**The regulatory isoform rPGRP-LC resolves immune activation through receptor clearance via ESCRT-mediated trafficking**

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# ABSTRACT

Innate immune systems need to distinguish between harmful and innocuous stimuli to respond adequately to the level of threat. How *Drosophila* mounts differential immune responses to dead and live Gram-negative bacteria using the single peptidoglycan receptor PGRP-LC is unknown. We describe an alternative splice variant of PGRP-LC, termed rPGRP-LC, which selectively dampens immune activation in response to dead bacteria. *rPGRP-LC* mutants cannot resolve immune activation following Gram-negative infection and die prematurely. The alternative exon of rPGRP-LC encodes an adaptor module targeting rPGRP-LC to membrane micro-domains. rPGRP-LC-mediated immune resolution requires degradation of activating and regulatory receptors via endosomal ESCRT sorting. We propose that rPGRP-LC selectively responds to peptidoglycans from dead bacteria thereby tailoring the immune response to the level of threat.

Decision-making in the immune system is based on correctly assessing the level of threat. The innate immune system is triggered when host pattern recognition receptors (PRRs) encounter microbe-associated molecular patterns (MAMPs) 1, 2. MAMPs are conserved structural motifs absent from the host, for example bacterial cell wall constituents such as peptidoglycan (PGN) 3. The PRR-MAMP conceptual framework however cannot explain how the immune system distinguishes between live and dead or between beneficial and pathogenic microbes. Additional immune checkpoints have been proposed that further specify immune decision-making, such as microbial viability, virulence or proliferation 4, 5. MAMPs derived from PGN can originate from the break-down of the cell wall of dying bacteria, but also from bacterial remodelling enzymes active during proliferation or deployment of bacterial secretion systems 6. The enzymatically released cell wall metabolites are anhydromuropeptides, monomeric building blocks of PGN, such as TCT (tracheal cytotoxin). PGN polymers on the contrary are hidden underneath the outer lipopolysaccharide layer in Gram-negative bacteria, and become accessible only upon bacterial death since the outer membrane is impermeable to large PGN fragments 7. How PRRs integrate information from MAMPs with different “threat content” into a coherent immune response remains, however, an unresolved issue.

Binding of PGN and TCT to peptidoglycan recognition receptors stimulates the *Drosophila* IMD pathway, culminating in activation of the NF-κB-like transcription factor Relish. The IMD pathway can be activated by two peptidoglycan recognition receptors, the plasma membrane-anchored PGRP-LC and the cytosolic PGRP-LE 12. PGRP-LC senses polymeric and monomeric breakdown products of DAP-type PGN from Gram-negative bacteria and Gram-positive bacilli 13, 14, 15. It is proposed that PGRP signalling relies on ligand-induced clustering of receptor tails, which results in the recruitment of a signalling complex leading to activation of NF-κB 16. The *PGRP-LC* gene encodes three isoforms with distinct ectodomains that differ in their PGN-binding capacity 13, 17, 18. Detection of polymeric PGN relies on homotypic clusters of PGRP-LCx receptor isoforms alone, while detection of the monomeric muropeptide TCT is dependent on LCx-LCa receptor heterodimers 13, 17, 19.

Although PGN and TCT activate the same downstream signalling complex, their immune activation and resolution kinetics greatly differ. While it is clear that polymeric PGN induces a more efficient immune resolution than TCT 17, 21, the underlying molecular mechanism remains unknown. PGN-triggered activation of the IMD pathway in *Drosophila* is regulated at multiple steps. Secreted enzymatic PGRPs can hydrolyse polymeric and monomeric PGN into non-immunogenic fragments, reducing signal input into the pathway 21, 22, 23. PGRP-LF is a transmembrane receptor with two PGRP domains and a non-signalling cytosolic tail that inhibits PGRP-LC activation through receptor competition 24, 25. A cytosolic adaptor, Pirk (also called Rudra or Pims), has been proposed to disassemble receptor-signalosomes by competing with IMD for PGRP-LC occupancy or by depleting PGRP-LC from the plasma membrane 26, 27, 28. As these negative regulators are transcriptionally controlled by the IMD pathway, they establish a feedback loop preventing excessive activation of the immune system. However, based on their mode of action, none of these regulators is able to distinguish between polymeric PGN and TCT. Here, we have identified a regulatory PGRP-LC isoform that carries a distinct cytosolic tail predicted to bind phosphoinositides, and whose specific recruitment to polymeric PGN contributes to the resolution phase of the immune response.

# RESULTS

### The *PGRP-LC* locus encodes a splice variant with a unique cytosolic tail

The kinetics of IMD pathway activation in response to Gram-negative bacteria can be assessed by monitoring the expression of the antibacterial peptide gene *Diptericin*, a target gene of Relish. We noticed that identical concentrations of live and dead Gram-negative bacteria caused differential responses, with dead bacteria inducing a significantly faster return to baseline (**Fig. 1a**). Injection of different doses of TCT triggered the IMD pathway with kinetics similar to live bacteria, consistent with TCT acting as a MAMP that responds to live bacteria (**Fig. 1b**). Under the same conditions, polymeric PGN mimicked immune responses to dead bacteria. The resolution rate appeared significantly faster for PGN than for TCT at any dose, suggesting a qualitative rather than a quantitative difference in the resolution mechanism. Since both PGN and TCT engage IMD pathway adaptors through PGRP-LC, we hypothesized that the receptor itself might harbour a discriminatory mechanism to distinguish between PGN and TCT.

PGRP-LC (subsequently referred to as LC) is a type-II transmembrane receptor, whose N-terminal cytosolic tail is identical in the three LC isoforms LCa, LCx and LCy 13, 29, 30. This tail lacks endocytic motifs, and no ligand-induced trafficking of the receptor has been reported so far. A closer look at the *PGRP-LC* locus revealed a potential alternative first exon, which might encode a functionally distinct cytosolic tail (**Fig. 1c**). We termed the cytosolic tail variant ‘regulatory PGRP-LC’ (rPGRP-LC or rLC). The previously unrecognized alternative exon 5 is present in the genomes of 11 out of 12 sequenced *Drosophila* species, with the exception of *D. willistoni 31*. Exon 5 contains both a Kozak sequence upstream of the translation start codon and a splice donor site at its 3’ end (**Supplementary Fig. 1a**). Similarly to the conventional exon 4, exon 5 was alternatively spliced to exons encoding the transmembrane domain and the PGRP domains x, y and a (**Supplementary Fig. 1b, c**). Thus, the *PGRP-LC* locus gives rise to six receptor isoforms (**Fig. 1c**). Exon 4- and exon 5-containing transcripts exhibited similar tissue specificity and expression levels (**Fig. 1d**), and comparable expression kinetics after immune challenge (**Fig. 1e**).

### Structure-function analysis of rPGRP-LC

To identify conserved motifs of rLC, we aligned its protein sequences from 11 *Drosophila* species and found two distinct conserved domains separated by a short variable linker (**Fig. 1f**). Although the linker somewhat resembled a RIP homotypic interaction motif (RHIM) in species of the *melanogaster* subgroup, this was not substantiated by detailed computational analysis (K. Hofmann, A. Kajava, personal communication). *In silico* analysis of amino acids (Aa) 1-65 predicted a plant homeodomain (PHD)-type zinc finger with a characteristic C4HC3 arrangement (**Fig. 1f** and **Supplementary Fig. 2a**). PHD domains belong to a larger family of FYVE and RING zinc fingers, protein folds acting in protein-lipid interaction and ubiquitination, respectively 32, 33. The rLC PHD domain clustered with known lipid-binding adaptors, predicting a role in membrane targeting (**Supplementary Fig. 2b**). Aa 76 to 97 is predicted to form coiled-coils (**Supplementary Fig. 2c, d**). Circular dichroism analysis of the cytosolic region of rLC supported these structural predictions (data not shown). Taken together, the domain architecture of the cytosolic region of rLC suggested that this portion might mediate lipid binding, protein-protein interactions, and/or homo- or heterodimerization.

### rLC suppresses IMD signalling in response to polymeric PGN

Next, we tested whether rLC might play a role in correctly adjusting immune responses to different ligands. Fat body-specific overexpression of rLCx potently reduced the immune response to polymeric PGN, but failed to significantly suppress the TCT-induced response (**Fig. 1g**). In contrast to the ability of rLC to modulate the response to specific ligands, overexpression of rLCx had no effect on NF-κB activation triggered by overexpression of PGRP-LCx, PGRP-LE or IMD (**Fig. 1h**). Our data indicate, therefore, that rLCx selectively modulates the response to specific ligands.

To study the physiological role of the rLC isoforms, we made use of two *PGRP-LC* mutant strains; Δ*LC*E12 flies lack the entire *PGRP-LC* locus, and Δ*LC*ird7(1) flies carry an insertion in exon 4, and hence only express the rLC isoforms (**Fig. 1c and Fig. 2c**) 29, 30. Both mutants failed to induce antibacterial peptide gene expression or survive Gram-negative infection (**Fig. 2d, e**), indicating that rLC alone cannot activate the IMD pathway. We then generated a *PGRP-LC* locus specifically deleted for the *rLC* isoforms. For this we complemented the Δ*LC*E12 deficiency with either an engineered transgene containing the entire wild-type locus 17 or with the same locus deleted for exon 5 (**Fig. 1c**). Both rescue transgenes were inserted into the φC31 landing site at 51C (**Fig. 2a**), which ensures identical transgene expression (**Supplementary Fig.3a**), and crossed to either Δ*LC*E12or Δ*LC*ird7(1)mutant flies (**Fig. 2b and Supplementary Fig. 3b**). The two Δ*LC*E12 complemented lines are referred to as resc(*LC*wt) and resc(*LC*Δex5)*.*

Resc(*LC*Δex5) flies that lack *rLC* isoforms displayed significantly stronger IMD pathway activation after injection of polymeric PGN, while the response to TCT was unchanged (**Fig. 2f**). Next, we engineered flies carrying all possible permutations of activating isoforms LCxand LCa and regulatory isoforms rLCx and rLCa, and monitored the immune response to polymeric and monomeric PGN (**Fig. 2g**). As previously published 17, 34, LCx was necessary and sufficient to relay signals after polymeric PGN injections. We found that the response to PGN was significantly enhanced in flies lacking *rLCx* and carrying *LCx*, regardless of the presence of *LCa* and *rLCa* isoforms. This indicates that rLCx is necessary and sufficient to resolve IMD activation in the presence of PGN (**Supplementary Fig. 3c**). After TCT injections, the heterodimeric LCx-LCa module was required to relay signal, in agreement with previous work (REF). However, no significant increase was observed when either *rLCx* or *rLCa* or both were missing, indicating that TCT signalling is mostly resistant to negative regulation by rLC.

Additional experiments monitoring immune activation in the whole gut (IMD signalling dependent on PGRP-LC and PGRP-LE) compared to midgut (IMD signalling dependent on PGRP-LE alone) showed that the regulatory isoforms exclusively blunted PGRP-LC- but not PGRP-LE-activated IMD signalling in response to indigenous gut bacteria 17, 35 (**Supplementary Fig. 3d**). Moreover, immune signalling in the fat body, in the absence of infection, was not significantly affected by loss of *rLC* (**Supplementary Fig. 3d**). Collectively, our observations demonstrate that rLC isoforms function as negative regulators of PGRP-LC but not PGRP-LE activated IMD signalling. In contrast to PGRP-LF or the nonaspanins TM9SF2/4, which prevent PGRP-LC-mediated IMD activation even in the absence of stimulus 24, 25, 36, regulation by rLC is ligand-dependent. Most notably, rLC isoforms seem to differentially affect the immune response to polymeric and monomeric PGN, i.e. to differentiate between varying levels of threat.

### rLC resolves IMD activation during bacterial infection

Next we tested whether rLC can specifically curtail immune signalling when the immune system is facing dead bacteria. While flies lacking rLC isoforms killed bacteria as efficiently as wild-type flies (**Fig. 3a**), we found that rLC was required for the resolution of IMD signalling following an infection. Accordingly, flies lacking *rLC* isoforms were significantly delayed in resolving their immune response, despite efficient bacterial killing (**Fig. 3b**). This delay was not due to the persistence of live bacteria as the same effect was observed after challenge with heat-killed bacteria (**Fig. 3b** and **Supplementary Fig. 3e**). Therefore, *rLC* contributes to the resolution of immune activation by sensing the presence of polymeric PGN from dead bacteria.

Despite efficient bacterial clearance, *rLC* mutants died significantly earlier than wild-type controls (**Fig. 3c**). Although this appears somewhat paradoxical, this observation is in line with previous findings indicating that deregulated IMD pathway activation can result in premature death 23, 25, 37. Alternatively, we cannot exclude that even small numbers of bacteria could flare up and kill the host. While loss of rLC function resulted in elevated IMD signalling, we found that overexpression of *rLCx* in the fat body significantly blunted the immune response after challenge with heat-killed Gram-negative bacteria (**Fig. 3d**). Accordingly, *rLCx*-overexpressing flies had a significantly lower survival rate to live bacteria, possibly because they down-regulated IMD activation too early (**Supplementary** **Fig. 3f**). Of note, fat-body specific expression of rLCx did not change the base-line levels of *Dpt* expression *per se* (**Fig. 3d**, time-point 0). Collectively, these data identify rLCx as a novel regulatory isoform of PGRP-LC that controls IMD pathway kinetics, thereby preventing immune-induced lethality from unresolved IMD activation.

### rLC interacts with Pirk through the PHD domain

We next investigated the mechanism by which rLC downregulates the IMD pathway. First we assessed the possibility that rLC regulates the availability of LC receptors on the cell surface. To this end we evaluated the levels of endogenous GFP-tagged LC in the presence (resc(*LC*wt)) and absence resc(*LC*Δex5) of rLC. We found that LC significantly accumulated in *rLC*-deficient flies when compared to control flies after challenge with dead bacteria, suggesting that rLC controls the levels of surface exposed LC receptors (**Fig. 4a**). We noted that LCx and rLCx co-immunoprecipitated with Pirk from cellular extracts (**Fig. 4b**). Pirk is a negative regulator of the IMD pathway that reportedly down-regulate the surface availability of PGRP-LC 26, 27, 28. Strikingly, the PHD domain of rLCx was indispensable for its interaction with Pirk since a PHD domain-deficient form of rLCx failed to bind to Pirk. Since Pirk can bind to different motifs in LC and rLC (RHIM and additional cytosolic motifs for LCx 26, 27, 28, PHD for rLCx, this study), it is likely that Pirk mediates its effect by binding to either receptor.

Next, we assessed whether rLC and Pirk cooperate in regulating the IMD pathway upon bacterial infection. Loss of either *pirk* or *rLC* alone significantly increased the immune response, and combined loss of both was linearly additive (**Fig. 4c**). Conversely, overexpression of either regulator reduced the immune response by about 50% and concomitant expression of *rLC* and *pirk* led to over 60% decrease, albeit with no significant additive effect (**Fig. 4d** and **Supplementary Fig. 4a**). Overexpressed *rLCx* was able to suppress IMD signalling in the absence of *pirk,* demonstrating that Pirk is not required for rLC-mediated regulation of IMD signalling (**Fig. 4e** and **Supplementary Fig. 4b**). Importantly, overexpressed Pirk suppressed pathway activation stimulated by either polymeric PGN or monomeric TCT, indicating that Pirk does not discriminate between live or dead bacteria. Thus, Pirk operates differently from rLC, with rLC exclusively regulating the response to dead bacteria (**Fig. 4f**).

Together, these data indicate that despite their physical interaction, Pirk and rLC can regulate IMD activation independently of one another. Nonetheless, under physiological conditions it is highly likely that Pirk and rLC work in concert to suppress IMD signalling, by promoting extracellular, PGN dependent, and intracellular clustering of activating and regulatory receptors.

### Endocytic mechanisms regulate the IMD pathway

To investigate the role of endocytic trafficking in the activation and the regulation of IMD signalling, we performed a targeted mini-screen of key endocytic trafficking components in the fat body, the main immune responsive organ of the fly (**Fig. 5a**, **Supplementary Tables 1 and 2**). While none of the tested genes prevented IMD pathway activation, interfering with the early endosomal compartment by overexpression of dominant-negative (DN) *Rab5* (*Rab5DN*) or *Rab5* RNA interference (RNAi) increased the amplitude and duration of IMD pathway activation. In addition, knock-down of the endosome maturation kinase *Fab1* also led to increased activation, but had no effect on the return to baseline. Lastly, knock-down of *vps28, which disrupts* the ESCRT-I complex, extended the duration of IMD pathway activation. IMD pathway activation seemed largely clathrin- and dynamin-independent, since neither overexpression of dominant-negative *shibire* (*shiDN*), the fly homolog of dynamin, nor knock-down of *shibire* or *clathrin light chain* (*Clc*) prevented *Dpt* induction upon Gram-negative infection. Our observation that inactivation of Rab5, Fab1, and ESCRT components results in deregulated IMD signalling suggests that accurate termination of IMD signalling requires the endocytic machinery, and in particular ubiquitin-dependent removal and lysosomal degradation of a membrane-bound receptor.

### Subcellular localization of rLCx depends on the PHD domain

To test whether rLCx undergoes endocytosis, we visualized the subcellular localization of rLC in the adult fat body, using tagged versions of *rLCx* (**Supplementary Fig. 5a**). Under the same conditions, overexpression of GFP-PGRP-LCx caused cell death, precluding the analysis of its trafficking routes (data not shown). As controls, we overexpressed known lipid-binding probes tagged with GFP (**Supplementary Fig. 5b**). In adult fat body cells, full-length GFP-rLCx localized to discontinuous membrane domains and to perinuclear vesicles, partially mimicking the distribution of the endosomal FYVE-GFP marker (**Fig. 5b**, compare to **Supplementary Fig. 5b**). A similar distribution occurred in hemocytes, where GFP-rLCx localised to plasma membranes and intracellular vesicles, and was particularly enriched in cell-cell contact sites (**Supplementary Fig. 5c**). GFP-rLCxΔPHD, lacking the PHD domain, distributed more uniformly along the plasma membrane, reminiscent of the plasma membrane localizing probe GFP-PLCδ-PH (**Fig. 5c**, compare to **Supplementary Fig. 5b**). This suggests that the PHD domain is involved in receptor localization to specific membrane sub-domains. In fat body cells, GFP-rLCx was located at membrane domains that partially overlapped with apico-lateral regions (labelled by cortical actin and β-catenin), a component of adherens junctions (**Fig. 5d, e**). Taken together, these observations suggest that rLCx is retained in specific membrane domains in a PHD domain-dependent fashion.

Next, we tested the role of endocytosis in regulating the localisation and function of rLCx and Pirk. We noticed that GFP-rLCx accumulated in flies in which Rab5 or ESCRT components Vps28 and Tsg101 were depleted (**Fig. 5f** and **Supplementary Fig. 5d**). Conversely, overexpression of *Pirk* or *vps28* reduced total GFP-rLCx protein, and increased the numbers of GFP-rLCx positive vesicles (**Fig. 5g, h and j**), suggesting enhanced endocytosis. In contrast, depletion of components of the ESCRT machinery led to a decrease in the numbers of such vesicles (**Fig. 5g, i and j**, and **Supplementary Fig. 5e, f, g**). These observations are in agreement with the notion that rLCx undergoes endocytic degradation via the ESCRT machinery, a process that is aided by Pirk.

### Failure to endocytose rLC overactivates IMD signalling

Given that LC accumulates in rLC-deficient flies, we tested the interdependency of rLC and ESCRT machinery in regulating LC levels. Due to genetic constraints, we used flies with only a single copy of the rescue construct. Endogenous GFP-PGRP-LC accumulated in an rLC- and Vps28-dependent manner in the fat bodies of flies challenged with dead bacteria (**Fig. 6a, b**). Immediately after infection, Relish is cleaved to release the transcriptionally active fragment Rel-49. Relish cleavage was not significantly affected by lack of rLC or Vps28, indicating that early activation of the IMD response was normal (**Fig. 6a,b**). At the transcriptional level, accumulation of LC was reflected by significantly delayed immune resolution when *vps28* was knocked down, alone or in combination with loss of the single copy of rLC (**Fig. 6c**). Based on this observation, it is likely that rLC and Vps28 act in concert to remove LC from the plasma membrane. Indeed, the regulatory function of overexpressed *rLCx* was significantly impaired upon *vps28* knock-down (**Fig. 6d** and **Supplementary Fig. 5h**), suggesting that rLC acts upstream of Vps28. Of note, LC was previously shown to interact with the ESCRT protein Vps28 in an S2 cell-based protein interaction network 45.

As receptor ubiquitylation can serve as signal for endocytic trafficking, we tested whether rLC recruits the ubiquitin ligase DIAP2, which acts at multiple steps in the IMD pathway 46, 47, 48. Indeed, co-immunoprecipitation experiments in S2 cells indicated that rLC bound to DIAP2, and that this interaction required the PHD domain of rLC (**Fig. 6e**). Together, these data suggest that resolution of IMD signalling is controlled by rLC-mediated endocytosis and ESCRT-dependent degradation of PGRP-LC (**Fig. 7**).

# DISCUSSION

While it is clear that PGRP-LC is the major signalling receptor sensing Gram-negative bacteria in flies, it remained elusive how this receptor contributes to the resolution phase, once bacteria are killed and release polymeric PGN. Here, we have uncovered a regulatory isoform of LC (rLC) that adjusts the immune response to the level of threat. We demonstrate that rLC specifically down-regulates IMD pathway activation in response to polymeric PGN, a hallmark of efficient bacterial killing. Our data are consistent with a model whereby the presence of rLC leads to efficient endocytosis of LC and termination of signalling via the ESCRT pathway. Trafficking mediated shut down of LC-dependent signalling ensures that LC receptors are switched off once the balance is tipped in favour of ligands signifying dead bacteria, allowing *Drosophila* to terminate a successful immune response. Failure to do so results in over-signalling leading to the death of the host despite bacterial clearance. Consistent with this model we find that defects in endosome maturation and in the formation of multivesicular bodies (MVBs) enhance immune activation and prevent immune resolution. In addition to regulating LC signalling via the ESCRT machinery, rLC can also inhibit LC signalling by forming non-productive rLC-LC hetero- or rLC-rLC homo-dimers. Since IMD recruitment requires dimerization of activating receptors, neither rLC-rLC nor rLC-LC dimers can activate the pathway. In this sense, rLC acts similarly to PGRP-LF with regards to TCT: it can form ligand-bound dimers with LC but cannot signal 24.

The early death of rLC deficient flies might be due to aberrant activation of apoptotic pathways downstream of IMD. Indeed, mammalian TNFR signalling, which is functionally similar to the IMD pathway, induces NF-κB and MAPK cascades from the plasma membrane, but triggers cell death from an intra-cellular compartment following endocytosis 49, 50. Possibly, rLC mutants exhibit erroneous trafficking of LC, and therefore might induce apoptosis following prolonged retention of LC at the plasma membrane.

Molecularly, rLC is characterized by a cytosolic PHD domain, predicted to bind to phosphoinositides. We find that the PHD domain of rLC targets rLC to distinct membrane domains, but cannot exclude that this localization additionally relies on protein-protein interactions. Furthermore, the PHD domain also mediates binding of rLC to the cytosolic regulator Pirk and the ubiquitin ligase DIAP2. The combined capability to control membrane localization and to recruit downstream signalling modulators is reminiscent of the “sorting/signalling adaptor paradigm” that is emerging for mammalian PRRs 51. Sorting adaptors are cytosolic signalling components with phosphoinositide binding domains that are selectively recruited to defined subcellular locations and thereby shape the signalling output of the receptors they interact with 51. In vertebrate immune signalling, bacterial sensing modules (e.g. TLRs), lipid-binding sorting modules (e.g. TIRAP or TRIF) and signalling modules (MyD88, TRAM) are carried on separate molecules and are assembled via transient interactions 52. *Drosophila* MyD88 combines sorting and signalling functions in a single molecule, by-passing the need for TIRAP 53. Strikingly, rPGRP-LC combines features of sensing/signalling receptors and sorting adaptors into one single molecule. The fact that *Drosophila* rLC has no immediate homologs in vertebrates with PGN-sensing and –signalling pathways suggests evolutionary uncoupling of sensing and sorting domains, possibly to increase the spectrum of signalling by combinatorial recruitment of adaptors to sensing receptors.

Recent evidence from vertebrates also implicates the ESCRT machinery in suppressing spurious NF-κB activation: the TNFR superfamily member Lymphotoxin-β Receptor, which activates a signalling cascade that is functionally similar to PGRP-LC signalling, is degraded in an ESCRT-dependent manner in zebrafish and human cells 54. Thus ESCRT-mediated clearance of receptors upstream of NF-κB seems remarkably well conserved throughout evolution.

# METHODS

Methods and associated references are available in the online version of the paper.

**Note:** Supplementary Information including 5 Supplementary Figures, 4 Supplementary Tables, Supplementary Figure Legends, Supplementary Discussion and Supplementary References are available in the online version of this paper.

# ACKNOWLEDGEMENTS

CN was funded by a postdoctoral grant from the National Research Fund Luxembourg (AFR08/037). PM acknowledges funding support from Breast Cancer Now (CTR-QR14-007) and NHS funding to the NIHR Biomedical Research Centre. We thank members of the Lemaitre lab for discussions, S. Boy, J. Rybniker (EPFL) and A. Kleino (University of Massachusetts) for sharing advice and reagents, C. Day, M. Miaczynska and N. Silverman for insightful comments, K. Hofmann and A. Kajava for help with RHIM analysis, M. Gonzalez-Gaitán (University of Geneva) for flies and advice, and the Bloomington, TRiP and VDRC stock centres for fly stocks.

# AUTHOR Contributions

CN conceived the project, carried out experiments and wrote the manuscript. FS assisted with experiments. CR provided additional experimental data. PM and BL advised on experimental design and commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

# Figure Legends

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**Figure 1. The *PGRP-LC* locus encodes cytosolic tail variant isoforms with regulatory potential.**

**a.** *Dpt* mRNA expression kinetics following infected with live or heat-inactivated *Ecc15*. Data shows mean ± SEM of n=3 repeats.

**b.** *Dpt* mRNA expression kinetics after injection with varying concentrations of synthetic monomeric (TCT) and polymeric peptidoglycan (PGN) from *E.coli*. The molarity of PGN is determined based on titration of its hydrolysed component parts (e.g. muramic acid, DAP) and refers to monomeric units (1 GlcNac-MurNac-tetrapeptide ~ 1000Da) in the PGN polymers (personal communication, Dominique Mengin-Lecreulx). Due to steric hindrance between PGRP domains, only every fifth monomeric unit can be bound in PGN 66, lowering the effective binding site concentration 5-fold [move this to material and methods]. Data shows mean ± SEM of n=3 repeats. Resolution after PGN occurs faster than after TCT (difference in normalized slopes PGN vs TCT, 8h vs 24h by linear regression, P=0.0027779).

**c.** Schematic representation of the *PGRP-LC* genomic locus (only protein-coding exons are numbered) and resulting polypeptides. Exon 4 (blue) encodes the previously known signalling and cytosolic tail of LC; exon 5 (orange) encodes the cytosolic tail of rLC. The coding sequence of *eGFP* (green) was inserted at the transcription start site in exon 4. Both exons 4 and 5 can be spliced to x, a and y ectodomains (greys), resulting in two sets of polypeptides with differing cytosolic tails and a complete range of PGN sensing domains (see also Supplementary Fig. 1). To create exon 5 deletion loci, exon 5 was removed as described in Experimental Procedures (deletion indicated by brackets), leaving the intronic sequences on either side intact. Exon 4 encodes the activating tail with an IMD binding domain and a RHIM motif (hatched). Exon 6 (grey) is present in both isoforms and encodes the transmembrane (TM) domain (hatched). Note that both rescue constructs carry an N-terminal GFP tag on exon 4-containing PGRP-LC isoforms that was previously shown not to affect LC function 17.

**d.** Tissue expression of rLCx quantified for adult fat bodies, whole guts or midguts dissected from unchallenged *w1118* flies (see also Supplementary Fig. 3d).

**e.** Expression kinetics of *LCx* and *rLCx* in *c564-Gal4/+* flies infected with heat-killed *Ecc15*. Data in d and e are mean ± SEM of n≥3 repeats*.*

**f.** Evolutionary conservation of rLC. Protein sequences (corresponding to Aa 1 to 97 in the *D. melanogaster* sequence) of 11 sequenced *Drosophilia* species were aligned using ClustalW. Orange blocks denote conserved residues defining predicted sequence elements (C4HC3 zinc finger, amphipathic α-helical coiled-coil, see also Supplementary Fig. 1).

**g.** Fat-body specific overexpression (driver: *c564-Gal4*) of rLCx selectively blunts immune responses to polymeric PGN but not to the monomer TCT. Injections as in b.

**h.** rLCx does not blunt IMD activation in the absence of bacterial ligands. *c564-Gal4* or *c564-Gal4;UAS-rLCx* driver lines were crossed to *UAS-PGRP-LCx*, *UAS-PGRP-LE* or *UAS-Imd*. *Dpt* mRNA was quantified 3 days after flies were switched to 29°C for gene induction. Data are mean ± SEM of n≥3 repeats, analysed by 2-way ANOVA with Bonferroni posttests (a, g, h).

**Figure 2. rLC is a ligand-specific regulator of IMD pathway activation.**

**a.** Genomic rescue of PGRP-LC mutants (chromosome III) by reinsertion of Pacman constructs carrying the full PGRP-LC locus (*resc[LCwt]*) or the locus lacking rLC (*resc[LCΔex5]*) into the φC31 landing site at position 51C on chromosome II.

**b.** Genotypes and phenotypes of rescued flies used in this study. Different rescue constructs on chromosome II were crossed to different *PGRP-LC* mutants. See also Suppl. Fig. 3c for immune response in different genetic contexts.

**c.** All rLC isoforms are absent from *ΔLCE12* but transcribed in *ΔLCird7(1)*. In *ΔLCird7(1)* an insertional mutation in exon 4 introduces a premature stop codon in LC but not rLC transcripts.

**d.** rLC alone fails to induce an immune response 8h after *Ecc15* infection.

**e.** rLC alone cannot confer resistance to infection with *Ecc15*. Data represent pooled survival counts of n=3 biological repeats. Log-rank test, *p*<0.0001 for mutants versus wt.

**f.** Loss of rLC results in significantly enhanced immune responses to PGN at 8h and 24h but no change in response to TCT. Injections as in Fig. 1b. Data are mean ± SEM of n=3, analysed by 2-way ANOVA and Bonferroni posttests, comparing *resc(LCwt)* and *resc(LCΔex5)*.

**g.** PGN-elicited responses (8h after injection) require rLCx for down-regulation; TCT-elicited responses (24h after injection) are unaffected by loss of rLCx or rLCa alone or in combination (presence of isoform is indicate by a +; see Supplementary Table 3 for full genotypes). Data represent mean ± SEM of n≥3, analysed by 1-way ANOVA and Tukey’s posttests (b) or Dunnett’s posttests (e).

**Figure 3. The rLC regulatory isoform resolves IMD pathway activation after infection.**

**a.** Bacterial clearance (live *Ecc15-GFP*) in flies lacking rLC. Fly lysates were plated for colony counts at the indicated times [this should be moved to material and methods]. Data (n=4 biological repeats) is represented as box plots (Tukey). Linear regression analysis gives clearance rates of 217 ± 114 bacteria/h (*wt*, ns), 280 ± 60 bacteria/h (*resc[LCwt],* p = 1.05e-05) and 313 ± 88 bacteria/h (*resc[LCΔex5]*, p= 0.000615).

**b.** Loss of rLC increases the amplitude and duration of IMD activation after Gram-negative challenge. *resc(LCwt)* and *resc(LCΔex5)* flies were infected by pricking with live (left panel) or heat-killed (right panel) *Ecc15* or heat-killed *E.coli* (Supplementary Fig. 3e) and collected at indicated time points for quantification of *Dpt* mRNA.

**c.** Despite an efficient immune response flies lacking rLC die faster than wt flies when infected with live *Ecc15*. Data represent pooled survival counts of n=3 biological repeats. Log-rank test, *p*<0.0001 for *resc(LCwt)* versus *resc(LCΔex5)*.

**d.** rLC overexpression reduces the amplitude and duration of the IMD response, as measured by *Dpt* mRNA expression in flies with or without fat body-specific (*c564-Gal4*) overexpression infected with heat-killed *Ecc15*. Data represent mean ± SEM of n≥3 repeats, analysed by 2-way ANOVA with Bonferroni posttests (b, e).

**Figure 4. rLC interacts with the negative regulator Pirk.**

**a.** Endogenous GFP-PGRP-LC accumulates after infection in flies lacking rLC. *resc(LCwt)* and *resc(LCΔex5)* flies were infected as in b. and fat bodies were dissected at 0, 1, 2 and 3 h after challenge. Relish cleavage was monitored as a read-out of IMD activation.

**b.** Pirk interacts with the PHD domain of rLC. Indicated constructs were transfected into S2 cells and HA-tagged Pirk was purified with anti-HA antibodies. Expression and presence of co-purified rLC proteins was analysed by immunoblotting with anti-V5 antibody. Cell lysates are indicated in the left panel, and IP-ed proteins are indicated in the right panel.

**c.** Loss of *rLC* and *pirk* are additive. Flies deficient for either *pirk* or *rLC* or both were assessed for *Dpt* mRNA 8h after challenge with heat-killed *Ecc15*.

**d.** Overexpression of *rLC* or *pirk* reduces the immune response in offspring from *w;c564-Gal4* or *w;c564-Gal4;UAS-rLC* driver lines crossed to *w* or *UAS-pirk* and challenged with heat-killed *Ecc15*.

**e.** Overexpression of *rLC* can compensate for loss of *pirk*. Flies with or without fat body-specific rLC overexpression in a *pirk* mutant backgroundwere tested for immune activation after infection with *Ecc15* (see also Supplementary Fig. 4a, b).

**f.** Overexpression of *Pirk* does not discriminate between polymeric PGN (left panel) and the TCT monomer (right panel). Data represent mean + SEM of n≥3 repeats, analysed by 1-way ANOVA with Bonferroni (c, e) or Tukey’s posttests (d), or 2-way ANOVA with Bonferroni posttests (f).

**Figure 5. Endocytic mechanisms modulate IMD pathway kinetics and rLC localization.**

**a.** Targeted mini-screen for endocytic modifiers of IMD pathway activation. *UAS-gene* or *UAS-RNAi-gene* lines (see Supplementary Tables 3 and 4) were crossed to the temperature-controlled, fat body-specific driver line *w;c564-Gal4;tub-Gal80ts* and adult progeny was tested for *Dpt* mRNA levels 8h (peak activation) and 24h (resolution) after infection. Data are mean + SEM of n≥3 repeats analysed by 2-way ANOVA with Bonferroni posttests, showing *Dpt*/*RpL32* mRNA ratios normalized to maximal IMD activation of control line (black) at 8h after infection. Control crosses (mis-expression of PGRP-LCx) are in blue, significant hits are in red.

**b.** GFP-rLCx localizes to discrete cell membrane domains and to intracellular vesicles in adult fat body cells. Panels b to e show overexpression of rLCx constructs using the *c564-Gal4* driver line. b is a section through the apical region of cells (facing hemolymph), b’ is a section through the cell body. Same annotation for panels c and c’. An open arrowhead indicates the location of the orthogonal section in panels a’, c’, d’’ and e’’.

**c.** GFP-rLCxΔPHD distributes uniformly along the plasma membrane in fat body cells.

**d.** GFP-rLCx is enriched in the cortical regions of fat body cells as visualized by phalloidin staining of cortical actin. d shows green channel, d’ shows magenta channel and d’’ shows merged channels. Same annotation for e, e’ and e’’.

**e.** GFP-rLCx partially overlaps with adherens junction marker β-catenin, as visualized by anti-armadillo staining. Full arrowheads indicate co-localization of pixels in green and magenta channels (apparent as white on the merged panels).

**f.** Hits from the screen in a. affect GFP-rLCx protein stability. *c564-Gal4;UAS-GFP-rLCx* flies were crossed to indicated genotypes and progeny was collected for Western Blot 3 days after induction at 29°C. Genotypes are loaded in duplicates, with each sample resulting from an independent cross.

See Suppl. Fig. 5d for quantification.

**g.** GFP-rLCx localizes to apical vesicles in wild-type fat bodies. A sum projection of 3 consecutive apical slices is shown in g, h and i. GFP channel is shown in white.

**h.** *Pirk* overexpression increases GFP-rLCx-positive vesicle number.

**i.** *vps28* knock-down leads to enlarged GFP-rLCx-positive vesicles with visible lumen (arrows).

**j.** Quantification of GFP-rLCx vesicle distribution for indicated genotypes. See also Supplementary Fig. 5g for quantification of total cell area. Shown are box plots with 10th and 90th percentiles, with number of cells analysed above each box. Data were analysed by 1-way ANOVA with Dunnett’s post test. Scale bar in all images, 10 μm. Nuclear staining is DAPI (blue).

**Figure 6. rLC and ESCRT synergize to degrade activating PGRP-LC and resolve IMD pathway activation.**

**a.** Loss of *rLCx,* knock-down of *vps28* and a combination of both lead to accumulation of endogenous GFP-PGRP-LC during infection. Heterozygous *resc(LCwt)* or *resc(LCΔex5)* flies carrying a *c564-Gal4* driver were crossed to *ΔLCE12* or *vps28-RNAi*,*ΔLCE12*flies. The offspring was challenged with heat-killed *Ecc15* and fat bodies were collected for Western Blot at indicated time points. Relish cleavage is shown as a read-out of IMD pathway activation. A representative blot of n=4 independent experiments is shown.

**b.** Quantification of endogenous GFP-PGRP-LC protein, or accumulation of Rel49 fragment, during early infection time points as in a. Data shows mean + SEM of n=4 independent Western blots as in a., analysed by Kruskal-Wallis test with Dunn’s post-test. For GFP expression, only *resc(LCwt),c564-Gal4>+* versus *resc(LCΔex5),c564-Gal4>vps28 RNAi* is significant (p<0.05). For Rel49 accumulation, no comparison reached significance.

**c.** rLC and Vps28 act together in resolving IMD activation after infection. Flies as in a. were challenged with heat-killed *Ecc15* and *Dpt* mRNA expression was monitored over time. Data represents mean + SEM of n=4 independent experiments, analysed by 2-way ANOVA and Bonferroni post-test. Note that all genotypes are single-copy rescues and therefore show a less pronounced effect of rLC.

**d.** rLCx acts upstream of Vps28. Regulatory capacity of overexpressed rLCx is affected in fat bodies with knock-down of *vps28*. IMD activation was quantified as *Dpt* mRNA levels in whole flies, and normalized to activation in control (*w;c564-Gal4* x *w1118*). Data represent mean + SEM of n≥3 repeats, analysed by 2-way ANOVA with Bonferroni post tests (see also Supplementary Fig. 5g).

**e.** dIAP2 interacts with the PHD domain of rLC. Indicated constructs were transfected into S2 cells and HA-tagged dIAP2 was purified with anti-HA antibodies. Expression and presence of co-purified rLC proteins was analysed by immunoblotting with anti-V5 antibody. Cell lysates are indicated in the left panel, and IP-ed proteins are indicated in the right panel.

**Figure 7. Proposed model for rLCx-mediated resolution of IMD pathway activation.**

[I agree to leave this in]

**a.** During Gram-negative infection, TCT released from live bacteria can activate the IMD pathway by engaging PGRP-LC homodimers (as opposed to PGRP-LC/rLC heterodimers). The regulatory rPGRP-LC isoforms are absent from these signalling dimers and cannot contribute to regulation. IMD pathway activation triggers transcriptional induction of antimicrobial peptide genes and the production of negative regulators of the pathway, such as rLC. **b.** Once bacteria are killed, the balance of available ligands is shifted from TCT monomers to PGN polymers, which can recruit both activating PGRP-LC and regulatory rPGRP-LC isoforms. rPGRP-LC promotes ESCRT-mediated clearance of signalling complexes. Components of the ESCRT machinery capture signalling receptors, trafficking them into multivesicular bodies, from where the cytosolic tails can no longer interact with signalling adaptors. Receptors are eventually degraded to resolve IMD pathway activation. Note that the intracellular negative regulator Pirk (not shown) contributes to regulation in both scenarios, regardless of the type of ligand.

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## ONLINE METHODS

### Fly lines and crosses

*CanS* and *w1118* strains were used as *wt* controls. Unless otherwise indicated, all crosses involving Gal4 drivers were grown at 18-22°C to avoid developmental effects, and after hatching Gal4-UAS mediated expression was induced for 1 day (microscopy) or 3 days (immune read-outs) at 29°C. UAS and RNAi lines and full genotypes for every figure with crosses are listed in **Supplementary Table 1**. *PGRP-LC* loss of function (Δ*LC*) flies were *w;;PGRP-LC*E12and *w;;PGRP-LC*ird7(1) 29, 30, complemented by crossing to *w;*P[acman]-*GFP::PGRP-LC*51C to give resc(*LC*wt) or to *w;*P[acman]-*GFP::PGRP-LC*Δex551C to give resc(*LC*Δex5) (17 and this work). Single isoforms were obtained with the following complementation lines: *w;*P[acman]-*LCx*51C, *w;*P[acman]-*LCa*51Cand *w;*P[acman]-*LCx*Δex551C, *w;*P[acman]-*LCa*Δex551C(17 and this work). *pirkEY00723* has been described in 28. For all experiments, 2-10 day old adults were used.

### Cloning and generation of new transgenic fly strains

Primers and brief cloning strategies for all constructs are listed in **Supplementary Table 3**. Sequencing was done in-house or by Microsynth. For endogenous expression, exon 5-less P[acman] vectors were generated from existing vectors containing the full *PGRP-LC* locus with or without GFP at the ATG in exon 4, or modified *PGRP-LC* loci containing only isoform-specific exons (*PGRP-LCx*, *PGRP-LCa*) published in 17. Full-length vectors were digested with SwaI, isoform-specific vectors were digested with SwaI and NheI. The released backbones with parts of the *PGRP-LC* sequence were gel-purified, and the insert was re-synthesized in two PCR fragments to lack exon 5. Backbone and exon 5-less inserts were then reassembled using InFusion cloning (Takara Clontech), and sequenced across the entire *PGRP-LC* locus. Correct constructs were injected by BestGene into BDSC stock number 24482, a second chromosome landing site at 51C validated for previous *PGRP-LC* constructs 17.

For Gal4-UAS-mediated overexpression in vivo, the CDS of rPGRP-LCx without stop (for C-terminal fusion constructs) or with stop and 3’UTR was assembled from DGRC cDNA clone IP15993 and BDGP Gold clone IP15793 using standard PCR fusion and integrated into an entry vector using TOPO cloning (Life Technologies). These 2 entry vectors were used as templates for all subsequent applications. Small deletions or point mutations were done with the QuikChange Site-directed mutagenesis kit (Agilent). Inserts were recombined from entry clones to appropriate Gateway expression vectors (pTW for no tag or entry clone encoded N-terminal GFP tag, pTWM for C-terminal Myc tag, pTWR for C-terminal mRFP tag) by LR Clonase II reaction (Life Technologies). UAS constructs were injected into embryos by BestGene or the Transgenics Facility of CBMSO, University of Madrid, using standard P-element integration.

For copper-inducible expression in S2 cells, the CDS of rLCx without stop generated above was transferred into the pMT-V5-6xHis expression vector by restriction digest cloning and subsequently modified as for UAS constructs above. pMT-Pirk-HA, pMT-PGRP-LCx-V5 and pMT-dIAP2-HA have been described before 28, 47.

### Infections and immune response read-outs

Infection protocols were done as previously described 17, 56. We used males for survival only, and females for all other experiments. Flies were infected by pricking with a tungsten needle dipped in bacterial pellets, or by injection with glass capillaries controlled by Nanoject (13.8 nl per fly). Elicitors were *E.coli* peptidoglycan 0.05 mg/ml (~0.05 mM) and synthetic TCT 0.42 mM (a generous gift from D. Mengin-Lecreulx, CNRS - Université de Paris-Sud). *Ecc15* and *Ecc15-GFP* were grown at 29°C in LB or LB-Rifampicin, respectively, and pelleted to OD600=200. *E.coli* was grown at 37°C in LB and pelleted to OD600=400. All infection experiments were carried out at 29°C to optimize bacterial growth. For infections with dead bacteria, bacterial pellets were heat-inactivated by boiling at 95°C for 10 minutes.

Immune responses were quantified as *Diptericin* (*Dpt*) transcription compared to a ribosomal gene, *RpL32*. All primers used in this study are listed in **Supplementary Table 4**. Total RNA was extracted from 10 females or 15 fat bodies by TriZol, reverse-transcribed using Superscript II RT enzyme and random hexamer primers unless otherwise indicated, and qPCR of cDNA was performed on a LightCycler 480 using the SYBR green kit from Roche. Note that because of variability between bacterial pellets, occasional batch effects are observed, which increases deviation between samples from different biological repeats of an experiment. To correct for this, where necessary, we normalized all data points of each experiment to an internal control (usually wild-type or driver line).

For bacterial killing, 3 flies per genotype were collected within 1h of infection (infectious dose) or at later time points after infection (clearance), and crushed in 150 μl sterile LB. 4-fold serial dilutions of total fly extracts were plated on LB-Rifampicin in 3 μl spots, and GFP-positive colonies were counted the next day. The detection limit of this assay is 1 bacterium per 3 μl, or an equivalent of <17 bacteria/fly.

Survival was carried out on batches of 30 males per genotype per tube, and tubes were flipped every 3-4 days.

### Tissue preparation, Microscopy and Image analysis

To observe fat body cells, abdomen of females were dissected, opened along the left of right commissure between dorsal and ventral cuticle, and emptied of all inner organs. Imaging always shows the dorsal fat body layer. Overexpression constructs were either visualized directly after quick DAPI staining in PBS (**Fig. 5b, c**), or fixed in 4% paraformaldehyde in PBS then stained with DAPI and rhodamine-phalloidin (1:300, Life Technologies) (**Fig. 5d, g, h, i**). Immunohistochemistry was done as described in 17, using the following antibodies: mouse anti-armadillo (1:100, N2 7A1, DSHB) and species-specific Alexa-labelled secondary antibodies (1:1000, Life Technologies). Fat bodies were mounted in Dako immunofluorescence medium, and visualized on a Zeiss confocal LSM700 with a 40x oil-immersion objective.

To observe hemocytes, wandering larvae were collected in PBS and bled in batches of 4-7 individuals into 25 μl Schneider’s medium droplets on coverslips. Hemocytes were left to adhere at room temperature in moisturised chambers for 10-20 min, then fixed and stained with DAPI in PBS. Hemocytes were visualized on a Zeiss confocal LSM700 with a 63x oil-immersion objective.

The FIJI/ImageJ software suite was used for image processing 57. To quantify GFP-rLCx vesicles, the following steps were implemented as macros and applied to fat body z-stacks:

1. Make actin mask of cells

//empty ROI manager

roiManager ("reset");

//get file name to reuse in results files

name=getTitle();

//make a mask from actin channel

run("Duplicate...", "duplicate channels=3 slices=8-16 title=" + name + "\_actin" );

run("Z Project...", "projection=[Sum Slices]");

setAutoThreshold("Mean");

setOption("BlackBackground", true);

run("Convert to Mask");

run("Median...", "radius=8");

run("Options...", "iterations=8 count=4 black do=Open");

run("Analyze Particles...", "size=200-1000 circularity=0.20-1.00 show=Outlines clear add in\_situ");

roiManager("Save", "C:\\Users\\Claudine\\Desktop\\\RoiSet" + name + "\_actin.zip");

Macro 1 was followed by a manual verification step to discard any ROI that contained more than one cell or overlapped a region with non-fat body tissues (e.g. trachea). This curated ROI set was saved.

2. Analyse GFP channel

Manual selection step: identify which 3 top slices to select (generally #4-6; modify accordingly in macro). Open corresponding ROI set.

// get file name to reuse in results files

name=getTitle();

//retrieve the top 3 slices of GFP channel

run("Duplicate...", "duplicate channels=2 slices=4-6 title=" + name + "\_GFP" );

run("Z Project...", "projection=[Sum Slices]");

//thresholding

setAutoThreshold("Otsu dark");

setOption("BlackBackground", true);

run("Convert to Mask", "method=Otsu background=Dark black");

//count vesicles in each ROI

run("Set Measurements...", "area integrated display redirect=None decimal=3");

n = roiManager("count");

for (i=0; i<n; i++)

{roiManager("select",i);

run("Analyze Particles...", "display");

}

### Immnoblotting

For quantification of overexpressed GFP-rLCx protein levels (**Fig. 5** and **Supplementary Fig. 5**), 10 whole flies were directly lysed in 200 μl 2X SDS-PAGE loading buffer. 15 μl of total fly lysate were separated on SDS-PAGE, then transferred to nitrocellulose membranes (iBlot, Life Technologies). For quantification of endogenous GFP-LC protein levels (**Figs. 4 and 6**), 10 fat bodies were dissected and lysed in 65 μl RIPA buffer. 25 μl lysate were added to 35 μl 2X SDS-PAGE loading buffer, and 15 μl were loaded per well. Blots were revealed with mouse anti-GFP (1:1000; clone mix 7.1 & 13.1, 11814460001; Roche), mouse anti-Relish (1:500; 21F3; DSHB 58) or mouse anti-tubulin (1:2000; clone DM1A, T9026; Sigma), followed by donkey anti-mouse HRP-coupled secondary antibody (1:2000; 715-035-150; Jackson Immuoresearch). ECL (GE Healthcare) was used for detection.

### S2 cell expression and co-immunoprecipitation

S2 cells were transfected with copper-inducible pMT vectors. Co-immunoprecipitation was carried out as described in 28.

### Statistical analysis

All analyses were done using GraphPad Prism. P values as follows: \* denotes *p<*0.05, \*\* denotes *p*<0.01 and \*\*\* denotes *p*<0.001. Whenever more than 2 groups were compared, one-way or two-way ANOVA with corresponding post tests were used, to correct for multiple comparisons. Survival analysis was done by log-rank test. Tests and number of experiments are indicated in each figure legend.

## Supplementary Figure Legends

**Supplementary Figure 1, related to Figure 1. The *PGRP-LC* locus encodes a subset of isoforms containing an alternative first exon.**

**a.** Clustal Omega multiple sequence alignment of 5’ and 3’ regions of exon 5 in 11 sequenced *Drosophila* species indicate a conserved Kozak sequence upstream of the potential translation start site, and a splice donor site in 3’.

**b.** Exon 5 encoding the rLC cytosolic domain is spliced to all ectodomain-encoding exons (x, a and y isoforms). See also **Fig. 1c** for details of PGRP-LC locus architecture. Sequence alignments from RT-qPCR products on DNase treated total RNA from the *white* strain, reverse-transcribed using oligo dT primers. Sequenced products were blasted against the UCSC Genome Browser *D. melanogaster* Apr. 2006 (BDGP R5/dm3) Assembly. Note that the only exon 5-containing splice isoform listed in FlyBase is the one containing the x ectodomain, and is referred to as PGRP-LC-RE.

**c.** PCR products as sequenced in b.

**Supplementary Figure 2, related to Figure 1. Predicted protein domains in the cytosolic tail of rLC.**

**a.** Domain analysis in the cytosolic tail encoded by exon 5. The rLC cytosolic tail contains a PHD motif according to motif prediction using Phyre2 (not shown) and InterProScan (hits and E-values shown) 59, 60, 61.

**b.** Phylogram of *Drosophila* proteins with known function containing the RING/FYVE/PHD domain found in rLC. Boxed area highlights phosphoinositide-binding membrane adaptors. rLC clusters with this group, suggesting lipid binding potential. Clustering was done with ClustalW, using protein sequences corresponding to the RING/FYVE/PHD (IPR013083) domain of each protein.

**c.** Helical wheel diagram of the conserved residues 76 to 91, built using HeliQuest 62. Positively charged residues are in orange, negatively charged residues are in blue, and aliphatic residues are in green. Colour clustering indicates amphipathic properties.

**d.** The resulting α-helix is predicted to have a strong propensity for forming coiled-coils (prediction method: Coils 63.

**Supplementary Figure 3, related to Figures 2 and 3. Physiological and immune consequences of rLC loss-of-function.**

**a.** Deletion of exon 5 from the engineered P[acman]-*GFP::PGRP-LC* locus does not affect PGRP-LC expression in cis. Tested on unchallenged flies (initial insertion lines, carrying a wild-type *PGRP-LC* locus on chromosome III, plus one genomic copy of P[acman]-*GFP::PGRP-LC* at the *attP* landing site 51C on chromosome II). *eGFP* mRNA represents *LC* transcripts from P[acman] constructs only. Data are mean + SEM of n=4, analysed by Student’s t test.

**b.** Reproductive output is not significantly affected in rLC loss-of-function strains. Data shows mean ± SEM of hatched adults per female per 24h egg lay. Each dot is one tube; no significant difference was observed.

**c.** rLCx is necessary and sufficient for resolution of IMD activation quantified as *Dpt* mRNA 24h after infection with *Ecc15*, and rLCx-mediated regulation is dose-dependent. The extent of immune modulation appeared to depend on the gene dose of *rLC* isoforms, since efficiency of regulation correlated with genomic copy number of *rLC* (compare efficiency of rescue constructs in the Δ*LC*E12 background, which has zero genomic *rLC* copies, versus rescue in the Δ*LC*ird7(1) background, which has two genomic *rLC* copies). See also **Fig. 2a,b** for a visual explanation of genotypes.

**d.** Loss of rLC has no impact on PGRP-LE-dependent basal IMD activation by commensals in the midgut of conventionally reared flies, but leads to increased immune activation in whole guts where PGRP-LC contributes to sensing. Loss of rLC does not cause any significant changes in baseline IMD activation in the fat body of conventionally reared but uninfected flies. Adult fat bodies, whole guts or midguts were dissected from unchallenged flies and *Dpt* mRNA was quantified in each tissue (see also Fig. 1d).

**e.** Immune response kinetics to infection with *E.coli* as in **Fig. 3b.**

**f.** rLC overexpressing flies die faster after infection with *Ecc15*. Survival data are pooled from n=4 independent survivals. Log-rank test, p=0.0002. For all other panels, data represent mean ± SEM of n≥3, analysed by 1-way ANOVA and Tukey’s posttest (b, c) or by 2-way ANOVA with Bonferroni posttests (d,e).

**Supplementary Figure 4, related to Figure 4. rLC interacts with the negative regulator Pirk.**

**a.** Overexpression of *rLC* or *pirk* reduces the immune response. See **Fig. 4d** for experimental details.

**b.** Overexpression of *rLC* can compensate for loss of *pirk*. See **Fig. 4e** for experimental details.

**Supplementary Figure 5, related to Figure 5. rLCx overexpression constructs for subcellular localization.**

**a.** Schematic representation of all UAS constructs generated in this study. To test functionality, constructs were over-expressed in the fat body with *c564-Gal4* and their regulatory potential was assessed by measuring *Dpt* mRNA (left panel) 8h after infection with heat-killed *Ecc15*. Right panel shows fold induction for each construct, compared to wild-type. Unfortunately, mutational domain analysis using overexpression of rLC deletion constructs is hampered by the structural resemblance of truncated rLC versions with PGRP-LF. Indeed, any large truncation of the rLC cytosolic tail will leave a short cytosolic stump coupled to a peptidoglycan-binding PGRP ectodomain, strongly resembling PGRP-LF. It is likely that overexpression of such constructs will still regulate IMD activation, albeit by receptor competition mechanistically resembling regulation through PGRP-LF rather than through rLC-specific (or PHD-domain specific) mechanisms. An adequate but time-intensive approach would be to replace wild-type rLC with deletion mutants in situ.

**b.** Subcellular localization of overexpressed FYVE-GFP (left panel, single z slice through cell body) and PLCδ-PH-GFP (right panels, single z slices close to cell surface and through cell body). Scale bars 20 μm.

**b.** Subcellular localization of GFP-rLCx in fixed and DAPI-stained hemocytes from *hml-Gal4>UAS-GFP-rLCx* larvae. Filled arrowheads indicate plasma membrane localization, open arrowheads indicate localization to cell-cell contact points, arrow indicates localization to filopodia. Image is an average intensity z-projection of 10 consecutive z-slices. Scale bar 10 μm.

**c.** Quantification of Western Blot in **Fig. 5f**. GFP-rLCx accumulates when the endocytic machinery is impaired.

**d.** *Tsg101* knock-down leads to enlarged GFP-rLCx-positive vesicles. Compare to **Fig. 5g**.

**e.** *Rab5* knock-down reduces GFP-rLCx-positive vesicle number. Scale bar in d and e 10 μm.

**f.** Quantification of fat body cell size for indicated genotypes. See **Fig. 5j** for analysis details. Only *Rab5* knock-down significantly affects cell size. To correct for this, vesicle numbers were normalized to respective cell area in **Fig. 5j**.

**g.** Effect of RNAi against endocytic components on efficiency of rLC-mediated IMD pathway control (8h after infection with heat-killed *Ecc15*). Fab1 is a PI3P-5 kinase, Vps28 and Tsg101 are components of the ESCRT machinery, UbcD10 is the unique *Drosophila* E1 ligase involved in protein ubiquitination. Data show the reduction in IMD activation occurring after overexpression of rLC in various backgrounds, and represent mean + SEM of n=3 repeats (except for *Rab5* RNAi which caused significant developmental lethality) analysed by one-way ANOVA and Dunnett’s posttests.

## Supplementary Tables

**Supplementary Table 1.** Fly strains for targeted screen of IMD pathway modifiers in **Figure 5**.

|  |  |  |  |
| --- | --- | --- | --- |
| ***c564Gal4;tubGal80TS x …*** | | | **gift from/stock centre accession code** |
| **control** | **-** | *CanS* |  |
| **overexpression** | **LCx** | *w;UAS- GFP-LCx* |  |
| **shiDN** |  | BL5822 |
| **Rab5** | *w;;UAS-Rab5-GFP/TM6c* | Marcos Gonzalez-Gaitán |
| **Rab5CA** |  | BL9773 |
| **Rab5DN** | *w;;UAS-Rab5DN/TM3ser* | Marcos Gonzalez-Gaitán |
| **Rab11** | *w;UAS-Rab11-GFP* | Marcos Gonzalez-Gaitán |
| **Rab11CA** |  | BL9792 |
| **Rab11DN** |  | BL9791 |
| **Rab4** | *w;UAS-Rab4-GFP/CyO* | Marcos Gonzalez-Gaitán |
| **Rab4CA** |  | BL9768 |
| **Rab4DN** |  | BL9770 |
| **FYVE** | *w;UAS-2xFYVE-GFP* | Marcos Gonzalez-Gaitán |
| **RNAi** | **LC** | *w;;UAS-RNAi-rLC(1)* |  |
| **clc** |  | VDRC 106632 |
| **LF** | *w;UAS-RNAi-PGRP-LF* | Julien Royet |
| **shi** |  | VDRC 105971 |
| **Rab5** |  | VDRC 34096 |
| **Rab4** |  | VDRC 24672 |
| **Rab11** |  | VDRC 22198 |
| **Fab1** |  | VDRC 27591 |
| **tsg101** |  | VDRC 23944 |
| **vps25** |  | VDRC 108105 |
| **vps28** |  | VDRC 31894 |

Additional fly strains: *UAS-vps28* was a gift from Prof. Helmut Krämer (UT Southwestern). UAS-GFP-PGRP-PGRP-LCx and UAS-Flag-PGRP-LCx were gifts from the Tang lab 62.

|  |  |
| --- | --- |
|  | **genotypes** |
| Figure 1 | Panels a, b, d: *w1118*.  Panel e: *w;c564-Gal4/+*.  Panel g: *w;c564-Gal4/+* and *w;c564-Gal4/+;+/UAS-rLCx*.  Panel h: *w; c564-Gal4/+* and *w; c564-Gal4/+;+/UAS-rLC* and *w; c564-Gal4/UAS-LE;+/UAS-rLC* or *w; c564-Gal4/UAS-LE* and *w; c564-Gal4/+;UAS-IMD/UAS-rLC* or *w; c564-Gal4/+;UAS-IMD/+.* |
| Figure 2 | Panel d: *w;P[acman]-GFP::LC;LCE12* and *w;P[acman]::GFP-LCΔex5;LCE12*.  Panel e bars on histogram correspond to  1 - *w;;LCE12*.  2 - *w;P[acman]-LCa;LC E12*.  3 - *w;P[acman]-LCaΔex5;LC E12*.  4 - *w;P[acman]-LCx;LC E12*.  5 - *w;P[acman]-LCxΔex5;LC E12*.  6 - *w;P[acman]-LCx/P[acman]-LCa;LC E12*.  7 - *w;P[acman]-LCx/P[acman]-LCaΔex5;LC E12*.  8 - *w;P[acman]-LCxex5/P[acman]-LCaΔ;LC E12*.  9 - *w;P[acman]-LCxΔex5/P[acman]-LCaΔex5;LC E12*. |
| Figure 3 | Panels a, b, c: *w1118* and *w;;LCE12* and *w;P[acman]-GFP::LC;LCE12* and *w;P[acman]-GFP::LCΔex5;LCE12*.  Panel d: *w;c564-Gal4* and *w;c564-Gal4;UAS-rLC* |
| Figure 4 | Panel a: *w;P[acman]-GFP::LC;LCE12* and *w;P[acman]-GFP::LCΔex5;LCE12*  Panel c:  1 - *w;P[acman]-GFP-LC;LCE12*.  2 - *w;P[acman]-GFP-LCΔex5;LCE12*.  3 - *w;PirkEY00723,P[acman]-GFP-LC;LCE12*  4 - *w;PirkEY00723,P[acman]-GFP-LCΔex5;LCE12*.  Panel d:  1 - *w;c564-Gal4/+.*  2 - *w;c564-Gal4/+;+/UAS-rLCx*.  3 - *w;c564-Gal4/UAS-Pirk*.  4 - *w;c564-Gal4/UAS-Pirk;+/UAS-rLCx*.  Panel e:  1 - *w;c564-Gal4*.  2 - *w;c564-Gal4/+;+/UAS-rLCx.*  3 - *w;PirkEY00723,c564-Gal4/PirkEY00723*.  4 - *w;PirkEY00723,c564-Gal4/PirkEY00723;+/UAS-rLCx*.  Panel f:  *w;c564-Gal4/+* and *w;c564-Gal4/UAS-Pirk.* |
| Figure 5 | Panel a: see Supplementary Table 4.  Panels b, d, e, g: *w;c564-Gal4;UAS-GFP-rLCx*.  Panel c: *w;c564-Gal4;UAS-GFP-rLCxΔPHD*.  Panel f and j:  1 - *w;c564-Gal4/+;UAS-GFP-rLCx /+*  2 - *w;c564-Gal4/UAS-Pirk;UAS-GFP-rLCx /+*  3 – *w;c564-Gal4/UAS-vps28;UAS-GFP-rLCx /+*  4 – *w;c564-Gal4/vps28 RNAi KK;UAS-GFP-rLCx /+*  5 – *w;c564-Gal4/vps28 RNAi GD;UAS-GFP-rLCx /+*  6 – *w;c564-Gal4/tsg101 RNAi;UAS-GFP-rLCx /+*  7 – *w;c564-Gal4/Rab5 RNAi;UAS-GFP-rLCx /+*  Panel h: *w;c564-Gal4/UAS-Pirk;UAS-GFP-rLCx /+*  Panel i: *w;c564-Gal4/vps28 RNAi KK;UAS-GFP-rLCx /+* |
| Figure 6 | Panels a, b, c: *w;c564-Gal4,P[acman]-GFP::LC/+;LCE12* and *w; c564-Gal4,P[acman]-GFP::LCΔex5;LCE12* and *w; c564-Gal4,P[acman]-GFP-LC/vps28 RNAi;LCE12* and *w; c564-Gal4,P[acman]-GFP::LCΔex5/vps28 RNAi;LCE12*  Panel d: *w;c564-Gal4/+* and *w;c564-Gal4/+;+/UAS-rLCx* and *w;c564-Gal4/UAS-RNAi-vps28;tub-Gal80ts/+* and *w;c564-Gal4/UAS-RNAi-vps28;tub-Gal80ts/UAS-rLCx.* |
| Supplementary Figure 3 | Panel a: *w;P[acman]-GFP::LC/CyO* and *w;P[acman]-GFP::LCΔex5/CyO.*  Panels b, d, e : *w1118* and *w;;LCE12* and *w;P[acman]-GFP::LC;LCE12* and *w;P[acman]-GFP::LCΔex5;LCE12*.  Panel c: *w;;LCE12* and *w;P[acman]- LCx;LCE12* and *w;P[acman]-LCxΔex5;LCE12*.  Panel f: *w;c564-Gal4* and *w;c564-Gal4;UAS-rLC* |
| Supplementary Figure 4 | see Figure 4 d and e |
| Supplementary Figure 5 | Panel a uses *w;c564-Gal4* to cross to UAS constructs as listed.  Panel b: *w;c564-Gal4/UAS-GFP-myc2x-FYVE* (BL42712) and *w;c564-Gal4/+;UAS-PLCδ-PH/+* (BL 39693)  Panel c: *w;hml-Gal4/+;+/UAS-GFP-rLCx*.  Panels d and g: as in Fig. 5f  Panel e: *w;c564-Gal4/tsg101 RNAi;UAS-GFP-rLCx /+*  Panel f: *w;c564-Gal4/Rab5 RNAi;UAS-GFP-rLCx /+*  Panel h: *w;c564-Gal4;tub-Gal80ts*crossed to *w1118* or *w;UAS-RNAi-Fab1* or *w;UAS-RNAi-vps28* or *w;UAS-RNAi-tsg101* or *w;UAS-RNAi-UbcD10* or w;UAS-RNAi-*Rab5* with or without *UAS-rLCx* on third chromosome. |

**Supplementary Table 2.** *Diptericin* mRNA levels at 8h and 24h after infection, from screen in **Fig. 5a**.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| 8h after infection | | | | 24h after infection | | | |
|  | mean | SD | N |  | mean | SD | N |
| RNAi-LC | 0.380 | 0.231 | 4 | RNAi-LC | 0.053 | 0.015 | 3 |
| UAS-Rab5CA | 0.773 | 0.092 | 3 | UAS-Rab5CA | 0.083 | 0.018 | 3 |
| wt | 1.000 | 0.000 | 5 | UAS-Rab4CA | 0.085 | 0.012 | 3 |
| RNAi-clc | 1.050 | 0.496 | 3 | UAS-Rab11CA | 0.086 | 0.014 | 2 |
| UAS-shiDN | 1.110 | 0.508 | 3 | UAS-Rab4DN | 0.093 | 0.012 | 3 |
| RNAi-vps25 | 1.121 | 0.249 | 4 | UAS-Rab11DN | 0.098 | 0.020 | 2 |
| UAS-Rab4CA | 1.149 | 0.331 | 3 | UAS-Rab4 | 0.117 | 0.029 | 3 |
| RNAi-shi | 1.167 | 0.461 | 4 | UAS-Rab5 | 0.126 | 0.084 | 3 |
| UAS-Rab4DN | 1.207 | 0.079 | 3 | RNAi-Rab11 | 0.131 | 0.054 | 4 |
| UAS-Rab11CA | 1.207 | 0.196 | 3 | RNAi-clc | 0.137 | 0.034 | 3 |
| UAS-Rab11DN | 1.230 | 0.267 | 3 | wt | 0.141 | 0.056 | 4 |
| UAS-FYVE | 1.230 | 0.057 | 2 | RNAi-LF | 0.148 | 0.052 | 4 |
| UAS-Rab4 | 1.362 | 0.159 | 3 | UAS-shiDN | 0.152 | 0.037 | 3 |
| RNAi-vps28 | 1.427 | 0.646 | 4 | UAS-Rab11 | 0.172 | 0.109 | 2 |
| UAS-Rab11 | 1.471 | 0.289 | 3 | UAS-FYVE | 0.195 | 0.006 | 2 |
| RNAi-tsg101 | 1.483 | 0.223 | 3 | RNAi-Fab1 | 0.235 | 0.143 | 3 |
| RNAi-Rab11 | 1.486 | 0.320 | 4 | RNAi-Rab4 | 0.288 | 0.174 | 3 |
| UAS-Rab5 | 1.509 | 0.711 | 3 | RNAi-shi | 0.467 | 0.674 | 4 |
| RNAi-LF | 1.556 | 0.481 | 4 | RNAi-vps25 | 0.568 | 0.689 | 4 |
| RNAi-Rab4 | 1.673 | 0.435 | 4 | RNAi-tsg101 | 0.823 | 0.280 | 3 |
| RNAi-Fab1 | 2.183 | 0.476 | 2 | UAS-Rab5DN | 0.840 | 0.246 | 3 |
| UAS-Rab5DN | 3.628 | 0.750 | 3 | RNAi-Rab5 | 1.266 | 0.748 | 5 |
| RNAi-Rab5 | 3.712 | 1.149 | 4 | RNAi-vps28 | 1.529 | 0.859 | 4 |
| UAS-LCx | 3.807 | 1.457 | 3 | UAS-LCx | 2.471 | 0.385 | 3 |

Values are listed in ascending order per time point, as on graph in **Fig. 5a**.

**Supplementary Table 3.** Cloning strategies and primers

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Template vector** | **Strategy** | **Primer pairs** | **Primer sequences** | **Construct** |
| **P[acman] rescue constructs** | | | | |
| P[acman]-PGRP-LC and P[acman]-GFP-PGRP-LC | Restriction digest, PCR, InFusion | LCf & LCr\_int | LCf 5’-ttttaaggaaaatttaaatcattgca-3’  LCr\_int 5’-ttcttactctccgatcgctttatatgaac-3’  LCf\_int 5’-atcggagagtaagaagaaatactgaatatatagcaagct-3’  LCFLr 5’-ttatctccaatatttaaatctaattaaattaatg-3’  LCiso\_r 5’-tttgaaagaagctagctcatcttt-3’ | P[acman] with exon 5-less PGRP-LC loci |
| LCf\_int & LCFLr |
| P[acman]-PGRP-LCx and P[acman]-PGRP-LCa | LCf & LCr\_int |
| LCf\_int & LCiso\_r |
| **pENTR-D-TOPO entry clones for Gateway/Gal4-UAS expression** | | | | |
| Clone IP15993 | Fusion PCR, TOPO cloning | rLCf | 5’-**CACC**ATGTCGAGGAACACGCTTGAAATTTGCCTAA-3’ | CDS rLCx with or without stop in pENTR-D-TOPO |
| rLCr\_int | 5’-CCTCGATTCGCAACCCTC-3’ |
| Gold clone | rLCf\_int | 5’-GTTGCGAATCGAGGGAAGT-3’ |
| rLCr(with\_stop) | 5’-GTTTGTCATATTTCTTAAGACTCTATTTACTTG-3’ |
| rLCr(no\_stop) | 5’-GATTTCGTGTGACCAGTGC-3’ |
| pENTR-D-TOPO-rLCx | TOPO cloning | rLCf & rLCxrV5 | rLCxrV5 5’-ttattattacgtagaatcgagaccgaggagagggttagggataggcttaccGATTTCGTGTGACCAGTGC-3’ | rLCx with C-terminal V5 |
| pENTR-D-TOPO-rLCx | PCR, TOPO cloning | rLCxΔPHDf (& rLCr(with\_stop) or rLCr(no\_stop)) | 5’-**CACC**ATGAAATGTGACAACTGTCTGAATGG-3’ | CDS of rLCx without PHD domain (aa 1-57) |
| pENTR-D-TOPO-rLCx (with or without stop) | QuikChange | delHEKRLEf & delHEKRLLEr | delHEKRLEf 5’-GAAAAGTCACTGCGGGAAATCCTAGAACAGGGGC-3’  delHEKRLEr 5’-GCCCCTGTTCTAGGATTTCCCGCAGTGACTTTTC-3’ | CDS of rLCx without helix core aa 83-88 |
| pENTR-D-TOPO-rLCx (with or without stop) | Restriction digest (NotI,EcoRV), InFusion cloning | NotI-GFPf & GFPr for GFP; GFP-PHD.f or GFP-ΔPHD-LC.f &  EcoRVrev | NotI-GFPf 5’-gcaggctccgcggccATGgtgagcaagggcg-3’  GFPr 5’-cttgtacagctcgtccat-3’  GFP-PHD.f 5’-acgagctgtacaagtcgaggaacacgctt-3’  GFP-ΔPHD-LC.f 5’-gacgagctgtacaagaaatgtgacaactgtctgaa-3’  EcoRVrev 5’-ctcactataggggatatcagctggatggcaaataatg-3’ | GFP-rLCx and GFP-rLCx ΔPHD and GFP-rLCx-mRFP |
| **pMT-V5-6xHis clones for S2 cell expression** | | | | |
| pENTR-D-TOPO-rLCx | Restriction digest (KpnI, EcoRI) | pMT\_rLCx\_KpnI & pMT\_rLCx\_EcoRInostop | pMT\_rLCx\_KpnI 5’-CGGGGTACCATGTCGAGGAACACGCTTG-3’and pMT\_rLCx\_EcoRInostop 5’-GCAGAATTCGATTTCGTGTGACCAGTGCG-3’ | pMT-rLCx-V5-6xHis |
| pMT-rLCx-V5-6xHis | Restriction digest (KpnI, EcoRI) | rLCxΔPHDf & pMT\_rLCx\_EcoRInostop | See above | pMT-rLCx ΔPHD-V5-6xHis |
| pMT-rLCx-V5-6xHis | Restriction digest (KpnI, EcoRI) | pMT\_PE\_KpnI & pMT\_FYVE\_EcoRI | pMT\_PE\_KpnI 5’-CGGGGTACCATGTCGAGGAACACGCTTG-3’  pMT\_FYVE\_EcoRI 5’-CGAGCAGAATTCCCCATTCAGACAGTTGTCAC-3’ | pMT-PHD-V5-6xHis (aa 1-65) |
| pMT\_PE\_KpnI & pMT\_FYVEHelix\_EcoRI | pMT\_FYVEHelix\_EcoRI 5’-CGAGCAGAATTCcactagcccctgttctagg-3’ | pMT-PHD-Helix-V5-6xHis (aa 1-95) |
|  | | | | |
|  |  |  |  |  |

**Supplementary Table 4.** Sequencing and qPCR primers

|  |  |
| --- | --- |
| f5 (rLC-specific) | ttggagcacaaatgagcaag |
| r9 (x isoform-specific) | CTTGTCGTGAAGGTTCC |
| r12 (y isoform-specific) | GCCTGTGATTTGCCGA |
| r14 (a isoform-specific) | TTCTATATCGTAGGTCT |
| *LCx forward* (genotyping) | GGTGAATGTCGTCCAATCG |
| *LCx reverse* (genotyping) | ATTTCGTGTGACCAGTGCG |
| *LCa forward* (genotyping) | TGGACAACATTGGTGGTGG |
| *LCa reverse* (genotyping) | GACCAATGAGTCCAGTTGGC |
| Exon5 deletion forward# | TGTGACGTTCGGTGATAAGC |
| Exon5 deletion reverse# | CCGAAGAGATTTGTGGTGGT |
| *RpL32* reverse | AAACGCGGTTCTGCATGAG |
| *Dpt* forward | GCTGCGCAATCGCTTCTACT |
| *Dpt* reverse | TGGTGGAGTGGGCTTCATG |
| *rLC* forward (Fig. 1d)\* | Tattgtggtcggtcctgtca |
| *rLC* reverse (Fig. 1d)\* | GTTTTTCATGTTCCCGCAGT |
| *LC* forward (Fig. 1d)\* | TGTGACGTTCGGTGATAAGC |
| *LC* reverse (Fig. 1d)\* | CCGAAGAGATTTGTGGTGGT |
| *LCx* forward (Fig. 1e)\* | TTTGTCCTTTTCTGCCCAAC |
| *LCx* reverse (Fig. 1d,e)\* | atccaagtccgTTTGGTTCA |
| *rLCx* forward (Fig. 1e)\* | ttggagcacaaatgagcaag |
| *Pirk* forward | AAGAGCACGAGCAGGGTAAA |
| *Pirk* reverse | GTCTGGTGCTATTGCCGATT |

# wt fragment 2079bp, Δexon5 fragment 1790bp from gDNA. \* Requires DNase treatment before reverse transcription to prevent amplification from trace gDNA contamination.

## Supplementary Discussion

Internalization of receptor-ligand complexes begs the question whether peptidoglycan is fully degraded in the endo-lysosomal compartment or rather fragmented and released into the cytosol for sensing by PGRP-LE. In mammalian dendritic cells, Gram-negative bacteria access a late endosomal compartment from which muropeptides are exported into the cytosol. Export depends on solute carrier transporters of the SLC15A family, which also promote signalling assemblies of the peptidoglycan sensor NOD2 and the signalling adaptor RIPK2 on endosomal membranes 63. Whether similar assemblies happen downstream of rLCx and involve the functional homologs PGRP-LE and IMD remains to be investigated. Fly homologs within the SLC15A transporter family have been identified in phagolysosomes of *Drosophila* hemocytes 64, but a direct role in immune sensing in vivo has yet to be shown. Relevant to this hypothesis might be that overexpression of rLCx promoted rather than decreased TCT responses in the fat body (see **Fig. 1g**, right panel), although this was not significant. Mechanistic coupling of PGRP-LC-dependent peptidoglycan endocytosis and PGRP-LE-dependent cytosolic sensing of exported peptidoglycan fragments would contribute to explaining the partial cooperation between the two receptors 17, 34, 65.

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