The *Legionella pneumophila* effector LpdA is a palmitoylated phospholipase D virulence factor

Running title: The palmitoylated *L. pneumophila* lipase effector LpdA

Gunnar N. Schroeder[*,1], Philipp Aurass[†], Clare V. Oates[‡], Edward W. Tate[§], Elizabeth L. Hartland[‡], Antje Flieger[†], Gad Frankel[*,1]

[*]MRC Centre for Molecular Bacteriology and Infection, Department of Life Sciences, Imperial College, London, SW7 2AZ, UK

[†]Division of Enteropathogenic Bacteria and *Legionella* (Fg11), Robert Koch-Institute, Burgstr. 37, 38855 Wernigerode, Germany

[‡]Department of Microbiology and Immunology, University of Melbourne, Peter Doherty Institute for Infection and Immunity, Victoria 3010, Australia

[§]Department of Chemistry, South Kensington Campus, Imperial College, London, SW7 2AZ, UK

[1]For correspondence: MRC Centre for Molecular Bacteriology and Infection, Flowers Building, Imperial College London, London SW7 2AZ, UK

Tel: 44 20 7594 5253

Email: g.frankel@imperial.ac.uk or g.schroeder@imperial.ac.uk

Abstract

Legionella pneumophila is a bacterial pathogen which thrives in alveolar macrophages causing a severe pneumonia. Virulence of *L. pneumophila* depends on its Dot/Icm type IV secretion system (T4SS), which delivers more than 300 effector proteins into the host, where they rewire cellular signaling to establish a replication-permissive niche, the *Legionella*-containing vacuole (LCV). Biogenesis of the LCV requires substantial redirection of vesicle trafficking and remodeling of intracellular membranes. In order to achieve this, several T4SS effectors target regulators of membrane trafficking, while others resemble lipases. Here, we characterized LpdA, a phospholipase D effector, which was previously proposed to modulate the lipid composition of the LCV. We found that ectopically expressed LpdA was targeted to the plasma-membrane and Rab4- and Rab14-containing vesicles. Subcellular targeting of LpdA required a C-terminal motif, which is post-translationally modified by S-palmitoylation. Substrate specificity assays showed that LpdA hydrolyzed phosphatidyl-inositol, -inositol-3- and -4-phosphate, and -phosphatidyl-glycerol to phosphatidic acid (PA) *in vitro*. In HeLa cells LpdA generated PA at vesicles and the plasma membrane. Imaging of different phosphatidyl-inositol phosphate (PIP) and organelle markers revealed that while LpdA did not impact on membrane association of various PIP probes it triggered fragmentation of the Golgi apparatus. Importantly, although LpdA is translocated inefficiently into cultured cells a *L. pneumophila* Δ*lpdA* mutant displayed reduced replication in murine lungs, suggesting that it is virulence factor contributing to *L. pneumophila* infection *in vivo*.

Introduction

Legionella pneumophila is a bacterial pathogen which infects and replicates in protozoa as well as in human alveolar macrophages and lung epithelial cells causing a severe pneumonia, called Legionnaires' disease (1). Several secretion systems allow *L. pneumophila* to export proteins into its environment and interact with host cells (1, 2). The *Legionella* type I and type II secretion systems (T1SS/T2SS) have been shown to contribute to *L. pneumophila* virulence (3, 4); however only the Dot/Icm type IV secretion system (T4SS) is essential for intracellular replication and pathogenesis (5, 6). The Dot/Icm T4SS delivers more than 300 effector proteins into the host cell, where they manipulate cell signaling (7, 8). This enables the bacteria to evade phago-lysosomal degradation and instead establish a replication-permissive endoplasmic-reticulum (ER)-derived niche, the *Legionella*-containing vacuole (LCV) (9-11).

The biogenesis of the LCV is a multistep process and involves substantial remodeling of the membrane of the nascent *Legionella* phagosome (12, 13). Consequently, a large number of effectors, which are secreted by *Legionella,* are membrane associated proteins which target cellular regulators of membrane trafficking such as Rab GTPases (14, 15) or directly exploit and manipulate host lipids (16-18). To achieve membrane association, *Legionella* effectors highjack at least two common host cell mechanisms. Several effectors contain a classical C-terminal CAAX-box motif, which is recognized by prenyltransferases and post-translationally modified with a geranylgeranyl (20-carbon) or a farnesyl (15-carbon) lipid anchor (19, 20). Following the lipid transfer Ras-converting enzyme-1 (RCE-1) removes the amino acid residues after the modified cysteine and isoprenyl cysteine carboxyl methyltransferase (IcmT) methylates the new C-terminus. Together these irreversible modifications increase the hydrophobicity and membrane affinity of the effectors facilitating their membrane association. *Legionella* ensures the efficient modification of effectors by recruiting host proteins, such as farnesyl transferase, RCE-1 and IcmT to the LCV in a T4SS-dependent manner (20).

Alternatively to using a covalently attached lipid anchor, several *Legionella* effectors associate with membranes by binding phosphatidylinositol-phosphates (PIPs) (16, 21), negatively charged

glycerophospholipids which contain a mono- or polyphosphorylated *myo*-inositol ring (22). PIPs are versatile signaling molecules and specific enrichment of one subspecies gives identity to organelle membranes, leading to selective recruitment of PIP-binding proteins. *L. pneumophila* actively modulates the PIP composition of membranes. The effectors SidF and SidP are PIP phosphatases, which hydrolyze the D3 phosphate of different 3-phosporylated inositol headgroups (23, 24). This conversion of PIP species is important for the transition of the PI3P-rich early *Legionella*-containing phagosome to the mature PI4P-rich LCV. In addition, VipD removes PI3P from early endosomes, contributing to the decoupling of the LCV from the normal phagosomal maturation pathway (25).

VipD belongs to the large family of phospholipases, which hydrolyze either the carboxylester (subfamily A and B), the glycerol- (subfamily C) or the alcohol-oriented (subfamily D) phosphodiester bonds of phospholipid substrates. *L. pneumophila* encodes at least 15 putative phospholipase A (PLA) enzymes (18, 25) but other than VipD and PlaB, which was implicated in virulence *in vivo* (26), their functions in infection are not well defined. Additional effectors belonging to the other phospholipase subfamilies exist. The effector PlcC/CegC1 is a novel Zn^{2+}-dependent phospholipase C (PLC) and, together with the two homologues PLCs PlcA and PlcB, contributes to virulence in the *Galleria mellonella* infection model (27). The effector LpdA was first recognized in a bioinformatic search for effectors with potential function in phospholipid biosynthesis due to its homology with eukaryotic PLDs (28). Eukaryotic PLDs use mainly phosphatidylcholine (PC), but can also accept other phospholipid substrates, to generate the free lipid headgroup and the important signalling molecule phosphatidic acid (PA) (29). LpdA localizes to the LCV and perturbs PA distribution upon ectopic expression in cells, suggesting that it might modulate PA levels on the LCV (28). However, important features of LpdA such as substrate specificity and the consequences of its activity on host cell physiology remain unknown. In this study, we characterized the activity of LpdA, investigated its mode of membrane association and determined its effect on host cells *in vitro* and during mouse infection *in vivo*, to further develop our understanding of its function in *L. pneumophila* infection.

Material and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were cultured using LB broth or agar. *L. pneumophila* 130b (AA100) was grown at 37 °C on buffered charcoal yeast extract (CYE) plates or in *N*-(2-acetamido)-2-aminoethanesulfonic acid buffered yeast extract (AYE) broth (30, 31). If required, *Legionella* media were supplemented with 25 µg/ml kanamycin and 6 µg/ml chloramphenicol. All chemicals and reagents were obtained from Sigma Aldrich Ltd. if not indicated otherwise.

Cell culture and transfection. The human alveolar epithelial A549 cells, the cervical epithelial HeLa cells and HEK293E cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum, GlutaMAX™ (Invitrogen) and non-essential amino acids in a humidified with atmosphere of 5% CO_2. THP-1 human monocytic and Raw264.7 murine macrophage-like cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, GlutaMAX™ (Invitrogen). THP-1 cells were differentiated by the addition of 50 ng/mL phorbol 12-myristate 13-acetate (PMA) for 72 h. A549, HeLa, THP-1 and Raw264.7 cells were obtained from the American Type Culture Collection (ATCC) and subjected to minimal passaging. HEK293E cells were a kind gift from Avinash R. Shenoy (Imperial College London, UK). The transfection of HeLa and A549 lung epithelial cells with eukaryotic expression plasmids was performed according to manufacturer's instructions using GeneJuice (Novagen) or jetPRIME® (Polyplus Transfection) reagent respectively.

Plasmid and strain construction. All plasmids, primers and restriction enzymes (NEB Inc.) used and created in this study are described in Table 2. Gene sequences were extracted from the draft genome sequence of *L. pneumophila* strain 130b ((32); ftp://ftp.sanger.ac.uk/pub/pathogens/Legionella/pneumophila/130b/) or obtained by sequence homology search using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). If not indicated otherwise, chromosomal DNA of *L. pneumophila* 130b was used as PCR template. The mutagenesis of the catalytic lysine residues of LpdA was performed sequentially using the QuikChange II site-directed mutagenesis kit (Agilent Technologies) and pRK5 Myc LpdA (pICC1565) as starting

template. The mutated gene was then subcloned in other plasmids backbones. The expression plasmid for ER-targeted mCherry protein was obtained by introducing a C-terminal KDEL ER-targeting and an N-terminal Calreticulin ER-retention signal into pmCherry-C1 (Clontech) by reverse PCR. Standard molecular biology techniques were employed and the identity of all plasmids confirmed by sequencing. Plasmids were transformed into *L. pneumophila* by electroporation (33).

To create the *L. pneumophila* 130b Δ*lpdA* mutant strain, a PCR deletion cassette consisting of the kanamycin resistance (kan^r) cassette from pSB315 (34) embedded in the 3' and 5' chromosomal flanking regions of the *lpdA* gene was produced and transformed into *L. pneumophila* 130b by natural transformation (35). Transformants were selected by plating on CYE agar containing 25 µg/ml kanamycin. Replacement of the *lpdA* gene with the kan^r cassette and integrity of the chromosomal flanking regions was confirmed by sequencing of the genomic region.

Generation of stable A549 cell lines by transduction. Viral transductions were performed as described previously (36). Briefly, HEK293E cells were transfected with pICC1583 and the pCMV-VSV-G envelope and pCMV-MMLV-gag-pol packaging plasmids using Lipofectamine 2000 (Life Technologies), medium replaced after 24 h and cell supernatant containing virions harvested 48 h post transfection. The supernatant was passed through a 0.45 µm filter and added to A549 cells. After 24 h, the cells were washed with Dulbecco's (D)-PBS and incubated with growth medium supplemented with 1.5 µg/mL puromycin (Gibco). After one week of selection expression of the GFP-reporters throughout the cell population was confirmed by IF and cells were frozen or further passaged without selection.

Lipase activity assay. The substrate specificity of recombinant His_6-tagged LpdA was assessed as described previously (27). As solubility tests showed LpdA-His_6 was poorly soluble, assays were carried out with lysates of bacteria. For generation of lysates, strains were grown until exponential growth and expression was induced by addition of 1mM IPTG for 4 hours. Expression of the recombinant proteins with the expected apparent molecular weight (52 kDa) was confirmed by SDS PAGE. Subsequently, pelleted bacteria were suspended in 1/20 volume of the original culture volume

of 10 mg/ml of lysozyme and 1 µl/ml of Triton X-100 in 40 mM Tris-HCl pH 7.5 (25°C) at 37°C for 30 min. After repeated passage through a 26-gauge needle, the lysates were resuspended in one-half of the original culture volume of 40 mM Tris-HCl (pH 7.5) (25°C). 0.2 mM lipid substrates (dipalmitoylphosphatidyl-choline (PC) 99% purity, dipalmitoylphosphatidyl-glycerol (PG) 99% purity, dipalmitoylphosphatidyl-ethanolamine (PE) 99% purity, dipalmitoylphosphatidyl-serine (PS) 99% purity) or 0.25 mM PI and PIPs (Phosphatidyl-inositol diC16 (PIC16), Phosphatidyl-inositol 3-phosphate diC16 (PI3P), Phosphatidyl-inositol 4-phosphate diC16 (PI4P), Phosphatidyl-inositol 5-phosphate diC16 (PI5P), Phosphatidyl-inositol 3,4-bisphosphate diC16 (PI3,4P), Phosphatidyl-inositol 3,5-bisphosphate diC16 (PI3,5P), Phosphatidyl-inositol 4,5-bisphosphate diC16 (PI4,5P), Phosphatidyl-inositol 3,4,5-trisphosphate diC16 (PI3,4,5P)) were incubated with buffer (40 mM Tris-HCl pH 7.5 (25°C)) or lysates of BL21 or BL21 expressing LpdA-His6 or LpdAKK165/376RR-His6 inactive mutant in a 1:1 ratio. After 4-5h incubation at 37°C with agitation, lipids were extracted (37) and analyzed by TLC using chloroform / methanol / water (65:25:4 (v/v/v)) as the running phase and pure lipids substrates and phosphatidic acid (1,2-Dipalmitoyl-sn-glycero-3-phosphate (PA)1,2-Dimyristoyl-sn-glycero-3-phosphate monosodium salt (PA)) as references. The TLC plate was developed using 0.2% naphthol blue-black. PI, PA, and PIPs were purchased from Echelon Biosciences, all other lipid substrates were purchased from Avanti Polar Lipids, Inc. or Sigma-Aldrich.

Metabolic labelling and isolation of palmitoylated proteins. HeLa cells (one well/condition in 6-well plate) were transfected with expression plasmids for LpdA or its variants, grown over night and then incubated with 25 µM 17-octadecynoic acid (ODYA, Cambridge Biosciences). After 7 h the medium was removed, cells washed with PBS and scraped into 100 µL lysis buffer containing 1% Triton X-100, 0.1% SDS and complete EDTA-free protease inhibitor (Roche). After 15 min incubation large debris was removed by centrifugation (20000*g*, 10 min, 4 °C) and protein concentration in the supernatant measured. Click-chemistry was then performed on equal amounts of protein. Briefly, 1 mM $CuSO_4$, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 100 µM

Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) and 100 µM of a trifunctional azido-TAMRA-PEG-biotin (AzTB) capture reagent (38, 39) were added to the clarified lysate and the mix incubated for 45 min at room temperature. The reaction was stopped by adding 10 mM EDTA and proteins were precipitated by addition of 1 mL chilled methanol and centrifugation (20000*g*, 30 min, 4 °C). The pellet was washed three times with 1 mL methanol, air-dried and subsequently resuspended in 100 µL PBS (0.2% SDS, 1 mM EDTA). 10 µl of the solution was kept as input control. The biotinylated proteins were isolated using Dynabeads® MyOne™ Streptavidin C1 (Invitrogen; 50 µL slurry per 1 mg of protein), washed three times with at least 0.5 mL PBS (0.2% SDS) and subsequently boiled in 50 µL 1x SDS-gel sample buffer. Equal volumes of supernatant were subjected to SDS-PAGE and Western blotting. LpdA and control proteins were labelled with mouse anti-HA.11 (clone 16B12; MMs-101P, Cambridge Bioscience), mouse anti-Myc tag (05-724, Millipore), rabbit anti-calnexin (SPA-860F, Stressgen) and anti-tubulin (T6199, Sigma) antibodies in combination with appropriate horse radish peroxidase labelled secondary antibodies (Jackson Immuno Research). Labelled proteins were visualized using EZ-ECL detection reagent (Geneflow) and a Fuji Las3000 imager.

Infection of eukaryotic cells and intracellular growth assay. *L. pneumophila* strains were cultured for infection as described previously (32). The bacteria were suspended in cell culture media, supplemented with chloramphenicol (6 µg/ml) and 1 mM IPTG if required, and added to the cells at a multiplicity of infection (MOI) of 15-100 for 24 well plate A549 cell infections. To synchronize infection, samples were centrifuged for 5 min at 600 *g.*

For growth assays individual wells of a black 96 well plate with translucent bottom were seeded with $8.5x10^4$ THP-1 cells. Following differentiation for 72 h the medium was replaced with 100 µL growth medium supplemented with 6 µg/mL chloramphenicol and 1 mM IPTG. The cells were infected with *L. pneumophila* strains containing the pXDC31 GFP expression plasmid (40) at an MOI of 0.1 – 0.5 in triplicate. 1 h post infection the medium was replaced with one containing 100 µg/mL gentamicin, the cells incubated for a further 1 h and then washed three times with PBS. The infected cells were

incubated in 100 µL supplemented growth medium without phenol red and GFP fluorescence recorded over 92 h in a FLUOstar Omega plate reader (BMG Labtech) equipped with atmospheric control unit. Reads were taken every 2 h. Fluorescence measurements were corrected for the background fluorescence signal of uninfected cells and each curve normalized to zero starting value.

β-Lactamase (TEM1)-translocation assay. The translocation of LpdA was tested as described previously (32). Briefly, Raw264.7 cells were infected with *L. pneumophila* 130b expressing TEM1-fusions of LpdA, LpdA inactive mutant or control proteins, after 1h incubation and washing 20 µL freshly prepared CFF2-AM β-lactamase substrate (LiveBLAzer™ FRET-B/G Loading Kit, Invitrogen) was added, and finally the fluorescence emission at 450 nm and 520 nm was measured from the bottom using a Fluostar Optima plate reader 3 h post infection. The translocation rate was expressed as fold-increase of the 450 nm/520 nm emission ratio of each infected in correlation to the emission ratio of uninfected cells.

Immunofluorescence microscopy. Cells on coverslips were fixed with 3.2% paraformaldehyde (PFA), washed with D-PBS, incubated with 50 mM ammonium chloride, washed and permeabilized with 0.1% Triton X-100. After blocking with PBS containing 2% (w/v) BSA and 2% natural donkey serum samples were stained sequentially with primary and secondary antibodies and mounted using ProLong antifade reagent (Invitrogen). The used primary antibodies (including catalogue numbers) were: mouse anti-HA.11 (clone 16B12; MMs-101P, Cambridge Bioscience), mouse anti-Myc tag (05-724, Millipore), rabbit-anti-*Legionella* LPS (PA1-7227, Affinity BioReagents), rabbit anti-Giantin (ab24586, Abcam), anti-c-Myc FITC conjugate (F 2047, Sigma) and anti-HA-TRITC (H9037, Sigma). Secondary antibodies were: donkey anti-rabbit or anti-mouse IgG Alexa Fluor 488; donkey anti-rabbit or anti-mouse IgG Rhodamine Red-X (RRX) (all Jackson Immunoresearch). DNA was visualized with Hoechst 33342 dye. To visualize mitochondria transfected cells were treated with MitoTacker®Red (Life Technologies) prior to fixation. Samples were analyzed using an Axio Z1 Imager microscope. Typically 50-100 cells were accessed per condition and at least two independent

biological repeats carried out per experimental series. Representative images were deconvoluted and processed using AxioVision software (Carl Zeiss).

Infection of mice. All mouse procedures were approved by the University of Melbourne Animal Ethics Committee and carried out as described previously (41). Briefly, groups of mice were inoculated separately with approximately 2.5 x 10^6 colony forming units (CFU) of each *L. pneumophila* 130b strain under investigation. For consistency *L. pneumophila* 130b wild type (WT) or Δ*lpdA* mutant both carried the empty p4HA (pICC562) plasmid. 72 hours post inoculation, mice were sacrificed and their lung tissue extracted. Tissue was homogenized and complete host cell lysis was ensured by incubation in 0.1 % saponin for 15 min at 37 °C. Serial dilutions of the homogenate were plated onto plain CYE agar plates to determine the number of viable bacteria.

Results

L. pneumophila infection causes drastic changes to the host cell PA distribution

LpdA is conserved across the most commonly used sequenced prototype *L. pneumophila* isolates [130b (32), Philadelphia (42), Corby (43), Alcoy (44), Lens, Paris (45); 97-98 % identity; Fig. S1]. In order to investigate if LpdA affects cellular distribution of PA we generated an A549 lung epithelial cell line stably expressing a PA biosensor consisting of the PA-binding domain of the yeast protein Spo20 fused to GFP (A549-SPO) (46). In unchallenged cells GFP-Spo20 exclusively localizes to the nucleus (Fig. 1A), but upon stimulation of production it is rapidly recruited to sites where PA is generated (28, 46). A549-SPO cells were infected with wild type *L. pneumophila*, Δ*lpdA* or Δ*lpdA* complemented with a plasmid encoding 4HA-tagged LpdA. Infection with the T4SS-deficient *L. pneumophila* Δ*dotA* was used as a control. Immunofluorescence (IF) microscopy at 6 h post infection revealed that wild type *L. pneumophila* but not Δ*dotA* induced redistribution of GFP-Spo20 from the nucleus, resulting in a diffuse signal throughout the cells (Fig. 1A). A similar redistribution of GFP-Spo20 was observed as early as 2 h, and remained after 24 h, post infection (data not shown). The quantification of this effect by visual counting showed that at 6 h post infection about 80% of cells infected with wild type *L. pneumophila* had no nuclear GFP-Spo20 signal (Fig. 1B). In contrast, at the same time more than 80% of the cells infected with the *L. pneumophila* Δ*dotA* mutant retained a clear nuclear GFP-Spo20 signal. Analysis and quantification of the PA redistribution in the cells infected with either *L. pneumophila* Δ*lpdA* or the complemented strain showed that both induced migration of GFP-Spo20 from the nucleus with the same kinetics and to the same extent as wild type *L. pneumophila* (Fig. 1B and data not shown). These results show that upon infection, *L. pneumophila* induces rapid T4SS-dependent, but LpdA-independent, changes to the PA levels of host cells. The lack of obvious LpdA-dependent PA generation was not due to impaired translocation of the effector by *L. pneumophila* 130b into host cells as TEM1-β lactamase translocation assays confirmed delivery of LpdA, and an inactive LpdA KK165/376RR mutant in which the two catalytically lysine residues

were mutated (28), into host cells (Fig. S2). This suggests that any impact of LpdA during infection might be subtle and masked by other effectors.

LpdA is targeted to membranes by post-translational palmitoylation

As the PA reporter assay (Fig. 1) suggested that the function of LpdA during infection is masked by redundant effectors, we continued our study using ectopic-expression of HA- or Myc-tagged LpdA or inactive mutants. In HeLa (Fig. 2A) and A549 lung epithelial cells (data not shown) HA-LpdA (Fig. 2A) or Myc-LpdA (data not shown) localized to small vesicular structures, which were distributed throughout the cell and to less extent to the plasma membrane. As a similar distribution pattern for LpdA was observed in both cell lines, further experiments were carried out with HeLa cells because of their higher and more reproducible transfection efficiency. Co-transfection of these constructs with the GFP-Spo20 showed redistribution of the reporter from the nucleus to vesicular structures and plasma membrane sites, which were enriched in LpdA. The catalytically inactive mutant LpdA KK165/376RR failed to induce the redistribution of GFP-Spo20, confirming that LpdA generates PA at the plasma membrane and vesicular structures (Fig. 2B).

Bioinformatic sequence analysis of LpdA using CSS-Palm 2.0 (47) revealed that the five C-terminal amino acid residues of LpdA contain three cysteines, which represent a potential site for post-translational S-palmitoylation (Fig. 3A). Two of these cysteines are conserved across LpdA homologues of the most commonly used *L. pneumophila* isolates (Fig. S1). In order to determine the role of these residues for the membrane association of LpdA, we created an LpdAΔC5aa truncation mutant and visualized its localization upon ectopic expression by IF. To examine the role of a non-conserved cysteine in the localization of LpdA a C440F mutant, corresponding to the amino acid replacement found in other LpdA homologues, was included. While full length LpdA and LpdA C440F were indistinguishable and localized to vesicles and the plasma membrane the LpdAΔ5aa mutant showed a diffuse cytoplasmic distribution (Fig. 3B). Notably, similarly to the wild type effector, LpdAΔ5aa induced depletion of the GFP-Spo20 reporter from the nucleus (Fig. S3),

indicating that the truncated protein has lipase activity and that the loss of membrane association is not due to misfolding.

To elucidate if the C-terminal membrane association motif of LpdA is palmitoylated, we performed bio-orthogonal metabolic labelling with a lipid reporter, an emerging strategy used to profile protein lipidation (48). HeLa cells expressing HA-LpdA or HA-LpdAΔC5aa were incubated with growth medium supplemented with 17-octodecynoic acid (ODYA), a palmitic acid analogue which is readily incorporated into proteins by palmitoyltransferases (Fig. 3C; (49)). After cell lysis the trifunctionalized AzTB capture reagent (38, 39) was ligated to the ODYA moiety in a Cu(I)-catalyzed cycloaddition, allowing the isolation and analysis of modified proteins by Streptavidin pull down (PD), Western Blot (WB) (Fig. 3D) and in-gel fluorescence detection (not shown). Anti-tubulin Western Blot confirmed equal concentrations of the PD input lysates and the effective elimination of unmodified proteins and cytosolic content from the isolated PD fractions. The detection of similar amounts of the endogenous, palmitoylated protein Calnexin (Fig. 3D, (49)) in all 17-ODYA treated, but not the unlabeled control sample, validated comparable and specific metabolic labelling and PD of modified proteins. Anti-HA WB revealed weak background binding of HA-LpdA in absence and to similar extent of HA-LpdAΔC5aa in presence of ODYA to the beads. Importantly, compared to HA-LpdAΔC5aa and the controls HA-LpdA was enriched in the isolated PD fraction (Fig. 3D). This showed that LpdA is palmitoylated in the host cell and that the last five residues are critical for this modification.

LpdA localizes to Rab4- and Rab14-containing vesicles

To determine which subcellular membranes LpdA targets, we examined the co-localization of LpdA with a panel of marker proteins for different cellular membranes (GFP-Rab 1, 2, 4, 5, 6, 7, 10, 11, 14 and -LC3) and the mitochondria. The vesicular structures formed by LpdA co-localized with GFP-Rab4 and GFP-Rab14 positive membranes (Fig. 4, Inset). The association was lost when the C-terminal palmitoylation motif of LpdA was removed (Fig. S4). Sporadic association of LpdA with GFP-Rab7 and -Rab5 positive late and early endosomes was also observed (Fig. S5A). Notably,

expression of LpdA did not seem to change the distribution Rab4 or Rab14-positive vesicles or early and late endosomes compared to cells transfected with the LpdA inactive mutant (data not shown), suggesting that LpdA localizes to, but does not compromise, these transport vesicles. No co-localization with the mitochondria or GFP-Rab1, 2, 6, 10, 11 or the autophagosome marker GFP-LC3 was observed (Fig. S5A - C). Noteworthy, Rab1, 2 and 6, which are usually found at the Golgi apparatus, showed in some LpdA-expressing cells a more diffuse or vesicular distribution rather than a compact perinuclear localization (data not shown). Our data shows that vesicular LpdA mainly localizes to GFP-Rab4 and -Rab14-positive vesicles, which are involved in recycling transport processes between the plasma membrane and endosomes and endosomes and the Golgi-apparatus.

LpdA hydrolyzes phosphatidyl-inositol, -inositol-3- and -4-phosphate, and phosphatidyl-glycerol

The plasma membrane, Rab4 and Rab14-positive vesicles, which are targeted by LpdA, are made up of several different lipid species (50). In order to define the substrate specificity of LpdA we performed activity assays with a selection of purified lipids. We first attempted to purify recombinant His_6-tagged LpdA and the inactive mutant LpdA KK165/376RR. However, as the recombinant proteins were insoluble, PLD activity was determined using *E. coli* lysates containing wild type or mutant His_6-LpdA. Bacterial lysates were incubated with phosphatidyl-choline (PC), -serine (PS), -ethanolamine (PE) and -glycerol (PG) for 5 h (Fig. 5A) or phosphatidyl-inositiol and phosphatidyl-inositiol-monophosphates (Fig. 5B) and -polyphosphates (data not shown) for 4 h and the samples analyzed by thin-layer chromatography as described previously (27). Incubation of PG, but not PC (Fig. 5A), PE or PS (data not shown) with lysates containing wild type LpdA revealed PA generation. Furthermore, wild type LpdA efficiently released PA from PI, PI3P and PI4P but not PI5P or polyphosphorylated PIPs (Fig 5B). Importantly, substrate turn over did not occur in incubations with lysates of bacteria containing the empty plasmid or expressing His_6-LpdA KK165/376RR mutant. These results confirm that LpdA has PLD activity, specifically releasing PA from PI, PI3P, PI4P and PG *in vitro*.

LpdA induces disruption of the Golgi-apparatus

PI3P and PI4P are important determinants of the maturation of phagolysosomes, Golgi function and LCV biogenesis (16, 51, 52). As LpdA can hydrolyze these PIPs *in vitro*, we analyzed if LpdA affects the distribution of PIP-binding reporter proteins in the cell. We co-expressed the active lipase or the inactive mutant with the PI3P and PI4P reporters 2xFYVE-GFP (Fig. 6A) or Myc-SidC PIP4 (Fig. 6B), respectively (21). 2xFYVE-GFP displayed strong association with LpdA-containing vesicles (Fig. 6A); however, its distribution was similar in Mock-, LpdA- (Fig. 6A) or LpdA KK165/376RR- (data not shown) transfected cells. The Myc-SidC PIP4 reporter localized to perinuclear structures which were reminiscent of the Golgi and to very small vesicles throughout mock-transfected cells (Fig. 6B). Ectopically-expressed LpdA did not co-localize with the PI4P reporter or induce its loss from membranes; however in LpdA expressing cells the perinuclear Golgi-like SidC PI4P structures seemed to have fragmented into small and medium sized vesicles, showing that LpdA's activity impacts PI4P-containing compartments.

In order to elucidate if the action of LpdA has consequences for the integrity of the Golgi-apparatus, we immunostained LpdA transfected cells for the Golgi marker protein Giantin. This revealed that LpdA did not co-localize with the Golgi-marker (Fig.7A); however in cells expressing the wild type LpdA, but not in cells expressing the catalytically inactive mutant, the normal compact, tight stacks of Golgi-cisternae had disappeared and instead disintegration into separated stacks or even small, vesicle-like fragments was apparent. At the same time, LpdA did not localize to or affect the integrity of the ER, indicating that the fragmentation of the Golgi was not due to action of the effector at the ER which could interfere with early steps of the secretory pathway (Figure S6). Quantification of the Golgi damage revealed that about 90% of the LpdA KK165/376RR- or GFP-expressing cell, but only about 30 % of the LpdA expressing cells retained an intact Golgi (Fig. 7B). Notably, the Golgi disruption induced by the LpdA mutant lacking the C-terminal palmitoylation motif was significantly higher (*p=0.0243, two-tailed, unpaired t-test) than for the wild type enzyme, suggesting that spatial control can restrict the activity of LpdA. IF analysis of infected A549 cells revealed a T4S-dependent,

but LpdA-independent, disruption of the Golgi-apparatus into mini stacks and vesicles as early as 2 h 30 min post infection (Fig. S7), indicating that several effectors are involved in triggering the loss of integrity. Taken together, our data indicate that, although LpdA does not directly associate with Golgi-membranes, its activity can impact on the integrity of the Golgi-apparatus, most likely by perturbing PA levels and the lipid and protein transport by Rab14-positive vesicles between plasma membrane, endosomes and the Golgi.

LpdA contributes to the virulence of *L. pneumophila* in A/J mice

The infection data (Fig. 1 and Fig. S7) suggested that the activity of LpdA in cells might be masked by functionally redundant effectors. In line with this, a *L. pneumophila* LpdA null mutant was previously reported to show no growth defect in the environmental host *Acanthamoeba castellanii* (28). Similarly, we found that the *L. pneumophila* 130b Δ*lpdA* mutant was not attenuated for growth in THP-1 macrophages (Fig. S8). We reasoned therefore that the contribution of LpdA to virulence might only become apparent under the highly selective pressure encountered in the mammalian lung. In order to determine if LpdA has a role in virulence, we infected A/J mice with *L. pneumophila* 130b, Δ*lpdA* or Δ*lpdA* complemented with a plasmid allowing expression of 4HA-tagged LpdA, and examined the bacterial growth in the murine lung after 72 h. The *L. pneumophila* Δ*lpdA* mutant showed reduced bacterial load in murine lungs compared to *L. pneumophila* and *L. pneumophila* Δ*lpdA* 4HA-LpdA in two independent experiments (Fig. 8A & B). This difference between Δ*lpdA* mutant and complemented strain was statistically significant in both experiments (Fig. 8 $p = 0.0041$ and $p = 0.0355$; Mann Whitney U); however due to a higher variation in the number of recovered wild type bacteria, it was only significant between Δ*lpdA* mutant and wild type once (Fig. 8A, $p = 0.0025$, Mann Whitney U). There was no statistically significant difference between the wild type and the complemented strain. In order to test the role of the PLD activity of LpdA for its function in the murine infection model, a *L. pneumophila* Δ*lpdA* strain expressing 4HA-LpdA KK165/376RR inactive mutant was included. This showed that in contrast to expression of wild type 4HA-LpdA, the inactive LpdA mutant did not restore full virulence of the Δ*lpdA* mutant (Fig. 8B, $p = 0.0482$,

Mann Whitney U). Taken together, our data indicates that, although functionally redundant effectors seem to exist, LpdA contributes to optimal survival of *L. pneumophila in vivo* and that its lipase activity is required for fulfilling this function.

Discussion

L. pneumophila employs several effectors, such as SidF, LecE and LegS2, which have enzymatic activities that directly modulate membrane lipid composition and metabolism (23, 28, 53). Moreover, many other effectors share homology with eukaryotic lipases (18). The high abundance of lipid-converting enzymes indicates that subversion of host cell lipids is key for *L. pneumophila* infection; however our knowledge about the biology of most of these effectors remains limited. Here we report that LpdA is a *L. pneumophila* effector that exploits host cell mediated post-translational S-palmitoylation as membrane-targeting mechanism. To our knowledge LpdA is the first palmitolylated *L. pneumophila* effector, although other T4SS effectors have previously shown to exploit post-translational prenylation for membrane targeting (19, 20). Bacterial type III secretion system effectors, such as *Salmonella* SspH2 and SseI or *Pseudomonas* AvrPphB as well as several viruses have been shown to use post-translational palmitoylation for subcellular targeting (54-56), suggesting that its exploitation is an ancient strategy in the co-evolution of pathogens and hosts.

LpdA is a lipolytic effector, which has been proposed to be a PLD modulating phospholipid biosynthesis on the LCV (28). Our study revealed that LpdA confers a moderate advantage for survival and replication in the lungs of A/J mice to wild type *L. pneumophila* and the complemented strain over the Δ*lpdA* mutant. Importantly, our data indicates that this requires the catalytic activity of LpdA. The difference between the Δ*lpdA* and the complemented Δ*lpdA* strains was more consistent across experiments than the one between Δ*lpdA* and wild type *L. pneumophila*, as the latter showed larger variation in bacterial load compared to the complemented mutant. The increased variation could be due to higher heterogeneity of LpdA expression in the wild type population. Notably, hardly any *L. pneumophila* effector null mutants, and not even strains lacking up to one third of the known effectors, are attenuated in growth in most *L. pneumophila* cell infection models (57). A *L. pneumophila* Philadelphia-1 Δ*lpdA* mutant was previously reported to replicate as well as the wild type in amoeba (28). Similarly, we found that the *L. pneumophila* 130b Δ*lpdA* strain was not attenuated for replication in THP-1 macrophages. In the murine lung, the bacteria are confronted with

the diverse antibacterial mechanisms of macrophages and neutrophils, creating a highly selective environment in which the impact of individual effectors, such as LpdA, might become more apparent than in monoculture infections.

To gain a deeper understanding of the function of LpdA on a molecular level, we performed *in vitro* substrate specificity assays. These showed that LpdA has PLD activity towards PI and its derivatives, PI3P and PI4P, which all are important signaling molecules. PI3P is also hydrolyzed by the PLA effector VipD, which leads to loss of the PI3P-binding proteins from the membrane (25). Ectopic co-expression of LpdA with PI3P and PI4P reporter proteins did not cause any loss of the reporters from the membrane, suggesting that LpdA does not hydrolyze these two lipids or that the amounts which are removed are too small to impact on membrane anchoring of the reporter proteins.

In vitro, LpdA also hydrolyzed PG, which is a minor lipid constituent of eukaryotic membranes but can amount to up to 20% of bacterial membranes. Overexpression of LpdA could therefore have adverse effects on the integrity of the bacteria and causes unspecific release of LpdA. However, using the inactive LpdA KK165/376RR mutant, we demonstrated that the translocation of LpdA occurs independently of its activity but requires a functional T4SS. In eukaryotic cells PG is mainly found in mitochondrial membranes. However, neither our experiments nor the work of Viner and colleagues (28) provided any indication that LpdA is targeted to mitochondria or affects their integrity. In infected cells LpdA localizes to the LCV (28). The exact lipid composition of the LCV is unknown. However, as *L. pneumophila* releases outer membrane vesicles (OMVs), which can fuse with the LCV membrane (58), it is likely that the LCV membrane also contains PG of bacterial origin, which could serve as substrate for LpdA. OMVs released by *L. pneumophila* have been shown to interfere with phagosome-lysosome maturation and fusion of endosomes with the LCV (58), which is desirable for the bacteria at early stages of the infection. However, the maturation into the replication permissive LCV relies on fusion of ER-derived vesicles with the LCV, for which the transition to a more ER-like lipid composition could be favorable. LpdA could function in this adjustment process.

Our data show that ectopic-expression of LpdA induces drastic changes to cellular PA levels and has a detrimental effect on the integrity of the Golgi. Ectopic-expression levels of proteins might be substantially higher than during infection and therefore cause unspecific alterations of the cell physiology; however our data show that *L. pneumophila* infection induces drastic redistribution of a PA reporter in host cells as well as a rapid dispersal of the Golgi, a phenomenon which was also reported earlier by Rothmeier et al. (59). Both phenotypes were T4S-dependent, but not affected by deletion of LpdA, suggesting that LpdA has a small or very specific, tightly controlled role and that other redundant effectors mask its effect. A large number of effectors which were shown to target host cell mediators of the secretory pathway (60) could contribute to the Golgi disruption. Additional lipases encoded in *Legionella* genomes and effectors such as LecE, which activates host cell PA phosphatase, leading to the conversion of PA into diacylglycerol (28), could contribute to generation and turnover of PA, explaining why no accumulation of our PA reporter on any specific cellular membrane or the LCV was detected. In light of the involvement of redundant effectors during infection, only ectopic expression could be used to unravel the contribution of LpdA to these phenotypes in infection.

Using ectopic expression the data show that the lipase activity of LpdA is required for PA generation and the disruption of the Golgi. PA is an important signaling molecule and was implicated in the regulation of membrane tubulation and vesicle formation at recycling endosomes and the Golgi (61-63). In particular the structure and function of the Golgi was shown to be sensitive to disturbance of the cellular PA homeostasis. The inhibition of phosphatidic acid synthesis was previously shown to disrupt the Golgi structure (64). Robust activation of human PLD, which generates PA and regulates membrane trafficking at various cellular sites including the Golgi, is required for induction of Golgi fragmentation by the drug ilimaquinone (65, 66).

LpdA triggers Golgi disruption without localizing to the organelle which suggests that changes in lipid composition and PA levels in distant membranes sites adversely affect membrane transport processes to and/or the lipid composition of the Golgi, which result its breakdown. The main PA

466 generation sites are the plasma membrane and Rab4 and 14 containing vesicles, to which LpdA
467 localizes; however, the observation that LpdA lacking the palmitoylation motif, which does not
468 specifically localize to these sites anymore, induces even more pronounced Golgi disruption, leads to
469 the hypothesis that LpdA-mediated perturbation of the cellular PA homeostasis rather than the
470 modulation of a specific vesicular trafficking pathway can cause this effect.

471 Taken together, here we established that the effector LpdA is a PLD which can hydrolyze several
472 lipid substrates to modulate cellular PA levels. It contributes to virulence of *L. pneumophila* in the
473 murine lung and discovered that *Legionella* exploits host cell mediated post-translational S-
474 palmitoylation to exert spatial control over LpdA's activity in the cell. Moreover, our work revealed
475 that *L. pneumophila* infection induces rapid disruption of the Golgi-apparatus as well as dramatic
476 changes in host cell PA levels, with unknown benefits for the infection and involvement of
477 unidentified effectors besides LpdA, warranting future research in the interdependence of these
478 phenotypes and the role of phospholipase effectors in *L. pneumophila* pathogenesis.

Acknowledgments

We thank Professor Miguel Seabra (Imperial College (IC), UK) for the Rab GTPase expression plasmids, Dr. Nicolas Vitale (Institute of Cellular and Integrative Neurosciences, University of Strasbourg, France) for the GFP-SPO reporter plasmids, Dr. Xavier Charpentier for the pXDC31 plasmid (Université Lyon 1, France), Dr. Xin Li (IC, UK) for the PLCδ1 template DNA, Dr. Avinash Shenoy (IC, UK) for providing reagents for the creation of stable cell lines and Julian Rayner and Matthew Jones (Wellcome Trust Sanger Institute Cambridge, UK) for initial advice on the analysis of palmitoylation.

Funding

This work was supported by grants from the Medical Research Council UK, the German Research Foundation (DFG) grants DFG FL 359/6-1, 6-2 and grants to ELH from the Australian National Health and Medical Research Council (APP606788).

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Table 1. Bacterial strains used in the study

Strain	Serogroup/ genotype	Reference
Legionella pneumophila		
130b (AA100, ATCC BAA-74)	O1; clinical isolate	(30, 31)
130b Δ*dotA*	*dotA* gene disrupted with a Kan^r cassette	(32)
130b Δ*lpdA (ICC1080)*	*lpdA (LpdA/lpw_19211)* gene disrupted with a Kan^r cassette	This study
E. coli		
BL21(DE3) Star		Novagen
Top10		Invitrogen

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Table 2. Plasmids and primers used and created in this study.

Plasmid	**Description**			**Reference**
pRK5	**Vector for the expression of proteins with N-terminal Myc tag in mammalian cells**			**Clontech**
Derivatives	*Expressed protein*	*Primers 5' -> 3'*	*RS*	
pICC1565	Myc-LpdA	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAAGAACAAACACAACAGGTTC	BamHI EcoRI	This study
pICC1566	Myc-LpdAΔC5aa	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAGGTTCTTTTAGGCGATATC	BamHI EcoRI	This study
pICC1585	Myc-SidC PI4P	GTCATAGGATCCAAATATTCCTCCAAGCCATTATTGG GATACCCTGCAGCTATTTCTTTATAACTCCCGTGTAC	BamHI PstI	This study
pICC1341	**pRK5-HA, Vector for the expression of proteins with N-terminal HA tag in mammalian cells**			(21)
Derivatives	*Expressed protein*	*Primers 5' -> 3'*	*RS*	
pICC1567	HA-LpdA	as for pICC1565		This study
pICC1568	HA-LpdAΔC5aa	as for pICC1566		This study
pICC1569	HA-LpdA C440F	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAAGAACAAACAAAACAGGTTC	BamHI EcoRI	This study
pEGFP-N1	**pEGFP-N1, Vector for the expression of proteins with C-terminal GFP tag in mammalian cells**			**Clontech**
pICC1586	hs PLCδ1-PH-GFP	ATGCGCTCGAGATGGACTCGGGCCGGG AGCATGGATCCTCGATGTTGAGCTCCTTCAGGAAG	XhoI BamHI	This study
pICC562	**pMMB207c-4HA; Vector for the expression of proteins with four N-terminal HA-tags in *L. pneumophila***			(67)
Derivatives	*Expressed protein*	*Primers 5' -> 3'*	*RS*	
pICC1570	4HA-LpdA	TCGCACTGAGGTACCATGCTCCAATTTTTTGAGAAAGGT TCGCATCTAGATCAAGAACAAACACAACAGGTTC	KpnI XbaI	This study
pXDC61	**pXDC61; Vector for the expression of proteins with N-terminal β-lactamase TEM1-tag in *L. pneumophila***			(68)
Derivatives	*Expressed protein*	*Primers 5' -> 3'*	*RS*	
pICC1571	TEM1-LpdA	as for pICC1570		This study
pXDC31	**pXDC31; Vector for the inducible expression of GFP in *L. pneumophila***			(40)
pET28a(+)	**Vector for the expression of a 6 x His tag fusion protein in *E. coli***			**Novagen**
Derivatives	*Expressed protein*	*Primers 5' -> 3'*	*RS*	
pICC1572	His-LpdA	CGCACTGAGGATCCATGCTCCAATTTTTTGAGAAAGGT GCGTCGCAGAATTCTCAAGAACAAACACAACAGGTTC	BamHI EcoRI	This study
pICC1573	LpdA-His	CATGACCATGGGACTCCAATTTTTTGAGAAAGGT TCATGCTCGAGAGAACAAACACAACAGGTTC	NcoI XhoI	This study
Plasmids created by site-directed mutagenesis				
	Mutation	*Primers 5' -> 3'*		
	K165R	ATAATTCGATTGCGTCCAACCATAATAGAGCCATCTGGATTG CAATCCAGATGGCTCTATTATGGTTGGACGCAATCGAATTAT		This study
	K376R	GAAAAAAGAGGTTATTCATATGAGATTTATGTCAATTGATGGGCAGG CCTGCCCATCAATTGACATAAATCTCATATGAATAACCTCTTTTTTC		This study

Mutations were sequentially introduced into pICC1567, which was used for subcloning with the primers indicated above. (All this study).

Plasmid	Description	Plasmid	Description	Plasmid	Description
pICC1574	pRK5-HA-LpdA K165R	pICC1577	pRK5-HA-LpdA K376R	pICC1580	pRK5-HA-LpdA KK165/376RR
pICC1575	pRK5-Myc-LpdA K165R	pICC1578	pRK5-Myc-LpdA K376R	pICC1581	pRK5-Myc-LpdA KK165/376RR

pICC1576	pMMB207c-4HA-LpdA KK165/376RR	pICC1579	pXDC61 LpdA KK165/376RR	pICC1582	pET28a(+)-LpdA KK165/376RR
Plasmids for the transduction of eukaryotic cells					
pMXs-IRES-Puro	**pMXs-IP; vector including viral packaging signal, transcription and processing elements as well as a insertion site for a gene of interest allowing incorporation in retroviruses**				**Clontech**
Derivatives	*Expressed protein*	*Primers 5' -> 3'*			
pICC1583	GFPSPO reporter	CTAGGGATCCGCCACCATGGTGAGCAAGG GCATGAATTCTTAACTAGTCTTAGTGGCGTCATC			This study
Plasmid for the expression of ER-targeted mCherry					
pICC1584	pmCherryER	mCherry protein includes KDEL C-terminal ER targeting and N-terminal Calreticulin ER-retention signal			This study
	Calreticulin retention signal	GAGCAGCAGCGGCACGGATAGCAGCATGGTGGCGACCGGTAG GGCCTTCTTGGCCTAGCAGTAGCAGTGAGCAAGGGCGAGGAG			
	KDEL-ER targeting signal	GAGCTGTAACTGATCATAATCAGCCATACC ATCCTTTCTAGATCCGGTGGATCCC			
Primers for the construction of the Δ*lpdA* strain ICC1080					
Amplification of the 5' flanking region		CATAATGACAGGTAAGGCTTC CAGTGGGTACCGATTCACTCATAAATTAATTTTACCTAAT			This study
Amplification of the 3' flanking region		GCGACTCTAGATTTTAGGCTGTTTTGATCAAGCC ATCTCAATTACAGGAAATGCAAAC			This study
Primers for sequencing of the *lpdA* genomic region		CTTGCCATGTTCTGCTATATATTTC GTGATATTGTATGCACTTCCATC			This study

RS = restriction site

677

678 **Figure legends**

679 **Figure 1. *L. pneumophila* infection causes drastic changes to the cellular PA distribution.** A549
680 cells stably expressing the PA reporter GFP-SPO were left uninfected or infected for 6 h with *L.*
681 *pneumophila* 130b wild type (WT), Δ*dotA* or Δ*lpdA* carrying the empty p4HA or p4HA LpdA
682 plasmids and immunostained using an anti-*L. pneumophila* antibody (Red). DNA was visualized with
683 Hoechst DNA dye (blue). (A) Samples were examined by IF microscopy. Images are representative
684 of at least three independent experiments. Scale bar 5 μm. (B) Quantification of the effect of infection
685 on the cellular distribution of the GFP-SPO PA reporter by IF counting. Error bars represent the mean
686 standard deviation of three independent experiments.

687 **Figure 2. Ectopically-expressed LpdA localizes to the plasma membrane and small vesicles**
688 **where it generates PA.** Immunofluorescence images of HeLa cells transfected with pRK5 HA, pRK5
689 HA-LpdA WT or LpdA KK165/376RR (KKRR) mutant alone (A) or together with the GFP-SPO PA
690 reporter (B). Results are representative of at least three independent experiments. Scale bar 10 μm.

691 **Figure 3. Post-translational palmitoylation targets LpdA to membranes.** (A) The putative C-
692 terminal palmitoylation site of LpdA; the three cysteines predicted to be modified with a palmitic
693 acid moiety are underlined. (B) HA-LpdA, HA-LpdA C440F and HA-LpdAΔC5aa were ectopically
694 expressed in HeLa cells, immunostained and their localization analyzed by IF microscopy. Shown
695 are representative images of at least three independent experiments. Scale bar 10 μm. (C) Metabolic
696 labelling and protein isolation strategy. HeLa expressing LpdA were metabolically-labeled with 25
697 μM of the palmitic acid analogue 17-octadecynoic acid (ODYA). Following cell lysis a Biotin-moiety
698 and a fluorophore were ligated using click-chemistry. Post-translationally modified proteins were
699 isolated using Streptavidin pull down (PD) and analyzed by Western Blotting (WB). (D)
700 Representative result of the WB analysis of PD and Input fractions using HA-, Calnexin (endogenous
701 metabolic labelling control) and Tubulin (separation control) antibodies.

Figure 4. Vesicular LpdA is mainly localized to Rab14-containing vesicles. HeLa cells were co-transfected with pRK5 HA or pRK5 HA-LpdA and plasmids for the expression of marker proteins of different cellular compartments and processed for IF microscopy. Rab14 and LpdA positive vesicles are highlighted with arrows in the magnified inset. Similar results were observed in at least two independent experiments. Scale bar 10 μm.

Figure 5. LpdA catalyzes the conversion of PG, PI, PI3P and PI4P to PA. Lysates of *E. coli* with or without expression of His_6 tagged LpdA or LpdA KK165/376RR (KKRR) or a buffer control were incubated with (A) 0.2 mM PC or PG for 5 h at 37 °C or (B) 0.25 mM PI_{C16} or mono-phosphorylated PI_{C16} for 4h at 37 °C. Lipids were extracted and analyzed by TLC. Corresponding pure PC, PG, PI_{C16} and PA standards were included. Similar results were obtained in at least two independent experiments.

Figure 6. LpdA induces rearrangement of PI4P-containing compartments. HeLa cells were co-transfected with pRK5 HA or pRK5 HA-LpdA and a plasmid expressing (A) the PI3P-binding protein FYVE fused to GFP or (B) the Myc-tagged PI4P binding domain of SidC, stained and analyzed IF microscopy. (A). Vesicles in which LpdA and the PI3P marker co-localize are highlighted with arrows in the magnified inset. Similar results were observed in at least three independent experiments. Scale bar 5 μm.

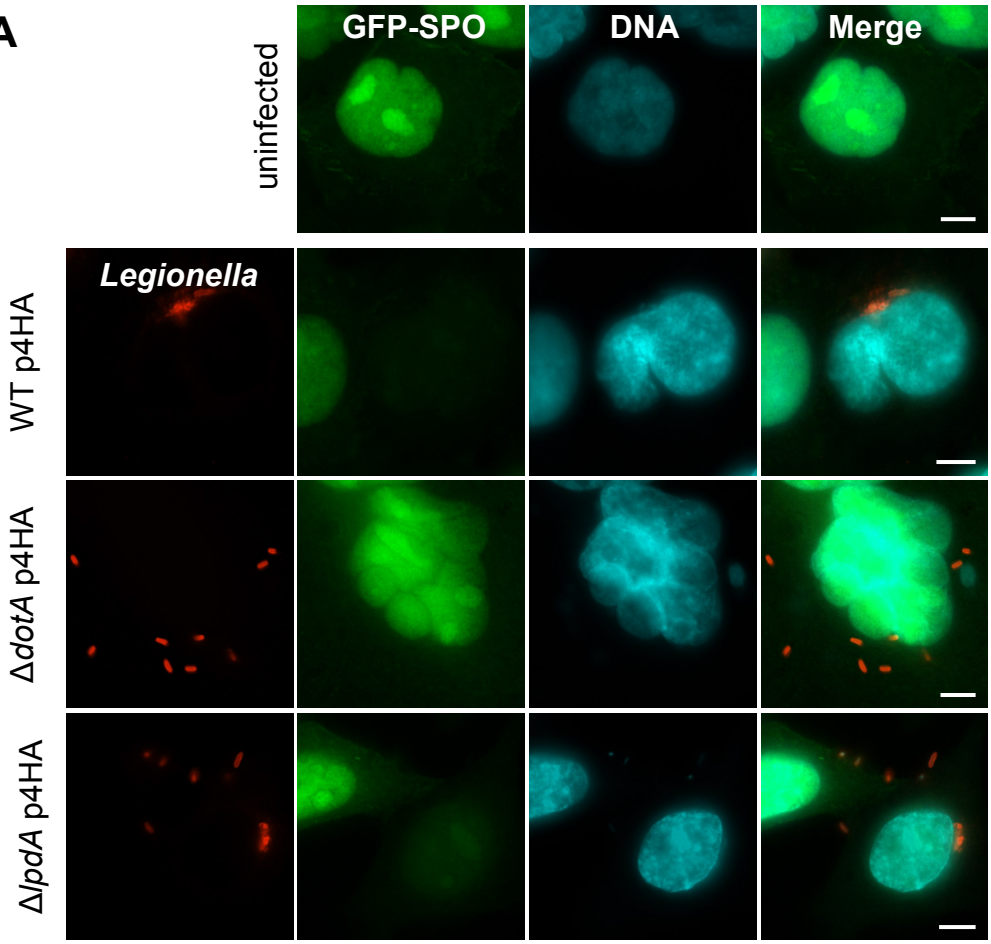
Figure 7. LpdA activity in the cell disrupts the Golgi. HeLa cells were transfected with pRK5 HA-LpdA WT, -LpdA KK165/376RR (KKRR) mutant, LpdAΔ5aa or -GFP and immunostained for HA and the Golgi-protein Giantin. Samples were imaged (A) and integrity of the Golgi apparatus was assessed in 100 transfected cells (B). (A) Arrows highlight Giantin-positive fragments which do not co-localize with LpdA. Images are representative of at least three independent experiments. Scale bar 10 μm. (B) Error bars represent the mean standard deviation of three independent experiments. Significance was analyzed using the two-tailed, unpaired t-test.

Figure 8. LpdA contributes to the fitness of *L. pneumophila* 130b during pulmonary infections of A/J mice. Results of two independent experiments (A & B). A/J mice were infected with *L.*

pneumophila wild type (WT) and Δ*lpdA* mutant strains by intranasal inoculation. At 72 h post infection the animals were sacrificed and the CFU in the lungs determined by plating. (A) Both wild type and complemented Δ*lpdA* strain grow significantly (p= 0.0025 & p= 0.0041, Mann Whitney U) better than mutant in the lungs of mice. (B) The Δ*lpdA* strain and the Δ*lpdA* strain expressing the inactive LpdA KK165/376RR (KKRR) mutant are significantly (p = 0.0355; p = 0.0482; Mann Whitney U test) attenuated compared to the complemented *L. pneumophila* Δ*lpdA* p4HA LpdA strain.

Figure 1

A


GFP-SPO
DNA
Merge
uninfected
Legionella
WT p4HA
ΔdotA p4HA
ΔlpdA p4HA

B

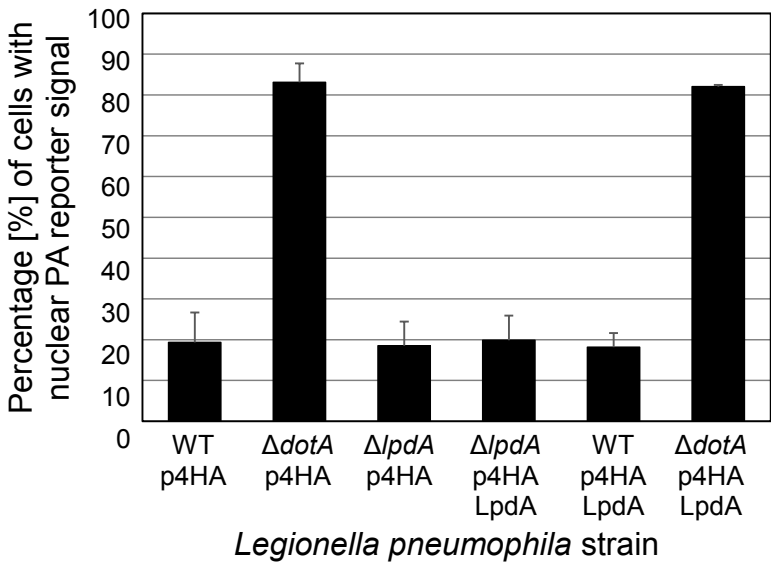

Percentage [%] of cells with nuclear PA reporter signal
100
90
80
70
60
50
40
30
20
10
0
WT p4HA
ΔdotA p4HA
ΔlpdA p4HA
ΔlpdA p4HA LpdA
WT p4HA LpdA
ΔdotA p4HA LpdA
Legionella pneumophila strain

Figure 2

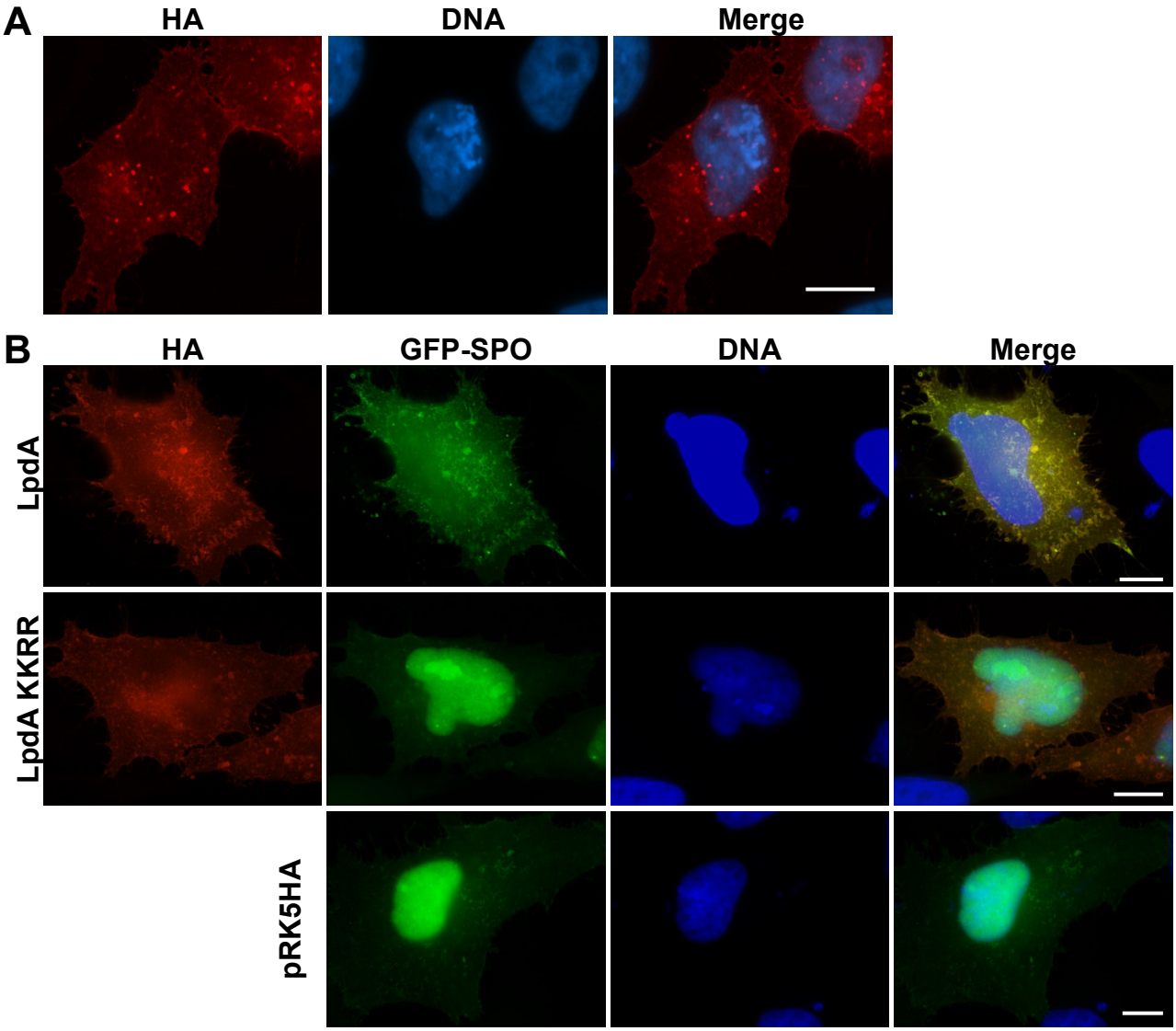
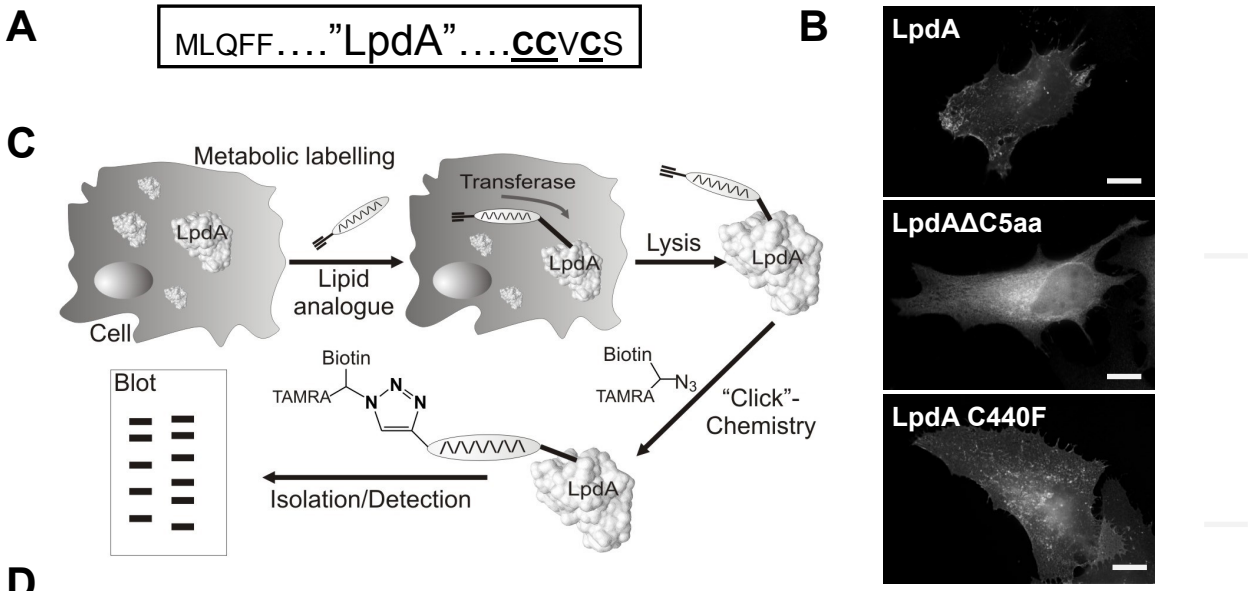


Figure 3


A
MLQFF….”LpdA”….ccVCS
B
LpdA
LpdAΔC5aa
LpdA C440F
C
Metabolic labelling
Transferase
Lysis
LpdA
Cell
Lipid analogue
Blot
Biotin
TAMRA
Biotin
TAMRA
N3
“Click”-Chemistry
Isolation/Detection

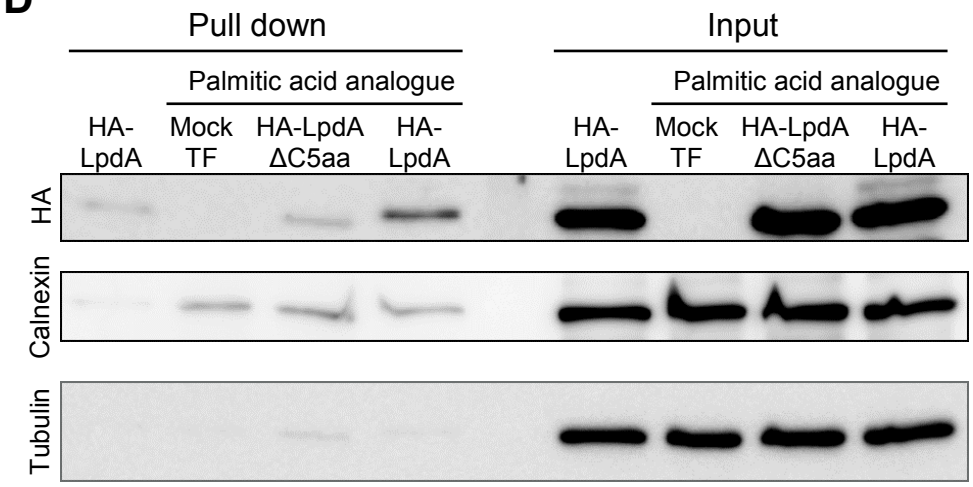

D
Pull down
Input
Palmitic acid analogue
Palmitic acid analogue
HA-LpdA
Mock TF
HA-LpdA ΔC5aa
HA-LpdA
HA-LpdA
Mock TF
HA-LpdA ΔC5aa
HA-LpdA
HA
Calnexin
Tubulin

Figure 4

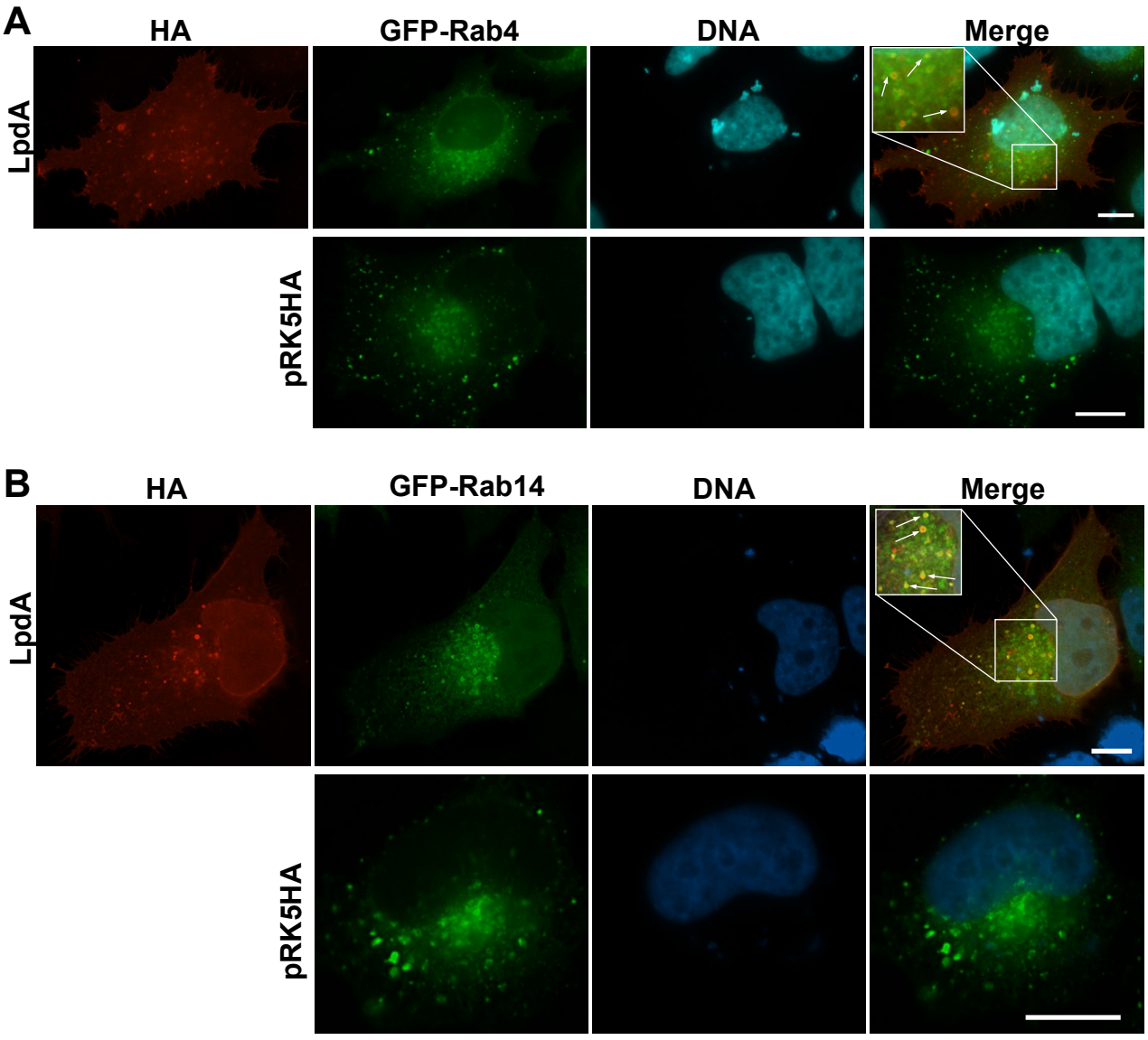
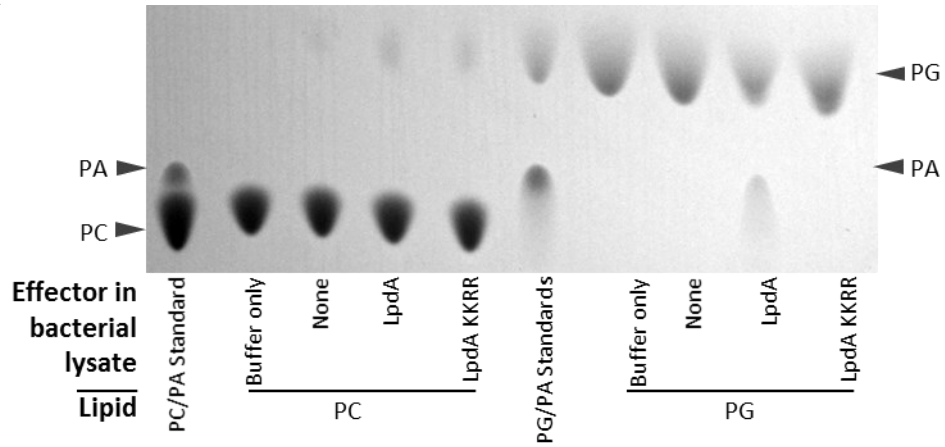

A
HA
GFP-Rab4
DNA
Merge
LpdA
pRK5HA
B
HA
GFP-Rab14
DNA
Merge
LpdA
pRK5HA

Figure 5

A


PG
PA
PA
PC
Effector in bacterial lysate
Lipid
PC/PA Standard
Buffer only
None
LpdA
LpdA KKRR
PG/PA Standards
Buffer only
None
LpdA
LpdA KKRR
PC
PG

B

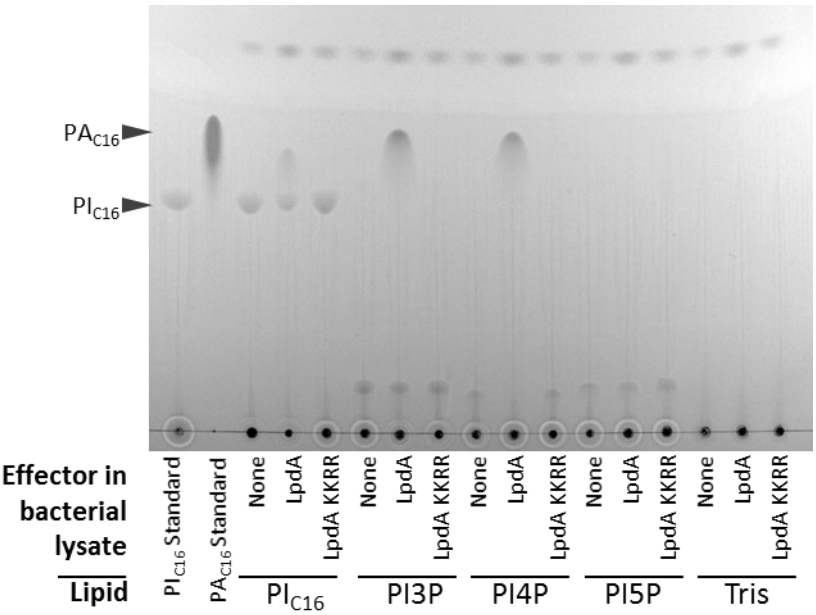

PA_{C16}
PI_{C16}
Effector in bacterial lysate
Lipid
PI_{C16} Standard
PA_{C16} Standard
None
LpdA
LpdA KKRR
None
LpdA
LpdA KKRR
None
LpdA
LpdA KKRR
None
LpdA
LpdA KKRR
None
LpdA
LpdA KKRR
PI_{C16}
PI3P
PI4P
PI5P
Tris

Figure 6

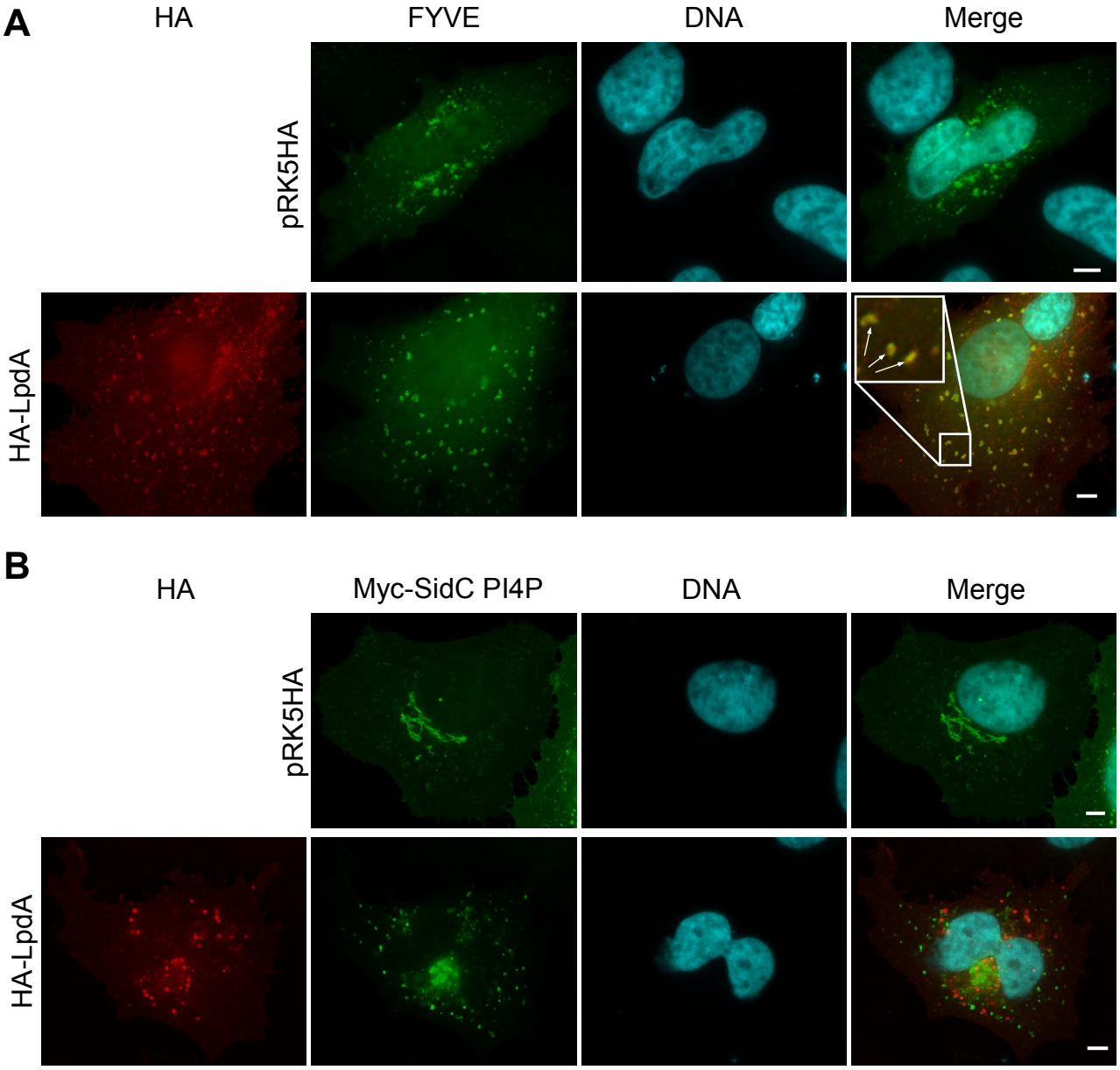

A
HA
FYVE
DNA
Merge
pRK5HA
HA-LpdA
B
HA
Myc-SidC PI4P
DNA
Merge
pRK5HA
HA-LpdA

Figure 7

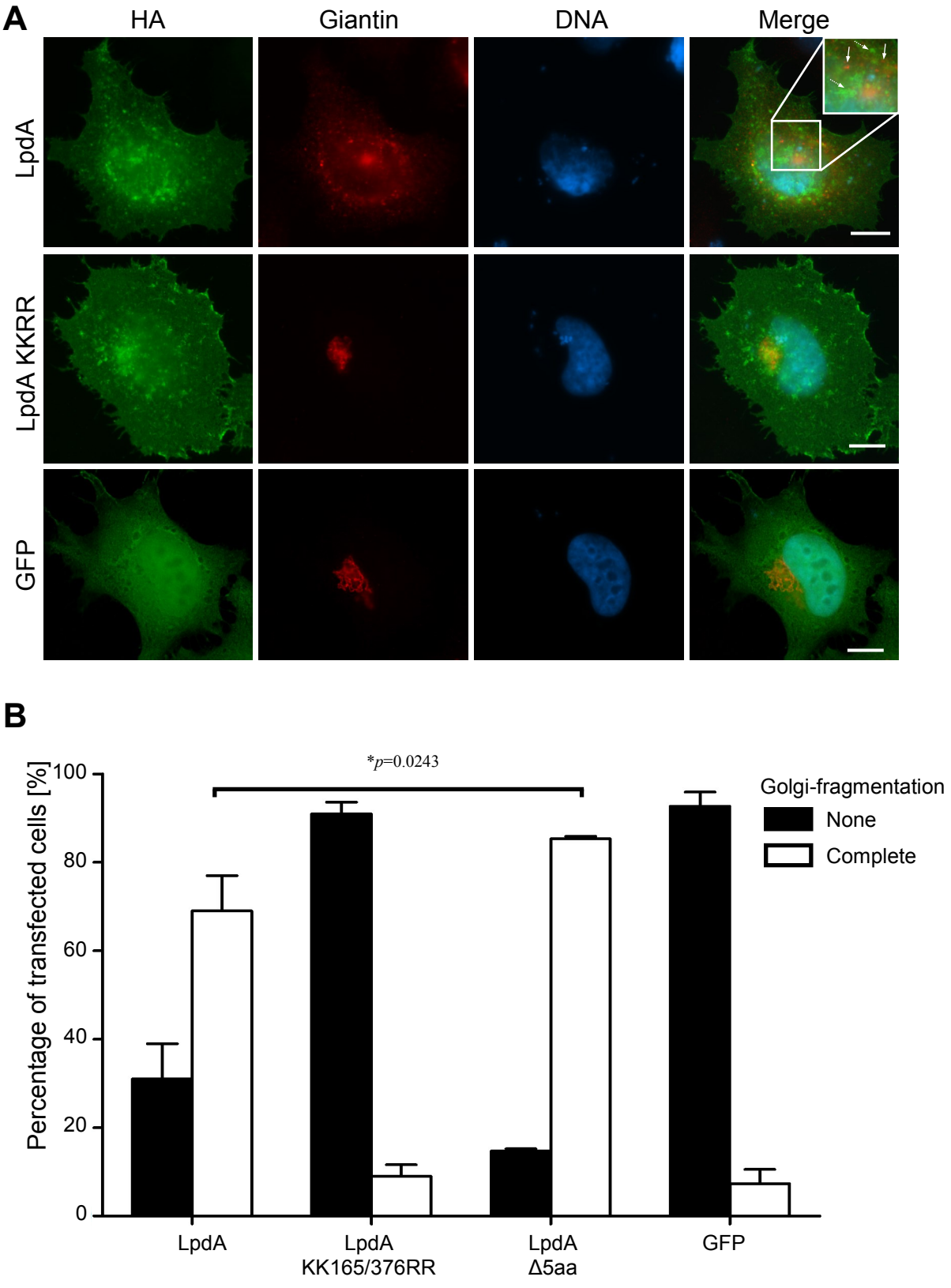
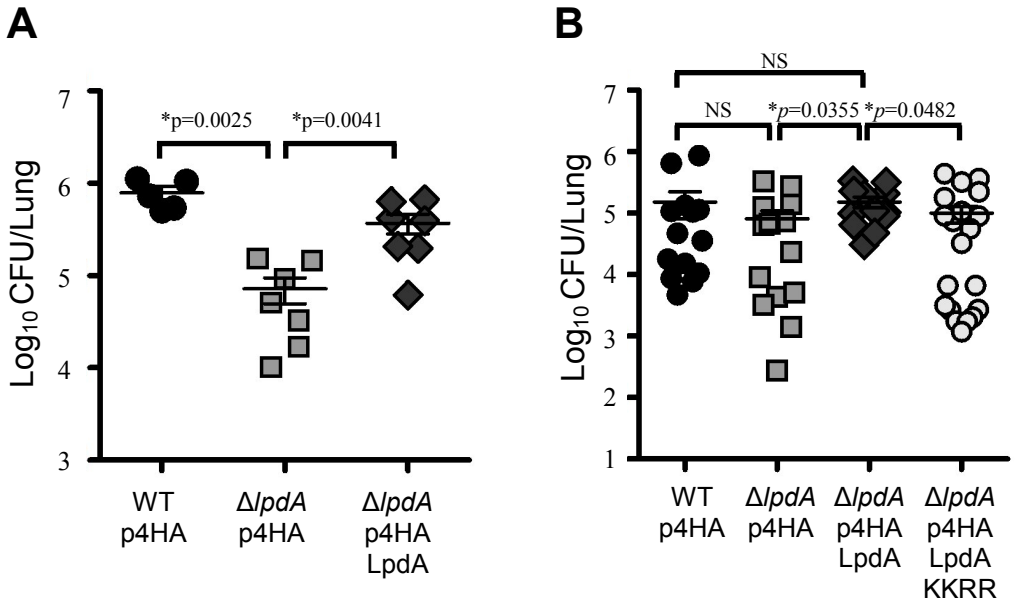

A
HA
Giantin
DNA
Merge
LpdA
LpdA KKRR
GFP
B
*p=0.0243
Golgi-fragmentation
None
Complete
Percentage of transfected cells [%]
100
80
60
40
20
0
LpdA
LpdA KK165/376RR
LpdA Δ5aa
GFP

Figure 8


A
B
Log10 CFU/Lung
*p=0.0025
*p=0.0041
NS
NS
*p=0.0355
*p=0.0482
WT p4HA
ΔlpdA p4HA
ΔlpdA p4HA LpdA
ΔlpdA p4HA LpdA KKRR