The inositol polyphosphate 5-phosphatase OCRL1 restricts intracellular growth of *Legionella*, localizes to the replicative vacuole and binds to the bacterial effector LpnE

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**Summary**

*Legionella pneumophila*, the causative agent of Legionnaires’ disease, replicates within a specific vacuole in amoebae and macrophages. To form these *Legionella*-containing vacuoles (LCVs), the bacteria employ the Icm/Dot type IV secretion system and effector proteins, some of which anchor to the LCV membrane via the host glycolipid phosphatidylinositol 4-phosphate [PtdIns(4)P]. Here we analysed the role of inositol polyphosphate 5-phosphatases (IP5Ps) during *L. pneumophila* infections. Bacterial replication and LCV formation occurred more efficiently in Dictyostelium discoideum amoebae lacking the IP5P Dd5P4, a homologue of human OCRL1 (Oculocerebrorenal syndrome of Lowe), implicated in retrograde endosome to Golgi trafficking. The phenotype was complemented by Dd5P4 but not the catalytically inactive 5-phosphatase. Ectopically expressed Dd5P4 or OCRL1 localized to LCVs in *D. discoideum* via an N-terminal domain previously not implicated in membrane targeting, and OCRL1 was also identified on LCVs in macrophages. Dd5P4 was catalytically active on LCVs and accumulated on LCVs harbouring wild-type but not *ΔicmT* mutant *L. pneumophila*. The N-terminal domain of OCRL1 bound *L. pneumophila* LpnE, a Sel1-like repeat protein involved in LCV formation, which localizes to LCVs and selectively binds PtdIns(3)P. Our results indicate that OCRL1 restricts intracellular growth of *L. pneumophila* and binds to LCVs in association with LpnE.

**Introduction**

*Legionella pneumophila* is an environmental bacterium that parasitizes various species of predatory amoebae in water and soil, including the social amoeba *Dictyostelium discoideum* (Fields et al., 2002; Steinert and Heuner, 2005). The bacteria are accidentally transmitted to the human lung via contaminated aerosols and thus may cause the severe pneumonia Legionnaires’ disease, or a milder illness termed Pontiac fever. *L. pneumophila* replicates in alveolar macrophages (Nash et al., 1984), using a mechanism likely adopted through its longstanding evolutionary interaction with amoebae (Greub and Raoult, 2004; Molmeret et al., 2005; Hilbi et al., 2007). To transfer between host cells, *L. pneumophila* switches from a replicative to a transmissive phase, which involves a complex regulatory network including an apparent quorum sensing system (Spirig et al., 2008; Tiaden et al., 2007, 2008).

Within macrophages and amoebae, *L. pneumophila* forms a replicative compartment, the *Legionella*-containing vacuole (LCV). LCVs avoid fusion with lysosomes (Horwitz, 1983), instead intercept early secretory trafficking at endoplasmic reticulum (ER) exit sites (Kagan and Roy, 2002) and likely also communicate with other trafficking routes (Dorer et al., 2006). In the course of their maturation, LCVs accumulate the v-SNARE Sec22b, as well as the small GTPases Arf1 and Rab1, the activity of which is required to form the replicative niche (Kagan and Roy, 2002; Derre and Isberg, 2004; Kagan et al., 2004).

At later stages, LCVs fuse with the ER and acquire the ER resident protein calnexin (Swanson and Isberg, 1995; Tilney et al., 2001; Robinson and Roy, 2006), and calnexin-positive LCVs undergo a transition from ‘tight’ to ‘spacious’ vacuoles (Lu and Clarke, 2005). Uptake of *L. pneumophila* and the formation of LCVs in macrophages and amoebae requires a type IV secretion system (T4SS), the Icm/Dot apparatus (Segal et al., 1998; Vogel et al., 1998; Segal and Shuman, 1999; Solomon et al., 2000; Hilbi et al., 2001; Watarai et al., 2001; Otto et al., 2004). More than 70 Icm/Dot substrates (*effector* proteins) have been identified, some of which interfere with host cell processes such as apoptosis, GTP cycles,
IP5Ps (Dd5P1–4), namely Dd5P4/OCRL1. Defects in OCRL1 cause Oculocerebrorenal syndrome of Lowe (Attree et al., 1992), a rare X-linked disorder characterized by mental retardation, congenital cataracts and renal failure.

OCRL1 and INPP5B are 45% identical and share a catalytically active inositol polyphosphate 5-phosphatase domain and an inactive Rho GAP domain. This domain architecture is conserved in one out of four distinct D. discoideum IP5Ps (Dd5P1–4), namely Dd5P4 (Loovers et al., 2003). While the deletion of either Dd5P1, -2 or -3 did not produce obvious phenotypes, the ablation of D. discoideum Dd5P4 caused developmental defects, lower growth rates in axenic medium as well as on bacterial lawns and impaired phagocytosis. OCRL1 is a close mammalian orthologue of Dd5P4 sharing 33% overall amino acid identity with the amoeba enzyme (Fig. 1). Upon expression of OCRL1 in D. discoideum lacking Dd5P4, phagocytosis, growth and developmental defects were partially restored, and thus, OCRL1 can functionally replace Dd5P4 (Loovers et al., 2007).

Here we analyse the role of IP5Ps during phagocytosis and intracellular replication of L. pneumophila. We find that Dd5P4/OCRL1 attenuates intracellular replication of L. pneumophila, localizes to LCVs via its N-terminus and binds to the L. pneumophila PtdIns(3)P-binding effector protein LpnE.

**Results**

**Dd5P4 attenuates intracellular replication of wild-type L. pneumophila**

IP5Ps play a role in membrane dynamics and vesicle trafficking in mammals as well as in D. discoideum (Lowe, 2005; Loovers et al., 2007). To assess whether IP5Ps are involved in the uptake of L. pneumophila and formation of LCVs, we used D. discoideum mutant strains lacking individual IP5Ps (ΔDd5P1, -2, -3 or -4). Compared with wild-type D. discoideum, the uptake of wild-type L. pneumophila was reduced in ΔDd5P1 or ΔDd5P4 by approximately 40% and not affected in ΔDd5P2 or ΔDd5P3 (Fig. 2A). Phagocytosis of L. pneumophila lacking a functional Icm/Dot T4SS (ΔicmT) was not affected by the absence of any of the four IP5Ps. Moreover, as described previously for wild-type D. discoideum
(Weber et al., 2006), amoebae lacking the IP5Ps phagocytosed approximately 10 times more wild-type *L. pneumophila* than ΔicmT mutant bacteria.

Interestingly, approximately 100- to 1000-fold more wild-type *L. pneumophila* were released from ΔDd5P4 compared with wild-type *D. discoideum* or compared with mutant amoebae lacking the other IP5Ps (Fig. 2B), indicating that *L. pneumophila* replicates much more efficiently in absence of Dd5P4. To quantify intracellular *L. pneumophila*, infected amoebae were lysed with saponin (Fig. 2C). Under these conditions, intracellular replication of *L. pneumophila* was found to initiate earlier and to proceed at a higher rate in ΔDd5P4 compared with wild-type *D. discoideum*. A more efficient intracellular replication in absence of Dd5P4 was also observed by flow cytometry using GFP-labelled *L. pneumophila* (Fig. 2D). The degradation of *L. pneumophila* ΔicmT by amoebae was not affected in the absence of Dd5P4 (Fig. 2D) or any other IP5P (data not shown).

In order to analyse complementation of the intracellular growth phenotype, GFP fusion constructs of Dd5P4 and fragments thereof were constitutively expressed in *D. discoideum* ΔDd5P4 under the control of the act15 promoter. The production of full-length Dd5P4–GFP reduced intracellular growth of wild-type *L. pneumophila* in Dd5P4-deficient host cells almost to the level observed in wild-type *D. discoideum* (Fig. 2E). In contrast, upon production of the catalytically inactive mutant protein Dd5P41–787ΔGFP, *L. pneumophila* grew as efficiently as in ΔDd5P4. The production of Dd5P41–465ΔGFP containing the catalytic 5-phosphatase domain in ΔDd5P4 suppressed the growth of wild-type *L. pneumophila* at early time points, while the production of Dd5P4466–787–GFP in Dd5P4-deficient host cells did not affect intracellular growth of the bacteria (Fig. 2F). These results indicate that catalytic activity of Dd5P4 is required to attenuate intracellular replication of *L. pneumophila*. The ectopic expression of OCRL1 in ΔDd5P4 did not result in reduced intracellular replication (data not shown). This lack of complementation might be due to less efficient production of the mammalian protein in *D. discoideum* amoebae. Flow cytometry analysis revealed that Dd5P4–GFP was produced by all amoebae (>99%) and at higher levels compared with OCRL1–GFP, which was produced by only a fraction of the amoebae (approximately 40%) and at lower levels (Fig. S1).

**Dd5P4 attenuates the maturation and dynamics of LCVs**

The maturation of LCVs involves the acquisition of the ER-resident protein calnexin (Swanson and Isberg, 1995; Tilney et al., 2001; Robinson and Roy, 2006) and a transition of calnexin-positive LCVs from ‘tight’ to ‘spacious’ vacuoles (Lu and Clarke, 2005). In wild-type *D. discoideum*, approximately 70% LCVs turned calnexin-positive within 1 h post infection (Fig. 3A). Throughout this time period, LCVs recruited calnexin–GFP more efficiently in absence of Dd5P4, indicating that Dd5P4 attenuates the interaction and fusion of ER with LCVs. Moreover, within 4.5 h approximately 25%–40% LCVs underwent a transition from tight to spacious vacuoles in wild-type *D. discoideum*, regardless of whether Dd5P4–GFP or a catalytically inactive mutant protein was ectopically expressed or not (Fig. 3B). In contrast, the LCV transition was almost completely blocked in strain ΔDd5P4, as in absence of Dd5P4 less than 10% spacious LCVs were observed 4.5 h post infection. The transition from tight to spacious LCVs was complemented by ectopically expressed OCRL1–GFP (Erdmann et al., 2007).

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**Fig. 1.** Domain architecture of the *D. discoideum* inositol polyphosphate 5-phosphatase Dd5P4 and the mammalian orthologue OCRL1. Highlighted are the domains (i) LVA (*Legionella* vacuole association), (ii) IP5P (inositol polyphosphate 5-phosphatase) including the catalytically essential aspartic acid residues (arrows), (iii) ASH (ASPM-SPD2-Hydin), and (iv) Rho GAP (GTPase activating protein) including the catalytically essential arginine residue (arrow), which is replaced by isoleucine or glutamine in Dd5P4 and OCRL1 respectively. The percentage identity of the full-length proteins and the individual domains are indicated. OCRL1 binds to vacuoles containing icm/Dot-proficient *L. pneumophila* (LVA domain), to α-adaptin of the AP2 complex (FEDNF motif) (Ungewickell et al., 2004), to clathrin (LIDLE motif, amino acids 539–600) (Choudhury et al., 2005; Erdmann et al., 2007), to the GTPases Rab1, -5, -6 (amino acids 540–726) (Hyvola et al., 2006), Arf1 and Arf6 (Rho GAP domain) (Lichter-Konecki et al., 2006), as well as to the Rab5 effector APPL1 (ASH, Rho GAP) (Erdmann et al., 2007).
Fig. 2. Dd5P4 attenuates intracellular replication of wild-type *L. pneumophila.*
A. Phagocytosis of GFP-labelled wild-type (black bars) or ΔicmT mutant *L. pneumophila* (grey bars) by wild-type *D. discoideum* or ΔDd5P1–4 was determined by flow cytometry.
B. Release of wild-type *L. pneumophila* into the supernatant of infected wild-type *D. discoideum* ( ), ΔDd5P1 ( ), ΔDd5P2 (△), ΔDd5P3 ( ) or ΔDd5P4 ( □) was quantified by colony forming units (cfu).
C. Single round infections of wild-type *L. pneumophila* in wild-type *D. discoideum* ( ) or ΔDd5P4 ( □) were assessed by lysing the infected cells with 0.8% saponin and determining bacterial cfu.
D. Quantification by flow cytometry of intracellular growth of GFP-labelled wild-type *L. pneumophila* or degradation of GFP-labelled ΔicmT within wild-type *D. discoideum* or ΔDd5P4.
E. Release of wild-type *L. pneumophila* into the supernatant of infected wild-type *D. discoideum* ( ), ΔDd5P4 ( □), ΔDd5P4 producing Dd5P41-787-GFP ( ), or ΔDd5P4 producing Δd5P41-787(D319A)-GFP ( ) was quantified by determining cfu.
F. Release of wild-type *L. pneumophila* into the supernatant of infected wild-type *D. discoideum* ( ) or ΔDd5P4 producing Δd5P41-787-GFP ( ), or Δd5P4 producing Δd5P41-787(D319A)-GFP ( ) was quantified by determining cfu.

*P (two-tailed Student's t-test) < 0.02 (A), < 0.002 (B), < 0.01 (C).
expressing functional Dd5P4–GFP but not by the catalytically inactive mutant protein Dd5P4 1-787(D319A)–GFP (Fig. 3B). The defect in LCV transition was also partially complemented by ectopical expression of the human enzyme OCRL1 1-893 in Dd5P4 (Fig. 3C), indicating that OCRL1 at least partially substitutes for Dd5P4 with regard to LCV dynamics. The functional redundancy among the two enzymes is reflected by an identical domain organization and an overall identity of 33% (Fig. 1).

Dd5P4 binds to LCVs via an N-terminal domain and is catalytically active on LCVs

The effects of Dd5P4 on intracellular replication of L. pneumophila and on the maturation and dynamics of LCVs suggest that Dd5P4 might directly bind to LCVs. To test this hypothesis, we produced GFP fusion proteins of Dd5P4 and fragments thereof in wild-type and Dd5P4 D. discoideum (Fig. 4A). Flow cytometry analysis revealed that the different fusion proteins or GFP alone were produced by more than 95% of the amoebae (Fig. S1, data not shown). The GFP fusion proteins were distributed throughout the host cell cytoplasm in intact wild-type D. discoideum (Fig. S2) as well as in Dd5P4 amoebae (data not shown) infected with L. pneumophila. However, upon homogenization of infected D. discoideum, the wild-type fusion protein Dd5P4 1-787–GFP and...
the catalytically inactive mutant protein DdSP41–787(D319A)GFP were found to localize to LCVs (Fig. 4B), indicating that DdSP4 indeed accumulates on LCVs, and the catalytic activity of the phosphatase is not required for this localization. Furthermore, all GFP fusion proteins containing the N-terminal 132 amino acids of DdSP4 localized to LCVs, while fusion proteins lacking the N-terminus or GFP alone did not bind to LCVs. These results identify the 132 amino acid N-terminal fragment of DdSP4 as an LCV-localizing domain. This DdSP4 fragment has not been previously implicated in membrane targeting, and thus, we term the domain ‘LVA’ (Legionella vacuole association).

Identical results were obtained for the recruitment of DdSP4 or fragments thereof to LCVs in lysates of the D. discoideum ∆DdSP4 mutant strain (Fig. S3). In wild-type or ∆DdSP4 mutant D. discoideum approximately 80% of LCVs stained positive for ectopically produced DdSP41–787-GFP 1 h post infection (Fig. 4C). Furthermore, in all our experiments aimed at the quantification of DdSP4 or fragments thereof on LCVs, we did not observe obvious differences in the amount of the proteins on either tight or spacious vacuoles (data not shown).

To test whether DdSP4 not only binds to LCVs but is catalytically active on this subcellular compartment, we quantified on the vacuoles PtdIns(4)P, putatively produced from PtdIns(4,5)P2 by the 5-phosphatase activity of DdSP4. To this end, we determined by immunofluorescence microscopy the amount of the PtdIns(4)P-binding lcIm/Dot substrate SidC (Weber et al., 2006) on LCVs in wild-type or ∆DdSP4 D. discoideum. LCVs in wild-type D. discoideum (i.e. in presence of DdSP4) accumulated 1.4 times more SidC (P < 0.001; Mann–Whitney U-test), compared with LCVs in the ∆DdSP4 mutant strain (Fig. 4D). This result is in agreement with the notion that DdSP4 is catalytically active on LCVs and thus increases the local concentration of PtdIns(4)P by using PtdIns(4,5)P2 as a substrate.

OCRL1 binds to LCVs via an N-terminal domain in D. discoideum and decorates LCVs in macrophages

The defective transition from tight to spacious LCVs observed in D. discoideum ∆DdSP4 was partially complemented by ectopical expression of the human enzyme OCRL1 (Fig. 3C), suggesting that OCRL1 might localize to LCVs similar to DdSP4. To address this question, we ectopically expressed full-length OCRL11,893–GFP as well as OCRL11,235–GFP in wild-type and ∆DdSP4 D. discoideum. Compared with the full-length fusion protein OCRL11,893–GFP, the fragment OCRL11,235–GFP was produced by more amoebae (approximately 76%) and at higher levels, yet both mammalian fusion proteins were less efficiently generated than the DdSP4 fusion proteins (Fig. S1). In intact D. discoideum infected with L. pneumophila, ectopically expressed full-length OCRL11,893–GFP yielded a punctuate staining, while the N-terminal OCRL11,235–GFP fragment localized throughout the host cell cytoplasm (Fig. 5A). However, upon homogenization of infected amoebae, full-length OCRL1–GFP, and even more pronounced OCRL11,235–GFP, was found to accumulate on LCVs in wild-type D. discoideum (Fig. 5B), as well as in the ∆DdSP4 mutant strain (data not shown). These results indicate that, similar to DdSP4, also OCRL1 localizes to LCVs via an N-terminal LVA domain.

In RAW264.7 macrophages infected with L. pneumophila the localization of endogenous OCRL1 was analysed by immunofluorescence microscopy. Similar to DdSP4 in D. discoideum, immunolabelling of OCRL1 in intact macrophages yielded a punctuate staining (data not shown), and upon homogenization of the infected macrophages, OCRL1 was identified on LCVs containing single or several bacteria (Fig. 5C). In summary, DdSP4 and OCRL1 are recruited via their N-termini to LCVs in D. discoideum or macrophages respectively.

Accumulation of DdSP41–132 on LCVs harbouring Icm/Dot-proficient L. pneumophila

Upon fixation of infected D. discoideum with paraformaldehyde, the recruitment of DdSP4 to LCVs was not visible in intact cells (Fig. S2). Thus, we tried methanol fixation to visualize the association of DdSP4 to LCVs in intact amoebae (Fig. 6A and B). Under these conditions, an accumulation of DdSP41–132–GFP on LCVs was clearly visible in intact infected cells, while longer fusion constructs were distributed throughout the cytoplasm similar to paraformaldehyde-fixed amoebae (Fig. S2, data not shown). To analyse the subcellular distribution of DdSP41–132–GFP in D. discoideum infected with L. pneumophila in more detail, we used the ER protein disulfide isomerase (PDI) and the endosome/lysosome protein common antigen 1 (CA1) as markers. One hour post infection, 62% or 50% LCVs harbouring wild-type L. pneumophila stained positive for DdSP41–132–GFP or double-positive for DdSP41–132–GFP and PDI respectively (Fig. 6A). Furthermore, 65% or 28% of LCVs harbouring wild-type L. pneumophila stained positive for DdSP41–132–GFP or double-positive for DdSP41–132–GFP and CA1 respectively (Fig. 6B). In contrast, DdSP41–132–GFP was not detectable on LCVs harbouring ∆icmT mutant L. pneumophila, 13% of which stained positive for PDI (Fig. 6A) and 56% were positive for CA1 (Fig. 6B).

An analysis of the recruitment kinetics revealed that the percentage of DdSP41–132–positive LCVs harbouring wild-type L. pneumophila continuously increased over 2 h, at which time point close to 100% of the vacuoles stained positive for DdSP41–132–GFP (Fig. 6C). At 1 h post infection, approximately 80% of the LCVs stained positive for
Dd5P4\textsubscript{1-132}–GFP, similar to the recruitment kinetics of full-length Dd5P4\textsubscript{1-787}–GFP (Fig. 4C). Thus, the recruitment kinetics of the N-terminal Dd5P4\textsubscript{1-132} fragment and full-length Dd5P4\textsubscript{1-787} are very similar. In contrast, LCVs harbouring ΔicmT mutant bacteria never acquired detectable amounts of Dd5P4\textsubscript{1-132}–GFP. In summary, these results indicate that Dd5P4 is selectively recruited via its N-terminal domain to LCVs harbouring wild-type but not icm/dot mutant \textit{L. pneumophila}.

The N-terminal domains of Dd5P4 or OCRL1 have not been previously implicated in membrane targeting of these IP5Ps. To test whether Dd5P4\textsubscript{1-132} localizes to membranes by binding to phosphoinositides or other lipids, we performed a protein-lipid overlay assay using purified GST–Dd5P4\textsubscript{1-132} (41.3 kDa) and as a positive control GST–SidC\textsubscript{609-917} (62.5 kDa). While GST–Dd5P4\textsubscript{1-132} did not bind to any of the lipids spotted onto nitrocellulose membranes (Fig. 6D), GST–SidC\textsubscript{609-917} specifically bound to PtdIns(4)P as described (Weber \textit{et al.}, 2006; Ragaz \textit{et al.}, 2008). Thus, Dd5P4\textsubscript{1-132} might bind to LCVs via a bacterial or a host cell protein receptor rather than via a lipid.

\textit{L. pneumophila} \textit{LpnE} binds to OCRL1\textsubscript{1-236}, LCV membranes and PtdIns(3)P

As Dd5P4\textsubscript{1-132} apparently does not bind to a lipid, we tested whether Dd5P4 or OCRL1 bind to a protein receptor on LCVs. To assay whether the N-terminal domain of OCRL1 binds to a \textit{L. pneumophila} protein, OCRL1\textsubscript{1-236} was covalently coupled to Affigel-10 beads, incubated...
with *L. pneumophila* lysate, and bound proteins were identified by LC-ESI-MS/MS (Fig. 7A). Using this protocol, a prominent protein band specifically retained by OCRL1-236 coupled to beads was identified as the 41.4 kDa *L. pneumophila* protein LpnE (Newton et al., 2006; 2007). The most prominent protein band eluting from OCRL1-236 beads incubated with either *L. pneumophila* lysate or binding buffer corresponded to the 27.3 kDa OCRL1-236 fragment.

To confirm the interaction between OCRL1-236 and LpnE, purified GST–OCRL1-236 was incubated with *L. pneumophila* lysate, precipitated with glutathione beads, and LpnE was identified by Western blot using an antibody against LpnE (Fig. 7B). In the reciprocal approach, purified GST–LpnE was incubated with *L. pneumophila* lysate, precipitated with glutathione beads, and binding of OCRL1-236–GFP was visualized by Western blot using an antibody against GFP (Fig. 7C). These immunoprecipitation experiments confirmed the interaction between OCRL1-236 and LpnE. In a similar experimental setup the interaction of LpnE with Dd5P4-132 was analysed. However, neither did LpnE associate with GST–Dd5P4-132, nor Dd5P4-132–GFP with GST–LpnE. Perhaps, the additional N-terminal amino acids 1–122 of OCRL1 contribute to or stabilize the interaction with LpnE.
If the interaction between OCRL1 and LpnE is relevant in vivo, LpnE should localize to LCV membranes. Indeed, LpnE was identified by immunofluorescence microscopy on LCVs in RAW264.7 macrophages infected with wild-type but not icmT mutant L. pneumophila (Fig. 7D). As expected, LCVs harbouring an L. pneumophila ΔlpnE mutant strain did not stain with the antibody. These results are in agreement with a function for LpnE in binding or stabilizing OCRL1 on LCVs. We also tried to visualize LpnE on LCVs harbouring wild-type L. pneumophila in D. discoideum. However, the affinity-purified anti-LpnE antibody used was not suitable in this case, as Western blot experiments revealed cross-reactions with D. discoideum proteins (data not shown).

Next, we analysed whether the L. pneumophila ΔlpnE mutant strain shows a phenotype regarding the recruitment of OCRL1/Dd5P4 in D. discoideum. At 1 h post infection LCVs harbouring the L. pneumophila ΔlpnE mutant strain stained positive for OCRL1\(^{1-236}\)-GFP (Fig. 8A) or Dd5P4\(^{1-132}\)-GFP (Fig. 8B) to a similar extent as LCVs harbouring wild-type bacteria. Furthermore, the acquisition kinetics of Dd5P4\(^{1-132}\)-GFP were very similar regarding LCVs harbouring ΔlpnE or wild-type L. pneumophila (Fig. 8C). While approximately 90% of the LCVs containing ΔlpnE or wild-type L. pneumophila stained positive for Dd5P4\(^{1-132}\)-GFP 2 h post infection, none of the LCVs containing a ΔicmT strain acquired the fusion protein during a 4 h infection period. We also tested

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**Fig. 7.** L. pneumophila LpnE binds to OCRL1\(^{1-236}\) and LCV membranes.

A. For pull-down experiments, OCRL1\(^{1-236}\) covalently linked to Affigel-10 beads or control beads were incubated with L. pneumophila lysate or buffer, and proteins bound to the washed beads were separated by SDS-PAGE, stained with Coomassie brilliant blue and identified by LC-ESI-MS/MS. A 41.4 kDa band was identified as L. pneumophila LpnE.

B. Purified GST–OCRL1\(^{1-236}\) was incubated with L. pneumophila lysate or (C) purified GST–LpnE was incubated with lysate from D. discoideum producing OCRL1\(^{1-236}\)-GFP, respectively, precipitated with glutathione beads, and the proteins bound to washed beads were visualized by Western blot using antibodies against LpnE or GFP respectively (composed images from one experiment; representative of three independent assays).

D. Confocal laser scanning micrographs of lysed macrophages infected with GFP-labelled wild-type, ΔicmT or ΔlpnE L. pneumophila. Calnexin and LpnE were visualized using antibodies.
whether LpnE modulates the catalytic activity of Dd5P4. As shown above (Fig. 4D), more PtdIns(4)P-binding SidC accumulated on LCVs in presence of Dd5P4, indicating that the phosphatase is catalytically active. However, in absence or presence of lpnE, the amount of SidC on LCVs was similar, indicating that LpnE does not affect the activity of Dd5P4 (Fig. S4). Taken together, these results suggest that LpnE is apparently dispensable for...
the recruitment of OCRL1/Dd5P4 to LCVs, and the \textit{L. pneumophila} protein does not modulate the catalytic activity of the phosphatase.

Finally, as LpnE localizes to the LCV membrane (Fig. 7D), we addressed the question whether the protein might bind to a lipid. Using a protein-lipid overlay assay, LpnE was found to specifically bind to PtdIns(3)P but not to other phosphoinositides or lipids \textit{in vitro} (Fig. 8D). This result suggests that LpnE anchors to LCVs via PtdIns(3)P. SidC, used as a control, specifically bound to PtdIns(4)P and less efficiently to PtdIns(3)P, as observed previously (Weber et al., 2006).

Discussion

An analysis of the role of IP5Ps during phagocytosis and intracellular replication of \textit{L. pneumophila} revealed that \textit{D. discoideum} Dd5P4 impairs intracellular growth of \textit{L. pneumophila} and the interaction of LCVs with the ER (Figs 2 and 3). Furthermore, Dd5P4 and OCRL1 were found to be recruited to LCVs in \textit{D. discoideum} and macrophages (Figs 4 and 5). Dd5P4 is the closest homologue of mammalian OCRL1, and both IP5Ps are classified as GAP domain-containing inositol phosphatases (Loovers et al., 2007) (Fig. 1). Dd5P4 and OCRL1 share a number of properties, including a catalytic activity as type II IP5Ps (Zhang et al., 1995; Loovers et al., 2003). Upon heterologous expression of OCRL1 in \textit{D. discoideum} \textit{ΔDd5P4}, growth and developmental defects of the mutant strain are fully complemented, and a phagocytosis defect is partially complemented (Loovers et al., 2003; 2007). Similarly, the impaired transition from tight to spacious LCVs observed in the \textit{ΔDd5P4} mutant strain is partially complemented upon heterologous expression of OCRL1 (Fig. 3C). Together, these results suggest that the orthologues Dd5P4 and OCRL1 share similar functions, and thus, \textit{D. discoideum} Dd5P4 is a valid model to study OCRL1. Compared with Dd5P4–GFP lower amounts of OCRL1–GFP bound to LCVs (Figs 4B and 5B), corresponding to the finding that OCRL1–GFP was produced by fewer \textit{D. discoideum} cells and in lower amounts (Fig. S1). Therefore, a less efficient production and LCV targeting of OCRL1 in \textit{D. discoideum} likely accounts for the only partial complementation of phenotypes observed for strain \textit{ΔDd5P4} infected with \textit{L. pneumophila}.

OCRL1 shares the domain architecture and 45% identity with INPP5B, another mammalian type II IP5P. While OCRL1 and INPP5B are structurally similar, some evidence supports a role for OCRL1 rather than INPP5B during intracellular replication of \textit{L. pneumophila}: (i) ectopically expressed OCRL1 localizes to LCVs in \textit{D. discoideum} (Fig. 5B); (ii) the anti-OCRL1 antibody used in our study was raised against the N-terminal amino acid residues 1–237 (Choudhury et al., 2005), which are only 18% identical with INPP5B, against which no specific antibody could be raised (Williams et al., 2007); and (iii) OCRL1 (but not INPP5B) binds clathrin (Ungewickell et al., 2004; Choudhury et al., 2005; Williams et al., 2007). Clathrin is a pivotal component of early endosomes to TGN vesicle trafficking and was recently identified in the proteome of purified LCVs (Urwyler et al., 2008). INPP5B, on the other hand, is implicated in the retrograde pathway from the ERGIC to ER (Williams et al., 2007), which does not involve clathrin.

Dd5P4/OCRL1 directly affects the formation and maturation of LCVs, as in absence of Dd5P4 LCVs interact with the ER more rapidly, and the transition from tight to spacious vacuoles is impaired (Fig. 3). The significance of this morphological transition for \textit{L. pneumophila} infection is not clear. The transition is inhibited due to the absence of different \textit{D. discoideum} or \textit{L. pneumophila} factors, yet the efficiency of intracellular bacterial replication is either increased (in \textit{D. discoideum} \textit{ΔDd5P4} or \textit{ΔPI3K1/2}) (Fig. 2; Weber et al., 2006), decreased (in \textit{D. discoideum} \textit{ΔRtoA}) (Li et al., 2005) or not affected (by \textit{L. pneumophila \textit{AsidC-sdcA}}) (Ragaz et al., 2008). The spacious phagosomes likely do not represent compartments harbouring ‘sick’ bacteria that no longer form a replication-permissive vacuole, as the recruitment to LCVs of calnexin is dependent on the paralogous Icm/Dot substrates SidC and SdcA, and this process precedes the morphological transition (Ragaz et al., 2008). In absence of these effectors, the number of spacious vacuoles decreased by more than 50%, yet \textit{L. pneumophila \textit{ΔsidC-sdcA}} mutant bacteria replicate at wild-type rate (and not more efficiently).

OCRL1 regulates retrograde vesicle trafficking between endosomes and the TGN (Ungewickell et al., 2004; Choudhury et al., 2005; Williams et al., 2007), and therefore, a functional retrograde pathway might restrict intracellular replication of \textit{L. pneumophila}. The bacteria also replicate more efficiently in \textit{D. discoideum} in absence of PI3Ks (Weber et al., 2006), which have also been implicated in endosomal vesicle trafficking, including the retrograde endosome to TGN transport (Lowe, 2005; Bonifacino and Rojas, 2006). However, while in absence of Dd5P4 lower amounts of the PtdIns(4)P-binding Icm/Dot substrate SidC are bound to LCVs (Fig. 4D), higher amounts of SidC are bound to LCVs in absence of PI3Ks (Weber et al., 2006). Thus, binding of SidC to LCVs, and as a corollary the amount of PtdIns(4)P on LCVs is apparently not the rate-limiting factor for intracellular growth of \textit{L. pneumophila}.

Accumulating evidence implicates trafficking between the TGN and endosomes in the formation of LCVs. Recently, the secretory and endosomal small GTPases Rab1, Rab7, Rab8 and Rab14 were identified in the proteome of purified LCVs, and these GTPases were
recruited to vacuoles harbouring wild-type but not ΔicmT mutant L. pneumophila (Urwyler et al., 2008). Rab8 localizes to endosomes and regulates the late secretory pathway through endosomes by promoting fusion of TGN exit transport vesicles with endosomes (Henry and Sheff, 2008). Thus, Rab8 and OCRL1 share a subcellular localization and might accumulate on LCVs by direct fusion of the LCVs with endosomes at early retrograde transport exit sites and/or late secretory entry sites.

OCRL1 and Dd5P4 anchor to LCVs harbouring Icm/ Dot-proficient bacteria via an N-terminal domain, which we term ‘LVA’ (Legionella vacuole association) to reflect its novel role in membrane binding of the IP5Ps. Except the N-terminal 21 amino acid residues of Dd5P4, the LVA domains of Dd5P4 and OCRL1 are fairly similar and share 21% identity (Fig. 1). However, the N-terminus of OCRL1 includes another 122 amino acids, the function of which is unknown. These additional amino acids might contribute to the interaction of OCRL11-236 with the L. pneumophila effector LpnE (Fig. 7), in agreement with the finding that the shorter N-terminus of Dd5P4 apparently does not interact with LpnE (data not shown). LpnE localizes to LCVs harbouring wild-type but not ΔicmT mutant L. pneumophila (Figs 7D and 8C), suggesting that the effector is an Icm/Dot substrate. Moreover, LpnE selectively binds to PtdIns(3)P (Fig. 8D) and thus is the first member of a second family of phosphoinositide-binding L. pneumophila effectors, in addition to the PtdIns(4)P-binding Icm/ Dot substrates represented by SidC (Weber et al., 2006; Ragaz et al., 2008).

OCRL11-236 and Dd5P41-132 are recruited to LCVs harbouring an L. pneumophila strain lacking lpnE (Fig. 8), indicating that LpnE is not required for the recruitment of the IP5Ps. LpnE might stabilize OCRL1 on LCVs, or other L. pneumophila Sel1-like repeat proteins might substitute for the protein. Many effector proteins of L. pneumophila form families of (functionally redundant) paralogues (Luo and Isberg, 2004). Indeed, the four L. pneumophila genomes sequenced to date encode the same 6 or 7 Sel1-like repeat proteins (Fig. S5), including LpnE (Newton et al., 2006; 2007), EnhC (Cirillo et al., 2000; Liu et al., 2008) and LidL (Conover et al., 2003). The four Sel1-like repeat proteins most closely related to LpnE are EnhC, LidL, Lpg1062 and Lpg1356, which share 28–37% identity with LpnE. Sel1-like repeats are implicated in protein–protein interactions, and the proteins may serve as adaptors for the assembly of macromolecular complexes (Mittl and Schneider-Brachert, 2007).

Binding of OCRL1/Dd5P4 on LCVs by LpnE or other Sel1-like repeat proteins might promote the restriction of intracellular growth of L. pneumophila. Thus, the interaction between LpnE and OCRL1 possibly represents a bacterial mechanism to downregulate intracellular replication, thereby sustaining a protective niche, which would be lost upon rapid killing of the host cell. Alternatively, the accumulation of OCRL1/Dd5P4 on LCVs harbouring Icm/ Dot-proficient but not icm/dot mutant L. pneumophila (Fig. 6) suggests that OCRL1 and/or the retrograde transport pathway play a role in growth restriction of vacuolar pathogens by the host cell. Thereby, this pathway might contribute to containment and clearance of pathogenic intracellular microorganisms.

OCRL1 is recruited and activated by Rab GTPases of the Golgi apparatus (Rab1, Rab6) or early endosomes (Rab5) (Hyvola et al., 2006) (Fig. 1). The small GTPases Arf1 and Rab1 localize to LCVs and promote intracellular replication of L. pneumophila (Kagan and Roy, 2002; Nagai et al., 2002; Derre and Isberg, 2004; Kagan et al., 2004), and therefore, these GTPases might recruit and activate OCRL1 (Fig. 4D). The ASH and Rho GAP domains of OCRL1 have been proposed to orient the catalytic IP5P domain to the membrane (Erdmann et al., 2007). However, as the LVA domain of OCRL1/Dd5P4 is sufficient to bind the phosphatases to LCVs, this domain also contributes to or is even sufficient to properly position the catalytic IP5P domain on LCVs. In agreement with this hypothesis, an N-terminal Dd5P4 fragment including only the LVA and IP5P domains is recruited to LCVs (Fig. 4A and B) and partially complements the intracellular growth phenotype of L. pneumophila in the ΔDd5P4 mutant strain (Fig. 2F).

Legionella pneumophila is taken up by macrophages and amoebae by ‘efficient phagocytosis’, an Icm/Dot T4SS-dependent process resembling macropinocytosis (Hilbi et al., 2001; Watarai et al., 2001; Weber et al., 2006). While PI3Ks are dispensable for efficient phagocytosis of L. pneumophila (Weber et al., 2006), the absence of Dd5P1 and Dd5P4 reduced the number of infected D. discoideum by approximately 40% (Fig. 2A). These findings are in agreement with a role of Dd5P4 in promoting phagocytosis by removing PtdIns(4,5)P2 at the plasma membrane (Loovers et al., 2007). Along the same line, at early time points post infection (< 30 min) already 20–45% of the LCVs stained positive for the N-terminal fragment Dd5P41-132 (Fig. 6C), again indicating a role for Dd5P4 in early steps of the uptake process. OCRL1 was found to translocate to membrane ruffles (resembling macropinocytosis) upon stimulation of quiescent cells with growth factors and activation of the small GTPase Rac (Faucher et al., 2003; 2005). Furthermore, OCRL1 binds to the GTPases Rac, Cdc42 and Rap5 present in ruffles (Hyvola et al., 2006; Erdmann et al., 2007). Therefore, L. pneumophila possibly facilitates its uptake by recruiting OCRL1/ Dd5P4 to the entry sites. An analogous mechanism has been reported for the enteropathogenic bacteria Shigella flexneri (Niebuhr et al., 2002) and Salmonella enterica (Terebiznik et al., 2002), which facilitate their entry by translocating into host cells the bacterial inositol...
Experimental procedures

Bacteria, cells and growth conditions

The L. pneumophila wild-type strain JR32 (Sadosky et al., 1993) and the isogenic ΔicmT mutant strain GS3011 (Segal et al., 1998) were grown for 3 days on CYE agar plates. Liquid cultures were inoculated in AYE medium at an OD600 of 0.1 and grown for 21 h at 37°C (Weber et al., 2006). To maintain plasmids, chloramphenicol was added at 5 μg ml⁻¹. As ‘input’ controls for phagocytosis experiments, 20 μl of a 10⁵ ml⁻¹ bacterial solution was plated and counted after 3 days.

The D. discoideum wild-type strain Ax3 and mutant strains lacking individual IP5Ps (ΔDd5P1–4) were obtained from P.J.M. van Haastert (University of Groningen) (Lovers et al., 2003). The amoebae were grown axenically at 23°C in 75 cm² tissue culture flasks in HLS liquid medium (10 g of glucose, 5 g of yeast extract, 5 g of proteose peptone, 5 g of thionine E peptone, 2.5 mM Na₂HPO₄, 2.5 mM KH₂PO₄ in 1 l of H₂O, pH 6.5), supplemented with 20 μg ml⁻¹ G418 or 10 μg ml⁻¹ blasticidin S when necessary, split twice a week and fed with fresh HL5 medium 24 h before use. Transfection of D. discoideum with expression plasmids for GFP fusion constructs was performed as described (Faix et al., 2004). Briefly, exponentially growing cells were washed once in SorC (2 mM Na₂HPO₄, 15 mM KH₂PO₄, 50 μM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and then in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 50 mM CaCl₂, pH 6.0) and once in electroporation buffer (EB; 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indicated, the number of bacteria released into the supernatant
was quantified by plating aliquots of appropriate dilutions on
CYE plates. Due to the relatively high moi of 1 used, L. pneu-
mothila grew with moderate efficiency in wild-type D. discoi-
deum, as observed in previous studies (Weber et al., 2006;
Ragaz et al., 2008).

Intracellular bacterial growth before host cell lysis was quanti-
fied by counting cfu after selectively lysing infected D. discoi-
deum with saponin (‘single round replication’) as described
(Weber et al., 2006). Briefly, at the time points indicated, the MB
medium was replaced by 100 µl of 0.8% saponin and incubated
for 20 min. The cells were lysed by vigorously pipetting, and
aliquots were plated in appropriate dilutions.

Intracellular trafficking of L. pneumophila, and localization of
DD5P4, OCRL1 or LpnE on LCVs

Dictyostelium discoideum or macrophages were processed for
fluorescence microscopy as described (Albers et al., 2005; Weber
et al., 2006). At the time points indicated, the infected phagocytes
were washed 3 times with cold SorC buffer (D. discoideum) or
PBS (macrophages) and fixed with 4% paraformaldehyde in PBS
at 4°C or with methanol at −20°C for 30 min. The fixed cells were
washed, permeabilized (0.1% Triton X-100, 10 min) and blocked
with 2% normal human AB serum in SorC or PBS for 30 min.
Alternatively, the infected phagocytes were washed with cold SorC
or PBS, re-suspended in homogenization buffer (20 mM Hepes,
250 mM sucrose, 0.5 mM EGTA, pH 7.2) (Derre and Isberg, 2004)
and lysed by 11 passages through a ball homogenizer (Isobiotech)
onto coverslips coated with poly-L-lysine, immobilized by 4% paraformaldehyde for 30 min at 4°C and blocked with 2% normal
human AB serum in SorC or PBS for 30 min.
The coverslips with fixed intact cells or lysates were incubated
for 1 h at room temperature on parafilm with 30 µl of primary
antibody diluted in blocking buffer (affinity purified rabbit anti-SidC
(Weber et al., 2006), 1:1000; affinity purified rabbit anti-LpnE
(Newton et al., 2006, 2007), 1:30; affinity purified sheep anti-
OCRL11 (Choudhury et al., 2005), 1:400; goat anti-calnexin
(Santa Cruz, 1:100); anti-D. discoideum common antigen 1 (CA1),
1:1000; anti-D. discoideum protein disulfide isomerase (PDI)
(Monnat et al., 1997), 1:10 000; anti-L. pneumophila
common antigen 1 (CA1), 1:1000) and washed 3 times. Secondary antibodies (FITC-conjugated goat anti-rabbit IgG [H+L], Cy5-conjugated goat anti-
rabbit IgG [H+L]; rhodamine-conjugated bovine anti-rabbit IgG;
FITC-conjugated donkey anti-sheep IgG [H+L], Cy5-conjugated
donkey anti-goat IgG [H+L], Cy5-conjugated goat anti-mouse IgG

Table 1. Plasmids used in this study.

| Plasmid       | Characteristics                                         | PCR oligonucleotides (template)                          | Reference                        |
|---------------|--------------------------------------------------------|----------------------------------------------------------|----------------------------------|
| pCR2          | gat–sidC in pGEX-4T-1                                   |                                                          | Weber et al. (2006)              |
| pCR44         | gat–sidC039 in pGEX-4T-1                                |                                                          | Ragaz et al. (2008)              |
| pCalnexin–GFP | CalnexinN–RSSSLK-gfp(S65T); P<sub>art5</sub>            |                                                          | Müller-Taubenberger et al. (2001) |
| pDDSP4–GFP    | DdSP4–gfp in pDM6                                       |                                                          | Loovers et al. (2007)            |
| pDDSP4<sub>2031A</sub>–GFP | DdSP4<sub>2031A</sub>–gfp in pDM6 |                                                          | Loovers et al. (2007)            |
| pEGFP-N1      | Expression of GFP in mammalian cells                    |                                                          | Amersham                         |
| pGEX-4T-1     | Expression of N-terminal gat fusions; P<sub>art5</sub>  |                                                          | Amersham                         |
| pGEX-6P-1     | Expression of N-terminal gat fusions; P<sub>art5</sub>  |                                                          | Amersham                         |
| pUC4K         | pUC4K oriR (pBR322), MCS::Km                            |                                                          | Amersham                         |
| pGEM-T-Easy   | Cloning of PCR products                                 |                                                          | Promega                          |
| pLAW344       | oriT (RK2), oriR (ColE1), sacB                           |                                                          | Water et al. (1994)              |
| pMMB207–Km14–gfp | Gfp in pMMB207-Km14, Δlac<sup>+</sup>                     |                                                          | Mampel et al. (2006)             |
| pMMB207C–RBS–M45 | M45–MCS in pMMB207; P<sub>x</sub>                      |                                                          | Weber et al. (2006)              |
| pOCRL1        | Ocr1 in pMB74                                           |                                                          | Loovers et al. (2007)            |
| pSW001        | DsRedExpress in pMMB207C, Δlac<sup>+</sup>               | oSW5/24 (pSW105)                                         | This study                       |
| pSW009        | gat–DdSP4<sub>319</sub>–132 in pGEX-4T-1                |                                                          | This study                       |
| pSW010        | gat–OCRL1<sub>12</sub>–25 in pGEX-6T-1                  |                                                          | This study                       |
| pSW011        | gat–OCRL1<sub>12</sub>–25 in pGEX-4T-1                  |                                                          | This study                       |
| pSW013        | gat–lpeN in pGEX-4T-1                                   |                                                          | This study                       |
| pSW014        | M45–LpnE in pMMB207C; P<sub>x</sub>                     | oSW26/27 (L. pneumophila genomic DNA)                     | This study                       |
| pSW015        | Genomic region of lpeN in pGEM-T Easy                    |                                                          | This study                       |
| pSW016        | Genomic region of lpeN in pLAW344                        |                                                          | This study                       |
| pSW101        | MCS-m45 in pDXA                                         | oSW3/4                                                   | This study                       |
| pSW102        | MCS–gfp in pDXA                                         | oSW1/2 (pEGFP-N1)                                        | This study                       |
| pSW103        | Ocr1<sub>12</sub>–gfp in pDXA                           | oSW7/8 (pOCRL1)                                          | This study                       |
| pSW105        | DdSP4<sub>319–25</sub>–gfp in pDXA                      | oSW5/12 (pDDSP4–GFP)                                     | This study                       |
| pSW106        | DdSP4<sub>300–319</sub>–gfp in pDXA                     | oSW5/12 (pDDSP4<sub>2031A</sub>–GFP)                     | This study                       |
| pSW107        | DdSP4<sub>319</sub>–gfp in pDXA                         | oSW5/17 (pDDSP4–GFP)                                     | This study                       |
| pSW108        | DdSP4<sub>319</sub>–gfp in pDXA                         | oSW18/12 (pDDSP4–GFP)                                    | This study                       |
| pSW111        | DdSP4<sub>319</sub>–gfp in pDXA                         | oSW5/19 (pSW105)                                         | This study                       |
| pSW112        | DdSP4<sub>319</sub>–gfp in pDXA                         | oSW20/17 (pSW105)                                        | This study                       |
| pSW113        | Ocr1<sub>12</sub>–gfp in pDXA                           | oSW7/23 (pSW103)                                         | This study                       |
| pSW114        | DdSP4<sub>319</sub>–gfp in pDXA                         | oSW20/12 (pSW105)                                        | This study                       |
| pVatM–GFP     | VatM–gfp in pDXA                                        |                                                          | Lu and Clarke (2005)             |
[H+L], or Cy3-conjugated goat anti-mouse IgG [H+L]; Jackson Immunoresearch) were diluted in blocking buffer and incubated for 1 h at room temperature. Finally, the coverslips were washed and mounted using Vectashield (Vector Laboratories) supplemented with 1 μg ml−1 DAPI to stain DNA.

The amount of SidC on LCVs harbouring DsRed-labelled *L. pneumophila* in wild-type or ΔDsSP4 *D. discoideum* was quantified by immunofluorescence using affinity anti-SidC and Cy5-conjugated secondary antibodies (Weber et al., 2006). The fluorescence intensity of an area identical for all samples and covering a single LCV was quantified using the ‘Quantity One’ software (Bio-Rad) after background correction (averaged intensity of 3 areas within the infected amoeba). ‘Tight’ and ‘spacious’ vacuoles were scored exactly as described (Weber et al., 2006; Ragaz et al., 2008). Briefly, ‘spacious’ vacuoles were defined as vacuoles where the SidC-labelled membrane surrounding *L. pneumophila* was clearly detached, leaving a space between the membrane and the bacterium. All other LCVs observed were scored as ‘tight’.

The samples were viewed with an inverted confocal microscope (Axiovert 200 M; Zeiss), equipped with a 100× oil phase contrast objective (Plan Neofluar; Zeiss), an ‘Ultraswell’ confocal head (Perkin Elmer) and a krypton/argon laser (643-RYB-A01; Melles Griot). Data processing was performed with the ‘Volocity’ 2.6.1 software (Improvision).

**Binding of GST–DdSP4 or GST–LpnE to phosphoinositides and other lipids in vitro**

The GST–DdSP4 or GST–LpnE fusion proteins were produced in *E. coli* BL21(DE3) (Novagen) induced at a cell density of OD600 0.6 with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at 30°C in LB medium. The soluble fusion proteins were purified from lysates prepared by French press using glutathione sepharose beads in a batch procedure according to the manufacturer’s recommendations (Amersham), and purity of the protein preparations was analysed by SDS-PAGE.

Direct binding of GST–DdSP4 or GST–LpnE to phosphoinositides and other lipids was tested in a protein-lipid overlay assay as described (Weber et al., 2006; Dowler et al., 2002), using commercially available PIP-strips and PIP-arrays (Echelon) and the PtdIns(4)P-binding probes GST–SidC or GST–SidC809–917 as a control.

**Pulldown experiments**

The N-terminal fragment OCRL11–236 was purified from *E. coli* BL21(DE3) and covalently linked to Affigel-10 beads as described (Ragaz et al., 2008), incubated with 1 ml *L. pneumophila* lysate prepared by sonication, and washed 4 times with binding buffer (20 mM Hepes, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, pH 7.5). As a comparison, the *L. pneumophila* protein SidM covalently linked to Affigel-10 beads was used. Proteins bound to the washed beads were separated by 12% SDS-PAGE, stained with Coomassie brilliant blue and identified by LC-ESI-MS/MS. To confirm binding of *L. pneumophila* LpnE to OCRL11–236, GST–OCRL11–236 or GST–LpnE was produced and purified as described above, incubated with lysates prepared by sonication of *L. pneumophila* or *D. discoideum* producing OCRL11–236–GFP, respectively, and precipitated with glutathione beads. The proteins bound to beads washed with binding buffer were separated by 12% SDS-PAGE and visualized by Western blot using antibodies against LpnE (1:500; (Newton et al., 2006; 2007) or GFP (1:2500; Roche Diagnostics) respectively.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Ectopic production of GFP and fusion proteins of Dd5P4, OCRL1 or fragments in wild-type *D. discoideum*. Flow cytometry data of *D. discoideum* wild-type strain Ax3 producing (A) no ectopical protein, (B) GFP, (C) Dd5P41-787–GFP, (D) Dd5P41-132–GFP, (E) OCRL11-402–GFP, or (F) OCRL11-236–GFP. While all amoebae (> 99%) produced GFP or fusion proteins of Dd5P4 or Dd5P41-132 at high levels, only a portion of the amoebae (42–76%) produced GFP fusion proteins with mammalian OCRL1 or OCRL11-236 at lower levels.

**Fig. S2.** Distribution of ectopically produced GFP fusion proteins of Dd5P4 and fragments in *D. discoideum* wild-type. Confocal laser scanning micrographs of paraformaldehyde-fixed wild-type *D. discoideum* producing the Dd5P4–GFP fusion proteins indicated (green), infected with DsRed-labelled *L. pneumophila* (red) at an moi of 50 (1 h) and stained with an anti-SidC antibody (blue) for LCVs. DNA was visualized with DAPI. The Dd5P4–GFP fusion proteins localized throughout the host cell cytoplasm.

**Fig. S3.** Binding of GFP fusion proteins of Dd5P4 and fragments to LCVs in infected *D. discoideum*. Confocal laser scanning micrographs of *D. discoideum* ΔDd5P4 expressing the Dd5P4–GFP fusion constructs indicated (green), and infected with DsRed-labelled wild-type *L. pneumophila* at an moi of 100 (1 h). LCVs in lysates were visualized using an affinity purified anti-SidC antibody (blue). Only Dd5P4–GFP fusion constructs containing the N-terminal 132 amino acids of Dd5P4 localize to LCVs.

**Fig. S4.** The catalytic activity of Dd5P4 on LCVs is not affected by LpnE. Dot plot of SidC fluorescence intensity (median and variance) on LCVs in calnexin–GFP-labelled wild-type or ΔDd5P4 *D. discoideum*. The amoebae were infected with DsRed-labelled JR32 wild-type or ΔlpnE mutant *L. pneumophila* and immunolabelled for SidC, which binds to PtdIns(4)P on LCVs. In presence of the inositol polyphosphate 5-phosphatase Dd5P4, the median SidC fluorescence intensity was higher, indicating that the enzyme was catalytically active on the LCV membrane producing PtdIns(4)P. In absence of *lpnE*, Dd5P4 was still catalytically active, indicating that LpnE does not affect the activity of the phosphatase.

**Fig. S5.** Overview and alignment of *L. pneumophila* proteins containing Sel1-like repeats. (A) Overview of *L. pneumophila* Sel1-like repeat proteins. The genes encoding Sel1-like repeat proteins are present in the genomes of all *L. pneumophila* strains sequenced to date (Philadelphia, Paris, Lens, Corby) with the exception of *lpg1062*. LpnE (Lpg2222, Lpp2174, Lpl2147, Lpc1689), EnhC (Lpg2639, Lpp2692, Lpl2564, Lpc0501), LidL (Lpg1172, Lpp1174, Lpi1180, Lpc0638), Lpg1062 (Lpl1059, Lpc2212), Lpg1356 (Lpp1310, Lpl1307, Lpc0770), Lpg2485 (Lpp2549, Lpl2405, Lpc1993), Lpg0896 (Lpp0957, Lpl0927, Lpc2397). Lpc0165 is unique to *L. pneumophila* strain Corby. The following domains are highlighted: Sel1 (grey diamonds), signal peptide (black bar), TPR (dark grey diamonds), methyltransferase (grey bar), HNH nuclease (dark grey bar). (B) Alignment of the closest LpnE homologues: EnhC (30.1% identity), LidL (32.5%), Lpg1062 (28.4%) and Lpg1356 (37.3%). Additional amino acids present in EnhC or LidL that do not align with the other homologues are indicated in parentheses.

Table S1. Oligonucleotides used in this study.

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