Amp^R	Invitrogen
pDXA-HC	*Dictyostelium* expr. vector, P_{act15}, Neo^R, Amp^R	(Manstein *et al.*, 1995)
pEGFP-N1	Mammalian expr. vector for GFP-fusion constructs, PCMV, Neo^R, Kan^R	Clontech Laboratories
pEGFP-C1	Mammalian expr. vector for GFP-fusion constructs, PCMV, Neo^R, Kan^R	Clontech Laboratories
pEGFP-C1-SNX1	Mammalian expr. vector for GFP-SNX1	(Bujny *et al.*, 2008)
pEGFP-C3-2FYVE	Mammalian expr. vector for GFP-2xFYVE	(Gillooly *et al.*, 2000)
pGEM-t easy	Cloning of PCR products, Amp^R	Promega
pGEX-4T-1	N-terminal *gst* fusion; P_{act15}; Amp^R	Amersham
pIF007	pMMB207-C-P_{tac}-RBS-*dsred*-RBS-MCS-*ridL*	(Finsel *et al.*, 2013b)
pIF009	pMMB207-C-RBS-*gfp*-P_{ridL}-*ridL* (constitutive *gfp*, derivative of pNT29)	(Finsel *et al.*, 2013b)
pMMB207-C	*Legionella* expr., vector, Δ*mobA*, no RBS, Cam^R	(Weber *et al.*, 2006)
pNT28	pMMB207-C-RBS-*gfp* (constitutive *gfp*)	(Tiaden *et al.*, 2007)
pNT29	pMMB207-C-RBS-*gfp*-MCS (constitutive *gfp*)	(Tiaden *et al.*, 2007)
pSU12	pDXA-HC-*gfp-RAB8A*	(Urwyler *et al.*, 2009)
pSU22	pDXA-HC-*gfp-RAB1A*	(Urwyler *et al.*, 2009)
pSU23	pDXA-HC-*gfp-RAB7A*	(Urwyler *et al.*, 2009)
pSU31	pDXA-HC-*gfp-RAB14*	(Urwyler *et al.*, 2009)
pSW001	pMMB207-C-RBS-*dsred* (constitutive *dsred*)	(Mampel *et al.*, 2006)
pSW102	pDXA-HC-MCS-eGFP	(Weber *et al.*, 2006)
pSW111	pDXA-HC-*Dd5P4*$_{1\text{-}132}$-*gfp*	(Weber *et al.*, 2006)

2.1.3 Oligonucleotides Used for Cloning

Oligo	Sequence (5' - 3')*	Comments
oCR5	AAAAACGCGGATCCATGATTCTCGAGGAGTACATCC	5' of *ridL*; *Bam*HI
oCR6	AAAAACGCGTCGACTTACTTACGCATCCCTGTACTC	3' of *ridL*; *Sal*I
oCR18	CATGAGTAAACCCGAGGC	seq. primer of *ridL*
oCR19	CAAATTTACTTCCGGGAACC	seq. primer of *ridL*
oIF12	AAAAACGCGGATCCATTCACCTTATCCTTGCTATACTTCT	5' of *ridL* (incl. P_{ridL}); *Bam*HI
oIF13	CGGGGATCCATTCACCTTAT	seq. primer of *ridL*
oIF14	TTGTACCGCTTGAACCACTG	seq. primer of *ridL*

*=Restriction sites are underlined.

2.1.4 Oligonucleotides Used for RNAi

Target Gene	Gene description	Entrez Gene ID	Product Name	Product ID
ARF1	ADP-ribosylation factor 1	375	Hs_ARF1_1	SI00299250
			Hs_ARF1_10	SI02757272
			Hs_ARF1_11	SI02757279
			Hs_ARF1_8	SI02654470
VPS26A	Vacuolar protein sorting 26A	9559	Hs_VPS26A_1	SI03122273
			Hs_VPS26A_2	SI04197914
			Hs_VPS26A_3	SI04227587
			Hs_VPS26A_4	SI04300856
VPS26B	Vacuolar protein sorting 26B	112936	Hs_VPS26B_2	SI04153660
			Hs_VPS26B_3	SI04183886
			Hs_VPS26B_4	SI04230982
			Hs_MGC10485_3	SI00631281
VPS29	Vacuolar protein sorting 29	51699	Hs_VPS29_2	SI00760606
			Hs_VPS29_5	SI04161773
			Hs_VPS29_6	SI04170313
			Hs_VPS29_7	SI04339293

VPS35	Vacuolar protein sorting 35	55737	Hs_VPS35_5	SI04268131
			Hs_VPS35_6	SI04279296
			Hs_VPS35_7	SI04287605
			Hs_VPS35_8	SI04316914
CIMPR	Cation-independent mannose 6-phosphate receptor, insulin-like growth factor 2 receptor (IGFR2)	3482	Hs_IGF2R_1	SI00017549
			Hs_IGF2R_3	SI00017563
			Hs_IGF2R_4	SI00017570
			Hs_IGF2R_5	SI03112963
OCRL	Oculocerebrorenal Syndrome of Lowe	4952	Hs_OCRL_2	SI00006034
			Hs_OCRL_3	SI00006041
			Hs_OCRL_4	SI00006048
			Hs_OCRL_5	SI03074799
INPP5B	Inositol polyphosphate-5-phosphatase	3633	Hs_INPP5B_5	SI02658579
			Hs_INPP5B_6	SI02658586
			Hs_INPP5B_10	SI04435494
			Hs_INPP5B_2	SI00447783
PI3K	Phosphatidylinositol-3-kinase, class 3	5289	Hs_PIK3C3_5	SI00605822
			Hs_PIK3C3_6	SI00605829
			Hs_PIK3C3_7	SI03649520
			Hs_PIK3C3_8	SI03649527
RAB5A	Rab5A, RAS oncogene family member	5868	Hs_RAB5A_5	SI00301588
			Hs_RAB5A_8	SI02655037
			Hs_RAB5A_10	SI03111115
			Hs_RAB5A_6	SI02632602
RAB8A	Rab8A, RAS oncogene family member	51762	Hs_RAB8A_5 HP	SI02662254
			Hs_RAB8A_1 HP	SI00076090
			Hs_RAB8A_3 HP	SI00076104
			Hs_RAB8A_4 HP	SI00076111

2.1.5 Chemicals and Consumables

Item	Supplier
1 Kb Plus DNA Ladder	Life Technologies (Grand Island)
ACES	AppliChem (Darmstadt)
Acrylamid/Bisacrylamid	Serva (Heidelberg)
Activated charcoal powder	Fluka (Buchs)
Agar	BD Biosciences (Franklin Lakes)
Agarose	Biozym (Hessisch Oldendorf)
Bacteriological Peptone	Oxoid (Wesel)
Bacto Proteose Peptone	BD Biosciences (Franklin Lakes)
Bacto Yeast Extract	BD Biosciences (Franklin Lakes)
BBL Yeast Extract	BD Biosciences (Franklin Lakes)
β-mercaptoethanol	AppliChem (Darmstadt)
cOmplete, Mini Protease Inhibitor	Roche (Basel)
CTxB-AlexaFluor555	Molecular Probes (Eugene)
D(+)-Glucose-Monohydrate	Fluka (Buchs)
Dextran-AlexaFluor647	Molecular Probes (Eugene)
DNA purification kit	Qiagen (Germantown)
DNA purification kit	Macherey-Nagel (Dueren)
DNase	Roche (Basel)
DreamTaq Green PCR Master Mix (2x)	Thermo (Waltham)
ECL detection kit	GE Healthcare (Chalfont St Giles)
FCS	Life Technologies (Grand Island)
FeN_3O_9 x 9 H_2O	Sigma (St. Louis)
Gene Pulser Cuvette	Bio-Rad (Munich)
Glutathione Sepharose 4B	GE Healthcare (Chalfont St Giles)

Glycine	MP Biomedicals (Eschwege)
KH_2PO_4	Fluka (Buchs)
LB Agar	Life Technologies (Grand Island)
LB Broth Base	Life Technologies (Grand Island)
L-cysteine	Sigma (St. Louis)
L-glutamine	Life Technologies (Grand Island)
Lipofectamine 2000	Life Technologies (Grand Island)
Lipofectamine RNAiMAX	Life Technologies (Grand Island)
MACS-MS separation columns	Miltenyi Biotec (Bergisch-Gladbach)
Na_2HPO_4	Fluka (Buchs)
Nonidet-P40 (99%)	AppliChem (Darmstadt)
Nucleofection reagents	Lonza (Basel)
PageRuler Prestained Protein Ladder 10-170K	Thermo (Waltham)
PFA	Sigma (St. Louis)
Pfu polymerase	Promega (Fitchburg)
Phusion polymerase	Thermo (Waltham)
PIP strips	Echelon Biosciences (Salt Lake City)
Plastic containers	TPP (Trasadingen)
Plastic Luer-Lock syringes (3 ml)	BD Biosciences (Franklin Lakes)
PMSF	Sigma (St. Louis)
poly-L-lysine	Sigma (St. Louis)
Restriction enzymes	Thermo (Waltham)
RPMI 1640	Life Technologies (Grand Island)
SDS	Serva (Heidelberg)
Sodium-Citrate x 2 H_2O	Fluka (Buchs)
STxB-Cy3	gift from L. Johannes (Paris)
T4 DNA-Ligase	New England Biolabs (Ipswich)

TEMED	Biomol Feinchemikalien (Hamburg)
TRIS	MP Biomedicals (Santa Ana)
Trypsin	Life Technologies (Grand Island)
Vectashield mounting medium	Vector Laboratories (Cambridgeshire)

Materials not listed above were obtained from Roth (Karslruhe).

2.1.6 Antibodies

2.1.6.1 Primary Antibodies

Antibody	**Origin**	**Supplier**
anti-CIMPR	rabbit	gift from S. Höning (Cologne)
anti-EEA1	rabbit	Abcam (Cambridge)
anti-giantin	rabbit	Abcam (Cambridge)
anti-GM130	mouse	BD Biosciences (Franklin Lakes)
anti-GST	mouse	Sigma (St. Louis)
anti-RidL	rabbit	affinity-purified (Finsel *et al.*, 2013b)
anti-SidC	rabbit	affinity-purified (Weber *et al.*, 2006)
anti-SNX1	goat	Santa Cruz (Dallas)
anti-SNX1	mouse	BD Biosciences (Franklin Lakes)
anti-SNX2	goat	Santa Cruz (Dallas)
anti-SNX3	goat	Santa Cruz (Dallas)
anti-SNX5	goat	Abcam (Cambridge)
anti-TfR	rabbit	Abcam (Cambridge)
anti-Vps26	rabbit	Abcam (Cambridge)
anti-Vps35	goat	Abcam (Cambridge)

2.1.6.2 Secondary Antibodies

Antibody	Origin	Label	Supplier
anti-goat IgG	donkey	FITC	Santa Cruz (Dallas)
anti-goat IgG	donkey	HRPO	Santa Cruz (Dallas)
anti-mouse IgG	goat	FITC	Jackson ImmunoResearch (West Grove)
anti-mouse IgG	goat	Cy3	Jackson ImmunoResearch (West Grove)
anti-mouse IgG	sheep	HRPO	GE Healthcare (Chalfont St Giles)
anti-rabbit IgG	goat	Cy5	Jackson ImmunoResearch (West Grove)
anti-rabbit IgG	goat	FITC	Jackson ImmunoResearch (West Grove)
anti-rabbit IgG	bovine	Rhodamine	Santa Cruz (Dallas)
anti-rabbit IgG	donkey	HRPO	GE Healthcare (Chalfont St Giles)
anti-rabbit IgG	goat	MicroBeads	Miltenyi Biotec (Bergisch Gladbach)

2.1.7 Standard Buffers

PBS (phosphate-buffered saline) (10 x)

Component	Per liter buffer	Supplier
NaCl	80 g	Roth
KCl	2 g	Roth
Na_2HPO_4	14.2 g	Fluka
KH_2PO_4	2.4 g	Fluka

The pH was adjusted to 7.4 with with 1 M NaOH or 1 M HCl and the buffer autoclaved and stored at room temperature.

SorC (Malchow *et al.*, 1972)

Component	**Per liter buffer**	**Supplier**
Na_2HPO_4	0.28 g	Fluka
KH_2PO_4	2.04 g	Fluka
$CaCl_2$ x 2 H_2O	7.35 mg (50 µM)	Roth

The pH was adjusted to 6.0 with 1 M KOH or 1 M HCl and the buffer autoclaved and stored at room temperature.

TBS (TRIS-buffered saline) (10 x)

Component	**Per liter buffer**	**Supplier**
TRIS	6.5 g	MP Biomedicals
NaCl	80 g	Roth

The pH was adjusted to 7.5 with HCl and the buffer autoclaved and stored at 4 °C.

2.2 Methods

2.2.1 *Legionella pneumophila*

2.2.1.1 Growth on CYE Agar Plates

L. pneumophila requires a complex nutritional mix and grows optimally at 37 °C. The activated charcoal in the CYE-agar plates absorbs toxic substances released from the agar during autoclaving.

CYE agar plates (charcoal yeast extract agar plates) (Feeley *et al.*, 1979)

Component	**Per liter medium**	**Supplier**
ACES	10 g	AppliChem
Bacto Yeast Extract	10 g	BD Biosciences
Activated charcoal puriss p.a.; powder	2 g	Fluka
Agar	15 g	BD Biosciences
L-cysteine	0.4 g in 10 mL H_2O	Sigma
FeN_3O_9 x 9 H_2O	0.25 g in 10 mL H_2O	Sigma

ACES and the yeast extract were dissolved in H_2O and the pH adjusted to 6.9 with 10 M KOH. After addition of the activated charcoal, autoclaving and cooling to 50 °C the filter-sterilised cysteine- and iron-solutions were added. If necessary chloramphenicol was supplemented to an end concentration of 5 mg/l . Plates were stored at 4 °C.

2.2.1.2 Growth in Liquid Culture

3 ml AYE liquid medium were inoculated with *L. pneumophila* grown on CYE agar plates for 3 days (**Section 2.2.1.1**). Starting with an OD_{600} of 0.1, the bacteria reached their most infectious state after approximately 21-22 h (37 °C) on a turning wheel at an OD_{600} of 3.0.

AYE medium (ACES yeast extract medium) (Horwitz, 1983)

Component	Per liter medium	Supplier
ACES	10 g	AppliChem
Bacto Yeast Extract	10 g	BD Biosciences
L-cysteine	0.4 g	Sigma
FeN_3O_9 x 9 H_2O	0.25 g	Sigma

ACES was mixed with yeast extract in 900 mL H_2O. Cysteine and iron were separately dissolved in 10 ml H_2O each and slowly added while stirring. The pH was adjusted to 6.9 with 10 M KOH and the medium passaged through a glass fiber filter 6-8 times and filter-sterilised afterwards. The medium was stored at 4 °C.

2.2.1.3 *L. pneumophila* Glycerol Stocks

L. pneumophila liquid cultures (ca.18 h at 37 °C) (**Section 2.2.1.2**) mixed 1:2 with sterile 50% glycerol were used as storage stocks at −80 °C.

2.2.1.4 Electrocompetent *L. pneumophila*

1 ml of a pre-stationary phase *L. pneumophila* culture was added to 30 ml AYE (**Section 2.2.1.2**). At an OD_{600} between 0.3 and 0.5 the bacteria were cooled and washed 3 times with sterile, ice-cold 10% glycerol (10 ml, 2.5 ml, 160 µL). Aliquots of 25 µL were snap-frozen and stored at −80 °C.

2.2.1.5 Transformation of *L. pneumophila* by Electroporation

100 ng of plasmid was added to electrocompetent *L. pneumophila* (**Section 2.2.1.4**) on ice. After transfer into a cuvette with a 2 mm electrode gap (Gene Pulser Cuvette) and electroporation (2.5 kV, 200 Ω, 25 µF), 450 µL AYE were added. The bacteria were incubated for 5 h at 37 °C on a turning wheel and plated onto selective CYE agar (**Section 2.2.1.1**).

2.2.2 *Escherichia coli*

2.2.2.1 *E. coli* Growth Media

E. coli was grown in LB liquid medium (20 g/l LB Broth Base) or on LB agar plates (32 g/l LB Agar) at 37 °C.

2.2.2.2 *E. coli* Glycerol Stocks

E. coli liquid cultures (**Section 2.2.2.1**) mixed 1:2 with sterile 50% glycerol were used as storage stocks at −80 °C.

2.2.2.3 Chemically Competent *E. coli*

1 ml of an *E. coli* overnight culture (TOP10, BL21) was added to 100 ml LB medium (**Section 2.2.2.1**). At an OD_{600} of 0.5 the bacteria were cooled and washed with 40 ml ice-cold TFB1 and subsequently with 4 ml ice-cold TFB2. Aliquots of 50 µL were snap-frozen and stored at −80 °C.

TFB1

Component	Per liter buffer	Supplier
Potassium acetate	2.82 g (30 mM)	Roth
KCl	7.46 g (100 mM)	Roth
$CaCl_2$ x 2 H_2O	1.48 g (10mM)	Roth
$MnCl_2$	6.3 g (50 mM)	Roth
Glycerol	150 mL (15%)	Roth

The pH was adjusted to 5.8 with 0.2 N acetic acid and the buffer filter-sterilised and 40 ml aliquots stored at −20 °C.

TFB2

Component	Per 100 ml buffer	Supplier
MOPS	0.42 g (10 mM)	Roth
$CaCl_2$ x 2 H_2O	1.11 g (75 mM)	Roth
KCl	0.074 g (10 mM)	Roth
Glycerol	15 ml (15%)	Roth

The pH was adjusted to 6.5 with KOH and the buffer filter-sterilised and 4 ml aliquots stored at −20 °C.

2.2.2.4 Transformation of *E. coli* by Heat Shock

50 - 100 ng of plasmid was added to chemically competent *E. coli* (**Section 2.2.2.3**) on ice and incubated for 20 min. The bacteria were heat-shocked for 1 min at 42 °C and put back on ice for 2 min. After addition of 450 µL LB medium and incubation at 37 °C for 1 h, the bacteria were plated onto selective LB agar (**Section 2.2.2.1**).

2.2.3 Mammalian Cell Lines

2.2.3.1 Cultivation of Mammalian Cell Lines

Murine RAW 264.7 macrophages, human A549 lung epithelial cells and human HeLa cervix carcinoma cells were cultivated in RPMI 1640 medium supplemented with 10% FCS and 2 mM L-glutamine at 37 °C under 5% CO_2. During routine cell culture, the cells were washed once with PBS (**Section 2.1.7**) and detached from the surface using trypsin.

2.2.3.2 Storage of Mammalian Cell Lines

The cells of a 80% confluent 75 cm^2 culture vessel were harvested and resuspended in 3-4 ml freezing medium (70% RPMI 1640, 20% FCS, 10% DMSO). Cryo vials were filled with 1 ml aliquots, placed in a freezing box (filled with isopropanol and precooled for 1 h at 4 °C), frozen at −80 °C overnight and stored in liquid N_2.

When thawing the cells, the toxic DMSO-containing freezing solution was replaced as quickly as possible by standard culture medium (**Section 2.2.3.1**).

2.2.3.3 Transfection of Mammalian Cell Lines

Experiments for the uptake of DNA into mammalian cells were performed according to the manufacturer's guideline. RAW 264.7 macrophages were nucleofected with the Amaxa system whereas A549 and HeLa cells were transfected using Lipofectamine.

2.2.4 *Dictyostelium discoideum*

2.2.4.1 Cultivation of *D. discoideum*

D. discoideum was grown axenically at 23 °C in culture flasks in HL5 medium as nutritional source. Detachment of the amoeba for routine cell culture was achieved by mechanical force.

HL5 medium (Watts and Ashworth, 1970)

Component	Per liter medium	Supplier
D(+)-Glucose-Monohydrate	11 g	Fluka
BBL Yeast Extract	5 g	BD Biosciences
Bacto Proteose Peptone	5 g	BD Biosciences
Bacteriological Peptone	5 g	Oxoid
Na_2HPO_4	0.355 g (2.5 mM)	Fluka
KH_2PO_4	0.34 g (2.5 mM)	Fluka

The pH was adjusted to 6.5 with 1 M KOH or 1 M HCl and the medium autoclaved and stored at 4 °C.

2.2.4.2 Storage of *D. discoideum*

The axenically grown amoeba of one confluent 75 cm^2 flask were resuspended in 9 ml freezing medium (80% HL5, 10% FCS, 10% DMSO) (**Section 2.2.4.1**). Cryo vials were

filled with 1 ml aliquots, placed in a freezing box (filled with isopropanol and precooled for 1 h at 4 °C), frozen at −80 °C overnight and stored in liquid N_2.

When thawing the cells, the toxic DMSO-containing freezing solution was replaced as quickly as possible by standard culture medium.

2.2.4.3 Transformation of *D. discoideum* by Electroporation

Transfection of *D. discoideum* with plasmids was achieved by electroporation (Faix *et al.*, 2004). Briefly, exponentially growing cells were washed once in SorC and once in electroporation buffer (**Section 2.1.7**). The cell pellet was resuspended in electroporation buffer and the suspension was gently mixed with 25 µg plasmid DNA and transferred to a 4 mm electroporation cuvette. Electroporation conditions were 1 ms at 1 kV and 10 µF applied twice 5 s apart using the Gene Pulser Xcell apparatus. After electroporation, the cells were transferred to a six-well plate and incubated for 15 min at 23 °C. The suspension was supplemented to 2 mM $CaCl_2$ and 2 mM $MgCl_2$ with the healing solution and incubated again for 15 min at 23 °C. Finally, 3 ml HL5 medium was added and the cells were allowed to recover for 24 h (**Section 2.2.4.1**). Finally, the medium was replaced by HL5 containing 20 µg G418 per ml to select for transfectants.

Electroporation Buffer

Component	Per liter buffer	Supplier
Sucrose	17.11 g (50 mM)	Roth
KH_2PO_4	1.36 g (10 mM)	Fluka

The pH was adjusted to 6.1 with KOH. After filter sterilisation 50 ml aliquots were stored at −20 °C.

Electroporation Healing Solution

Component	Per liter buffer	Supplier
$MgCl_2$ x 6 H_2O	20.32 g (0.1 M)	Roth
$CaCl_2$ x 2 H_2O	14.7 g (0.1 M)	Roth

After filter-sterilisation 12 ml aliquots were stored at −20 °C. The stock solution was used at a 1:50 dilution resulting in end concentrations of 2 mM each.

2.2.5 *Acanthamoeba castellanii*

2.2.5.1 Cultivation of *A. castellanii*

A. castellanii was grown at 23 °C in culture flasks in PYG medium as nutritional source (Moffat and Tompkins, 1992; Segal and Shuman, 1999). Detachment of the amoeba for routine cell culture was achieved by mechanical force.

PYG medium

Component	Per liter medium	Supplier
BBL Yeast Extract	1 g	BD Biosciences
Bacto Proteose Peptone	20 g	BD Biosciences
$MgSO_4$ x 7 H_2O (0.4 M)	10 ml	Roth
$CaCl_2$ x 2 H_2O (0.05 mM)	8 ml	Roth
Sodium-Citrate x 2 H_2O (1 M)	3.4 ml	Roth
$Fe(NH_4)_2$ x 7 H_2O (0.25 M)	10 ml	Roth
Na_2HPO_4 (0.250 M)	10 ml	Fluka
KH_2PO_4 (0.250 M)	10 ml	Fluka

The pH was adjusted to 6.5 with 1 M KOH or 1 M HCl. After addition of 50 ml 2 M Glucose the medium was filter-sterilised and stored at 4 °C.

2.2.5.2 Storage of *A. castellanii*

The amoeba of one confluent 75 cm^2 flask were resuspended in 9 ml freezing medium (80% PYG, 10% FCS, 10% DMSO) (**Section 2.2.5.1**). Cryo vials were filled with 1 ml aliquots, placed in a freezing box (filled with isopropanol and precooled for 1 h at 4 °C), frozen at −80 °C overnight and stored in liquid N_2.

When thawing the cells, the toxic DMSO-containing freezing solution was replaced as quickly as possible by standard culture medium.

2.2.6 *Cloning of Plasmids*

Standard protocols were used for cloning (PCR amplification, restriction digest, ligation), all PCR fragments were sequenced and plasmids isolated using kits from Qiagen or Macherey-Nagel.

Plasmid pIF007 expressing the *ridL* gene under control of P_{tac} was constructed by amplification of *ridL* by PCR with the oligos oCR5 and oCR6 (**Section 2.1.3**), restriction digest with *Bam*HI/*Sal*I and ligation into pMMB207-C-RBS-*dsred*-RBS-MCS (pCR77) cut with the same enzymes (**Section 2.1.2**).

To construct plasmid pIF009, the *ridL* gene and its endogenous promoter were amplified by PCR with the oligos oIF12 and oCR6 (**Section 2.1.3**), cut with *Bam*HI/*Sal*I and inserted into pNT29 cut with the same enzymes (**Section 2.1.2**).

2.2.7 *Purification of Proteins*

For the production of GST fusion proteins *E. coli* BL21(DE3) containing pGEX-derivatives were induced at a cell density (OD_{600}) of 0.6 with 0.5 mM isopropyl-1-thio-β-D-galacto-pyranoside (IPTG) for 4 h at 37 °C in LB medium and lysed by French press. GST fusion proteins of RidL (pCR90), DdVps26 (pCR115) and DdVps29 (pCR116) were purified using glutathione-sepharose beads in a batch procedure according to the manufacturer's recommendations (**Section 2.1.2**). The solubility and purity of the protein preparations was analysed by SDS-PAGE and the concentration spectrophotometrically determined with the NanoDrop.

2.2.8 *Pull-down Experiments*

For pull-down experiments using GST-Vps26 or GST-Vps29 as baits, RidL from a lysate of *L. pneumophila* overproducing RidL (pIF007) was used (**Section 2.1.2**). The lysate was obtained by spinning down 9 ml of an overnight culture, resuspension in lysis buffer, lysis by French press, and clearing of the lysate by centrifugation (7000 x g, 10 min, 4 °C). 50 µg of the lysate was incubated on a rotation wheel (1.5 h, 4 °C) with 20 µg GST-Vps26 or GST-Vps29 and 20 µL washed glutathione-sepharose beads in a total volume of 50 µL binding buffer (**Section 2.2.7**). The beads were washed 4 times with 1 ml binding buffer, suspended in 20 µL SDS-PAGE loading dye, boiled and centrifuged. The supernatant was analysed by Western blot using a polyclonal

anti-RidL antibody (1:1000) (**Section 2.1.6**). Beads only with bacterial lysate were used as pull-down control, and 1 µL lysate was used as input control.

Lysis buffer

Component	Per 50 ml buffer	Supplier
TRIS (1 M)	2.5 ml (50 mM)	MP Biomedicals
$MgCl_2$ (1 M)	0.5 ml (10 mM)	Roth
DTT (0.5 M)	0.1 mL (1 mM)	Roth
PMSF (0.2 M)	0.125 mL (0.5 mM)	Sigma
DNase	50 U	Roche
Glycerol (99%)	5 ml (10%)	Roth

The pH was adjusted to 7.5 with 1 M KOH or 1 M HCl and the solution filter-sterilised and stored at 4 °C.

Binding buffer

Component	Per 50 ml buffer	Supplier
TRIS (1 M)	2.5 ml (50 mM)	MP Biomedicals
NaCl (1 M)	1 ml (20 mM)	Roth
$MgCl_2$ (1 M)	0.5 ml (10 mM)	Roth
DTT (0.5 M)	0.1 mL (1 mM)	Roth
EDTA (0.5 M)	0.1 mL (1 mM)	Roth
Nonidet-P40 (99%)	0.25 ml (0.5%)	AppliChem

The pH was adjusted to 7.5 with 1 M KOH or 1 M HCl and the solution filter-sterilised and stored at 4 °C.

2.2.9 *Protein-Lipid Interaction Studies*

Binding of GST-RidL and GST-SidC (**Section 2.2.7**) to PIs and other lipids was tested in protein-lipid overlay assays using commercially available PIP strips. To this end, the

nitrocellulose membranes were blocked with 4% fat-free milk powder in TBS-Tween (0.1% Tween-20 [v/v], pH 8.0) (**Section 2.1.7**) for 1 h at room temperature and incubated with 500 pmol affinity-purified GST-fusion protein in blocking buffer overnight at 4 °C. Binding was visualised with the ECL kit using a monoclonal anti-GST antibody (1:1000) and a secondary anti-mouse peroxidase-labelled antibody (**Section 2.1.6**).

2.2.10 *LCV Isolation*

Purification of LCVs from phagocytes has been previously described in various publications (Finsel *et al.*, 2013a; Hoffmann *et al.*, 2012, 2013; Urwyler *et al.*, 2009).

Briefly, *D. discoideum* amoebae producing calnexin-GFP or RAW 264.7 macrophages were grown to 80% confluency in 75 cm^2 flasks (**Section 2.2.4.1**). The phagocytes were infected with *L. pneumophila* expressing DsRed (pSW001) for 1 h at 25 °C or 37 °C, respectively (3 flasks per bacterial strain) (**Sections 2.1.2**, **2.2.1.2** and **2.2.3.1**), washed three times with SorC (amoebae) or PBS (macrophages) (**Section 2.1.7**) and lysed in homogenisation buffer with a ball homogeniser using an exclusion size of 8 µm.
For the immuno-magnetic separation step, the homogenate was blocked with 2% FCS and incubated with antibodies against the LCV-specific marker SidC (1:3000) and subsequently with a secondary antibody coupled to magnetic beads (20 µL/0.5 ml homogenate) (**Section 2.1.6**). The labelled LCVs were retained on a magnetic cell sorting column during washing with homogenisation buffer and were finally eluted with 0.5 ml homogenisation buffer after removal of the magnetic field. The eluate was added on top of a Histodenz gradient (10% to 35%, 11.5 ml) and centrifuged at 3350 x g for 1 h at 4 °C.

The LCV isolation was either followed by fluorescence microscopy (**Section 2.2.11**) or Western blot analysis using 4% milk powder in TBS-Tween (0.1% Tween-20 [v/v], pH 8.0) as blocking reagent and anti-Vps26 (1:1000) or anti-SNX1 (1:500) antibodies for protein detection (**Section 2.1.7**, **Section 2.1.6**).

Homogenisation buffer (Derré and Isberg, 2004)

Component	Per liter buffer	Supplier
HEPES	4.77 g (20 mM)	Roth
Sucrose	85.58 g (250 mM)	Roth
EGTA	0.19 g (0.5 mM)	Roth

The pH was adjusted to 7.2 with 1 M HCl or 1 M NaOH and the buffer filter-sterilised and stored at 4 °C.

2.2.11 *(Immuno-)Fluorescence Experiments*

2.5 x 10^5 cells of *D. discoideum* RAW 264.7 or A549 cells in 0.5 ml medium were seeded onto sterile coverslips in 24-well plates and let grow over night (**Section 2.2.4.1**, (**Section 2.2.3.1**)). Infectious *L. pneumophila* overnight cultures (**Section 2.2.1.2**) were diluted in the eukaryotic cell medium to infect the phagocytes at a multiplicity of infection (MOI) of 25 (*D. discoideum*) 10 (RAW 264.7) or 50 (A549), respectively. The infection was synchronised by centrifugation, and the infected cells were incubated at 25 °C (*D. discoideum*) or 37 °C (mammalian cells). The intact cells were permeabilised using 0.1% Triton X-100 in SorC (*D. discoideum*) or PBS (mammalian cells) for 10 min at room temperature (**Section 2.1.7**).

When homogenisation of the infected cells was necessary for the immuno-fluorescence experiments, 8.5 x 10^5 cells per well were seeded in a 6-well plate, grown over night and infected with the desired strain (see above). To obtain homogenates, the phagocytes were washed 3 times with cold SorC or PBS after one hour of infection, suspended in homogenisation buffer (**Section 2.2.10**) and lysed by eleven passages through a ball homogeniser using an exclusion size of 8 µm. The homogenate was then centrifuged onto coverslips coated with poly-L-lysine.

Intact or lysed cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 4 °C and blocked with 5% BSA in SorC (*D. discoideum*) or PBS (mammalian cells) for 30 min. The coverslips were incubated on parafilm (1 h, RT) with 30 µL of a primary antibody diluted in blocking buffer (anti-SNX1, anti-SNX2, anti-SNX3, anti-SNX5 or anti-Vps35, 1:100; anti-EEA1 or anti-CIMPR, 1:200; anti-RidL or anti-SidC, 1:250; anti-giantin, 1:400) (**Section 2.1.6**), and washed 3 times. Appropriate secondary antibodies coupled to FITC, Cy5 or DyLight650 were diluted 1:200 in blocking buffer and incubated with the sample for 1 h at room temperature. Finally, the coverslips were washed and mounted using Vectashield supplemented with 1 µg/ml DAPI to stain DNA and ultimately analysed with a Leica TCS SP5 confocal microscope.

2.2.12 *Retrograde Trafficking Experiments*

For the analysis of retrograde trafficking in RAW 264.7 macrophages, the cells were infected with *gfp*-expressing wild-type or Δ*ridL L. pneumophila* strains at an MOI of 50 for 1 hour (**Section 2.2.1.2**). The cells were washed with PBS and 0.2 ml medium supplemented with fluorescently labelled CTxB-Cy3 (1:1000, stock solution 0.5 mg/ml) was added (5 min on ice, 10-60 min at 37 °C) (**Section 2.1.7**). Optionally, CTxB was incubated along with dextran-AlexaFluor647 (1:125). After fixation with 4% PFA (20 min, RT), the infected macrophages were immuno-stained with antibodies against GM130 (1:200) or TfR (1:100) and secondary Cy5-coupled antibodies (**Section 2.1.6**). Colocalisation of the toxin with different cellular compartments was analysed by fluorescence microscopy.

Alternatively, HeLa cells were lipofected with the plasmid pEGFP-C1-ridL (pCR094) encoding GFP-RidL (**Section 2.1.2**,**Section 2.2.3.3**) 24 hours prior to the addition of fluorescently labelled STxB-Cy3 for 30 minutes (Mallard *et al.*, 1998). Inhibition of retrograde trafficking was analysed by determining colocalisation between the toxin and the Golgi, which has been stained with anti-giantin (1:400) (**Section 2.1.6**).
Scoring of the colocalisation was done by calculating the Pearson's correlation coefficient. It is defined as the covariance of two variables (in this case intracellular localisation of proteins) divided by the product of their standard deviations. The value ranges from -1 (no colocalisation) over 0 (random colocalisation) to +1 (complete colocalisation).

2.2.13 *Co-Infection Experiments*

Co-infection competition assays were performed by infecting *A. castellanii* (5 x 10^4 per well, 96-well plate) in Ac buffer with a mixture of wild-type *L. pneumophila* and Δ*ridL* mutant bacteria at an MOI of 0.01 at the ratio of 1:10 (**Section 2.2.5.1**, **Section 2.2.1.2**). The infected amoebae were grown for 9 d at 37 °C. Every third day the supernatant and lysed amoebae (0.8% saponin) were diluted 1:1000, fresh amoebae were infected (50 µL homogenate per 200 µL amoebae culture volume), and aliquots were plated on CYE agar plates containing kanamycin or not to determine colony forming units (CFU) (**Section 2.2.1.1**) (Kessler *et al.*, 2013).

Ac buffer

Component	Per liter buffer	Supplier
$MgSO_4$ x 7 H_2O (0.4 M)	10 ml	Roth
$CaCl_2$ x 2 H_2O (0.05 mM)	8 ml	Roth
Sodium-Citrate x 2 H_2O (1 M)	3.4 ml	Fluka
$Fe(NH_4)_2$ x 7 H_2O (0.25 M)	10 ml	Roth
Na_2HPO_4 (0.250 M)	10 ml	Fluka
KH_2PO_4 (0.250 M)	10 ml	Fluka

The pH was adjusted to 6.5 with 1 M HCl and the buffer autoclaved and stored at 4 °C.

2.2.14 *RNA Interference*

Using RNA interference, selected target proteins were knocked down in HeLa cells. Experiments were performed with cells cultured in 96-well plates and treated with a final concentration of 20 nM of siRNA oligonucleotides (**Section 2.2.3.1**). The siRNA stock (10 µM) was diluted 1:75 in RNase-free water, and 15 µL of diluted siRNA (133.33 nM) was added per well (**Section 2.1.4**). Subsequently, 14.9 µL DMEM medium without FCS was mixed with 0.1 µL RNAiMax (not longer than 20 min), added to the well, mixed by pipetting and incubated for 1-4 h at room temperature (longer incubation times increased transfection efficiency). During the incubation time, the HeLa cells were diluted in DMEM medium containing 14% FCS, 70 µL of the diluted cells (4 x 10^3 cells) were added per well (final FCS concentration = 10%) and incubated for 72 h. The HeLa cells were then infected (MOI 20) with 100 µL per well *L. pneumophila* cultures grown to an OD_{600} of 3.0 (**Section 2.2.1.2**) and diluted in DMEM, centrifuged (10 min, 1500 rpm) and incubated for 1 h. Subsequently, the cells were washed 3 times with pre-warmed medium containing 10% FCS and incubated for 2 d (well plate was kept moist with water in extra wells).

To determine intracellular growth of *L. pneumophila*, the medium was collected, the infected cells were lysed with 100 µL 0.8% saponin (20 min, 37 °C), and 20 µL of the combined supernatant and lysate were plated on CYE plates (**Section 2.2.1.1**). The CFU obtained were standardised to the number of cells accounting for RNAi toxicity. The efficiency of RNA interference was checked by RT-PCR or by Western blot using a polyclonal anti-Vps35 antibody (1:125) (**Section 2.1.6**).

3 Results

3.1 The *L. pneumophila* Effector RidL Promotes Intracellular Replication

High throughput studies and bioinformatical analyses of the *L. pneumophila* protein RidL had shown it to be a substrate of the Icm/Dot T4SS (Huang *et al.*, 2011; Zhu *et al.*, 2011; Zusman *et al.*, 2007). This could be confirmed in the course of this thesis in experiments utilising adenylate cyclase fusion proteins or saponin detergent extraction followed by a subcellular fractionation (Finsel *et al.*, 2013b). Furthermore and characteristically for effectors, RidL was preferentially produced in the late exponential growth phase (Finsel *et al.*, 2013b). The protein was produced by nine clinical and environmental *L. pneumophila* isolates, but not by several other species of the *Legionella* genus (Finsel *et al.*, 2013b). Additional *in silico* investigations showed *ridL* to be also encoded by *L. longbeachae* and *L. drancourtii*.

L. pneumophila lacking the effector protein exhibited no growth phenotype in AYE liquid medium (observation) and uptake into macrophages or *D. discoideum* was also unaffected (Finsel *et al.*, 2013b).

To analyse the influence of the effector on intracellular replication, *A. castellanii* was co-infected with wild-type and RidL-deficient *L. pneumophila* at an MOI of 0.01 at the ratio of 1:10. Already after 3 days, the percentage of mutant bacteria decreased while after 6 days the effector-deficient strain was completely outcompeted by wild-type bacteria, indicating a replication-beneficial role for RidL in host cells (**Figure 3.1**). Utilising fluorescently labelled bacteria, impaired growth for the Δ*ridL* mutant strain was also reported in macrophages and *D. discoideum*. Additionally, the growth defect could be complemented by introducing a plasmid harbouring *ridL* (Finsel *et al.*, 2013b).

In summary, RidL is necessary for efficient intracellular replication of *L. pneumophila* in host phagocytes.

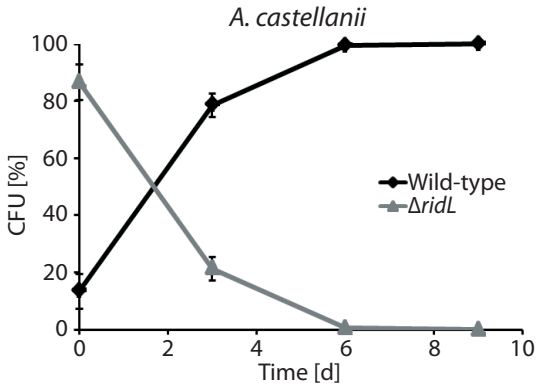

Figure 3.1: RidL Promotes Intracellular Replication.
A. castellanii was co-infected with wild-type and RidL-deficient *L. pneumophila* at the ratio of 1:10 (MOI 0.01). Every third day, the supernatant and lysed amoebae were diluted 1:1000 and fresh amoebae were infected (50 µl homogenate per 200 µl amoebae culture volume). Aliquots were plated on CYE agar plates with or without kanamycin to determine CFU. Data are means and standard deviations of triplicates. (Published in Finsel *et al.* (2013b))

3.2 Endogenous RidL Localises to the LCV

To determine the cellular distribution of translocated RidL, *D. discoideum* producing the GFP-tagged ER and LCV marker calnexin (CNX) was infected with different *L. pneumophila* strains at an MOI of 25 for 1 hour. After immuno-staining of the effector protein, RidL showed a bipolar localisation on the vacuole juxtaposed with the bacterial poles, whereas calnexin was distributed normally on the entire LCV (**Figure 3.2**). As expected, no RidL was detected on vacuoles harbouring the effector- or T4SS-deficient mutants, confirming its translocation to be strictly dependent on a functional Icm/Dot secretion system.

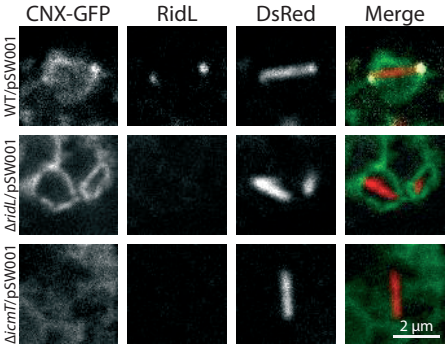

Figure 3.2: Bipolar Localisation of RidL on LCVs in *D. discoideum*.
D. discoideum producing calnexin-GFP was infected with DsRed-labelled wild-type, Δ*ridL* or T4SS-defective Δ*icmT* *L. pneumophila* (MOI 25, 1 h). RidL was visualised by immuno-fluorescence and the bacteria were stained with DAPI. (Published in Finsel *et al.* (2013b))

Furthermore, *D. discoideum* expressing *calnexin-gfp* infected with DsRed-labelled wild-type bacteria was used to analyse the temporal dynamics of RidL on the vacuole (MOI 10). SidC, which decorates the entire LCV and was used as a control (**Section 1.1.5**), and RidL were stained with antibodies and the effector-positive LCVs were counted in intact cells. Early after infection, the LCV-association kinetics of RidL were

similar to the kinetics of the effector SidC. The pathogen vacuoles showed the maximum percentage of recruitment of both effectors after 60 minutes of infection, with RidL localising to 80% and SidC to 90% of the LCVs (**Figure 3.3**). However, the amount of RidL-positive LCVs decreased to 45% after 6 h, whereas SidC was still detected on 80% of the vacuoles after this time.

This indicates that RidL is an effector which exhibits its function early after uptake into the host cell.

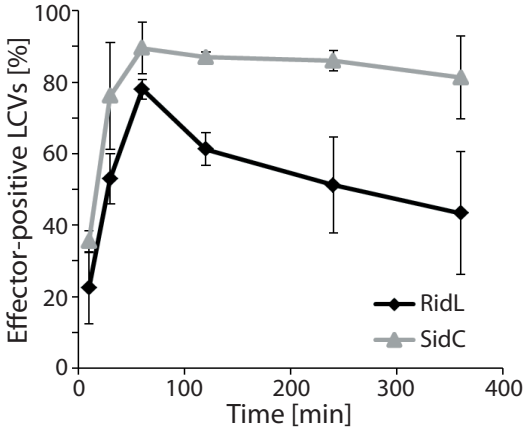

Figure 3.3: RidL and SidC dynamics on LCVs.
D. discoideum producing calnexin-GFP was infected with DsRed-labelled wild-type bacteria at an MOI of 10. RidL and SidC were visualised by immuno-fluorescence and the amount of effector-positive LCVs was counted after different points in time. Data represent means and standard deviations of two independent experiments (n = 50-100 LCVs each). (Published in Finsel *et al.* (2013b))

In a similar experiment, localisation of RidL was analysed in macrophages. The cells were infected with wild-type bacteria harbouring a plasmid encoding for M45-tagged SidC, which was used to stain the LCV (MOI 10, 1 h). After lysis of the cells with a ball homogeniser the amount of RidL-positive LCVs was determined by immunofluorescence (**Figure 3.4**). The effector localised to approximately 80% of the LCVs in macrophages, showing again a bipolar distribution on the poles. The same phenotype was observed when RidL was visualised in intact macrophages infected with wild-type bacteria producing the fluorophor DsRed (**Figure 3.4A**, lower panel).

Once more, these findings confirm the T4SS-dependent translocation of RidL and furthermore show the effector to accumulate polarly on the LCV in phagocytes.

3.3 RidL Can Decorate the Entire LCV

To analyse possible changes in RidL localisation upon overproduction, a *L. pneumophila* strain was used which carried a plasmid producing DsRed and about 200 times more RidL (pIF007) compared to wild-type bacteria (**Figure 3.5A**). Infection of *D. discoideum* producing calnexin-GFP and RAW 264.7 macrophages with this strain (MOI 25

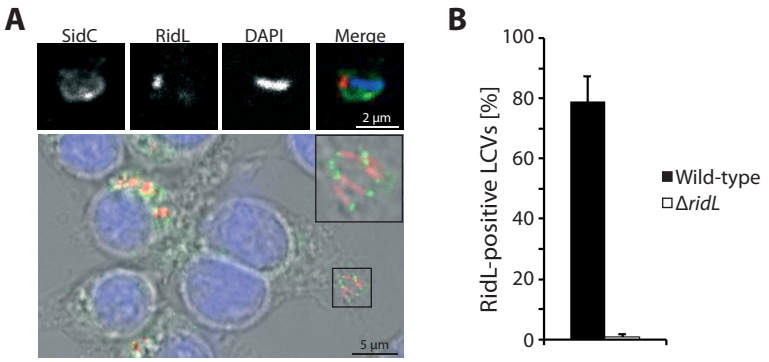

Figure 3.4: Bipolar Localisation of RidL on LCVs in Macrophages. (**A, upper panel; B**) Macrophages were infected with *L. pneumophila* producing M45-SidC as an LCV-marker (MOI 10, 1 h). After homogenisation RidL and M45-SidC were visualised by immuno-fluorescence, bacteria stained with DAPI and the amount of effector-positive LCVs was documented. Data represent means and standard deviations of three independent experiments (n > 50 LCVs each). (**A, lower panel**) Figure shows a representative image of intact macrophages, which were infected with DsRed-labelled wild-type baceria (MOI 20, 1 h), stained against RidL and treated with DAPI. (Published in Finsel *et al.* (2013b))

or MOI 10, respectively) and subsequent immuno-staining of RidL revealed the effector to decorate LCVs more extensively (**Figure 3.5B, C**). Quantification in macrophages showed 35% of the vacuoles to be covered nearly completely by RidL.

To confirm the finding that RidL is able to bind on the entire LCV membrane, *D. discoideum* amoebae producing RidL-GFP were infected with fluoresently-labelled wild-type bacteria (MOI 25). The recruitment of the fusion protein to the vacuoles was determined by immuno-fluorescence with SidC as an LCV marker. After 1 hour RidL was detected evenly distributed over the complete membrane on 80% of the vacuoles (**Figure 3.6**).

These results suggest that the receptor RidL binds to is not only present in the proximity of the bacterial poles, but can be found on the entire LCV membrane. Furthermore, localisation of the effector to the vacuole does not depend on a translocation by the T4SS.

3.4 RidL Binds PtdIns(3)*P* and Vps29

In search of this receptor and given that several T4SS substrates bind to host lipids (**Section 1.1.5**), GST-RidL and GST-SidC as a control were subjected to a protein-lipid overlay assay. This revealed RidL to specifically bind to PtdIns(3)*P* and SidC as

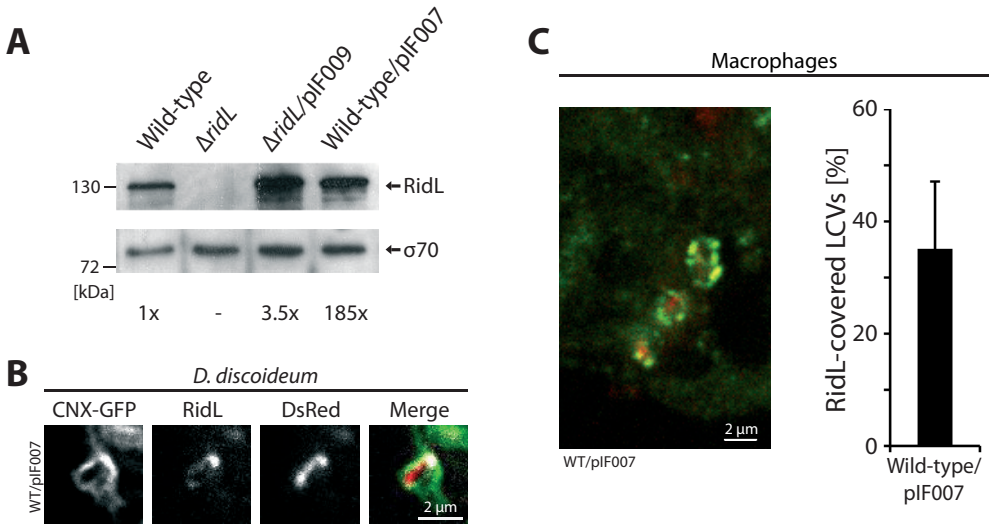

Figure 3.5: RidL Decorates Large Areas of the LCV Upon Overproduction. (**A**) The amount of RidL or the loading control sigma factor σ70 was quantified densitometrically after Western blotting with lysates of *L. pneumophila* wild-type, Δ*ridL*, Δ*ridL*/pIF009 (P_{ridL}-RidL) and wild-type/pIF007 (P_{tac}-RidL; diluted 1:100). (**B, C**) *D. discoideum* producing calnexin-GFP and RAW 264.7 macrophages were infected with DsRed-producing wild-type bacteria overexpressing *ridL* (pIF007) (MOI 25 and MOI 10, respectively; 1 h) and immuno-stained against the effector. Quantification data for macrophages represent means and standard deviations of three independent experiments (n > 40 LCVs each). (Published in Finsel *et al.* (2013b))

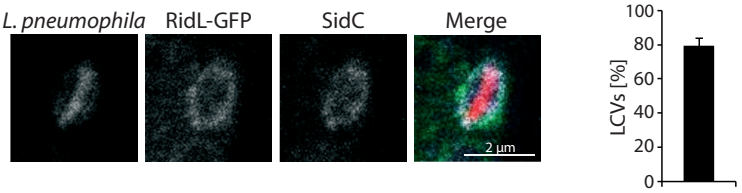

Figure 3.6: Ectopically Produced RidL Decorates the Entire LCV in *D. discoideum*. *D. discoideum* expressing *ridL-gfp* was infected with *L. pneumophila* producing DsRed (MOI 25, 1 h). The cells were immuno-stained against the vacuole marker SidC and the amount of LCVs positive for a complete RidL coverage documented. Data represent means and standard deviations of three independent experiments (n > 50 LCVs each). (Published in Finsel *et al.* (2013b))

previously shown to PtdIns(4)P (**Figure 3.7A**).

In order to find additional interaction partners, RidL was covalently linked to beads and incubated with lysates of *D. discoideum* or macrophages. Mass spectrometrical analysis determined the proteins that were specifically bound by the effector to be Vps26, Vps29 and Vps35, the core components of the cargo-selective subcomplex of the retromer (**Section 1.2.2**) (Finsel *et al.*, 2013b).

To identify the specific factor(s) of the retromer subcomplex RidL interacts with, a pulldown was performed with GST-purified Vps26 or Vps29 and lysates of *L. pneumophila* overproducing the effector. Western blot analysis revealed that Vps29, but not Vps26 specifically retained RidL (**Figure 3.7B**). A similar approach with His-RidL instead of cell lysates confirmed this specific interaction and indicates that this interaction is direct and independent of additional *L. pneumophila* factors (Finsel *et al.*, 2013b).

This finding and the observation that the effector is able to cover the entire LCV membrane (**Section 3.3**) led to its nomenclature 'RidL' (= Retromer interactor decorating LCVs).

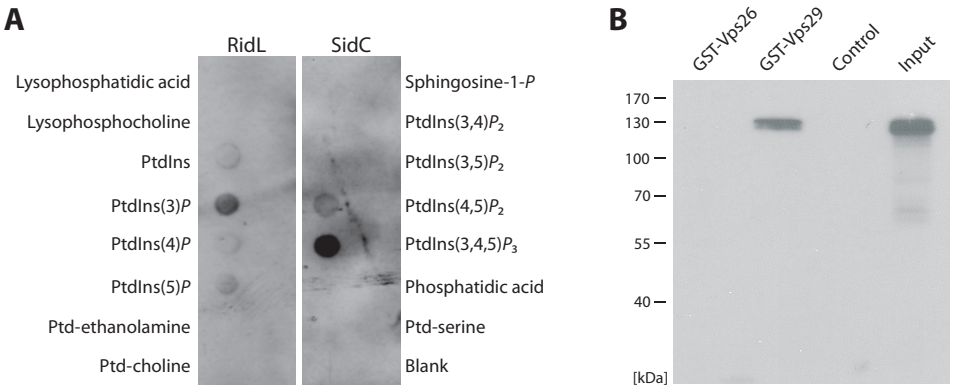

Figure 3.7: RidL Binds PtdIns(3)*P* and Vps29. (**A**) GST-RidL and GST-SidC were incubated with commercially available PIP-strips, showing specificity of the effectors to PtdIns(3)*P* or PtdIns(4)*P* after anti-GST Western blotting. (**B**) Pull-down analysis and subsequent anti-RidL Western blotting identified RidL from *L. pneumophila* lysates to be specifically retained by GST-Vps29, not Vps26. The control sample was *L. pneumophila* lysate incubated with glutathione beads without GST-tagged protein and the input was pure lysate. (Published in Finsel *et al.* (2013b))

3.5 Cargo-selective Subunits and Cargo Receptors Localise to LCVs

To determine whether components of the cargo-selective subcomplex and cargo receptors localise to the LCV and whether RidL influences their acquisition, *D. discoideum* amoebae producing GFP-fusion proteins were infected with DsRed-labelled *L. pneumophila* strains (MOI 25). After infection for 1 h and homogenisation of the cells, presence of the GFP-tagged proteins on LCVs was quantified by fluorescence microscopy (**Figure 3.8**). Calnexin-GFP was used as a control and was, like the analysed

retromer subunits, only detected on LCVs established by bacteria with a functional T4SS. In contrast, about 70% of the LCVs containing wild-type bacteria were positive for calnexin-GFP, Vps26-GFP or Vps29-GFP.

Interestingly, Vps10-GFP, a homologue of the mammalian hydrolase-binding sortilin (**Section 1.2.1**), showed reduced acquisiton frequency to LCVs (45%) and was the only protein whose recruitment was RidL-dependent. Approximately twice as many LCVs harbouring bacteria lacking *ridL* were positive for the cargo receptor compared to LCVs harbouring the wild-type (25%).

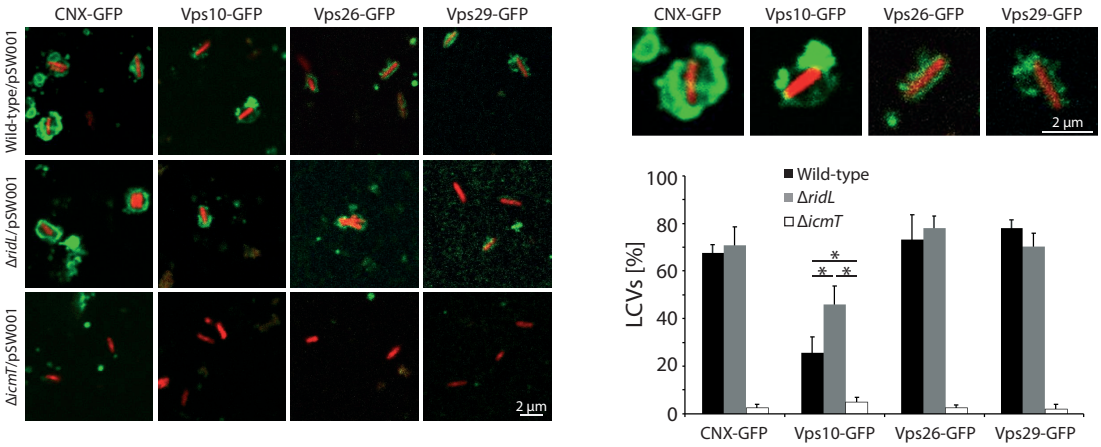

Figure 3.8: Acquisition of Retromer Components by LCVs in *D. discoideum*. *D. discoideum* producing GFP-fusion proteins were infected with *L. pneumophila* wild-typ, Δ*ridL* and Δ*icmT* strains (MOI 25, 1 h). After cell homogenisation, presence of the GFP-tagged proteins on LCVs was quantified by fluorescence microscopy. The left panel shows overview images and the upper right panel magnifications of LCVs positive for the fusion proteins. Data represent means and standard deviations of 3 independent experiments (n > 50 LCVs each; * = p < 0.05). (Published in Finsel *et al.* (2013b))

In a similar approach, RAW 264.7 macrophages were infected with fluorescently labelled *L. pneumophila* for 1 h (MOI 25) and homogenates of the cells immuno-stained against the early endosomal antigen 1 (EEA1), the hydrolase-binding CIMPR and the retromer component Vps35 (**Figure 3.9**). Again, detection of the proteins on the vacuoles was strictly Icm/Dot-dependent and the acquisition of neither the control (EEA1) nor the cargo-selective subunit (Vps35) was altered in presence of RidL (60% and 35%, respectively). However, and similar to Vps10-GFP in *D. discoideum* described above, an increased percentage of LCVs harbouring the Δ*ridL* strain was positive for the cargo receptor CIMPR compared to wild-type (40% vs. 30%).

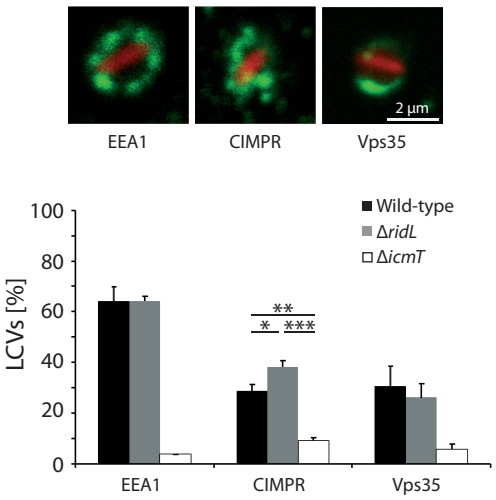

Figure 3.9: Acquisition of Retromer Components to LCVs in Macrophages.
Macrophages were infected with *L. pneumophila* wild-type, Δ*ridL* and Δ*icmT* strains (MOI 25, 1 h). After cell homogenisation, presence of EEA1, CIMPR and Vps35 on LCVs was quantified by immuno-staining and fluorescence microscopy. Data represent means and standard deviations of 3 independent experiments (n > 50 LCVs each; * = p < 0.05; ** = p < 0.01; *** = p < 0.001). (Published in Finsel *et al.* (2013b))

In summary, recruitment of the components of the cargo-selective subcomplex to LCVs depended on a functional T4SS and was not changed in presence of RidL, suggesting that the effector is dispensable for acquisition and assembly of the Vps heterotrimer on LCVs. However, RidL decreased the accumulation of the cargo receptors Vps10 and CIMPR on the LCV, indicating an influence of the effector on retrograde trafficking in host cells.

3.6 RidL Does Not Alter LCV Stability

To exclude the possibility that the acquisition phenotype of the cargo receptors is only a secondary effect caused by a modulation of LCV stability by RidL, pathogen vacuoles were isolated from *D. discoideum*.

The amoebae were infected with wild-type and Δ*ridL L. pneumophila* producing DsRed and the LCVs immuno-magnetically purified utilising an antibody against the vacuole marker SidC according to the standard protocol (**Figure 3.10**). Both strains yielded approximately 40% intact and less than 30% ruptured LCVs with an altogether comparable morphology and stability. These data indicate that RidL does not influence the overall integrity of the LCV membrane.

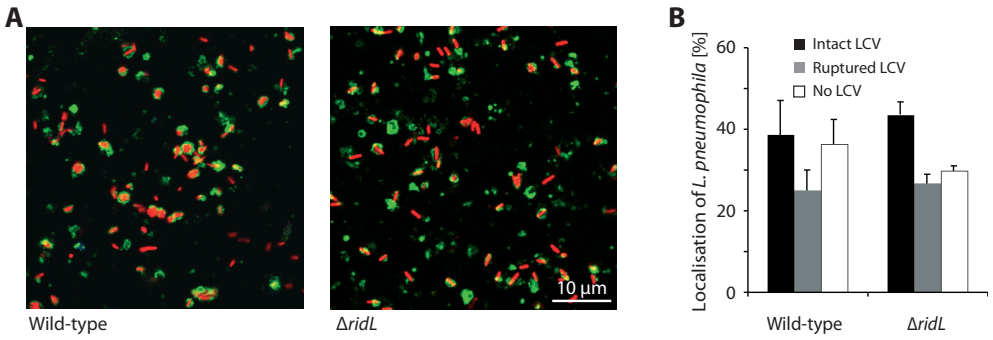

Figure 3.10: RidL Does Not Affect LCV Stability. *D. discoideum* producing calnexin-GFP was infected with wild-type and Δ*ridL L. pneumophila* expressing *dsred* (MOI 50, 1 h). After immunoaffinity purification of the LCVs according to the standard protocol (**A**), LCVs were quantified by fluorescence microscopy regarding morphology, stability and yield (**B**). Data represent means and standard deviations of 3 independent experiments (n > 50 LCVs each). (Published in Finsel *et al.* (2013b))

3.7 Influence of RidL on the Localisation of Various Host Proteins

Additional to the retromer components tested in **Section 3.5**, several other important host proteins were analysed regarding their putative RidL-dependent recruitment to LCVs. To this end, *D. discoideum* amoebae expressing *gfp*-fusion constructs were infected with *L. pneumophila* (MOI 25, 1 h) and the presence of these factors on LCVs was quantified by fluorescence microscopy (**Figure 3.11A**). All tested proteins had previously been detected on LCVs and comprised the ER marker calnexin as a control, the ER/Golgi-resident GTPase Rab1, the TGN-localised GTPase Rab8, the endosomal marker GTPases Rab7 and Rab14 and the PtdIns(4,5)P_2 5-phosphatase Dd5P4/OCRL, which was reported to play a role in retrograde trafficking (Choudhury *et al.*, 2005; Clemens *et al.*, 2000; Kagan *et al.*, 2004; Urwyler *et al.*, 2009; Weber *et al.*, 2009). Each of the proteins accumulated on the LCV in an Icm/Dot-dependent, but RidL-independent manner, indicating that the effector is not involved in the recruitment of these factors.

However, in a similar analysis with GFP-tagged lysosomal-associated membrane protein-1 (LAMP-1) produced in macrophages (MOI 25, 1 h), LCVs established by Δ*ridL L. pneumophila* associated twice as frequent with this factor compared to wild-type (**Figure 3.11B**). Considering that replication-defective LCVs accumulated the lysosomal marker (see Δ*icmT* in **Figure 3.11B** and Roy *et al.* (1998)), this phenotype corresponds to the decreased intracellular fitness of the effector mutant (**Section 3.1**).

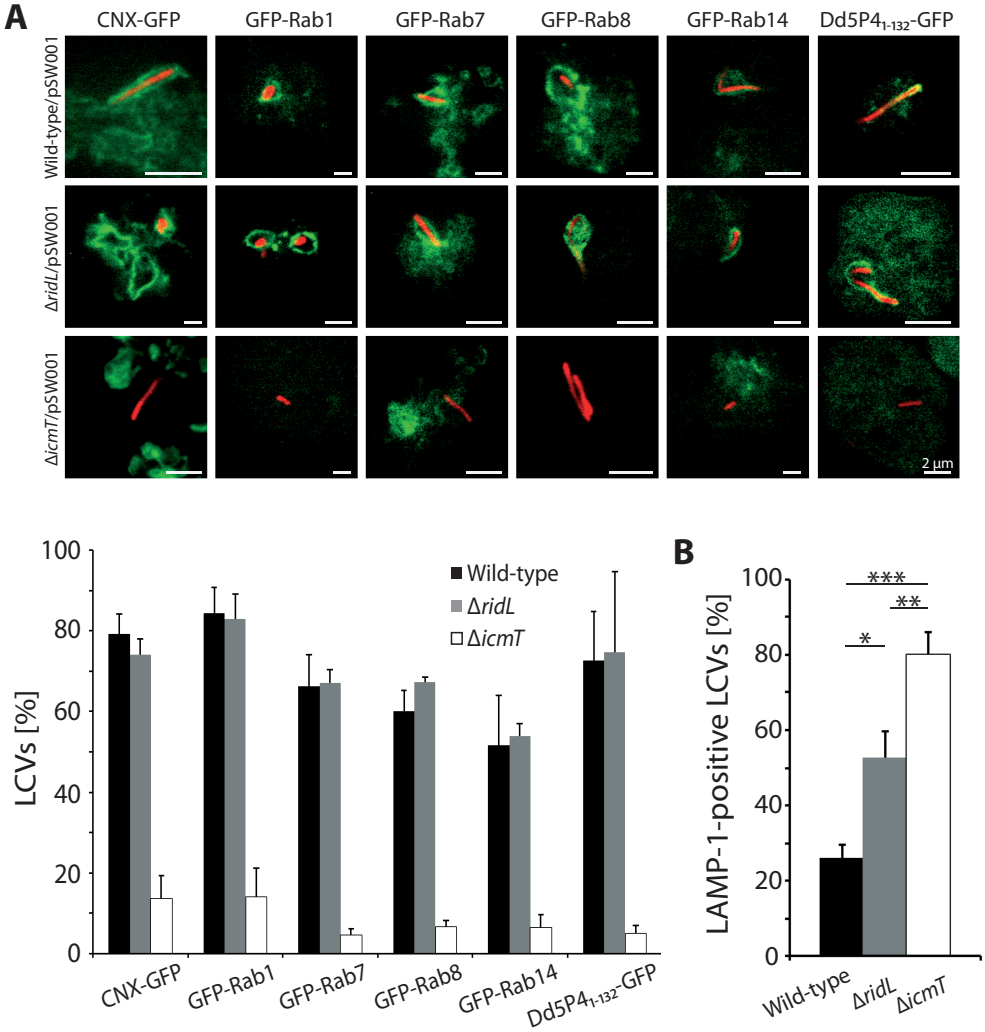

Figure 3.11: Acquisition of Host Proteins to LCVs in *D. discoideum*. (**A**) *D. discoideum* or (**B**) macrophages producing GFP-fusion proteins were infected with *L. pneumophila* wild-type, Δ*ridL* and Δ*icmT* strains (MOI 25, 1 h). Recruitment of the GFP-tagged proteins on LCVs in intact cells was quantified by fluorescence microscopy. Data represent means and standard deviations of 3 independent experiments (n > 50 LCVs each; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (Published in Finsel *et al.* (2013b))

3.8 RidL Decreases LCV Association of SNX Proteins

After determining the influence of RidL on the cargo-selective subcomplex (**Section 3.5**), the putative effector-dependent LCV recruitment of the membrane-deforming subcomplex was analysed (**Section 1.2.2**). To this end, macrophages were infected with *dsred*-expressing *L. pneumophila* wild-type or Δ*ridL* strains at an MOI of 20 for

1 h. After homogenisation of the cells, the LCVs were immuno-stained against several sorting nexins and the vacuole marker SidC (**Figure 3.12**). Interestingly, and in contrast to the Vps proteins, the overall percentage of SNX1- and SNX2-positive vacuoles was much lower. Furthermore, SNX3 was not detected on LCVs at all whereas sorting nexin 5 decorated half of the stained vacuoles.

However, when infecting RAW 264.7 with bacteria lacking *ridL*, the frequency of SNX1- and SNX2-positive pathogen vacuoles was twice as high as for the wild-type strain. On the other hand, the phenotype for SNX3 (not detected) and SNX5 (50% of the LCVs) remained unchanged.

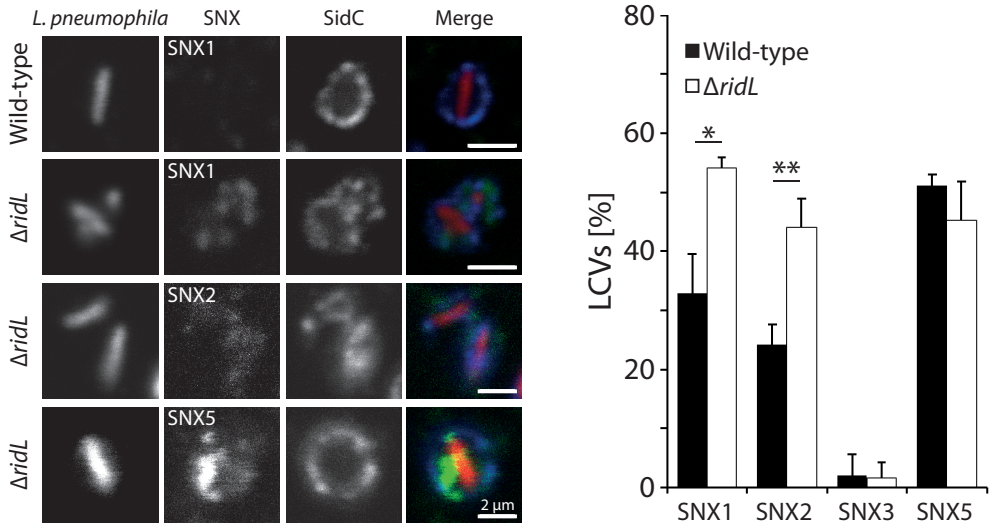

Figure 3.12: Acquisition of Sorting Nexins to LCVs in Macrophages. RAW 264.7 macrophages were infected with wild-type and Δ*ridL* strains producing DsRed (MOI 20, 1 h). After homogenisation, SNX1, SNX2, SNX3 or SNX5 were immuno-stained together with the vacuole marker SidC and the amount of sorting nexin-positive LCVs documented. Data represent means and standard deviations of 3 independent experiments (n > 40 LCVs each; * = p < 0.05; ** = p < 0.01;). (Published in Finsel *et al.* (2013b))

To confirm the RidL-dependent localisation phenotype of SNX1, LCVs were isolated from macrophages und subjected to Western blot analysis. Cell lysates before and after the purification process were stained against SNX1 and Vps26 as a control, which has been shown to be recruited to LCVs in a RidL-independent manner (**Figure 3.13**). Again, unlike the Vps protein, SNX1 was barely detected in samples of isolated vacuoles harbouring wild-type bacteria, but showed signal twice as strong for LCVs containing Δ*ridL L. pneumophila*. As previously documented, recruitment of Vps26 to vacuoles

was not influenced by the presence of the effector.

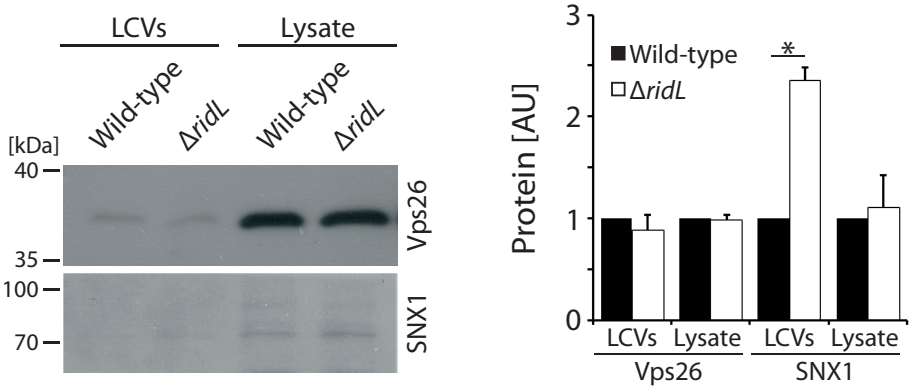

Figure 3.13: Binding of SNX1 to LCVs is Decreased by RidL. LCVs harbouring *dsred*-expressing wild-type or Δ*ridL* bacteria (MOI 50, 1 h) were isolated from RAW 264.7 macrophages. The amount of SNX1 and Vps26 was densitometrically quantified after Western blotting and compared to lysates of the cells. Data represent means and standard deviations of 3 independent experiments (AU = arbitrary units; * = $p < 0.05$;). (Published in Finsel *et al.* (2013b))

In an additional corroborative experiment, human A549 alveolar basal epithelial cells transiently expressing *gfp-SNX1* were infected with *L. pneumophila* at an MOI of 100 and after 1 h homogenised and immuno-stained against SidC as a marker of the pathogen vacuole (**Figure 3.14**). Once again, significantly more SNX1-decorated LCVs were observed after infection with the Δ*ridL* strain compared to wild-type (50% versus 30%).

As a summary, RidL has been shown to decrease the presence of SNX1 and SNX2 on LCVs indicating a putative removal of the sorting nexins by the effector.

3.9 RidL Competes with SNX1 for Binding to PtdIns(3)*P* on Membranes

A possible reason for the decreased SNX-levels on vacuoles is given by the affinity of both RidL and the SNX proteins for PtdIns(3)*P*, suggesting a competition for binding sites on the membrane. To test this hypothesis, HeLa cells were transfected to ectopically produce GFP-RidL and subsequently analysed regarding colocalisation of SNX1 and the endosomal marker transferrin receptor (TfR), which were visualised by immunostaining. Additional to the effector fusion protein, GFP-2xFYVE, the PtdIns(3)*P*-

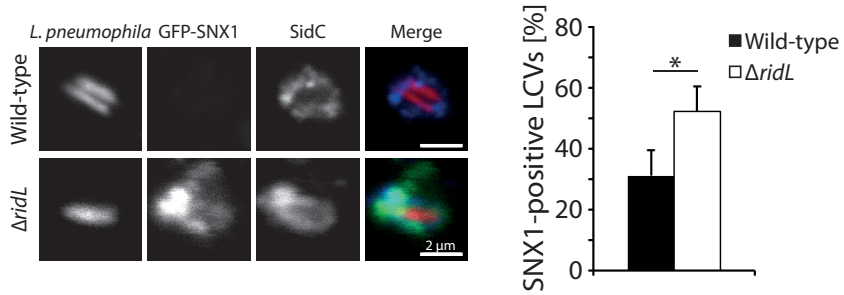

Figure 3.14: RidL Decreases Accumulation of SNX1-GFP on LCVs in A549 Cells. A549 lung epithelial cells producing GFP-SNX1 were infected with wild-type and Δ*ridL* strains expressing *dsred* (MOI 100, 1 h). After homogenisation, SidC was visualised by immuno-fluorescence and the percentage of GFP-SNX1-positive LCVs documented. Data represent means and standard deviations of 3 independent experiments (n > 40 LCVs each; * = p < 0.05;). (Published in Finsel *et al.* (2013b))

binding domain of the eukaryotic regulator protein Hrs, and GFP were used as positive and negative controls, respectively (**Figure 3.15**). Strikingly, in cells expressing *gfp-ridL* or *gfp-2xfyve* SNX1 exhibited significantly less colocalisaion with TfR compared to cells producing GFP only.

This suggests that RidL reduces the amounts of LCV-bound sorting nexins by competition for the binding to PtdIns(3)P on membranes. Notably and in accordance with these findings, SNX5, which does not bind to PtdIns(3)P, was reported to localise to LCVs unaffected by RidL (**Section 3.8**).

3.10 RidL Inhibits Retrograde Trafficking of Shiga Toxin in HeLa Cells

After having shown an influence of RidL on components of the retromer complex and cargo receptors (**Sections 3.5** and **3.8**), the putative modulation of retrograde trafficking by the effector was directly tested. For this purpose, HeLa cells were transiently transfected with the plasmid pEGFP-C1-*ridL* and correct production of the fusion protein GFP-RidL was verified by Western blot (Finsel *et al.*, 2013b).

To exclude an effect of the N-terminal GFP-tag on the intracellular localisation of the protein, HeLa cells were infected with DsRed-labelled wild-type *L. pneumophila* 1 d after transfection (MOI 100, 1 h) and the recruitment of the effector to LCVs

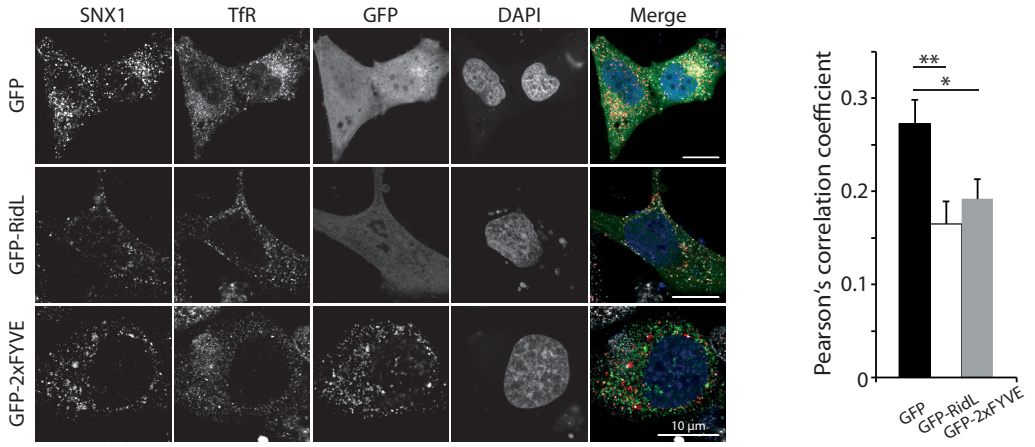

Figure 3.15: Competition of RidL or GFP-2xFYVE with SNX1 for Binding to PtdIns(3)*P*. Colocalisation of SNX1 with the endosomal marker TfR was documented in HeLa cells ectopically producing GFP, GFP-RidL or GFP-2xFYVE utilising the Pearson's correlation coefficient. Data represent means and standard deviations of 3 independent experiments (n = 10 cells each; * = $p < 0.05$; ** = $p < 0.01$;). (Published in Finsel *et al.* (2013b))

was analysed by immuno-fluorescence of homogenates with SidC as vacuole marker (**Figure 3.16**). Approximately 60% of the LCVs were GFP-RidL-positive indicating that the ectopic production of the fusion protein does not disturb the normal localisation of the effector. Furthermore, the presence of GFP-RidL had no impact on cell adherence, spreading or overall morphology of the epithelial cells (Finsel *et al.*, 2013b).

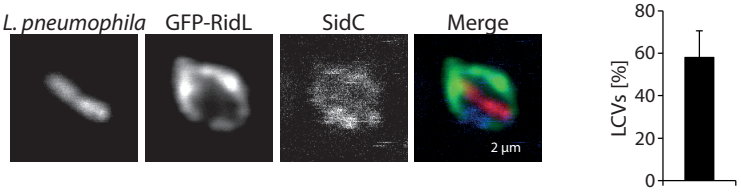

Figure 3.16: Ectopically Produced GFP-RidL Localises to LCVs in HeLa Cells. HeLa cells transiently transformed to produce GFP-RidL were infected with fluorescent wild-type *L. pneumophila* at an MOI of 100 for 1 h. After homogenisation presence of GFP-RidL on LCVs was quantified by immuno-fluorescence with SidC as vacuole marker. Data represent means and standard deviations of 3 independent experiments (n > 50 LCVs each). (Published in Finsel *et al.* (2013b))

To directly analyse the effect of RidL on the retrograde trafficking route, the transfected HeLa cells were treated with Cy3-labelled Shiga toxin subunit B (STxB), a well studied tool to investigate the retrograde pathway (**Section 1.2.5**). 30 min after addition of the toxin the intact cells were immuno-stained against the Golgi-marker giantin

and the colocalisation of STxB with this compartment documented (**Figure 3.17**). Compared to control cells producing GFP only, the toxin showed a significantly lower overlap with giantin in HeLa cells expressing *gfp-ridL*.

These results indicate a disruption of the retrograde transport of the toxin by RidL and therefore strongly suggests the effector to be an inhibitor of retrograde vesicle trafficking. Notably, the inhibition had taken place before StxB reached the Golgi apparatus.

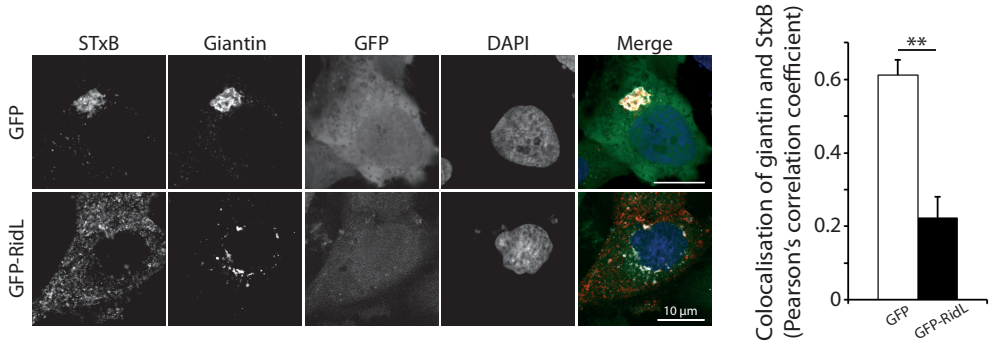

Figure 3.17: GFP-RidL Inhibits Retrograde Trafficking of STxB in HeLa Cells. HeLa cells ectopically producing GFP-RidL or GFP as a control were treated with STxB-Cy3. Colocalisation of the toxin with the Golgi marker giantin was analysed after 30 min by immuno-fluorescence and scored with the Pearson's correlation coefficient. Data represent means and standard deviations of 3 independent experiments (n = 10 cells each; ** = $p < 0.01$;). (Published in Finsel *et al.* (2013b))

3.11 RidL Inhibits Retrograde Trafficking of Cholera Toxin in Macrophages

To confirm the inhibitory effect of RidL on the retrograde pathway with endogenously instead of ectopically produced effector proteins, transport of cholera toxin subunit B (CTxB) was assessed in infected RAW 264.7 macrophages. CTxB was fluorescently labelled with AlexaFluor555 and represents another well established tool to study retrograde trafficking (**Section 1.2.5**). After infection of the macrophages with GFP-labelled *L. pneumophila* wild-type, Δ*ridL*, Δ*icmT* or a Δ*ridL* complementation mutant strain for 1 h (MOI 50), the cells were treated with the toxin for various intervals up to 1 h (Finsel *et al.*, 2013b). Compared to uninfected cells, trafficking of CTxB was disturbed in wild-type-infected cells, showing a cortical distribution of the toxin instead of a perinuclear localisation. Consistent with previos results, the T4SS-mutant Δ*icmT* and

the effector-deficient strain Δ*ridL* had no influence on the intracellular toxin transport. Upon expression of *ridL* in the *L. pneumophila* strain lacking this effector, inhibition of the retrograde pathway was restored to levels obtained for wild-type bacteria. Importantly, no strain-dependent differences in adherence, spreading or overall morphology of the macrophages were observed. Furthermore, the toxin was never detected on LCVs indicating that CTxB bypasses the vacuole when transported in a retrograde manner (Finsel *et al.*, 2013b).

In summary, these findings substantiate the claim that RidL is an inhibitor of the retrograde pathway and that endogenous amounts of the effector are sufficient to exhibit this function.

Moreover, the above described experimental setup was used in order to identify the intracellular site of retrograde inhibition. To this end, infected macrophages were treated with CTxB and either co-incubated with the lysosomal marker dextran-Alexa-Fluor647 for 45 min or immuno-stained against the Golgi-marker GM130 or the early/ recycling endosomal marker TfR (**Figure 3.18**). When infected with wild-type bacteria, CTxB colocalised clearly with TfR, but only to a low extent with dextran or GM130. For a better visualisation of the results, the fluorescence intensities of the toxin and the repective marker along a section were plotted in a diagram. Remarkably, upon infection with *L. pneumophila* lacking *ridL* colocalisation of the toxin was not only observed for the endosomal, but also for the Golgi marker. Again, dextran barely colocalised with CTxB.

These experiments confirm that RidL is indeed a retrograde trafficking inhibitor and reveal that the interruption of the pathway takes place at an post-endosomal, but pre-Golgi stage. Notably, this is the very step of the retrograde pathway the retromer complex catalyses and suggests that RidL modulates this cellular apparatus to exhibit its function.

3.12 Functional Retrograde Trafficking Restricts Intracellular Growth

Having shown that the *L. pneumophila* effector RidL inhibits retrograde vesicle trafficking (**Sections 3.10** and **3.11**), siRNA experiments were performed to investigate the putative benefits the bacteria gain by deactivating this host cell pathway. For this purpose, key factors of the retromer complex were knocked down by RNAi in HeLa cells (3 d) and the influence on replication of *L. pneumophila* determined by a CFU assay (MOI

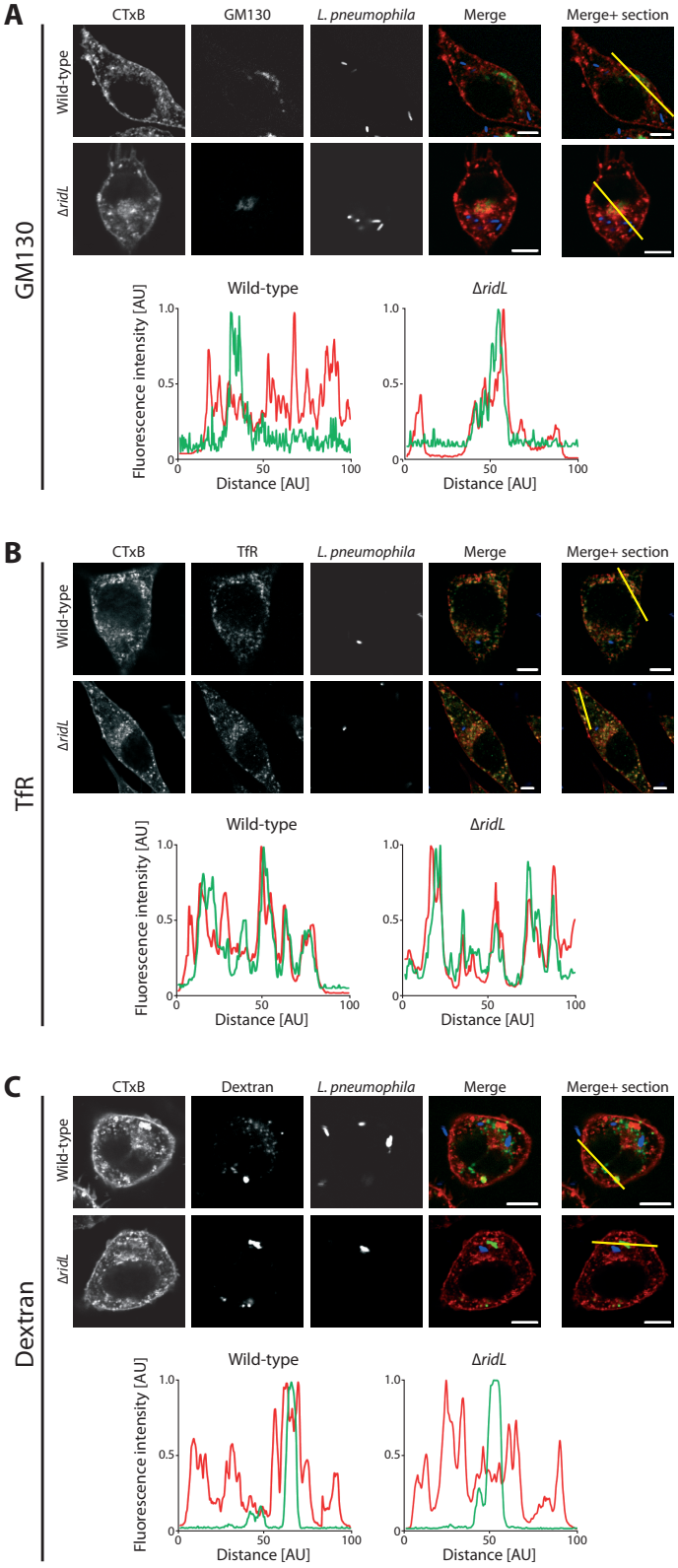

Figure 3.18: RidL Inhibits Retrograde Trafficking of CTxB in Macrophages. Macrophages were infected with *gfp*-expressing *L. pneumophila* wild-type or Δ*ridL* strains (MOI 50, 1 h; depicted in blue colour) and subsequently treated with CTxB-AlexaFluor555 (red) for 45 min. The infected cells were either immunostained against GM130 (**A**) or TfR (**B**) (green) or had been co-incubated with the toxin and dextran-AlexaFluor647 (**C**). Colocalisation was visualised in a diagram, where fluorescence intensities of CTxB (red) and the respective marker (green) are plotted along a section. Pictures show representative cells from 3 independent experiments (AU = arbitrary units; scale bar = 2 µm). (Published in Finsel *et al.* (2013b))

20, 2 d). The following experiments were conducted in collaboration with Dr. Curdin Ragaz (Basel, Switzerland).

Depletion of the GTPase Arf1 served as a positive control and reduced intracellular growth of the wild-type bacteria to a CFU count half as high as with non-silencing scrambled siRNA (**Figure 3.19**). In contrast, treatment of the host cells with siRNA against the cargo recognition subunits Vps26A/B or Vps29, the cargo receptor CIMPR, the PI 5-phosphatases OCRL or INPP5B or the PI kinase PI3K yielded in a doubling of the CFU. Consistently, the data obtained for PI3K confirm the results from Weber *et al.* (2006), where chemical inhibition of the kinase increased intracellular replication of *L. pneumophila*. The beneficial effect of the knock-down of OCRL or its paralogue INPP5B on bacterial growth was confirmed by replication assays in fibroblasts from LOWE syndrome patients, who carry loss-of-function mutations in their *OCRL* genes (Lowe, 2005). In accordance with the RNAi experiments, the CFU count was twice as high as upon infection of control cells (Finsel *et al.*, 2013b).

Surprisingly and in contrast to the other components of the cargo-selective subcomplex Vps26 and Vps29, treatment of cells with siRNA against Vps35 did not significantly affect replication of bacteria. However, Western blot analyses showed that despite successful *vps35* mRNA depletion 15-30% of the protein remained in the cells compared to the negative control, thus providing an explanantion for the lack of a phenotype (Finsel *et al.* (2013b) and **Figure 3.20**).

Unexpectedly, knock-down of SNX1, SNX2, SNX3 or SNX5 or combinations of 2 of the sorting nexins had no impact on intracellular replication of the bacteria (Finsel *et al.*, 2013b).

As a summary, depletion of key components of the retrograde pathway promoted bacterial growth upon infection with *L. pneumophila* suggesting that the pathogens inhibit this pathway by RidL to increase intracellular replication. Of note, all significant in- or decreases in replication efficiency of the wild-type upon siRNA treatment are similar for the Δ*ridL* effector mutant suggesting that the restriction of intracellular growth by retrograde trafficking is apparently epistatic to the effects of RidL on the retromer.

In conclusion, it was shown that *L. pneumophila* disrupts the retrograde vesicle pathway of host cells, which restricts intracellular growth of the bacteria, and that the Icm/Dot translocated protein RidL is indispensable for the inhibition. Detailed investigations revealed, that the effector binds to the retromer subunit Vps29 and the lipid PtdIns(3)P allowing conclusions and educated speculations about the mechanism with which the substrate modulates retrograde trafficking (**Section 4.1**).

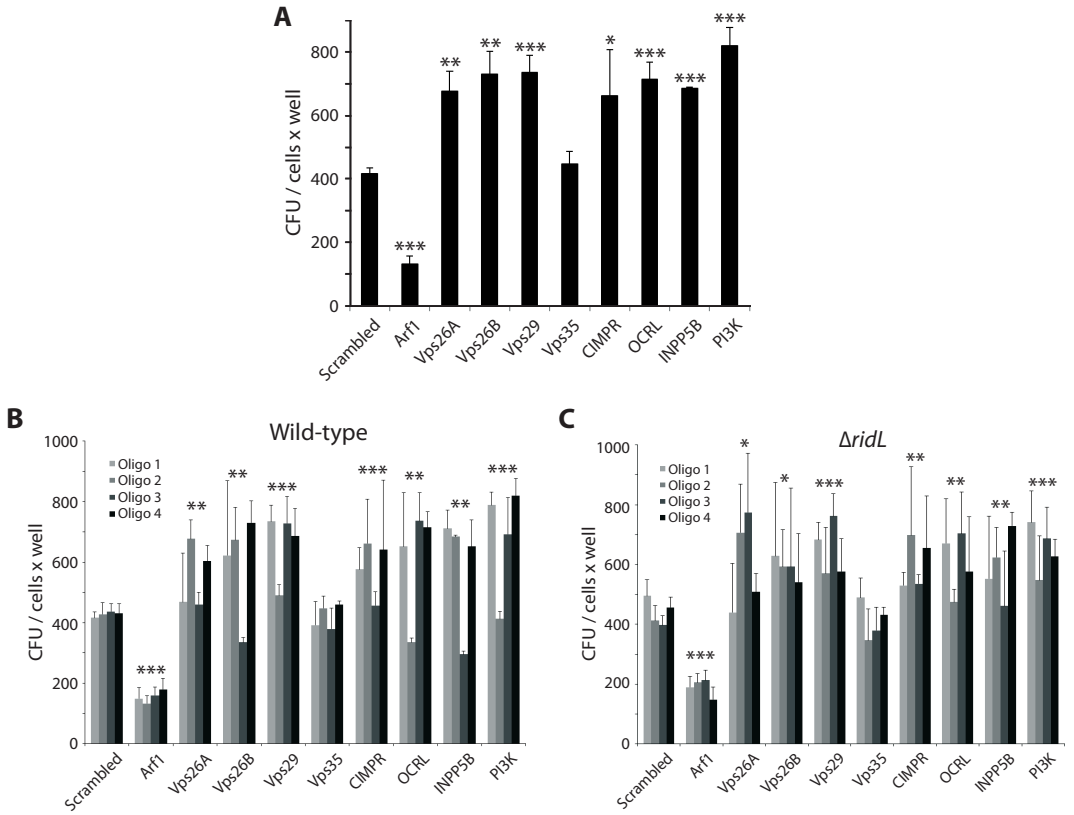

Figure 3.19: Retromer Inhibition Increases Intracellular Replication of *L. pneumophila*. HeLa cells were treated with siRNA against key factors of retrograde vesicle trafficking for 3 d and subsequently infected with *L. pneumophila* wild-type or Δ*ridL* strains (MOI 20). After 48 hours intracellular growth was quantified by counting CFU and corrected for cytotoxic effects. For a clearer visualisation of the results obtained for the wild-type, representative data of two to four of the four siRNAs used were chosen (**A**). The complete set of data is shown in (**B**) for wild-type and in (**C**) for the effector mutant and represent means and standard deviations of 3 independent experiments. (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (Published in Finsel *et al.* (2013b))

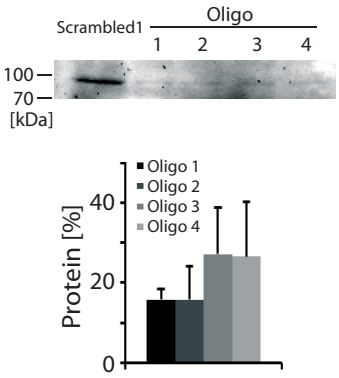

Figure 3.20: Quantification of Vps35 Depletion after RNAi. Efficiency of Vps35 protein depletion with the four different siRNAs was determined by Western blot using cells treated with scrambled siRNA as a control. The residual protein amount was determined densitometrically. Data represent means and standard deviations of 3 independent experiments. (Published in Finsel *et al.* (2013b))

4 Discussion

Infection by *Legionella pneumophila* fundamentally reprograms the host cell's signal pathways and vesicle trafficking to suit the needs of the pathogen. The bacteria have been shown to interact with the secretory as wells as the endocytic pathway and, as reported in this thesis, also with the retrograde pathway (**Chapter 3** and **Section 4.1**). The exact way in which *L. pneumophila* benefits from interfering with retrograde trafficking and the mechanistic background of the respective effector inhibiting this pathway (RidL) remain to be further elucidated. Only two other pathogens, *Salmonella enterica* serovar Typhimurium (McGourty *et al.*, 2012) and herpesvirus saimiri (Kingston *et al.*, 2011), have been shown to inhibit the retrograde pathway and few other viruses exploit it.

4.1 *Legionella pneumophila* Inhibits Retrograde Trafficking

As shown in **Chapter 3**, *L. pneumophila* inhibits retrograde trafficking to promote intracellular replication. This phenotype is dependent on the presence of the effector RidL, which binds PtdIns(3)P and the retromer protein Vps29.

First indications that retrograde trafficking may play a role during *Legionella* infection was the identification of Rab7, OCRL/Dd5P4 and Vps29 on LCVs (Clemens *et al.*, 2000; Shevchuk *et al.*, 2009; Urwyler *et al.*, 2009; Weber *et al.*, 2009). Vps29 is part of the retromer's cargo selective heterotrimer and Rab7 is essential for the recruitment of this subcomplex to membranes (Cullen and Korswagen, 2011; Rojas *et al.*, 2008) (**Section 1.2**). OCRL/Dd5P4 regulates retrograde MPR trafficking by its ability to dephosphorylate PtdIns(4,5)P_2, thereby modulating a signalling cascade comprising the protein cofilin and the RhoGTPase Rac1, both implicated in F-actin dynamics (Choudhury *et al.*, 2005; van Rahden *et al.*, 2012).

Increased levels of the cargo receptors Vps10 and CIMPR on LCVs in absence of RidL indicated this effector to be involved in the modulation of retrograde trafficking. The reduced percentage of cargo receptors on LCVs harbouring wild-type bacteria may be the result on an overall reduction of the cellular amount of theses proteins. This

could be caused by disruption of the endosome-TGN recycling process and subsequent degradation of Vps10 and CIMPR. In support of this notion, the retrograde transport of STxB and CTxB from endosomes to the TGN was inhibited by RidL. Notably, presence of the effector had no impact on the accumulation of the *Dictyostelium* OCRL homologue Dd5P4, Rab7 or other GTPases implicated in the secretory or endosomal trafficking. This suggests that the effector plays no fundamental role in these pathways.

Although it is now clear that RidL acts as retrograde trafficking inhibitor, its mechanism remains to be determined further. Specific binding of Vps29 could be a major hint and may indicate that the effector targets the retromer complex itself to prevent its interactions. Since presence of RidL had no impact on recruitment or assembly of the cargo selective subcomplex, at least on LCVs, blocking of subsequent events after membrane association may be essential. Furthermore, since tubulation from LCVs has never been observed, this could indicate that the process is stopped prior to the formation of vesicles emanating from this compartment. Comparison of the elements required for STxB, CTxB and CIMPR trafficking, which are all disturbed by RidL, might also provide clues for its mechansim. Overlapping components in these pathways are clathrin and its associated factors, the retromer core complex and factors mediating vesicle scission, like dynamin and proteins of the EHD-family (**Section 1.2** and **Figure 1.4**). Since it is assumed that RidL exhibits its function after Vps-trimer assembly, but prior to vesicle formation (see above), the retromer core complex is the likely target of the effector.

It is possible, that binding of RidL affects the known interaction of Vps29 with the Rab GAP TBC1D5. However, this could not be shown in a co-immunoprecipitation assay using Vps29 as bait (data not shown). Nevertheless, the experiment was performed *in vitro* under potentially unapt conditions. Interestingly, SNX1 has also been reported to directly bind to Vps29. This interaction is too weak to confer recruitment, but may play a role in subsequent events and therefore constitute a target for RidL interference.

Due to its high molecular size, RidL is likely to harbour different functional domains and/or to affect also proteins adjacent to Vps29. It is conceivable, that RidL posttranslationally modifies components of the retromer cargo-selective subcomplex thereby blocking their interactions. This would be similar to the PtdIns(4)*P*-binding effector SidM, which AMPylates Rab1 to inhibit its inactivation by the Rab1 GAP LepB (**Section 1.1.5**). Alternatively, the masking of binding sites without modification may also be a possibility to prevent retromer interactions. One of the likely targets is Vps35, which binds the WASH complex and EHD1 (**Section 1.2.4**). These components are necessary for vesicle formation, and interfering with their association with the Vps tri-

mer halts retrograde pathway progression. This would resemble the mechanism of Tip of herpesvirus saimiri (HVS), which renders the retromer inactive by binding Vps35 and thereby retains the complex on maturing endosomes (Kingston *et al.*, 2011) (**Section 4.3**).

However, HVS Tip did not change levels of associated SNX1, whereas RidL decreased the amount of SNX1 and SNX2 on LCVs. This may be caused by the affinity of RidL to PtdIns(3)P, the membrane anchors of SNX1 and SNX2. Consistently, RidL was able to displace SNX1 from transferrin receptor positive membranes. Interestingly, the percentage of SNX5-positive LCVs was not altered by RidL, although membrane recruitment depends on dimerisation with SNX1/2. This may be due to independent association of SNX5 with LCVs by binding either an adaptor such as a different SNX or directly PtdIns(4,5)P_2. However, the latter scenario is somewhat improbable due to the low levels of this PI on LCVs, which is thought to be converted to PtdIns(4)P by OCRL/Dd5P4 (Weber *et al.*, 2009). It is also conceivable, that SNX5 binds the membrane-localised RidL or that its weak affinity for PtdIns(3)P is sufficient to retain it on LCVs (**Figure 4.1**) (Liu *et al.*, 2006).

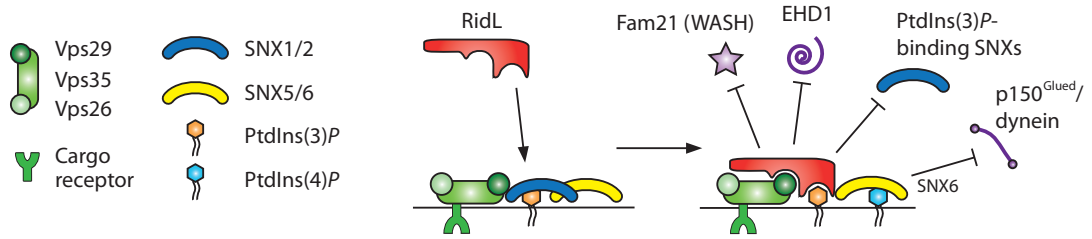

Figure 4.1: The *L. pneumophila* Effector RidL Inhibits Retrograde Trafficking. RidL is a retrograde trafficking inhibitor, which binds Vps29 and PtdIns(3)P. This causes an inhibition of retrograde trafficking. The effector might block this pathway by displacing sorting nexins and/or by preventing interaction of Vps35 with the WASH complex recruiting Fam21 and/or tubule stabilisers/scission factors of the EHD family. Moreover, competition of p150^{Glued} with PtdIns(4)P for the binding of SNX6 may contribute to retrograde pathway inhibition on LCVs. The prevention of STx or CTx transport is thought to be analogue to the blocking of the CIMPR recycling.

In this context it is noteworthy, that the PI composition of LCVs itself may contribute to a disruption of retrograde trafficking. The interaction of SNX6 with p150^{Glued} may be disturbed by the higher affinity of SNX6 for PtdIns(4)P, which is enriched on LCVs, causing disturbance of vesicle formation and transport (Niu *et al.*, 2013).

It is evident that RidL not only affects proteins on the LCV, but also on endosomes. Ectopic expression of RidL in HeLa cells disrupted transport of STxB indicating that the LCV is dispensable for inhibition of retrograde traficking. Furthermore, infection of macrophages and subsequent analyses of CTxB trafficking showed no colocaliation with LCVs and indicated that inhibition of the toxin takes place at early endosomes. Once again, this may be caused by the aforementioned competition of RidL with SNX1 for PtdIns(3)P, an early endosomal marker, or by RidL-dependent modulation of retromer activity.

Surprisingly, in contrast to knock-down of several other key components of retrograde traffcking, siRNA treatment of various SNXs and combinations thereof had no effect on intracellular *L. pneumophila* multiplication. The lack of phenotype could be explained by the interchangeability of SNX1 with SNX2 and of SNX5 with SNX6, requiring a simultaneous knock-down. However, even combined depletion of SNX1 and SNX2 did not increase growth efficiency. This may be caused by a requirement of these SNXs in other, retromer-independent processes, which cancel out the replication-beneficial inhibition of retrograde trafficking. For instance, SNX1 and SNX2 have been reported to interact with Rac1 and the RhoG GEF Kalirin-7, and SNX1 mediates sorting of the G-protein coupled receptors $P2Y_1$ and PAR1 (Gullapalli *et al.*, 2006; Nisar *et al.*, 2010; Prosser *et al.*, 2010). Alternatively, it is also possible, that the resolution of the experiment was insufficient to obtain significant results, as for instance even the control knock-down of Arf1 decreased replication only slightly.

Furthermore and also unexpectedly, depletion of factors of the retrograde pathway promoted growth of the Δ*ridL* strain similar to the wild-type strain. An explanation for this is, that the growth restriction by retrograde trafficking is epistatic to the effects of RidL on the retromer. For example, knock-down of the pathway components could be more effective than inhibition by the effector. Or silencing of the factors might cause the activation of compensatory effects in the host cells that promote intracellular bacterial growth.

Finally, RidL may also block productive interaction of the retromer with other sorting nexins, that are relevant for growth of *L. pneumophila*. Currently, there are 33 mammalian SNXs known, which are involved in endosomal sorting and show mostly affinity to PtdIns(3)P (Cullen, 2008). However, the exact cellular functions of most SNXs remain unclear.

Additional analyses were performed to obtain clues regarding RidL's putative enzymatic activity (or activities). However, *in silico* investigations of the amino acid sequence or predicted protein structure of the effector revealed no homologies to catalytic domains or other proteins. This suggests that the effector contains novel binding

motifs for Vps29 and PtdIns(3)P or even unknown domains for a putative enzymatic activity. *In vitro* experiments were performed to test for several catalytic activities, but failed to demonstrate GTPase activity for RidL or the ability of the effector to posttranslationally modify its binding partner Vps29 by ADP-ribosylation, guanylation, acetylation, ubiquitination or phosphorylation (data not shown). An attempt was made to exploit the fact, that related effectors tend to cluster in specific genomic regions of *Legionella*, which might allow deductions regarding their respective functions (Burstein *et al.*, 2009). However, as the two genes upstream of *ridL* encode metabolic enzymes and the function of the two downstream encoded proteins is unknown, the genome context of the effector gene did not provide any additional information on the activity of RidL.

The question remains, why *L. pneumophila* growth is promoted by inhibition of the retrograde pathway, either by siRNA knock-down or by RidL. The disruption of CIMPR and Vps10 trafficking suggests, that delivery of acid hydrolases might be affected. The result would be a decreased lysosomal activity of the cell, which in turn would lead to a higher survival rate of internalised bacteria. This is consistent with findings for *S.* Typhimurium, which inhibits the supply of lysosomes with degradative enzymes, thus increasing bacterial replication (**Section 4.2**) (McGourty *et al.*, 2012). Corroborating this hypothesis, depletion of CIMPR by siRNA and the subsequent disturbance of lysosomal activity also promoted growth of *L. pneumophila*.

However, in contrast to *Salmonella*, *Legionella* avoids fusion with the lysosomal compartment, where the acid hydrolases are activated due the low pH. Nevertheless, it is possible that the LCVs are supplied with these enzymes by vesicles from the TEN. Once the phagosome becomes acidified prior to intracellular replication, the presence of the now catalytically active hydrolases would be detrimental to the bacteria (Sturgill-Koszycki and Swanson, 2000; Tilney *et al.*, 2001).

In line with the notion that disruption of MPR trafficking is a major function of RidL are also results obtained after treatment of host cells with Retro-1. This specific inhibitor of STx1, STx2 and ricin retrograde transport did not affect growth of *L. pneumophila*, indicating that this Rab9-independent retrograde pathway is dispensable for intracellular replication (data not shown). The characteristic of RidL being an early effector is also coherent with this assumption, as the bacteria would benefit from retrograde inhibition at the earliest possible moment. Nevertheless, considering the multifunctional nature of the retromer, it remains possible that interference of RidL with other signalling or vesicle pathways contributes to *Legionella* fitness.

In summary, RidL inhibits retrograde trafficking at the endosomal stage and that inhibition of this pathway promotes intracellular replication of *L. pneumophila*. This

may be achieved by modulating interactions of the cargo recognition subcomplex and/or by competition with sorting nexins. This leads to a block in pathway progression and a replication-beneficial under-supply of lysosomes with hydrolases.

4.2 *Salmonella enterica* serovar Typhimurium Inhibits Retrograde Trafficking

Salmonella enterica serovar Typhimurium is a gram-negative, facultative intracellular pathogen that causes a self-limiting gastroenteritis (Coburn *et al.*, 2006; Haraga *et al.*, 2008). Pathogenesis of the bacteria is dependent on two T3SS encoded on the *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2), which translocate more than 40 effectors (Ramos-Morales, 2012). Uptake into epithelial cells is facilitated by the SPI-1 T3SS (Patel and Galan, 2005) whereas the SPI-2 T3SS enables the formation of the replication-permissive *Salmonella*-containing vacuole (SCV) (Figueira and Holden, 2012). Like *Legionella*, *Salmonella* manipulates host cell factors and subverts signalling pathways with its effector proteins to promote intracellular proliferation (Hilbi and Haas, 2012).

Recently, *S.* Typhimurium has been reported to inhibit retrograde retrieval of CD- and CI-MPRs from endosomes (McGourty *et al.*, 2012). This study was spurred by the paradox, that although SCVs are acidic (Rathman *et al.*, 1996) and harbour lysosomal membrane glycoproteins as well as the late endosomal markers Rab7, LAMP-1 and V-ATPase, MPRs and their target hydrolases are excluded from the vacuole (Garcia-del Portillo and Finlay, 1995; Hang *et al.*, 2006; Méresse *et al.*, 1999; Rathman *et al.*, 1997). McGourty *et al.* (2012) showed that the normal TGN-localisation of CD- and CIMPR is changed to a dispersed distribution throughout the cell upon infection with *S.* Typhimurium, suggesting interference with the receptors' pathways. Interestingly, retrograde trafficking of CTxB, which requires the SNARE syntaxin 6, instead of syntaxin 10 like MPRs, is not affected by the bacteria (**Sections 1.2.4** and **1.2.5**). The localisation of syntaxin 10 was not altered after infection, but CDMPR was redistributed to structures containing Vps26. This indicates, that the modulation of the MPR trafficking takes place between the Vps26-dependent retromer sorting and the syntaxin 10-dependent fusion of the vesicle with the TGN. This coincides with the localisation and function of Rab9, which is like syntaxin 10 required for MPR recycling (**Section 1.2.4**). This GTPase is known to bind to the eukaryotic protein SifA- and kinesin-interacting protein (SKIP), which in turn binds to the *S.* Typhimurium effector SifA and is crucial for membrane stability and motor dynamics at the SCV (Boucrot *et al.*,

2005; Jackson *et al.*, 2008). Infection with effector mutants and depletion of SKIP revealed that SifA and its interaction with SKIP are responsible for the redistribution of the MPR cargo receptors. With both SifA and SKIP present, the Rab9-CDMPR colocalistation percentage is decreased, suggesting SifA-SKIP to capture Rab9 and redirect it to the SCV, where SifA resides (**Figure 4.2**) (Brumell *et al.*, 2002). Formation of this complex prevents Rab9 from interacting with GCC185, which is resonsible for vesicle tethering at the TGN, and thus inhibits retrograde trafficking of MPRs (Ganley *et al.*, 2008). In summary, SifA recruits SKIP to the SCV, where it sequesters Rab9, which is no longer available for the MPR recycling process.

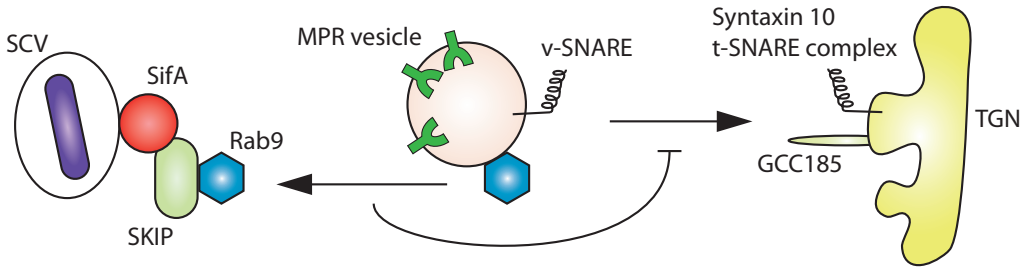

Figure 4.2: *S.* Typhimurium Inhibits Retrograde MPR Trafficking by Rab9 Sequestration. Transport of MPR containing vesicles from LE to the TGN requires Rab9. The GTPase binds the tethering factor GCC185, which enables membrane fusion mediated by the interaction of the v-SNARE with the Syntaxin 10-containing t-SNARE complex. Upon infection with *S.* Typhimurium, Rab9 is sequestered by SKIP, which is relocated to the SCV by the effector SifA. The reduction of available Rab9 leads to an inhibition of MPR trafficking and thus a decrease in lysosomal potency. Adapted from McGourty *et al.* (2012).

As reported previously by Ikeda *et al.* (2008), interference with the MPR-recycling leads to misrouting and secretion of newly synthesised lysosomal hydrolases. This is also the case for the acid hydrolases cathepsin B and D and β-hexosaminidase after infection with *S.* Typhimurium, resulting in a strong decrease of lysosome function. This suggests that the bacteria disturb the delivery of these enzymes to the lysosomes to protect themselves from degradation. This hypothesis is strengthened by experiments depleting either syntaxin 6 or syntaxin 10 from cells. Lack of syntaxin 6 increased lysosome potency and decreased replication of *S.* Typhimurium, whereas absence of syntaxin 10 reduced lysosomsal efficiency and enhanced bacterial growth. A similar result was obtained by direct downregulation of cathepsins which also led to increased intracellular multiplication. The disruption of retrograde MPR trafficking and thus the "disarming" of the lysosomes is a novel way for intracellular survival, even potentially

enabling *S.* Typhimurium to use the once harmful compartment as a supply for nutrients.

Notably, sorting nexins play an important role during SCV maturation. Bujny *et al.* (2008) reported, that SNX1 is recruited to the SCV and forms extensive, long-range tubules, termed spacious vacuole-associated tubules (SVATs), shortly after bacterial uptake. SVAT-formation coincides with a decrease of vacuolar size, suggesting membrane exit by tubulation. Depletion of SNX1 leads an overall delay of bacterial replication and the accumulation of CIMPR on SCVs, indicating the removal of this receptor via SVATs. However, it is still unclear whether this process involves the other components of the retromer complex.

Furthermore, SNX3 can be found on SCVs early after infection and is crucial for the induction of (SNX3-)tubules (Braun *et al.*, 2010). Of note, SNX3 possesses no membrane-deforming BAR-domain and SNX1 and SNX2 are required for tubulation. Moreover, depletion of SNX3 impairs Rab7 and LAMP-1 recruitment and causes changes in the formation of *Salmonella*-induced filaments (SIFs), indicating a role in the regulation of SCV maturation.

4.3 Herpersvirus saimiri Inhibits Retromer Activity

The rhadinovirus or γ_2-herpesvirus herpesvirus saimiri (HVS) naturally parasitises the squirrel monkey, but does not cause any disease or tumours. However, infections of rheses macaques or rabbits result in lethal fulminant lymphoma (Fickenscher and Fleckenstein, 2001). Notably, subgroup C of HVS can cause cell immortalisation, i.e. Interleukin-2 (IL-2)-independent, permanent growth of primary T cells from Old World and New World monkeys as well as human T cells.

The viral tyrosine kinase-interacting protein (Tip) is essential for this immortalisation process (Duboise *et al.*, 1998) and additionally for a downregulation of T-cell receptor (TCR) signal transduction (Biesinger *et al.*, 1995; Cho *et al.*, 2004; Jung *et al.*, 1995). The TCR translocates to lymphocyte-specific protein tyrosine kinase (Lck)-enriched lipid rafts after its interaction with major histocompatibility complex (MHC) class II molecules carrying antigenic peptides (Xavier *et al.*, 1998). Following phosphorylation of both components and TCR signal transduction, the complex is endocytosed and degraded in the lysosome (D'Oro *et al.*, 1997).

Tip has been reported to bind to the endosomal/lysosomal protein p80 and the kinase Lck, which binds tightly to the TCR coreceptor and sensitivity enhancer CD4

to prevent its internalisation (Foti *et al.*, 2002; Heck *et al.*, 2006). The interaction of Tip with Lck initiates recruitment of the TCR complex to lipid rafts and the interaction with p80 subsequently induces the aggregation and internalisation of the TCR containing lipid raft domain (Park *et al.*, 2002). This lysosmal vesicle formation leads to TCR downregulation and Lck degradation and thus lower levels of surface exposed CD4 (**Figure 4.3**) (Cho *et al.*, 2006; Park *et al.*, 2003).

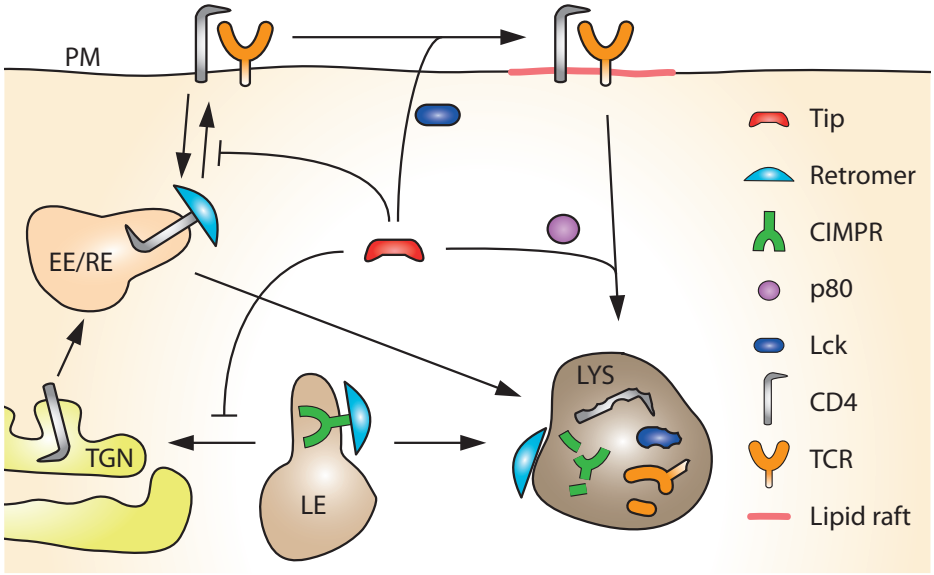

Figure 4.3: Herpersvirus saimiri Inhibits Retromer Activity by Binding Vps35. The HVS protein Tip interacts with Lck to recruit the TCR complex to lipid rafts. Internalisation and degradation of the TCR is subsequently mediated by the interaction of Tip with p80. Binding of Tip to Vps35 of the retromer inhibits its activity and its exit from endosomes. This leads to an accumulation of the complex at lysosomes and to a stop in the recycling process of CIMPRs ultimately resulting in their lysosomal degradation. Interaction of Tip with Vps35 also leads to a decrease in CD4 surface localisation and an overall reduction of the intracellular CD4 protein amount. This could be caused by an inhibition of a retromer-dependent anterograde transport of the receptor from either REs or EEs.

Recently, Kingston *et al.* (2011) reported Tip to interact with the retromer subunit Vps35 at two distinct regions of the protein. Upon expression of Tip, Vps35 is redistributed to large lysosomal compartments where both proteins colocalise. Furthermore, intracellular CIMPR levels and activity of its associated acid hydrolase β-glucuronidase are drastically reduced. Notably, Tip expression had no influence on protein levels of Vps proteins or SNX1 nor their assembly. This suggests, that the interference with

retrograde trafficking is mediated by binding of Tip only after retromer recruitment and that the protein interferes with the machinery's activity relating to downstream processess. More precisely, the redistribution of Vps35 to lysosomes indicates Tip to prevent the exit of the retromer from maturing endosomes by binding the complex. This leads to a disruption of CIMPR recycling and consequently a decreased delivery of acid hydrolases to endosomes. Due to the stable and prolonged nature of the Vps35-Tip binding, an enzymatic modification of Vps35 by Tip seems less likely than a sterical block of Vps35 interactions. Several factors have been shown to bind to Vps35, e.g. Fam21, which recruits WASH to endosomes, a complex that is required for tubule scission and/or actin-stabilisation of regulatory membrane microdomains (Gomez and Billadeau, 2009; Harbour *et al.*, 2010; Puthenveedu *et al.*, 2010). Further interactors of the cargo-selective subcomplex are EHD3 and its paralogue EHD1, which have been speculated to contribute to tubule stabilisation/scission or even induction of membrane curvature (Daumke *et al.*, 2007; Gokool *et al.*, 2007; Naslavsky *et al.*, 2009). All these trafficking steps take place after the initial retromer recruitment and this would be consistent with the notion, that Tip blocks the retromer exit from endosomes (**Figure 4.3**).

Interestingly, the ability of Tip to bind the retromer is also crucial for the *in vitro* immortalisation of primary T cells. It remains unclear what the exact mechanistic links are, but downregulation of CIMPR is suggested to play a role. The hydrolase receptor has been proposed to be a tumour suppressor protein, as mutations are numerous in breast and colon cancer cells (Braulke *et al.*, 1992). For instance CIMPR is able to regulate degradation of the insulin-like growth factor-II and facilitate activation of the growth inhibitor TGF-β1 (Ghosh *et al.*, 2003). Furthermore, abnormal localisation of acid hydrolases like cysteine cathepsins contribute to neoplastic progression (Mohamed and Sloane, 2006) and the Tip-induced swelling of the lysosome is similar to some cancer derived-cells (Kroemer and Jäättelä, 2005).

Furthermore, the above-mentioned decrease in CD4 surface localisation as well as an overall reduction of the intracellular CD4 protein amount is also dependent on a Tip-Vps35 interaction. It was shown that the transport of CD4 to the plasma membrane was inhibited by Tip and that the residual protein colocalised with Tip inside host cells. However, it remains unknown how inhibition of the retromer causes the specific downregulation of CD4. The finding of Temkin *et al.* (2011) could be helpful in this regard. The group reports that besides retrograde trafficking to the TGN, the retromer together with the WASH complex drive the anterograde transport from endosomes to the plasma membrane, in this case of the β2-adrenergic receptor. Interestingly, the small proportion of non-surface exposed CD4 resides in early/recycling endosomes (Woo *et al.*, 2010). This could indicate that normally I) CD4 is delivered

to endosomes where it is transported in a retromer-dependent way to the cell surface or II) that the recycling of endocytosed CD4 to the plasma membrane is mediated by this complex. In either case and consistent with the reported findings, blocking of the retromer by Tip would lead to an accumulation of CD4 in endosomal/lysosomal compartments (Figure 4.3). However, pathway II) is less likey, since the redistribution of CD4 to lysosomes seems to occur directly without a detour via surface exposure. On the other hand, interaction of Tip with Lck, which normally prevents endocytosis of this coreceptor, is required. Upon Tip-dependent degradation of Lck, internalisation of CD4 would increase and this would be consistent with pathway II).

In summary, inhibition of the retromer leads to immortalisation and CD4 downregulation and consequently weakens the host immune response. This facilitates persistence of HVS, and is a novel example of TCR deregulation by viruses (Callan and McMichael, 1999).

4.4 Viral Exploitation of the Retrograde Pathway without Inhibition

Not all intracellular pathogens that interfere with retrograde trafficking necessarily also inhibit it. Some viruses exploit this pathway for the intracellular transport of their components or the downregulation of surface receptors for immune evasion.

4.4.1 Human Papillomaviruses

One of these viruses interfering with the retromer-dependent pathway is the human papilloma-virus (HPV) type 16 (Lipovsky *et al.*, 2013). Papillomaviruses are small, nonenveloped DNA tumour viruses. They infect epithelial cells and are the most common sexually transmitted viruses (Stanley, 2010). After endocytosis HPV containing endosomes mature into acidic compartments (Letian *et al.*, 2010). This induces conformational changes of the major capsid protein L1 which in turn releases the minor capsid protein L2 and the genome (Day *et al.*, 2003; Modis *et al.*, 2002). The mechanism of the subsequent microtubule-dependent trafficking to the nucleus where the virus replicates is still not fully understood (Sapp and Bienkowska-Haba, 2009).

Very recently, a genome-wide siRNA screen identified the retromer and some additional retrograde pathway constituents, namely Rab6a, RAB6IP1, Rab7b and Rab9,

to be crucial for HPV entry (Lipovsky *et al.*, 2013). Knock-down of any of the three retromer Vps proteins strongly reduced infection efficiency of HPV5, HPV16 and HPV18. Pharmacological inhibition of retrograde trafficking with Retro-2, which selectively blocks transport of STx1, STx2 and ricin, also led to a decrease of the infection efficiency in a dose-dependent manner (Stechmann *et al.*, 2010). This treatment, as well as lack of Vps26, caused the capsid protein L1 to lose its perinuclear localisation and to redistribute to the cell periphery, but did not affect Golgi morphology. In infected cells the L1 and L2 proteins partially colocalised with the Golgi-marker GM130 or the TGN-marker TGN46, respectively, indicating transport of viral proteins to the Golgi. These perinuclear sites of colocalisation were termed Golgi-like compartments (GLCs), and transport of HPV16 to this compartment requires a functional retromer (**Figure 4.4**). Confocal microscopy also revealed colocalisation of L2 with Vps35, hinting a potential physical interaction in partially disassembled capsids. This hypothesis was strengthened by successful co-immunoprecipitations of L1 and L2 with the Vps trimer.

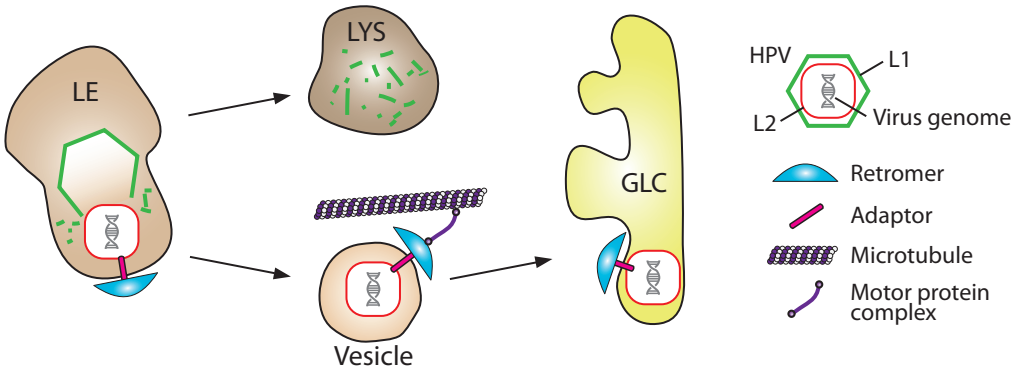

Figure 4.4: Human papillomavirus particles are transported to Golgi-like Compartments by the Retromer. After uptake into cells the major capsid proteins L1 of HPV undergo a pH-dependent conformational change in the LE, which releases the minor capsid L2 and the viral genome. L2 interacts with the retromer via an unknown adaptor and is transported together with the genome to a Golgi-like compartment, whereas the majority of L1 is degraded in the lysosome.

Of note, Day *et al.* (2013) also showed that L2 as well as the viral genome travel to the TGN, however in their studies L1 was retained at the LE. This is consistent with findings from other groups determining L1 to be degraded after dissociation from L2 (Bienkowska-Haba *et al.*, 2012). One explanation of this apparent discrepancy is that indeed most L1 is degraded and only a small proportion remains capsid-associated for

efficient HPV infection.

Interactive contacts of the retromer complex with the endocytosed virus must span the endosomal membrane. Several scenarios for this are conceivable, e.g. the viral hijacking of a transmembrane cargo receptor or conformational changes in the capsid itself which generate a membrane-protruding peptide. Such a domain is part of the L2 N-terminus. Additionally, its C-terminus contains a hydrophobic region which is able to disrupt membranes and direct membrane localisation of proteins (Bronnimann *et al.*, 2013; Kämper *et al.*, 2006). Interestingly, interaction of L2 with the cargo-adaptor SNX17 has been shown to be essential for intracellular HPV trafficking (Bergant Marušič *et al.*, 2012; Yin *et al.*, 2012). SNX17 may thus be the initial cytoplasmic binding partner of the virus and mediate further interaction with the retromer itself. Influence of Rab9 and Rab7b an viral entry, as shown in the siRNA screen, suggests late endosomes to be the origin of virus transport to the Golgi, whereas the TGN-resident Rab6a and the RAB6IP1 are factors likely to play a role in the vesicle docking process. However, inhibition of retrograde trafficking reduced virus infection only by 80%, indicating that also other pathways may play a role or that retrograde transport of the virus only enhances infection efficiency.

In contrast to the above-discussed work, which suggests vesicular transport of virus components to the Golgi, other studies describe the immediate viral escape from endosomes into the cytoplasm by membrane rupture (Sapp and Bienkowska-Haba, 2009). It is however possible that the reported membrane disruption occurs after delivery to the TGN. This may be necessary to utilise TGN-specific factors for further transport of the virus or delay a cytoplasmic immune response. Furthermore, there are also indications of HPV trafficking to the ER, placing the site of viral escape even closer to the nucleus (Laniosz *et al.*, 2009).

4.4.2 HIV

The human immunodeficiency virus (HIV), which targets cells of the human immune system and causes AIDS (acquired immunodeficiency syndrome), is another pathogen that exploits the retrograde transport route. The HIV-1 protein Env relies on TIP47, which is normally asociated with the recycling of CIMPR, for its transport to the TGN (Blot *et al.*, 2003) (**Section 1.2.4**). Moreover, interaction of Env with the viral Gag protein is mediated by TIP47, ensuring correct virus assembly (Lopez-Vergès *et al.*, 2006). Furthermore, a large siRNA screen revealed Rab6 and Vps53 to play a role in HIV infection (Brass *et al.*, 2008). As previously mentioned, Rab6 has been associated

with docking of vesicles at the TGN whereas Vps53 is a component of the tethering GARP complex (Conibear and Stevens, 2000) (**Section 1.2.4**). Finally, the HIV-1 protein Nef is reported to induce retrograde trafficking of MHC-I and co-stimulatory ligands to the TGN, hereby facilitating host immune evasion (Blagoveshchenskaya *et al.*, 2002; Chaudhry *et al.*, 2008).

4.4.3 Herpesviridae and SV40

A strategy similar to HIV is pursued by Herpesviridae. Their envelope glycoprotein M has been shown to direct relocalisation of surface proteins to the TGN in infected cells (Crump *et al.*, 2004). Once again, this interference presumably contributes to masking the affected cell from the immune system.

SV40 is another example for a virus hijacking retrograde pathways for its transport through the cell. Interestingly, this polyomavirus completely bypasses the Golgi on its way to the ER, where an interaction with the ERAD machinery is crucial for efficient infection (Norkin *et al.*, 2005; Schelhaas *et al.*, 2007).

4.5 Conclusion and Outlook

Investigations of the inhibition or subversion of the retrograde trafficking pathway by intracellular pathogens is an emerging field in microbiology. Given the importance of retrograde trafficking as a cell-autonomous defense pathway, it is not surprising that bacteria and viruses manipulate this pathway besides the well studied secretory and endosomal routes (**Figure 4.5**). This leads to an increased survival rate and infection efficiency of the microorganisms. The discoveries of RidL and other pathogen components modulating retrograde trafficking led not only to a better understanding of the pathogens, but these factors can also be used as tools to investigate basic cellular functions. Ultimately this could help treating or curing patients suffering from diseases, that can be caused by a dysregulation of retromer-dependent pathways, such as Alzheimer disease (Siegenthaler and Rajendran, 2012), Parkinson disease (Vilariño-Güell *et al.*, 2011; Zimprich *et al.*, 2011) and other neurodegenerative pathologies (Ropers *et al.*, 2011; Valdmanis *et al.*, 2007). Since studies on the modulation of retrograde trafficking by pathogens are still at the outset, many exciting discoveries may be anticipated.

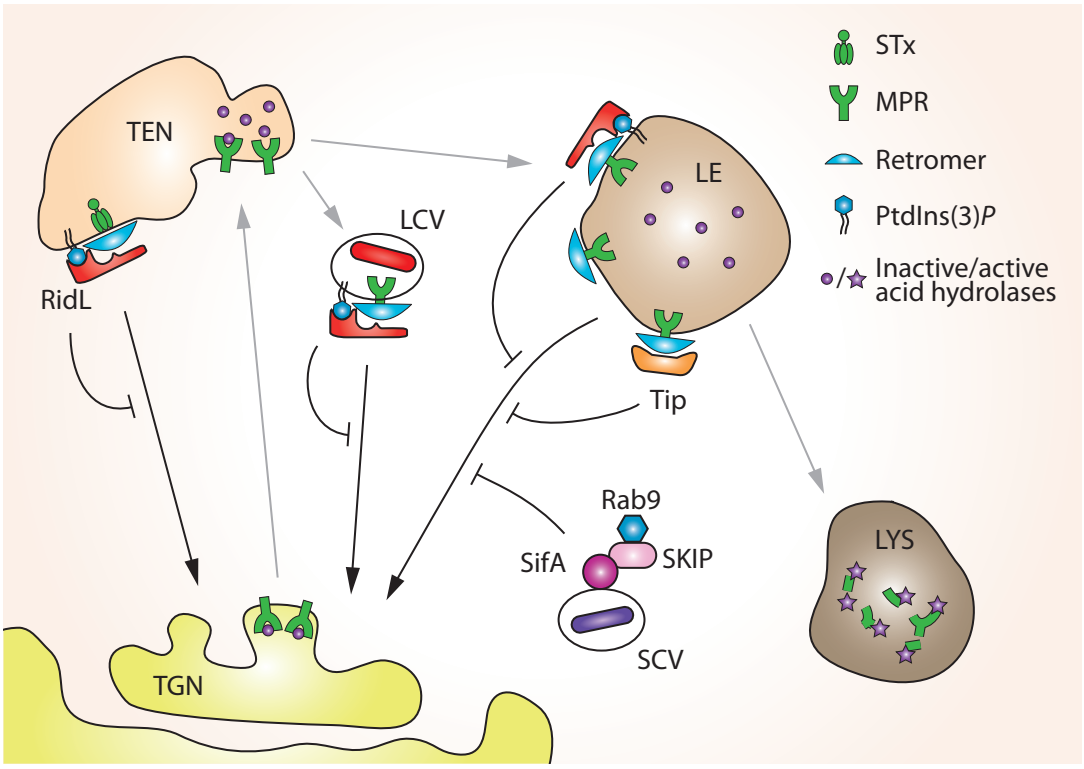

Figure 4.5: Intracellular Pathogens Inhibit MPR Recycling. RidL from *L. pneumophila* binds Vps29 and PtdIns(3)*P* and inhibits CTx and STx trafficking at the TEN and MPR recycling from LCVs and the LE. SifA from *S.* Typhimurium recruits Rab9-binding SKIP to the SCV, which also causes a block of the retrograde pathway of MPRs. Tip from HVS inhibits retromer activity and retains the complex on maturing endosomes. All theses processes lead to lysosomal MPR degradation and a misregulation of the intracellular supply with acid hydrolases.

Literature

Al-Khodor, Souhaila, Price, Christopher T, Habyarimana, Fabien, Kalia, Awdhesh, and Abu Kwaik, Yousef, 2008. A Dot/Icm-translocated ankyrin protein of *Legionella pneumophila* is required for intracellular proliferation within human macrophages and protozoa. *Molecular Microbiology*, 70(4):908–923.

Alli, OA Terry, Gao, Lian-Yong, Pedersen, Lisa L, Zink, Steven, Radulic, Marina, Doric, Miljenko, and Kwaik, Yousef Abu, 2000. Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila*. *Infection and Immunity*, 68(11):6431–6440.

Altman, Efrat and Segal, Gil, 2008. The response regulator CpxR directly regulates expression of several *Legionella pneumophila icm/dot* components as well as new translocated substrates. *Journal of Bacteriology*, 190(6):1985–1996.

Arasaki, Kohei, Toomre, Derek K, and Roy, Craig R, 2012. The *Legionella pneumophila* effector DrrA is sufficient to stimulate SNARE-dependent membrane fusion. *Cell Host & Microbe*, 11(1):46.

Arighi, Cecilia N, Hartnell, Lisa M, Aguilar, Ruben C, Haft, Carol R, and Bonifacino, Juan S, 2004. Role of the mammalian retromer in sorting of the cation-independent mannose 6-phosphate receptor. *The Journal of Cell Biology*, 165(1):123–133.

Bardill, J Patrick, Miller, Jennifer L, and Vogel, Joseph P, 2005. IcmS-dependent translocation of SdeA into macrophages by the *Legionella pneumophila* type IV secretion system. *Molecular Microbiology*, 56(1):90–103.

Bennett, Terry L, Kraft, Shannon M, Reaves, Barbara J, Mima, Joji, O'Brien, Kevin M, and Starai, Vincent J, 2013. LegC3, an effector protein from *Legionella pneumophila*, inhibits homotypic yeast vacuole fusion *in vivo* and *in vitro*. *PloS ONE*, 8(2):e56798.

Benson, Robert F, Fields, Barry S, *et al.* Classification of the genus *Legionella*. In *Seminars in Respiratory Infections*, volume 13, pages 90–99, 1998.

Bergan, Jonas, Dyve Lingelem, Anne Berit, Simm, Roger, Skotland, Tore, and Sandvig, Kirsten, 2012. Shiga toxins. *Toxicon*.

Bergant Marušič, Martina, Ozbun, Michelle A, Campos, Samuel K, Myers, Michael P, and Banks, Lawrence, 2012. Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. *Traffic*, 13(3):455–467.

Bienkowska-Haba, Malgorzata, Williams, Carlyn, Kim, Seong Man, Garcea, Robert L, and Sapp, Martin, 2012. Cyclophilins facilitate dissociation of the human papillomavirus type 16 capsid protein L1 from the L2/DNA complex following virus entry. *Journal of Virology*, 86(18):9875–9887.

Biesinger, Brigitte, Tsygankov, Alexander Y, Fickenscher, Helmut, Emmrich, Frank, Fleckenstein, Bernhard, Bolen, Joseph B, and Bröker, Barbara M, 1995. The product of the *Herpesvirus saimiri* open reading frame 1 (Tip) interacts with T cell-specific kinase $p56^{lck}$ in transformed cells. *Journal of Biological Chemistry*, 270(9):4729–4734.

Blagoveshchenskaya, Anastassia D, Thomas, Laurel, Feliciangeli, Sylvain F, Hung, Chien-Hui, and Thomas, Gary, 2002. HIV-1 Nef downregulates MHC-I by a PACS-1-and PI3K-regulated ARF6 endocytic pathway. *Cell*, 111(6):853–866.

Blot, Guillaume, Janvier, Katy, Le Panse, Sophie, Benarous, Richard, and Berlioz-Torrent, Clarisse, 2003. Targeting of the human immunodeficiency virus type 1 envelope to the *trans*-Golgi network through binding to TIP47 is required for env incorporation into virions and infectivity. *Journal of Virology*, 77(12):6931–6945.

Borner, Georg HH, Harbour, Michael, Hester, Svenja, Lilley, Kathryn S, and Robinson, Margaret S, 2006. Comparative proteomics of clathrin-coated vesicles. *The Journal of Cell Biology*, 175(4):571–578.

Boucrot, Emmanuel, Henry, Thomas, Borg, Jean-Paul, Gorvel, Jean-Pierre, and Meresse, Stephane, 2005. The intracellular fate of *Salmonella* depends on the recruitment of kinesin. *Science Signaling*, 308(5725):1174.

Brass, Abraham L, Dykxhoorn, Derek M, Benita, Yair, Yan, Nan, Engelman, Alan, Xavier, Ramnik J, Lieberman, Judy, and Elledge, Stephen J, 2008. Identification of host proteins required for HIV infection through a functional genomic screen. *Science*, 319(5865):921–926.

Braulke, Thomas, Mach, Lukas, Hoflack, Bernard, and Glössl, Josef, 1992. Biosynthesis and endocytosis of lysosomal enzymes in human colon carcinoma SW 1116 cells: impaired internalization of plasma membrane-associated cation-independent mannose 6-phosphate receptor. *Archives of Biochemistry and Biophysics*, 298(1):176–181.

Braun, Virginie, Wong, Anthony, Landekic, Marija, Hong, Wan Jin, Grinstein, Sergio, and Brumell, John H, 2010. Sorting nexin 3 (SNX3) is a component of a tubular endosomal network induced by *Salmonella* and involved in maturation of the *Salmonella*-containing vacuole. *Cellular Microbiology*, 12(9):1352–1367.

Brombacher, Eva, Urwyler, Simon, Ragaz, Curdin, Weber, Stefan S, Kami, Keiichiro, Overduin, Michael, and Hilbi, Hubert, 2009. Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of *Legionella pneumophila*. *Journal of Biological Chemistry*, 284(8):4846–4856.

Bronnimann, Matthew P, Chapman, Janice A, Park, Chad K, and Campos, Samuel K, 2013. A transmembrane domain and GxxxG motifs within L2 are essential for papillomavirus infection. *Journal of Virology*, 87(1):464–473.

Brüggemann, Holger, Cazalet, Christel, and Buchrieser, Carmen, 2006. Adaptation of *Legionella pneumophila* to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. *Current Opinion in Microbiology*, 9(1):86–94.

Brumell, John H, Goosney, Danika L, and Finlay, B Brett, 2002. SifA, a type III secreted effector of *Salmonella typhimurium*, directs *Salmonella*-induced filament (Sif) formation along microtubules. *Traffic*, 3(6):407–415.

Bujny, Miriam V, Popoff, Vincent, Johannes, Ludger, and Cullen, Peter J, 2007. The retromer component sorting nexin-1 is required for efficient retrograde transport of Shiga toxin from early endosome to the *trans* Golgi network. *Journal of Cell Science*, 120(12):2010–2021.

Bujny, Miriam V, Ewels, Phil A, Humphrey, Suzanne, Attar, Naomi, Jepson, Mark A, and Cullen, Peter J, 2008. Sorting nexin-1 defines an early phase of *Salmonella*-containing vacuole-remodeling during *Salmonella* infection. *Journal of Cell Science*, 121(12):2027–2036.

Burda, Patricie, Padilla, Steven M, Sarkar, Srimonti, and Emr, Scott D, 2002. Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *Journal of Cell Science*, 115(20):3889–3900.

Burns, Drusilla L, 2003. Type IV transporters of pathogenic bacteria. *Current Opinion in Microbiology*, 6(1):29–34.

Burstein, David, Zusman, Tal, Degtyar, Elena, Viner, Ram, Segal, Gil, and Pupko, Tal, 2009. Genome-scale identification of *Legionella pneumophila* effectors using a machine learning approach. *PLoS Pathogens*, 5(7):e1000508.

Callan, Margaret FC and McMichael, Andrew J. T cell receptor usage in infectious disease. In *Springer Seminars in Immunopathology*, volume 21, pages 37–54. Springer, 1999.

Cambronne, Eric D and Roy, Craig R, 2007. The *Legionella pneumophila* IcmSW complex interacts with multiple Dot/Icm effectors to facilitate type IV translocation. *PLoS Pathogens*, 3(12):e188.

Canuel, Maryssa, Lefrancois, Stephane, Zeng, Jibin, and Morales, Carlos R, 2008. AP-1 and retromer play opposite roles in the trafficking of sortilin between the Golgi apparatus and the lysosomes. *Biochemical and Biophysical Research Communications*, 366(3):724–730.

Carroll, Kate S, Hanna, John, Simon, Iris, Krise, Jeff, Barbero, Pierre, and Pfeffer, Suzanne R, 2001. Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. *Science*, 292(5520):1373–1376.

Cazalet, Christel, Rusniok, Christophe, Brüggemann, Holger, Zidane, Nora, Magnier, Arnaud, Ma, Laurence, Tichit, Magalie, Jarraud, Sophie, Bouchier, Christiane, Vandenesch, François, *et al.*, 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nature Genetics*, 36(11):1165–1173.

Chang, Bin, Kura, Fumiaki, Amemura-Maekawa, Junko, Koizumi, Nobuo, and Watanabe, Haruo, 2005. Identification of a novel adhesion molecule involved in the virulence of *Legionella pneumophila*. *Infection and Immunity*, 73(7):4272–4280.

Chaudhry, Ashutosh, Das, Suman Ranjan, Jameel, Shahid, George, Anna, Bal, Vineeta, Mayor, Satyajit, and Rath, Satyajit, 2008. HIV-1 Nef induces a Rab11-dependent routing of endocytosed immune costimulatory proteins CD80 and CD86 to the Golgi. *Traffic*, 9(11):1925–1935.

Chen, John, de Felipe, Karim Suwwan, Clarke, Margaret, Lu, Hao, Anderson, O Roger, Segal, Gil, and Shuman, Howard A, 2004. *Legionella* effectors that promote nonlytic release from protozoa. *Science*, 303(5662):1358–1361.

Cherla, Rama P, Lee, Sang-Yun, and Tesh, Vernon L, 2003. Shiga toxins and apoptosis. *FEMS Microbiology Letters*, 228(2):159–166.

Cho, Nam-Hyuk, Feng, Pinghui, Lee, Sun-Hwa, Lee, Bok-Soo, Liang, Xiaozhen, Chang, Heesoon, and Jung, Jae U, 2004. Inhibition of T cell receptor signal transduction

by tyrosine kinase-interacting protein of herpesvirus saimiri. *The Journal of Experimental Medicine*, 200(5):681–687.

Cho, Nam-Hyuk, Kingston, Dior, Chang, Heesoon, Kwon, Eun-Kyung, Kim, Jo-Min, Lee, Jung Hee, Chu, Hyuk, Choi, Myung-Sik, Kim, Ik-Sang, and Jung, Jae Ung, 2006. Association of herpesvirus saimiri Tip with lipid raft is essential for downregulation of T-cell receptor and CD4 coreceptor. *Journal of Virology*, 80(1):108–118.

Choudhury, Rawshan, Diao, Aipo, Zhang, Fang, Eisenberg, Evan, Saint-Pol, Agnes, Williams, Catrin, Konstantakopoulos, Athanasios, Lucocq, John, Johannes, Ludger, Rabouille, Catherine, *et al.*, 2005. Lowe syndrome protein OCRL1 interacts with clathrin and regulates protein trafficking between endosomes and the *trans*-Golgi network. *Molecular Biology of the Cell*, 16(8):3467–3479.

Christie, Peter J, 2001. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Molecular Microbiology*, 40(2): 294–305.

Clemens, Daniel L, Lee, Bai-Yu, and Horwitz, Marcus A, 2000. *Mycobacterium tuberculosis* and *Legionella pneumophila* phagosomes exhibit arrested maturation despite acquisition of Rab7. *Infection and Immunity*, 68(9):5154–5166.

Coburn, Bryan, Grassl, Guntram A, and Finlay, BB, 2006. *Salmonella*, the host and disease: a brief review. *Immunology and Cell Biology*, 85(2):112–118.

Conibear, Elizabeth and Stevens, Tom H, 2000. Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. *Molecular Biology of the Cell*, 11(1):305–323.

Cozier, Gyles E, Carlton, Jez, McGregor, Alex H, Gleeson, Paul A, Teasdale, Rohan D, Mellor, Harry, and Cullen, Peter J, 2002. The phox homology (PX) domain-dependent, 3-phosphoinositide-mediated association of sorting nexin-1 with an early sorting endosomal compartment is required for its ability to regulate epidermal growth factor receptor degradation. *Journal of Biological Chemistry*, 277(50):48730–48736.

Crump, Colin M, Bruun, Birgitte, Bell, Susanne, Pomeranz, Lisa E, Minson, Tony, and Browne, Helena M, 2004. Alphaherpesvirus glycoprotein M causes the relocalization of plasma membrane proteins. *Journal of General Virology*, 85(12):3517–3527.

Cullen, Peter J, 2008. Endosomal sorting and signalling: an emerging role for sorting nexins. *Nature Reviews Molecular Cell Biology*, 9(7):574–582.

Cullen, Peter J and Korswagen, Hendrik C, 2011. Sorting nexins provide diversity for retromer-dependent trafficking events. *Nature Cell Biology*, 14(1):29–37.

Daumke, Oliver, Lundmark, Richard, Vallis, Yvonne, Martens, Sascha, Butler, P Jonathan G, and McMahon, Harvey T, 2007. Architectural and mechanistic insights into an EHD ATPase involved in membrane remodelling. *Nature*, 449(7164):923–927.

Day, Patricia M, Lowy, Douglas R, and Schiller, John T, 2003. Papillomaviruses infect cells via a clathrin-dependent pathway. *Virology*, 307(1):1–11.

Day, Patricia M, Thompson, Cynthia D, Schowalter, Rachel M, Lowy, Douglas R, and Schiller, John T, 2013. Identification of a role for the *trans*-Golgi network in human papillomavirus 16 pseudovirus infection. *Journal of Virology*, 87(7):3862–3870.

De Buck, Emmy, Anné, Jozef, and Lammertyn, Elke, 2007. The role of protein secretion systems in the virulence of the intracellular pathogen *Legionella pneumophila*. *Microbiology*, 153(12):3948–3953.

de Felipe, Karim Suwwan, Pampou, Sergey, Jovanovic, Oliver S, Pericone, Christopher D, Senna, F Ye, Kalachikov, Sergey, and Shuman, Howard A, 2005. Evidence for acquisition of *Legionella* type IV secretion substrates via interdomain horizontal gene transfer. *Journal of Bacteriology*, 187(22):7716–7726.

de Felipe, Karim Suwwan, Glover, Robert T, Charpentier, Xavier, Anderson, O Roger, Reyes, Moraima, Pericone, Christopher D, and Shuman, Howard A, 2008. *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. *PLoS Pathogens*, 4(8):e1000117.

Derivery, Emmanuel, Sousa, Carla, Gautier, Jérémie J, Lombard, Bérangère, Loew, Damarys, and Gautreau, Alexis, 2009. The Arp2/3 activator WASH controls the fission of endosomes through a large multiprotein complex. *Developmental Cell*, 17 (5):712–723.

Derré, Isabelle and Isberg, Ralph R, 2004. *Legionella pneumophila* replication vacuole formation involves rapid recruitment of proteins of the early secretory system. *Infection and Immunity*, 72(5):3048–3053.

Di Paolo, Gilbert and De Camilli, Pietro, 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature*, 443(7112):651–657.

Diaz, Elva and Pfeffer, Suzanne R, 1998. TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. *Cell*, 93(3):433–443.

Diaz, Elva, Schimmöller, Frauke, and Pfeffer, Suzanne R, 1997. A novel Rab9 effector required for endosome-to-TGN transport. *The Journal of Cell Biology*, 138(2):283–290.

Donaldson, Julie G and Jackson, Catherine L, 2011. ARF family G proteins and their regulators: roles in membrane transport, development and disease. *Nature Reviews Molecular Cell Biology*, 12(6):362–375.

Dorer, Marion S, Kirton, Donald, Bader, Joel S, and Isberg, Ralph R, 2006. RNA interference analysis of *Legionella* in *Drosophila* cells: exploitation of early secretory apparatus dynamics. *PLoS Pathogens*, 2(4):e34.

D'Oro, Ugo, Vacchio, Melanie S, Weissman, Allan M, and Ashwell, Jonathan D, 1997. Activation of the Lck tyrosine kinase targets cell surface T cell antigen receptors for lysosomal degradation. *Immunity*, 7(5):619–628.

Duboise, S Monroe, Guo, Jie, Czajak, Sue, Desrosiers, Ronald C, and Jung, Jae U, 1998. STP and Tip are essential for herpesvirus saimiri oncogenicity. *Journal of Virology*, 72(2):1308–1313.

Endo, Yaeta, Tsurugi, Kunio, Yutsudo, Takashi, Takeda, Yoshifumi, Ogasawara, Toshihiro, and Igarashi, Kazuei, 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157: H7 and of Shiga toxin on eukaryotic ribosomes. *European Journal of Biochemistry*, 171(1-2):45–50.

Faix, Jan, Kreppel, Lisa, Shaulsky, Gad, Schleicher, Michael, and Kimmel, Alan R, 2004. A rapid and efficient method to generate multiple gene disruptions in *Dictyostelium discoideum* using a single selectable marker and the Cre-*loxP* system. *Nucleic Acids Research*, 32(19):e143–e143.

Feeley, James C, Gibson, Robert J, Gorman, George W, Langford, Nancy C, Rasheed, J Kamile, Mackel, Don C, and Baine, William B, 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. *Journal of Clinical Microbiology*, 10(4):437–441.

Feng, Yan, Jadhav, Ashutosh P, Rodighiero, Chiara, Fujinaga, Yukako, Kirchhausen, Tomas, and Lencer, Wayne I, 2004. Retrograde transport of cholera toxin from the plasma membrane to the endoplasmic reticulum requires the *trans*-Golgi network but not the Golgi apparatus in Exo2-treated cells. *EMBO reports*, 5(6):596–601.

Fernandez-Moreira, Esteban, Helbig, Juergen H, and Swanson, Michele S, 2006. Membrane vesicles shed by *Legionella pneumophila* inhibit fusion of phagosomes with lysosomes. *Infection and Immunity*, 74(6):3285–3295.

Fickenscher, Helmut and Fleckenstein, Bernhard, 2001. *Herpesvirus saimiri*. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 356(1408):545–567.

Fields, Barry S, 1996. The molecular ecology of *Legionellae*. *Trends in Microbiology*, 4 (7):286–290.

Figueira, Rita and Holden, David W, 2012. Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology*, 158(Pt 5):1147–1161.

Finsel, Ivo, Hoffmann, Christine, and Hilbi, Hubert. Immunomagnetic purification of fluorescent *Legionella*-containing vacuoles. In *Dictyostelium discoideum Protocols*, pages 431–443. Springer, 2013a.

Finsel, Ivo, Ragaz, Curdin, Hoffmann, Christine, Harrison, Christopher F, Weber, Stephen, van Rahden, Vanessa A, Johannes, Ludger, and Hilbi, Hubert, 2013b. The *Legionella* effector RidL inhibits retrograde trafficking to promote intracellular replication. *Cell Host & microbe*, 14(1):38–50.

Fjorback, Anja W, Seaman, Matthew, Gustafsen, Camilla, Mehmedbasic, Arnela, Gokool, Suzanne, Wu, Chengbiao, Militz, Daniel, Schmidt, Vanessa, Madsen, Peder, Nyengaard, Jens R, *et al.*, 2012. Retromer binds the FANSHY sorting motif in SorLA to regulate amyloid precursor protein sorting and processing. *The Journal of Neuroscience*, 32(4):1467–1480.

Foti, Michelangelo, Phelouzat, Marie-Anne, Holm, Åsa, Rasmusson, Birgitta J, and Carpentier, Jean-Louis, 2002. $p56^{Lck}$ anchors CD4 to distinct microdomains on microvilli. *Proceedings of the National Academy of Sciences*, 99(4):2008–2013.

Fraser, David W, Tsai, Theodore R, Orenstein, Walter, Parkin, William E, Beecham, H James, Sharrar, Robert G, Harris, John, Mallison, George F, Martin, Stanley M, McDade, Joseph E, *et al.*, 1977. Legionnaires' disease. *New England Journal of Medicine*, 297(22):1189–1197.

Fraser, Marie E, Chernaia, Maia M, Kozlov, Yuri V, and James, Michael NG, 1994. Crystal structure of the holotoxin from *Shigella dysenteriae* at 2.5 Å resolution. *Nature Structural & Molecular Biology*, 1(1):59–64.

Frost, Adam, Perera, Rushika, Roux, Aurélien, Spasov, Krasimir, Destaing, Olivier, Egelman, Edward H, De Camilli, Pietro, and Unger, Vinzenz M, 2008. Structural basis of membrane invagination by F-BAR domains. *Cell*, 132(5):807–817.

Frost, Adam, Unger, Vinzenz M, and De Camilli, Pietro, 2009. The BAR domain superfamily: membrane-molding macromolecules. *Cell*, 137(2):191–196.

Fry, Norman K, Warwick, Simon, Saunders, Nicholas A, and Embley, T Martin, 1991. The use of 16S ribosomal RNA analyses to investigate the phylogeny of the family Legionellaceae. *Journal of General Microbiology*, 137(5):1215–1222.

Ganley, Ian G, Espinosa, Eric, and Pfeffer, Suzanne R, 2008. A syntaxin 10-SNARE complex distinguishes two distinct transport routes from endosomes to the *trans*-Golgi in human cells. *The Journal of Cell Biology*, 180(1):159–172.

Garcia-del Portillo, Francisco and Finlay, B Brett, 1995. Targeting of *Salmonella typhimurium* to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors. *The Journal of Cell Biology*, 129(1): 81–97.

Garred, Oystein, van Deurs, Bo, Sandvig, Kirsten, *et al.*, 1995. Furin-induced cleavage and activation of Shiga toxin. *Journal of Biological Chemistry*, 270(18):10817–10821.

Gazdag, Emerich Mihai, Streller, Alexandra, Haneburger, Ina, Hilbi, Hubert, Vetter, Ingrid R, Goody, Roger S, and Itzen, Aymelt, 2013. Mechanism of Rab1b deactivation by the *Legionella pneumophila* GAP LepB. *EMBO Reports*.

Ghosh, Pradipta, Dahms, Nancy M, and Kornfeld, Stuart, 2003. Mannose 6-phosphate receptors: new twists in the tale. *Nature Reviews Molecular Cell Biology*, 4(3):202–213.

Gillooly, David J, Morrow, Isabel C, Lindsay, Margaret, Gould, Robert, Bryant, Nia J, Gaullier, Jean-Michel, Parton, Robert G, and Stenmark, Harald, 2000. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *The EMBO Journal*, 19(17):4577–4588.

Girod, Andreas, Storrie, Brian, Simpson, Jeremy C, Johannes, Ludger, Goud, Bruno, Roberts, Lynne M, Lord, J Michael, Nilsson, Tommy, and Pepperkok, Rainer, 1999. Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nature Cell Biology*, 1(7):423–430.

Glick, Thomas H, Gregg, Michael B, Berman, Bernard, Mallison, George, Rhodes, Wallace W, and Kassanoff, Ira, 1978. Pontiac fever: An epidemic of unknown etiology in a health department: I. Clinical and epidemiological aspects. *American Journal of Epidemiology*, 107(2):149–160.

Godi, Anna, Pertile, Paolo, Meyers, Rachel, Marra, Pierfrancesco, Di Tullio, Giuseppe, Iurisci, Cristiano, Luini, Alberto, Corda, Daniela, and De Matteis, Maria Antonietta, 1999. ARF mediates recruitment of PtdIns-4-OH kinase-β and stimulates synthesis of PtdIns(4,5)P_2 on the Golgi complex. *Nature Cell Biology*, 1(5):280–287.

Gokool, Suzanne, Tattersall, Daniel, and Seaman, Matthew NJ, 2007. EHD1 interacts with retromer to stabilize SNX1 tubules and facilitate endosome-to-Golgi retrieval. *Traffic*, 8(12):1873–1886.

Gomez, Timothy S and Billadeau, Daniel D, 2009. A FAM21-containing WASH complex regulates retromer-dependent sorting. *Developmental Cell*, 17(5):699–711.

Greub, Gilbert and Raoult, Didier, 2004. Microorganisms resistant to free-living amoebae. *Clinical Microbiology Reviews*, 17(2):413–433.

Gullapalli, Anuradha, Wolfe, Breann L, Griffin, Courtney T, Magnuson, Terry, and Trejo, JoAnn, 2006. An essential role for SNX1 in lysosomal sorting of protease-activated receptor-1: evidence for retromer-, Hrs-, and Tsg101-independent functions of sorting nexins. *Molecular Biology of the Cell*, 17(3):1228–1238.

Haft, Carol Renfrew, de la Luz Sierra, Maria, Barr, Valarie A, Haft, Daniel H, and Taylor, Simeon I, 1998. Identification of a family of sorting nexin molecules and characterization of their association with receptors. *Molecular and Cellular Biology*, 18(12):7278–7287.

Hägele, Sonja, Köhler, Rolf, Merkert, Hilde, Schleicher, Michael, Hacker, Jörg, and Steinert, Michael, 2000. *Dictyostelium discoideum*: a new host model system for intracellular pathogens of the genus *Legionella*. *Cellular Microbiology*, 2(2):165–171.

Hang, Howard C, Loureiro, Joana, Spooner, Eric, van der Velden, Adrianus WM, Kim, You-Me, Pollington, Annette M, Maehr, Rene, Starnbach, Michael N, and Ploegh, Hidde L, 2006. Mechanism-based probe for the analysis of cathepsin cysteine proteases in living cells. *ACS Chemical Biology*, 1(11):713–723.

Haraga, Andrea, Ohlson, Maikke B, and Miller, Samuel I, 2008. *Salmonellae* interplay with host cells. *Nature Reviews Microbiology*, 6(1):53–66.

Harbour, Michael E, Breusegem, Sophia YA, Antrobus, Robin, Freeman, Caroline, Reid, Evan, and Seaman, Matthew NJ, 2010. The cargo-selective retromer complex is a recruiting hub for protein complexes that regulate endosomal tubule dynamics. *Journal of Cell Science*, 123(21):3703–3717.

Harterink, Martin, Port, Fillip, Lorenowicz, Magdalena J, McGough, Ian J, Silhankova, Marie, Betist, Marco C, van Weering, Jan RT, van Heesbeen, Roy GHP, Middelkoop, Teije C, Basler, Konrad, *et al.*, 2011. A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. *Nature Cell Biology*, 13(8):914–923.

Heck, Elke, Friedrich, Ute, Gack, Michaela U, Lengenfelder, Doris, Schmidt, Monika, Müller-Fleckenstein, Ingrid, Fleckenstein, Bernhard, Ensser, Armin, and Biesinger, Brigitte, 2006. Growth transformation of human T cells by herpesvirus saimiri requires multiple Tip-Lck interaction motifs. *Journal of Virology*, 80(20):9934–9942.

Heidtman, Matthew, Chen, Emy J, Moy, Man-Yu, and Isberg, Ralph R, 2009. Large-scale identification of *Legionella pneumophila* Dot/Icm substrates that modulate host cell vesicle trafficking pathways. *Cellular Microbiology*, 11(2):230–248.

Hierro, Aitor, Rojas, Adriana L, Rojas, Raul, Murthy, Namita, Effantin, Grégory, Kajava, Andrey V, Steven, Alasdair C, Bonifacino, Juan S, and Hurley, James H, 2007. Functional architecture of the retromer cargo-recognition complex. *Nature*, 449 (7165):1063–1067.

Hilbi, Hubert and Haas, Albert, 2012. Secretive bacterial pathogens and the secretory pathway. *Traffic*, 13(9):1187–1197.

Hilbi, Hubert, Segal, Gil, and Shuman, Howard A, 2001. Icm/Dot-dependent upregulation of phagocytosis by *Legionella pneumophila*. *Molecular Microbiology*, 42(3): 603–617.

Hilbi, Hubert, Weber, Stephen, and Finsel, Ivo, 2011. Anchors for effectors: subversion of phosphoinositide lipids by *Legionella*. *Frontiers in Microbiology*, 2.

Hoffmann, C, Finsel, I, and Hilbi, H, 2012. Purification of pathogen vacuoles from *Legionella*-infected phagocytes. *Journal of Visualized Experiments*, (64).

Hoffmann, Christine, Finsel, Ivo, and Hilbi, Hubert. Pathogen vacuole purification from *Legionella*-infected amoeba and macrophages. In *Legionella*, pages 309–321. Springer, 2013.

Holmgren, J, Lönnroth, I, and Svennerholm, L, 1973a. Tissue receptor for cholera exotoxin: postulated structure from studies with GM1 ganglioside and related glycolipids. *Infection and Immunity*, 8(2):208–214.

Holmgren, J, Lönnroth, I, Svennerholm, L, *et al.*, 1973b. Fixation and inactivation of cholera toxin by GM1 ganglioside. *Scandinavian Journal of Infectious Diseases*, 5(1): 77.

Hong, Zhi, Yang, Yanrui, Zhang, Cheng, Niu, Yang, Li, Ke, Zhao, Xi, and Liu, Jia-Jia, 2009. The retromer component SNX6 interacts with dynactin p150$^{\text{Glued}}$ and mediates endosome-to-TGN transport. *Cell Research*, 19(12):1334–1349.

Horazdovsky, BF, Davies, BA, Seaman, MN, McLaughlin, SA, Yoon, S-H, and Emr, SD, 1997. A sorting nexin-1 homologue, Vps5p, forms a complex with Vps17p and is required for recycling the vacuolar protein-sorting receptor. *Molecular Biology of the Cell*, 8(8):1529.

Horwitz, Marcus A, 1983. Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. *The Journal of Experimental Medicine*, 158(4):1319–1331.

Horwitz, Marcus A and Maxfield, Frederick R, 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *The Journal of Cell Biology*, 99 (6):1936–1943.

Horwitz, Marcus A and Silverstein, Samuel C, 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *Journal of Clinical Investigation*, 66(3):441.

Hsu, FoSheng, Zhu, Wenhan, Brennan, Lucy, Tao, Lili, Luo, Zhao-Qing, and Mao, Yuxin, 2012. Structural basis for substrate recognition by a unique *Legionella* phosphoinositide phosphatase. *Proceedings of the National Academy of Sciences*, 109 (34):13567–13572.

Huang, Li, Boyd, Dana, Amyot, Whitney M, Hempstead, Andrew D, Luo, Zhao-Qing, O'Connor, Tamara J, Chen, Cui, Machner, Matthias, Montminy, Timothy, and Isberg, Ralph R, 2011. The E Block motif is associated with *Legionella pneumophila* translocated substrates. *Cellular Microbiology*, 13(2):227–245.

Hubber, Andree and Roy, Craig R, 2010. Modulation of host cell function by *Legionella pneumophila* type IV effectors. *Annual Review of Cell and Developmental Biology*, 26:261–283.

Ikeda, Kazuhiko, Hirayama, Masahiro, Hirota, Yuko, Asa, Erika, Seki, Jiro, and Tanaka, Yoshitaka, 2008. Drug-induced phospholipidosis is caused by blockade of mannose 6-phosphate receptor-mediated targeting of lysosomal enzymes. *Biochemical and Biophysical Research Communications*, 377(1):268–274.

Ingmundson, Alyssa, Delprato, Anna, Lambright, David G, and Roy, Craig R, 2007. *Legionella pneumophila* proteins that regulate Rab1 membrane cycling. *Nature*, 450 (7168):365–369.

Jacewicz, Mary, Clausen, Henrik, Nudelman, Edward, Donohue-Rolfe, Arthur, and Keusch, Gerald T, 1986. Pathogenesis of *Shigella* diarrhea. XI. Isolation of a Shigella toxin-binding glycolipid from rabbit jejunum and HeLa cells and its identification as globotriaosylceramide. *The Journal of Experimental Medicine*, 163(6):1391–1404.

Jackson, Laurie K, Nawabi, Parwez, Hentea, Cristiana, Roark, Everett A, and Haldar, Kasturi, 2008. The *Salmonella* virulence protein SifA is a G protein antagonist. *Proceedings of the National Academy of Sciences*, 105(37):14141–14146.

Jahn, Reinhard and Scheller, Richard H, 2006. SNAREs–engines for membrane fusion. *Nature Reviews Molecular Cell Biology*, 7(9):631–643.

Johannes, Ludger and Popoff, Vincent, 2008. Tracing the retrograde route in protein trafficking. *Cell*, 135(7):1175–1187.

Johannes, Ludger and Römer, Winfried, 2009. Shiga toxins–from cell biology to biomedical applications. *Nature Reviews Microbiology*, 8(2):105–116.

Jong, W Yu and Lemmon, Mark A, 2001. All phox homology (PX) domains from *Saccharomyces cerevisiae* specifically recognize phosphatidylinositol 3-phosphate. *Journal of Biological Chemistry*, 276(47):44179–44184.

Jung, Jae U, Lang, Sabine M, Jun, Toni, Roberts, Thomas M, Veillette, Andre, and Desrosiers, Ronald C, 1995. Downregulation of Lck-mediated signal transduction by Tip of herpesvirus saimiri. *Journal of Virology*, 69(12):7814–7822.

Kagan, Jonathan C and Roy, Craig R, 2002. *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nature Cell Biology*, 4(12):945–954.

Kagan, Jonathan C, Stein, Mary-Pat, Pypaert, Marc, and Roy, Craig R, 2004. *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. *The Journal of Experimental Medicine*, 199(9):1201–1211.

Kämper, Nadine, Day, Patricia M, Nowak, Thorsten, Selinka, Hans-Christoph, Florin, Luise, Bolscher, Jan, Hilbig, Lydia, Schiller, John T, and Sapp, Martin, 2006. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. *Journal of Virology*, 80(2):759–768.

Kessler, Aline, Schell, Ursula, Sahr, Tobias, Tiaden, André, Harrison, Christopher, Buchrieser, Carmen, and Hilbi, Hubert, 2013. The *Legionella pneumophila* orphan sensor kinase LqsT regulates competence and pathogen-host interactions as a component of the LAI-1 circuit. *Environmental Microbiology*.

Khelef, Nadia, Shuman, Howard A, and Maxfield, Frederick R, 2001. Phagocytosis of wild-type *Legionella pneumophila* occurs through a wortmannin-insensitive pathway. *Infection and Immunity*, 69(8):5157–5161.

Kingston, Dior, Chang, Heesoon, Ensser, Armin, Lee, Hye-Ra, Lee, Jongsoo, Lee, Sun-Hwa, Jung, Jae Ung, and Cho, Nam-Hyuk, 2011. Inhibition of retromer activity by herpesvirus saimiri tip leads to CD4 downregulation and efficient T cell transformation. *Journal of Virology*, 85(20):10627–10638.

Koharudin, Leonardus MI, Furey, William, Liu, Hao, Liu, Yong-Jian, and Gronenborn, Angela M, 2009. The phox domain of sorting nexin 5 lacks phosphatidylinositol 3-phosphate (PtdIns(3)P) specificity and preferentially binds to phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2. *Journal of Biological Chemistry*, 284(35):23697–23707.

Kroemer, Guido and Jäättelä, Marja, 2005. Lysosomes and autophagy in cell death control. *Nature Reviews Cancer*, 5(11):886–897.

Ku, Bonsu, Lee, Kwang-Hoon, Park, Wei Sun, Yang, Chul-Su, Ge, Jianning, Lee, Seong-Gyu, Cha, Sun-Shin, Shao, Feng, Do Heo, Won, Jung, Jae U, *et al.*, 2012. VipD of *Legionella pneumophila* targets activated Rab5 and Rab22 to interfere with endosomal trafficking in macrophages. *PLoS Pathogens*, 8(12):e1003082.

Kubori, Tomoko, Hyakutake, Akihiro, and Nagai, Hiroki, 2008. *Legionella* translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Molecular Microbiology*, 67(6):1307–1319.

Kubori, Tomoko, Shinzawa, Naoaki, Kanuka, Hirotaka, and Nagai, Hiroki, 2010. *Legionella* metaeffector exploits host proteasome to temporally regulate cognate effector. *PLoS Pathogens*, 6(12):e1001216.

Länger, F and Kreipe, HH, 2011. Immunodeficiency and immunocompromised patients. Opportunistic infection of the lungs. *Der Pathologe*, 32(5):411.

Laniosz, Valerie, Dabydeen, Sarah A, Havens, Mallory A, and Meneses, Patricio I, 2009. Human papillomavirus type 16 infection of human keratinocytes requires clathrin and caveolin-1 and is brefeldin a sensitive. *Journal of Virology*, 83(16):8221–8232.

Lauvrak, Silje U, Torgersen, Maria L, and Sandvig, Kirsten, 2004. Efficient endosome-to-Golgi transport of Shiga toxin is dependent on dynamin and clathrin. *Journal of Cell Science*, 117(11):2321–2331.

Letian, Tan, Tianyu, Zhang, *et al.*, 2010. Cellular receptor binding and entry of human papillomavirus. *Virology Journal*, 7(2).

Lipovsky, Alex, Popa, Andreea, Pimienta, Genaro, Wyler, Michael, Bhan, Ashima, Kuruvilla, Leena, Guie, Marie-Aude, Poffenberger, Adrian C, Nelson, Christian DS, Atwood, Walter J, *et al.*, 2013. Genome-wide siRNA screen identifies the retromer as a cellular entry factor for human papillomavirus. *Proceedings of the National Academy of Sciences*, 110(18):7452–7457.

Liu, Hao, Liu, Zu-Qiang, Chen, Carol X-Q, Magill, Stephen, Jiang, Yu, and Liu, Yong-Jian, 2006. Inhibitory regulation of EGF receptor degradation by sorting nexin 5. *Biochemical and Biophysical Research Communications*, 342(2):537–546.

Liu, Mingyu, Conover, Gloria M, and Isberg, Ralph R, 2008. *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor α-stimulated macrophages. *Cellular Microbiology*, 10(9):1906–1923.

Liu, Yancheng and Luo, Zhao-Qing, 2007. The *Legionella pneumophila* effector SidJ is required for efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome. *Infection and Immunity*, 75(2):592–603.

Lombardi, D, Soldati, T, Riederer, MA, Goda, Y, Zerial, M, and Pfeffer, SR, 1993. Rab9 functions in transport between late endosomes and the *trans* Golgi network. *The EMBO Journal*, 12(2):677.

Lomma, M, Dervins-Ravault, D, Rolando, M, Nora, T, Newton, HJ, Sansom, FM, Sahr, T, Gomez-Valero, L, Jules, M, Hartland, EL, *et al.*, 2010. The *Legionella pneumophila* F-box protein Lpp2082 (AnkB) modulates ubiquitination of the host protein parvin B and promotes intracellular replication. *Cellular Microbiology*, 12(9):1272–1291.

Lopez-Vergès, Sandra, Camus, Grégory, Blot, Guillaume, Beauvoir, Roxane, Benarous, Richard, and Berlioz-Torrent, Clarisse, 2006. Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. *Proceedings of the National Academy of Sciences*, 103(40):14947–14952.

Lowe, Martin, 2005. Structure and function of the Lowe syndrome protein OCRL1. *Traffic*, 6(9):711–719.

Lu, Hao and Clarke, Margaret, 2005. Dynamic properties of *Legionella*-containing phagosomes in *Dictyostelium* amoebae. *Cellular Microbiology*, 7(7):995–1007.

Lu, Lei, Tai, Guihua, and Hong, Wanjin, 2004. Autoantigen Golgin-97, an effector of Arl1 GTPase, participates in traffic from the endosome to the *trans*-Golgi network. *Molecular Biology of the Cell*, 15(10):4426–4443.

Luo, Zhao-Qing and Isberg, Ralph R, 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 101(3):841–846.

Machner, Matthias P and Isberg, Ralph R, 2006. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. *Developmental Cell*, 11(1): 47–56.

Machner, Matthias P and Isberg, Ralph R, 2007. A bifunctional bacterial protein links GDI displacement to Rab1 activation. *Science Signaling*, 318(5852):974.

Malchow, Dieter, Nägele, Brigitte, Schwarz, Heinz, and Gerisch, Günther, 1972. Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. *European Journal of Biochemistry*, 28(1):136–142.

Mallard, Frédéric, Antony, Claude, Tenza, Danièle, Salamero, Jean, Goud, Bruno, and Johannes, Ludger, 1998. Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of Shiga toxin B-fragment transport. *The Journal of Cell Biology*, 143(4):973–990.

Mallard, Frédéric, Tang, Bor Luen, Galli, Thierry, Tenza, Danièle, Saint-Pol, Agnès, Yue, Xu, Antony, Claude, Hong, Wanjin, Goud, Bruno, and Johannes, Ludger, 2002. Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *The Journal of Cell Biology*, 156(4):653–664.

Mamo, Aline, Jules, Felix, Dumaresq-Doiron, Karine, Costantino, Santiago, and Lefrancois, Stephane, 2012. The role of ceroid lipofuscinosis neuronal protein 5 (CLN5) in endosomal sorting. *Molecular and Cellular Biology*, 32(10):1855–1866.

Mampel, Jörg, Spirig, Thomas, Weber, Stefan S, Haagensen, Janus AJ, Molin, Søren, and Hilbi, Hubert, 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. *Applied and Environmental Microbiology*, 72(4):2885–2895.

Manstein, Dietmar J, Schuster, Hans-Peter, Morandini, Piero, and Hunt, Deborah M, 1995. Cloning vectors for the production of proteins in *Dictyostelium discoideum*. *Gene*, 162(1):129–134.

Marston, Barbara J, Lipman, Harvey B, and Breiman, Robert F, 1994. Surveillance for Legionnaires' disease: risk factors for morbidity and mortality. *Archives of Internal Medicine*, 154(21):2417.

McDade, Joseph E, Shepard, Charles C, Fraser, David W, Tsai, Theodore R, Redus, Martha A, Dowdle, Walter R, *et al.*, 1977. Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *The New England Journal of Medicine*, 297(22):1197.

McGough, Ian J and Cullen, Peter J, 2011. Recent advances in retromer biology. *Traffic*, 12(8):963–971.

McGough, Ian J and Cullen, Peter J, 2012. Clathrin is not required for SNX-BAR-retromer mediated carrier formation. *Journal of Cell Science*, 126(1):45–52.

McGourty, Kieran, Thurston, Teresa L, Matthews, Sophie A, Pinaud, Laurie, Mota, Luís Jaime, and Holden, David W, 2012. *Salmonella* inhibits retrograde trafficking of mannose-6-phosphate receptors and lysosome function. *Science*, 338(6109):963–967.

McKenzie, Jenna, Johannes, Ludger, Taguchi, Tomohiko, and Sheff, David, 2009. Passage through the Golgi is necessary for Shiga toxin B subunit to reach the endoplasmic reticulum. *FEBS Journal*, 276(6):1581–1595.

Méresse, Stéphane, Steele-Mortimer, Olivia, Finlay, B Brett, and Gorvel, Jean-Pierre, 1999. The rab7 GTPase controls the maturation of *Salmonella typhimurium*-containing vacuoles in HeLa cells. *The EMBO Journal*, 18(16):4394–4403.

Misra, Saurav, Puertollano, Rosa, Kato, Yukio, Bonifacino, Juan S, and Hurley, James H, 2002. Structural basis for acidic-cluster-dileucine sorting-signal recognition by VHS domains. *Nature*, 415(6874):933–937.

Modis, Yorgo, Trus, Benes L, and Harrison, Stephen C, 2002. Atomic model of the papillomavirus capsid. *The EMBO Journal*, 21(18):4754–4762.

Moffat, Jennifer F and Tompkins, LS, 1992. A quantitative model of intracellular growth of *Legionella pneumophila* in *Acanthamoeba castellanii*. *Infection and Immunity*, 60 (1):296–301.

Mohamed, Mona Mostafa and Sloane, Bonnie F, 2006. Cysteine cathepsins: multifunctional enzymes in cancer. *Nature Reviews Cancer*, 6(10):764–775.

Molmeret, Maelle, Alli, OA Terry, Zink, Steven, Flieger, Antje, Cianciotto, Nicholas P, and Kwaik, Yousef Abu, 2002. *icmT* is essential for pore formation-mediated egress of *Legionella pneumophila* from mammalian and protozoan cells. *Infection and Immunity*, 70(1):69–78.

Molmeret, Maëlle, Bitar, Dina M, Han, Lihui, and Kwaik, Yousef Abu, 2004. Disruption of the phagosomal membrane and egress of *Legionella pneumophila* into the cytoplasm during the last stages of intracellular infection of macrophages and *Acanthamoeba polyphaga*. *Infection and Immunity*, 72(7):4040–4051.

Monier, Solange, Jollivet, Florence, Janoueix-Lerosey, Isabelle, Johannes, Ludger, and Goud, Bruno, 2002. Characterization of novel Rab6-interacting proteins involved in endosome-to-TGN transport. *Traffic*, 3(4):289–297.

Moss, J and Vaughan, M, 1977. Mechanism of action of choleragen. Evidence for ADP-ribosyltransferase activity with arginine as an acceptor. *Journal of Biological Chemistry*, 252(7):2455–2457.

Mukherjee, Shaeri, Liu, Xiaoyun, Arasaki, Kohei, McDonough, Justin, Galán, Jorge E, and Roy, Craig R, 2011. Modulation of Rab GTPase function by a protein phosphocholine transferase. *Nature*, 477(7362):103–106.

Muller, Matthias P, Peters, Heide, Blumer, Julia, Blankenfeldt, Wulf, Goody, Roger S, and Itzen, Aymelt, 2010. The Legionella effector protein DrrA AMPylates the membrane traffic regulator Rab1b. *Science Signaling*, 329(5994):946.

Müller-Taubenberger, Annette, Lupas, Andrei N, Li, Hewang, Ecke, Mary, Simmeth, Evelyn, and Gerisch, Günther, 2001. Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *The EMBO Journal*, 20(23):6772–6782.

Mullins, Chris and Bonifacino, Juan S, 2001. Structural requirements for function of yeast GGAs in vacuolar protein sorting, α-factor maturation, and interactions with clathrin. *Molecular and Cellular Biology*, 21(23):7981–7994.

Murata, Takahiro, Delprato, Anna, Ingmundson, Alyssa, Toomre, Derek K, Lambright, David G, and Roy, Craig R, 2006. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nature Cell Biology*, 8(9):971–977.

Murga, Ricardo, Forster, Terri S, Brown, Ellen, Pruckler, Janet M, Fields, Barry S, and Donlan, Rodney M, 2001. Role of biofilms in the survival of *Legionella pneumophila* in a model potable-water system. *Microbiology*, 147(11):3121–3126.

Nagai, Hiroki, Kagan, Jonathan C, Zhu, Xinjun, Kahn, Richard A, and Roy, Craig R, 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. *Science*, 295(5555):679–682.

Nagai, Hiroki, Cambronne, Eric D, Kagan, Jonathan C, Amor, Juan Carlos, Kahn, Richard A, and Roy, Craig R, 2005. A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proceedings of the National Academy of Sciences of the United States of America*, 102(3):826–831.

Nash, TW, Libby, DM, and Horwitz, MA, 1984. Interaction between the Legionnaires' disease bacterium (*Legionella pneumophila*) and human alveolar macrophages. Influence of antibody, lymphokines, and hydrocortisone. *Journal of Clinical Investigation*, 74(3):771.

Naslavsky, Naava, McKenzie, Jenna, Altan-Bonnet, Nihal, Sheff, David, and Caplan, Steve, 2009. EHD3 regulates early-endosome-to-Golgi transport and preserves Golgi morphology. *Journal of Cell Science*, 122(3):389–400.

Neunuebel, M Ramona, Chen, Yang, Gaspar, Andrew H, Backlund Jr, Peter S, Yergey, Alfred, and Machner, Matthias P, 2011. De-AMPylation of the small GTPase Rab1 by the pathogen *Legionella pneumophila*. *Science Signaling*, 333(6041):453.

Neunuebel, M Ramona, Mohammadi, Sina, Jarnik, Michal, and Machner, Matthias P, 2012. *Legionella pneumophila* LidA affects nucleotide binding and activity of the host GTPase Rab1. *Journal of Bacteriology*, 194(6):1389–1400.

Newton, Hayley J, Sansom, Fiona M, Bennett-Wood, Vicki, and Hartland, Elizabeth L, 2006. Identification of *Legionella pneumophila*-specific genes by genomic subtractive hybridization with *Legionella micdadei* and identification of *lpnE*, a gene required for efficient host cell entry. *Infection and Immunity*, 74(3):1683–1691.

Newton, Hayley J, Sansom, Fiona M, Dao, Jenny, McAlister, Adrian D, Sloan, Joan, Cianciotto, Nicholas P, and Hartland, Elizabeth L, 2007. Sel1 repeat protein LpnE is a *Legionella pneumophila* virulence determinant that influences vacuolar trafficking. *Infection and Immunity*, 75(12):5575–5585.

Nichols, Benjamin J, Kenworthy, Anne K, Polishchuk, Roman S, Lodge, Robert, Roberts, Theresa H, Hirschberg, Koret, Phair, Robert D, and Lippincott-Schwartz, Jennifer, 2001. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *The Journal of Cell Biology*, 153(3):529–542.

Ninio, Shira, Zuckman-Cholon, Deborah M, Cambronne, Eric D, and Roy, Craig R, 2005. The *Legionella* IcmS-IcmW protein complex is important for Dot/Icm-mediated protein translocation. *Molecular Microbiology*, 55(3):912–926.

Nisar, Shaista, Kelly, Eamonn, Cullen, Pete J, and Mundell, Stuart J, 2010. Regulation of P2Y1 receptor traffic by sorting nexin 1 is retromer independent. *Traffic*, 11(4): 508–519.

Niu, Yang, Zhang, Cheng, Sun, Zhe, Hong, Zhi, Li, Ke, Sun, Demeng, Yang, Yanrui, Tian, Changlin, Gong, Weimin, and Liu, Jia-Jia, 2013. PtdIns(4)P regulates retromer–motor interaction to facilitate dynein–cargo dissociation at the *trans*-Golgi network. *Nature Cell Biology*.

Norkin, Leonard C, Kuksin, Dmitry, *et al.*, 2005. The caveolae-mediated SV40 entry pathway bypasses the golgi complex en route to the endoplasmic reticulum. *Virology Journal*, 2:38.

Norwood, Suzanne J, Shaw, Daniel J, Cowieson, Nathan P, Owen, David J, Teasdale, Rohan D, and Collins, Brett M, 2011. Assembly and solution structure of the core retromer protein complex. *Traffic*, 12(1):56–71.

O'Connor, Tamara J, Adepoju, Yewande, Boyd, Dana, and Isberg, Ralph R, 2011. Minimization of the *Legionella pneumophila* genome reveals chromosomal regions involved in host range expansion. *Proceedings of the National Academy of Sciences*, 108(36):14733–14740.

Oesterlin, Lena K, Goody, Roger S, and Itzen, Aymelt, 2012. Posttranslational modifications of Rab proteins cause effective displacement of GDP dissociation inhibitor. *Proceedings of the National Academy of Sciences*, 109(15):5621–5626.

Pan, Xiaoxiao, Luhrmann, Anja, Satoh, Ayano, Laskowski-Arce, Michelle A, and Roy, Craig R, 2008. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science Signaling*, 320(5883):1651.

Park, Junsoo, Lee, Bok-Soo, Choi, Joong-Kook, Means, Robert E, Choe, Joonho, and Jung, Jae U, 2002. Herpesviral protein targets a cellular WD repeat endosomal protein to downregulate T lymphocyte receptor expression. *Immunity*, 17(2):221–233.

Park, Junsoo, Cho, Nam-Hyuk, Choi, Joong-Kook, Feng, Pinghui, Choe, Joonho, and Jung, Jae U, 2003. Distinct roles of cellular Lck and p80 proteins in herpesvirus saimiri Tip function on lipid rafts. *Journal of Virology*, 77(16):9041–9051.

Patel, Jayesh C and Galan, Jorge E, 2005. Manipulation of the host actin cytoskeleton by *Salmonella*–all in the name of entry. *Current Opinion in Microbiology*, 8(1):10–15.

Paumet, Fabienne, Wesolowski, Jordan, Garcia-Diaz, Alejandro, Delevoye, Cedric, Aulner, Nathalie, Shuman, Howard A, Subtil, Agathe, and Rothman, James E, 2009. Intracellular bacteria encode inhibitory SNARE-like proteins. *PLoS ONE*, 4(10): e7375.

Pérez-Victoria, F Javier, Mardones, Gonzalo A, and Bonifacino, Juan S, 2008. Requirement of the human GARP complex for mannose 6-phosphate-receptor-dependent sorting of cathepsin D to lysosomes. *Molecular Biology of the Cell*, 19(6):2350–2362.

Peter, Brian J, Kent, Helen M, Mills, Ian G, Vallis, Yvonne, Butler, P Jonathan G, Evans, Philip R, and McMahon, Harvey T, 2004. BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science*, 303(5657):495–499.

Popoff, Vincent, Mardones, Gonzalo A, Tenza, Danièle, Rojas, Raúl, Lamaze, Christophe, Bonifacino, Juan S, Raposo, Graça, and Johannes, Ludger, 2007. The retromer complex and clathrin define an early endosomal retrograde exit site. *Journal of Cell Science*, 120(12):2022–2031.

Popoff, Vincent, Mardones, Gonzalo A, Bai, Siau-Kun, Chambon, Valérie, Tenza, Danièle, Burgos, Patricia V, Shi, Anbing, Benaroch, Philippe, Urbé, Sylvie, Lamaze, Christophe, *et al.*, 2009. Analysis of articulation between clathrin and retromer in retrograde sorting on early endosomes. *Traffic*, 10(12):1868–1880.

Prashar, Akriti, Bhatia, Sonam, Tabatabaeiyazdi, Zohreh, Duncan, Carla, Garduño, Rafael A, Tang, Patrick, Low, Donald E, Guyard, Cyril, and Terebiznik, Mauricio R, 2012. Mechanism of invasion of lung epithelial cells by filamentous *Legionella pneumophila*. *Cellular Microbiology*, 14(10):1632–1655.

Prosser, Derek C, Tran, Duvinh, Schooley, Allana, Wendland, Beverly, and Ngsee, Johnny K, 2010. A Novel, retromer-independent role for sorting nexins 1 and 2 in RhoG-dependent membrane remodeling. *Traffic*, 11(10):1347–1362.

Puthenveedu, Manojkumar A, Lauffer, Benjamin, Temkin, Paul, Vistein, Rachel, Carlton, Peter, Thorn, Kurt, Taunton, Jack, Weiner, Orion D, Parton, Robert G, and von Zastrow, Mark, 2010. Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. *Cell*, 143(5):761–773.

Ragaz, Curdin, Pietsch, Holger, Urwyler, Simon, Tiaden, André, Weber, Stefan S, and Hilbi, Hubert, 2008. The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cellular Microbiology*, 10(12):2416–2433.

Ramos-Morales, Francisco, 2012. Impact of *Salmonella enterica* type III secretion system effectors on the eukaryotic host cell. *ISRN Cell Biology*, 2012.

Rathman, Michelle, Sjaastad, Michael D, and Falkow, Stanley, 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. *Infection and Immunity*, 64(7):2765–2773.

Rathman, Michelle, Barker, Lucia P, and Falkow, Stanley, 1997. The unique trafficking pattern of *Salmonella typhimurium*-containing phagosomes in murine macrophages is independent of the mechanism of bacterial entry. *Infection and Immunity*, 65(4): 1475–1485.

Reddy, Jonathan V, Burguete, Alondra Schweizer, Sridevi, Khambhampaty, Ganley, Ian G, Nottingham, Ryan M, and Pfeffer, Suzanne R, 2006. A functional role for the GCC185 golgin in mannose 6-phosphate receptor recycling. *Molecular Biology of the Cell*, 17(10):4353–4363.

Ridenour, Dennis A, Cirillo, Suat LG, Feng, Sheng, Samrakandi, Mustapha M, and Cirillo, Jeffrey D, 2003. Identification of a gene that affects the efficiency of host cell infection by *Legionella pneumophila* in a temperature-dependent fashion. *Infection and Immunity*, 71(11):6256–6263.

Robinson, Camenzind G and Roy, Craig R, 2006. Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. *Cellular Microbiology*, 8 (5):793–805.

Rogers, Julie, Dowsett, AB, Dennis, PJ, Lee, JV, and Keevil, CW, 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Applied and Environmental Microbiology*, 60(5):1585–1592.

Rojas, Raul, Kametaka, Satoshi, Haft, Carol R, and Bonifacino, Juan S, 2007. Interchangeable but essential functions of SNX1 and SNX2 in the association of retromer with endosomes and the trafficking of mannose 6-phosphate receptors. *Molecular and Cellular Biology*, 27(3):1112–1124.

Rojas, Raul, van Vlijmen, Thijs, Mardones, Gonzalo A, Prabhu, Yogikala, Rojas, Adriana L, Mohammed, Shabaz, Heck, Albert JR, Raposo, Graça, van der Sluijs, Peter, and Bonifacino, Juan S, 2008. Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. *The Journal of Cell Biology*, 183(3): 513–526.

Römer, Winfried, Berland, Ludwig, Chambon, Valérie, Gaus, Katharina, Windschiegl, Barbara, Tenza, Daniele, Aly, Mohamed RE, Fraisier, Vincent, Florent, Jean-Claude, Perrais, David, *et al.*, 2007. Shiga toxin induces tubular membrane invaginations for its uptake into cells. *Nature*, 450(7170):670–675.

Ropers, Fabienne, Derivery, Emmanuel, Hu, Hao, Garshasbi, Masoud, Karbasiyan, Mohsen, Herold, Martin, Nürnberg, Gudrun, Ullmann, Reinhard, Gautreau, Alexis, Sperling, Karl, *et al.*, 2011. Identification of a novel candidate gene for non-syndromic autosomal recessive intellectual disability: the WASH complex member SWIP. *Human Molecular Genetics*, 20(13):2585–2590.

Rossier, Ombeline, Starkenburg, Shawn R, and Cianciotto, Nicholas P, 2004. *Legionella pneumophila* type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. *Infection and Immunity*, 72(1):310–321.

Roux, Aurélien, Koster, Gerbrand, Lenz, Martin, Sorre, Benoît, Manneville, Jean-Baptiste, Nassoy, Pierre, and Bassereau, Patricia, 2010. Membrane curvature controls dynamin polymerization. *Proceedings of the National Academy of Sciences*, 107(9): 4141–4146.

Rowbotham, Timothy J, 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *Journal of Clinical Pathology*, 33(12): 1179–1183.

Roy, Craig R, Berger, Karen H, and Isberg, Ralph R, 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. *Molecular Microbiology*, 28(3):663–674.

Ruck, Alexander, Attonito, John, Garces, Kelly T, Núnez, Lizbeth, Palmisano, Nicholas J, Rubel, Zahava, Bai, Zhiyong, Nguyen, Ken CQ, Sun, Lei, Grant, Barth D, *et al.*, 2011. The Atg6/Vps30/Beclin 1 ortholog BEC-1 mediates endocytic retrograde transport in addition to autophagy in *C. elegans*. *Autophagy*, 7(4):386–400.

Sadosky, Alesia B, Wiater, Lawrence A, and Shuman, Howard A, 1993. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infection and Immunity*, 61(12):5361–5373.

Saint-Pol, Agnès, Yélamos, Belén, Amessou, Mohamed, Mills, Ian G, Dugast, Marc, Tenza, Danièle, Schu, Peter, Antony, Claude, McMahon, Harvey T, Lamaze, Christophe, *et al.*, 2004. Clathrin adaptor epsinR is required for retrograde sorting on early endosomal membranes. *Developmental Cell*, 6(4):525–538.

Sánchez, Joaquín and Holmgren, Jan, 2011. Cholera toxin–a foe & a friend. *The Indian journal of medical research*, 133(2):153.

Sandvig, Kirsten and van Deurs, Bo, 2005. Delivery into cells: lessons learned from plant and bacterial toxins. *Gene Therapy*, 12(11):865–872.

Sapp, Martin and Bienkowska-Haba, Malgorzata, 2009. Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. *FEBS Journal*, 276(24):7206–7216.

Saxena, Shailendra K, O'Brien, AD, and Ackerman, EJ, 1989. Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28 S RNA when microinjected into *Xenopus* oocytes. *Journal of Biological Chemistry*, 264(1):596–601.

Schelhaas, Mario, Malmström, Johan, Pelkmans, Lucas, Haugstetter, Johannes, Ellgaard, Lars, Grünewald, Kay, and Helenius, Ari, 2007. Simian Virus 40 depends on ER protein folding and quality control factors for entry into host cells. *Cell*, 131 (3):516–529.

Schoebel, Stefan, Oesterlin, Lena Katharina, Blankenfeldt, Wulf, Goody, Roger Sidney, and Itzen, Aymelt, 2009. RabGDI displacement by DrrA from *Legionella* is a consequence of its guanine nucleotide exchange activity. *Molecular Cell*, 36(6):1060–1072.

Schwarz, Dana Gilbert, Griffin, Courtney T, Schneider, Elizabeth A, Yee, Della, and Magnuson, Terry, 2002. Genetic analysis of sorting nexins 1 and 2 reveals a redundant and essential function in mice. *Molecular Biology of the Cell*, 13(10):3588–3600.

Seaman, Matthew NJ, 2004. Cargo-selective endosomal sorting for retrieval to the Golgi requires retromer. *The Journal of Cell Biology*, 165(1):111–122.

Seaman, Matthew NJ, 2007. Identification of a novel conserved sorting motif required for retromer-mediated endosome-to-TGN retrieval. *Journal of Cell Science*, 120(14): 2378–2389.

Seaman, Matthew NJ, 2012. The retromer complex–endosomal protein recycling and beyond. *Journal of Cell Science*, 125(20):4693–4702.

Seaman, Matthew NJ, McCaffery, J Michael, and Emr, Scott D, 1998. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *The Journal of Cell Biology*, 142(3):665–681.

Seaman, Matthew NJ, Harbour, Michael E, Tattersall, Daniel, Read, Eliot, and Bright, Nicholas, 2009. Membrane recruitment of the cargo-selective retromer subcomplex is catalysed by the small GTPase Rab7 and inhibited by the Rab-GAP TBC1D5. *Journal of Cell Science*, 122(14):2371–2382.

Segal, Gil and Shuman, Howard A, 1998. Intracellular multiplication and human macrophage killing by *Legionella pneumophila* are inhibited by conjugal components of IncQ plasmid RSF1010. *Molecular Microbiology*, 30(1):197–208.

Segal, Gil and Shuman, Howard A, 1999. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infection and Immunity*, 67(5):2117–2124.

Sepulveda, JL and Wu, C, 2006. The parvins. *Cellular and Molecular Life Sciences*, 63 (1):25–35.

Shevchuk, Olga, Batzilla, Christoph, Hägele, Sonja, Kusch, Harald, Engelmann, Susanne, Hecker, Michael, Haas, Albert, Heuner, Klaus, Glöckner, Gernot, and Steinert, Michael, 2009. Proteomic analysis of *Legionella*-containing phagosomes isolated from *Dictyostelium*. *International Journal of Medical Microbiology*, 299(7):489–508.

Shi, Anbing, Sun, Lin, Banerjee, Riju, Tobin, Michael, Zhang, Yinhua, and Grant, Barth D, 2009. Regulation of endosomal clathrin and retromer-mediated endosome to Golgi retrograde transport by the J-domain protein RME-8. *The EMBO Journal*, 28(21):3290–3302.

Shiba, Tomoo, Takatsu, Hiroyuki, Nogi, Terukazu, Matsugaki, Naohiro, Kawasaki, Masato, Igarashi, Noriyuki, Suzuki, Mamoru, Kato, Ryuichi, Earnest, Thomas, Nakayama, Kazuhisa, *et al.*, 2002. Structural basis for recognition of acidic-cluster dileucine sequence by GGA1. *Nature*, 415(6874):937–941.

Shohdy, Nadim, Efe, Jem A, Emr, Scott D, and Shuman, Howard A, 2005. Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. *Proceedings of the National Academy of Sciences of the United States of America*, 102(13):4866–4871.

Siegenthaler, Barbara M and Rajendran, Lawrence, 2012. Retromers in Alzheimer's Disease. *Neurodegenerative Diseases*, 10(1-4):116–121.

Solomon, Jonathan M, Rupper, Adam, Cardelli, James A, and Isberg, Ralph R, 2000. Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. *Infection and immunity*, 68(5): 2939–2947.

Spooner, Robert A, Smith, Daniel C, Easton, Andrew J, Roberts, Lynne M, Lord, J Michael, *et al.*, 2006. Retrograde transport pathways utilised by viruses and protein toxins. *Virology Journal*, 3:26.

Stanley, Margaret, 2010. Pathology and epidemiology of HPV infection in females. *Gynecologic Oncology*, 117(2):S5–S10.

Stechmann, Bahne, Bai, Siau-Kun, Gobbo, Emilie, Lopez, Roman, Merer, Goulven, Pinchard, Suzy, Panigai, Laetitia, Tenza, Danièle, Raposo, Graça, Beaumelle, Bruno, *et al.*, 2010. Inhibition of retrograde transport protects mice from lethal ricin challenge. *Cell*, 141(2):231–242.

Stenmark, Harald, 2009. Rab GTPases as coordinators of vesicle traffic. *Nature Reviews Molecular Cell Biology*, 10(8):513–525.

Sturgill-Koszycki, Sheila and Swanson, Michele S, 2000. *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. *The Journal of Experimental Medicine*, 192(9):1261–1272.

Swanson, Michele S and Isberg, Ralph R, 1995. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infection and Immunity*, 63(9):3609–3620.

Swarbrick, James D, Shaw, Daniel J, Chhabra, Sandeep, Ghai, Rajesh, Valkov, Eugene, Norwood, Suzanne J, Seaman, Matthew NJ, and Collins, Brett M, 2011. VPS29 is not an active metallo-phosphatase but is a rigid scaffold required for retromer interaction with accessory proteins. *PloS ONE*, 6(5):e20420.

Tabuchi, Mitsuaki, Yanatori, Izumi, Kawai, Yasuhiro, and Kishi, Fumio, 2010. Retromer-mediated direct sorting is required for proper endosomal recycling of the mammalian iron transporter DMT1. *Journal of Cell Science*, 123(5):756–766.

Tai, Guihua, Lu, Lei, Wang, Tuan Lao, Tang, Bor Luen, Goud, Bruno, Johannes, Ludger, and Hong, Wanjin, 2004. Participation of the syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from the early/recycling endosome to the *trans*-Golgi network. *Molecular Biology of the Cell*, 15(9):4011–4022.

Tan, Yunhao, Arnold, Randy J, and Luo, Zhao-Qing, 2011. *Legionella pneumophila* regulates the small GTPase Rab1 activity by reversible phosphorylcholination. *Proceedings of the National Academy of Sciences*, 108(52):21212–21217.

Temkin, Paul, Lauffer, Ben, Jäger, Stefanie, Cimermancic, Peter, Krogan, Nevan J, and von Zastrow, Mark, 2011. SNX27 mediates retromer tubule entry and endosome-to-plasma membrane trafficking of signalling receptors. *Nature Cell Biology*, 13(6): 715–721.

Tesh, Vernon L, 2010. Induction of apoptosis by Shiga toxins. *Future Microbiology*, 5 (3):431–453.

Tiaden, André and Hilbi, Hubert, 2012. α-Hydroxyketone synthesis and sensing by *Legionella* and *Vibrio*. *Sensors*, 12(3):2899–2919.

Tiaden, André, Spirig, Thomas, Weber, Stefan S, Brüggemann, Holger, Bosshard, Rachel, Buchrieser, Carmen, and Hilbi, Hubert, 2007. The *Legionella pneumophila* response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. *Cellular Microbiology*, 9 (12):2903–2920.

Tilney, Lewis G, Harb, Omar S, Connelly, Patricia S, Robinson, Camenzind G, and Roy, Craig R, 2001. How the parasitic bacterium *Legionella pneumophila* modifies its

phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. *Journal of Cell Science*, 114(24):4637–4650.

Tsai, Billy and Rapoport, Tom A, 2002. Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *The Journal of Cell Biology*, 159(2):207–216.

Tsai, Billy, Rodighiero, Chiara, Lencer, Wayne I, and Rapoport, Tom A, 2001. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell*, 104(6):937–948.

Urwyler, Simon, Nyfeler, Yves, Ragaz, Curdin, Lee, Hookeun, Mueller, Lukas N, Aebersold, Ruedi, and Hilbi, Hubert, 2009. Proteome analysis of *Legionella* vacuoles purified by magnetic immunoseparation reveals secretory and endosomal GTPases. *Traffic*, 10(1):76–87.

Utskarpen, Audrun, Slagsvold, Hege H, Dyve, Anne Berit, Skånland, Sigrid S, and Sandvig, Kirsten, 2007. SNX1 and SNX2 mediate retrograde transport of Shiga toxin. *Biochemical and Biophysical Research Communications*, 358(2):566–570.

Valdmanis, Paul N, Meijer, Inge A, Reynolds, Annie, Lei, Adrienne, MacLeod, Patrick, Schlesinger, David, Zatz, Mayana, Reid, Evan, Dion, Patrick A, Drapeau, Pierre, *et al.*, 2007. Mutations in the *KIAA0196* gene at the *SPG8* locus cause hereditary spastic paraplegia. *The American Journal of Human Genetics*, 80(1):152–161.

van Rahden, Vanessa A, Brand, Kristina, Najm, Juliane, Heeren, Joerg, Pfeffer, Suzanne R, Braulke, Thomas, and Kutsche, Kerstin, 2012. The 5-phosphatase OCRL mediates retrograde transport of the mannose 6-phosphate receptor by regulating a Rac1-cofilin signalling module. *Human Molecular Genetics*, 21(23):5019–5038.

van Weering, Jan RT, Verkade, Paul, and Cullen, Peter J, 2012. SNX-BAR-mediated endosome tubulation is co-ordinated with endosome maturation. *Traffic*, 13(1):94–107.

Victor, L Yu, Plouffe, Joseph F, Pastoris, Maddalena Castellani, Stout, Janet E, Schousboe, Mona, Widmer, Andreas, Summersgill, James, File, Thomas, Heath, Christopher M, Paterson, David L, *et al.*, 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. *Journal of Infectious Diseases*, 186 (1):127–128.

Vilariño-Güell, Carles, Wider, Christian, Ross, Owen A, Dachsel, Justus C, Kachergus, Jennifer M, Lincoln, Sarah J, Soto-Ortolaza, Alexandra I, Cobb, Stephanie A, Wilhoite, Greggory J, Bacon, Justin A, *et al.*, 2011. *VPS35* mutations in Parkinson disease. *The American Journal of Human Genetics*, 89(1):162–167.

Vincent, Carr D, Friedman, Jonathan R, Jeong, Kwang Cheol, Buford, Emily C, Miller, Jennifer L, and Vogel, Joseph P, 2006. Identification of the core transmembrane complex of the *Legionella* Dot/Icm type IV secretion system. *Molecular Microbiology*, 62(5):1278–1291.

Vincent, Carr D, Friedman, Jonathan R, Jeong, Kwang Cheol, Sutherland, Molly C, and Vogel, Joseph P, 2012. Identification of the DotL coupling protein subcomplex of the *Legionella* Dot/Icm type IV secretion system. *Molecular Microbiology*, 85(2): 378–391.

Viner, Ram, Chetrit, David, Ehrlich, Marcelo, and Segal, Gil, 2012. Identification of two *Legionella pneumophila* effectors that manipulate host phospholipids biosynthesis. *PLoS Pathogens*, 8(11):e1002988.

Vogel, Joseph P, Andrews, Helene L, Wong, Swee Kee, and Isberg, Ralph R, 1998. Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science*, 279(5352):873–876.

Voges, D, Zwickl, P, and Baumeister, W, 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annual Review of Biochemistry*, 68(1): 1015–1068.

Wassmer, Thomas, Attar, Naomi, Bujny, Miriam V, Oakley, Jacqueline, Traer, Colin J, and Cullen, Peter J, 2007. A loss-of-function screen reveals SNX5 and SNX6 as potential components of the mammalian retromer. *Journal of Cell Science*, 120(1): 45–54.

Wassmer, Thomas, Attar, Naomi, Harterink, Martin, van Weering, Jan RT, Traer, Colin J, Oakley, Jacqueline, Goud, Bruno, Stephens, David J, Verkade, Paul, Korswagen, Hendrik C, *et al.*, 2009. The retromer coat complex coordinates endosomal sorting and dynein-mediated transport, with carrier recognition by the *trans*-Golgi network. *Developmental Cell*, 17(1):110–122.

Watarai, Masahisa, Derre, Isabelle, Kirby, James, Growney, Joseph D, Dietrich, William F, and Isberg, Ralph R, 2001. *Legionella pneumophila* is internalized by a

macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse Lgn1 locus. *The Journal of Experimental Medicine*, 194(8):1081–1096.

Watts, DJ and Ashworth, JM, 1970. Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem J*, 119(2):171–174.

Weber, Stefan S, Ragaz, Curdin, Reus, Katrin, Nyfeler, Yves, and Hilbi, Hubert, 2006. *Legionella pneumophila* exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. *PLoS Pathogens*, 2(5):e46.

Weber, Stefan S, Ragaz, Curdin, and Hilbi, Hubert, 2009. The inositol polyphosphate 5-phosphatase OCRL1 restricts intracellular growth of *Legionella*, localizes to the replicative vacuole and binds to the bacterial effector LpnE. *Cellular Microbiology*, 11(3):442–460.

White, Jamie, Johannes, Ludger, Mallard, Frédéric, Girod, Andreas, Grill, Stephan, Reinsch, Sigrid, Keller, Patrick, Tzschaschel, Barbara, Echard, Arnaud, Goud, Bruno, *et al.*, 1999. Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *The Journal of Cell Biology*, 147(4):743–760.

Wilcke, Mona, Johannes, Ludger, Galli, Thierry, Mayau, Véronique, Goud, Bruno, and Salamero, Jean, 2000. Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the *trans*-Golgi network. *The Journal of Cell Biology*, 151(6):1207–1220.

Woo, Seng-Ryong, Li, Nianyu, Bruno, Tullia C, Forbes, Karen, Brown, Scott, Workman, Creg, Drake, Charles G, and Vignali, Dario AA, 2010. Differential subcellular localization of the regulatory T-cell protein LAG-3 and the coreceptor CD4. *European Journal of Immunology*, 40(6):1768–1777.

Xavier, Ramnik, Brennan, Todd, Li, Qingqin, McCormack, Christine, and Seed, Brian, 1998. Membrane compartmentation is required for efficient T cell activation. *Immunity*, 8(6):723–732.

Xu, Li, Shen, Xihui, Bryan, Andrew, Banga, Simran, Swanson, Michele S, and Luo, Zhao-Qing, 2010. Inhibition of host vacuolar H^+-ATPase activity by a *Legionella pneumophila* effector. *PLoS Pathogens*, 6(3):e1000822.

Yin, Wenguang, Liu, Dapeng, Liu, Nian, Xu, Liangliang, Li, Song, Lin, Shuo, Shu, Xiaodong, and Pei, Duanqing, 2012. SNX17 regulates Notch pathway and pancreas development through the retromer-dependent recycling of Jag1. *Cell Regeneration*, 1 (1):4.

Yu, Min and Haslam, David B, 2005. Shiga toxin is transported from the endoplasmic reticulum following interaction with the luminal chaperone HEDJ/ERdj3. *Infection and Immunity*, 73(4):2524–2532.

Zhang, Peng, Wu, Yihui, Belenkaya, Tatyana Y, and Lin, Xinhua, 2011. SNX3 controls Wingless/Wnt secretion through regulating retromer-dependent recycling of Wntless. *Cell Research*, 21(12):1677–1690.

Zhu, Wenhan, Banga, Simran, Tan, Yunhao, Zheng, Cheng, Stephenson, Robert, Gately, Jonathan, and Luo, Zhao-Qing, 2011. Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of *Legionella pneumophila*. *PloS One*, 6(3):e17638.

Zimprich, Alexander, Benet-Pagès, Anna, Struhal, Walter, Graf, Elisabeth, Eck, Sebastian H, Offman, Marc N, Haubenberger, Dietrich, Spielberger, Sabine, Schulte, Eva C, Lichtner, Peter, *et al.*, 2011. A Mutation in *VPS35*, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. *The American Journal of Human Genetics*, 89(1):168–175.

Zusman, Tal, Aloni, Gali, Halperin, Einat, Kotzer, Hani, Degtyar, Elena, Feldman, Michal, and Segal, Gil, 2007. The response regulator PmrA is a major regulator of the *icm/dot* type IV secretion system in *Legionella pneumophila* and Coxiella burnetii. *Molecular Microbiology*, 63(5):1508–1523.

Zusman, Tal, Degtyar, Elena, and Segal, Gil, 2008. Identification of a hypervariable region containing new *Legionella pneumophila* Icm/Dot translocated substrates by using the conserved *icmQ* regulatory signature. *Infection and Immunity*, 76(10): 4581–4591.

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