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The Nonaspanins TM9SF2 and TM9SF4 Regulate the Plasma Membrane Localization and Signalling Activity of the Peptidoglycan Recognition Protein PGRP-LC in *Drosophila*

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Key Words

Drosophila · Innate immunity · Innate immune receptor · Innate immune signalling · Imd · Nonaspanins · Plasma membrane receptor · Phagocytosis · Transmembrane 9 proteins

Abstract

Transmembrane 9 (TM9) proteins, or nonaspanins, are a family of proteins conserved throughout evolution and characterized by 9 transmembrane domains. In *Drosophila*, TM9 superfamily protein member 4 (TM9SF4) and its closest parologue, TM9SF2, contribute to phagocytosis of various types of particles, while TM9SF4 displays non-redundant requirement in Gram-negative bacteria engulfment. In addition, the two TM9 proteins control the actin cytoskeleton in larval haemocytes and in *Drosophila* S2 cells. Here, we show that TM9SF4 and TM9SF2 co-immunoprecipitate with the peptidoglycan recognition protein (PGRP)-LC, which triggers the *Drosophila* immune response to bacterial infection. Furthermore, both TM9 proteins co-localize with this receptor in intracellular vesicles and at the plasma membrane in *Drosophila* S2 cells in culture and in the fly fat body. Silencing *TM9SF4* prevents plasma membrane localization of PGRP-LC, whereas silencing *TM9SF2* does not, which may account for the

non-redundant role of TM9SF4 in phagocytosis of Gram-negative bacteria. Finally, we provide a set of data suggesting that TM9 proteins can prevent inappropriate signalling from the unstimulated receptor.

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Introduction

Innate immune receptors detect microorganisms and altered-self signals to activate a set of stress and immune responses that result in clearance of the recognized entities, thus maintaining organism integrity [1]. They include various classes of receptors that may be secreted in body fluids, expressed at the surface of immune cells or in their cytoplasm. Among them, the peptidoglycan recognition proteins (PGRPs) recognize the bacterial cell wall component peptidoglycan. PGRPs are present in most invertebrates and vertebrates, but whereas in mammals they are mainly secreted and directly bactericidal, in flies they are either secreted or transmembrane proteins acting as receptor molecules that transmit a signal to the interior of the cell. In *Drosophila*, the transmembrane protein PGRP-LC is one major actor in the immune response to Gram-negative bacteria that activates the im-

immune deficiency (Imd) pathway in the fat body [2–4]. The signal from PGRP-LC is transmitted via the receptor-bound scaffolding protein Imd [5, 6]. Imd activation induces a signalling cascade resulting in the Relish (NF- κ B like transcription factor)-dependent activation of stress and immune response genes including those encoding antimicrobial peptides (AMPs) such as *Diptericin* (*Dipt*) and *Attacin A* (*AttA*) [7]. In addition, PGRP-LC specifically triggers phagocytosis of Gram-negative bacteria [4], while other phagocytic receptors in *Drosophila*, such as Eater and NimC1, contribute to the engulfment of various types of particles [8, 9].

The transmembrane 9 (TM9) proteins (also known as nonaspanins) are a group of highly conserved proteins with 9 transmembrane domains [10–13]. They include 3 members in the amoeba *Dictyostelium discoideum* (Phg1A, B, C), in the yeast *Saccharomyces cerevisiae* (TMN1–3) and in *Drosophila melanogaster* flies (TM9SF2, TM9SF3, TM9SF4) and 4 members in humans (TM9 superfamily proteins TM9SF1 to TM9SF4) [10, 14–17]. They are found in the endosomal compartments of yeast, *Dictyostelium* and human cells where they possibly contribute to cell migration, vesicular transport, endocytic trafficking and autophagy [11, 12, 17–21]. In *Dictyostelium*, TM9SF4/Phg1A is required for the phagocytosis and killing of bacteria [16, 22, 23]. Moreover, the two *Dictyostelium* TM9 proteins Phg1A and Phg1B synergistically contribute to the expression and/or localization of transmembrane proteins [14, 24]. The function of TM9SF4 in phagocytosis is conserved in human immune cells, where TM9SF4 overexpression contributes to enhanced phagocytic activity of metastatic tumour cells [13, 25], and in *Drosophila* [15, 26]. In *Drosophila*, TM9SF4 mutant macrophages and TM9SF4-silenced S2 cells are notably defective in phagocytosis of *Escherichia coli*-derived particles, a phenotype reminiscent of that of *pgrp-lc*-silenced cells [4, 15]. Also, the closely related protein TM9SF2 acts redundantly with TM9SF4 in the phagocytosis of various types of particles and in the control of the actin cytoskeleton [15].

In this study, we show that TM9SF2 and TM9SF4 are important for the subcellular localization and signalling activity of PGRP-LC in *Drosophila*. These two TM9 proteins interact with PGRP-LC and co-localize with the receptor in both intracellular punctate structures and at the plasma membrane. TM9SF4, but not TM9SF2, is required for PGRP-LC localization at the cell surface, which might account for the specific function of TM9SF4 in internalization of Gram-negative bacteria. Moreover, TM9SF2 and to a lesser extent TM9SF4 mutant flies showed constitutive activation of AMP gene expression, suggesting a negative

regulatory function of these two TM9 proteins on the unstimulated receptor. Since expression of both TM9SF2 and TM9SF4 inhibits PGRP-LC but not Imd signalling activity, mediated by their overexpression in S2 cells, these two TM9 proteins likely directly prevent inappropriate PGRP-LC signalling activity by interacting with the receptor.

Materials and Methods

Fly Strains

Flies were raised at 25°C. The TM9SF4¹ null mutant is described in the report of Bergeret et al. [15]. P[UAS-PGRP-LCx-Flag] (lines 16B and 77A) are described in the report of Schmidt et al. [27]. The P[EP]CG9318^{EP2088} designed in this study as TM9SF2^{EP2088} was obtained from the Exelixis Collection at the Harvard Medical School (<https://drosophila.med.harvard.edu/>). The transgenic lines P[UAS-TM9SF2-GFP] and P[UAS-TM9SF4-GFP] were obtained by germ-line-mediated integration using standard methods. With regard to the FLPout GAL4/UAS method, spontaneous activation of the GAL4 transcription factor without heat shock has been reported by Hennig et al. [28].

Cell Culture

Drosophila S2 cells were maintained in Schneider's medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). Gene inactivation was achieved as described by Clemens et al. [29]. The methodology and primers used are described in the legend to online supplementary figure 2 (for all online suppl. material, see www.karger.com/doi/10.1159/000365112). Activation of the *Attacin* promoter in S2 cells and induction of AMP genes in vivo were monitored as described by Thevenon et al. [30].

DNA Constructs

cDNA clones for TM9SF2 (LD44273) and TM9SF4 (GH02822) were purchased from Drosophila Genomics Resource Center (DGRC). The following primer sets were used for PCR amplification: TM9SF2 forward, 5'-ggggtaccATGATCCTGCTATCCGGA CTT-3', TM9SF2 reverse, 5'-ctagtctagaATCCACCTTGACAAC ACTGTA-3'; TM9SF4 forward, 5'-ggggaattcCACTCCCACACA CCACCAACA-3', and TM9SF4 reverse, 5'-gcgatccGTCGATC TTCACAGCTCCGTA-3'.

Full-length PCR products were cloned into pAc5.1/V5/HisB vector (Invitrogen) or pAc-GFP vectors, allowing for the expression of corresponding tagged proteins. Full-length and truncated pAc-PGRP-LC-V5 constructs and pAc-Imd-V5 constructs were made from the corresponding pMT vectors described in the report of Choe et al. [5].

Immunoprecipitation

Co-immunoprecipitation of GFP-tagged TM9 protein with V5- or Flag-tagged PGRP-LC was performed following standard procedures.

Immunofluorescence Microscopy and Clonal Analysis

Immunofluorescence microscopy of S2 cells and dissected fat body were performed as described by Bergeret et al. [15] and Taillebourg et al. [31], respectively.

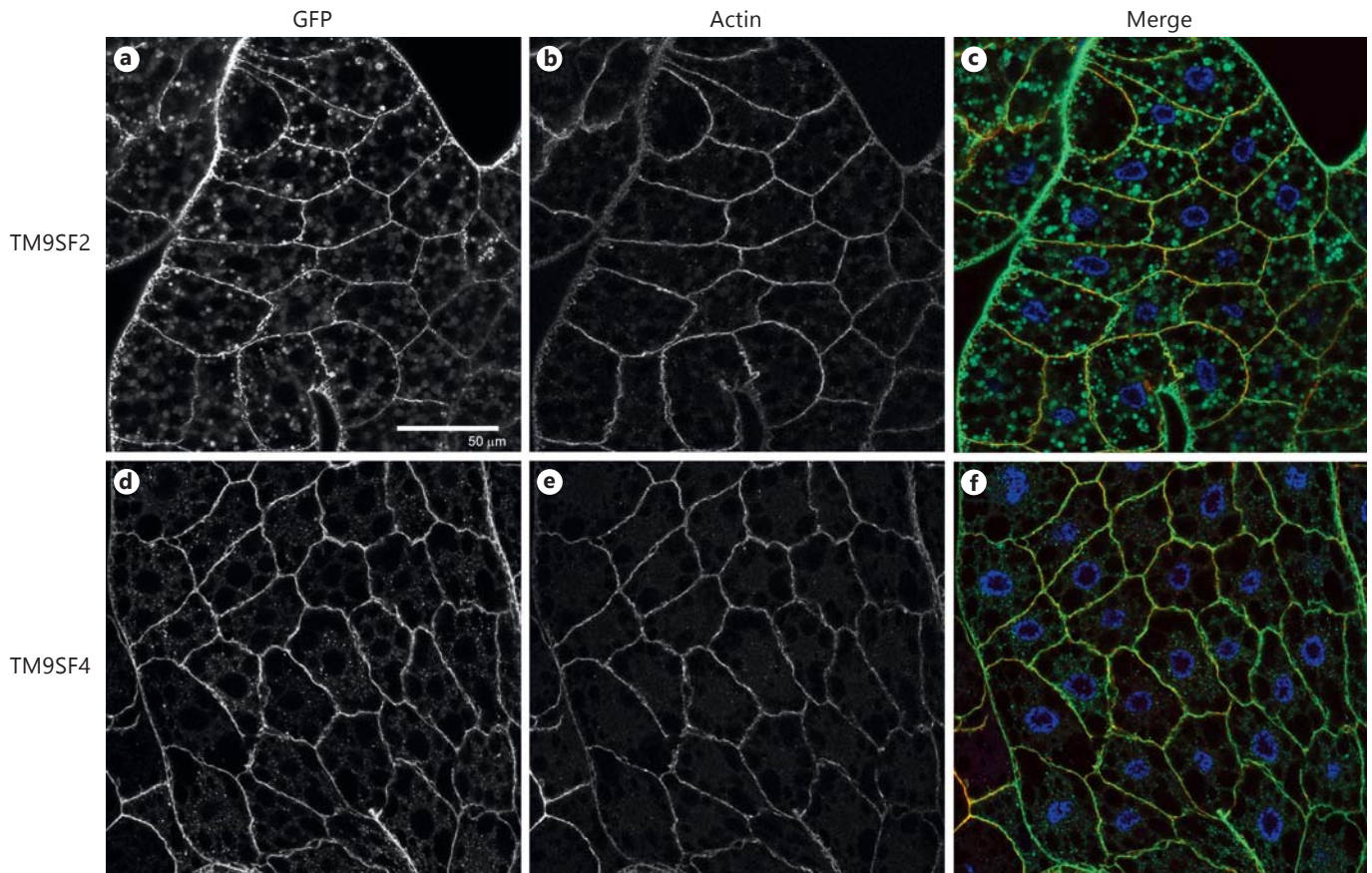


Fig. 1. TM9SF2 and TM9SF4 subcellular localization in fat body cells. Confocal microscopy images of fat bodies from third instar larvae expressing TM9SF2-GFP (**a-c**) or TM9SF4-GFP (**d-f**) in the fat body cells through the CgGal4 driver line. **a, d** TM9SF-GFP fusion proteins are visualized through GFP fluorescence. **b, e** The

F-actin network was labelled with Texas red-phalloidin. **c, f** Merge images in which GFP is green, the actin network is red and the nuclei are stained with Hoechst in blue. Genotypes: *w¹¹¹⁸;CgGal4/+;UAS-TM9SF2-GFP* (**a-c**), *w¹¹¹⁸;CgGal4/+;UAS-TM9SF4-GFP* (**d-f**). Scale bar = 50 μ m.

Fluorescence-Activated Cell Sorting Analysis

S2 cells were transfected with pAc-PGRP-LC-V5 and double-stranded RNA (ds*TM9SF4*, ds*GFP* or ds*PGRP-LC*). Then, 48 h after transfection, cells were washed in PBS buffer containing 3% fetal calf serum and 0.05% NaN₃ and incubated for 30 min at 4°C with anti-V5 antibody (Invitrogen) then with secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). Samples of 20,000 cells suspended in PBS buffer with 0.05% NaN₃ were analysed using a FACSCalibur flow cytometer (Becton Dickinson).

Results

TM9SF2 and TM9SF4 Display Similar Subcellular Localization to PGRP-LC

To examine the expression and the subcellular localization of TM9SF proteins in vivo, we constructed transgenic lines expressing TM9SF2-GFP or TM9SF4-GFP fu-

sion proteins. When expressed specifically in the fat body through the specific CgGal4 driver line, both TM9-GFP fusion proteins were present mainly at the plasma membrane and to a lesser extent in cytoplasmic punctate structures (fig. 1). We used the FLPout GAL4/UAS method [32] to induce the expression of the Flag-tagged PGRP-LC (PGRP-LC-Flag) [27] concomitantly with either TM9SF2-GFP or TM9SF4-GFP specifically in clones of fat body cells. PGRP-LC-Flag, TM9SF2-GFP or TM9SF4-GFP fusion proteins were all observed both at the cell surface and intracellularly in these clones (online suppl. fig. 1). Partial co-localization of PGRP-LC with TM9SF2 and TM9SF4 was observed mainly at the plasma membrane (online suppl. fig. 1). We then co-expressed fluorescent TM9SF-GFP fusion proteins (TM9SF2-GFP or TM9SF4-GFP) with a PGRP-LC protein tagged with a V5 epitope (PGRP-LC-V5) [5] in cultured *Drosophila* S2

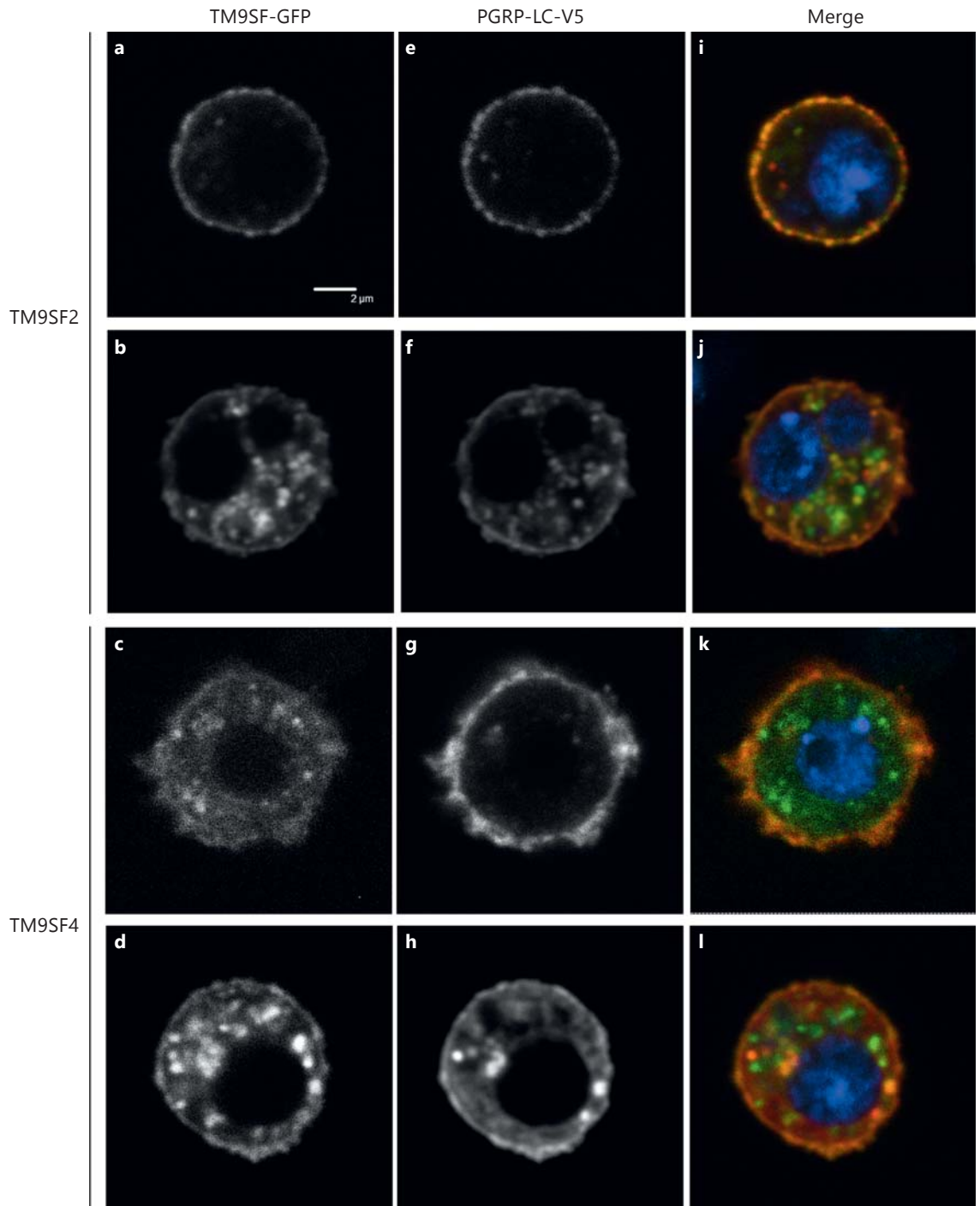


Fig. 2. TM9SF2 and TM9SF4 co-localize with PGRP-LC in S2 cultured cells. Confocal microscopy images of fixed *Drosophila* S2 cells expressing either TM9SF2-GFP (**a, b, e, f, i, j**) or TM9SF4-GFP (**c, d, g, h, k, l**) together with PGRP-LC-V5. **a-d** TM9SF-GFP fusion proteins are visualized through GFP autofluorescence.

e-h PGRP-LC-V5 tagged protein is visualized through immunofluorescence staining with an anti-V5 antibody. **i-l** Merge images with GFP in green, PGRP-LC-V5 in red and DNA stained with Hoechst in blue. Scale bar = 2 μ m.

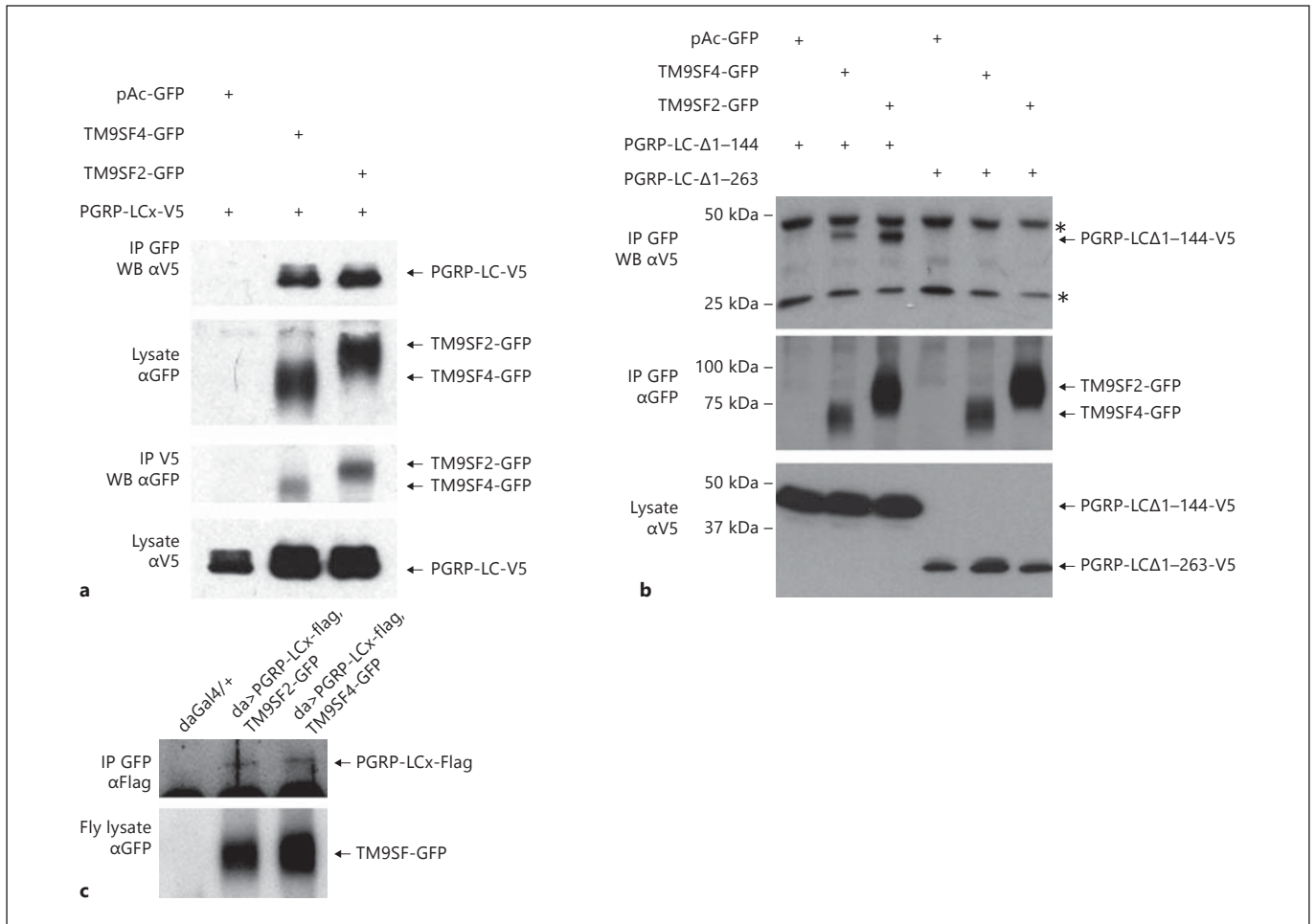


Fig. 3. TM9SF2 and TM9SF4 interact with PGRP-LC. **a, b** *Drosophila* S2 cells were transfected with pAc-PGRP-LC-V5 expressing full (**a**) or truncated (**b**) forms of the isoform PGRP-LCx and either pAc-TM9SF4-GFP or pAc-TM9SF2-GFP, as indicated. Cell extracts were immunoprecipitated (IP) with either anti-GFP (α GFP) or anti-V5 (α V5) antibodies, separated on SDS-PAGE and Western blotted (WB) with α V5 or α GFP, as indicated. **b** Recombinant protein expression was assessed by Western blot of cell lysates with α GFP or α V5. Asterisks indicate unspecific bands. **c** Extracts of

flies expressing the Flag-tagged PGRP-LCx isoform with either TM9SF2-GFP or TM9SF4-GFP were immunoprecipitated with an anti-GFP antibody. Co-immunoprecipitated product was revealed on a Western blot with anti-Flag (α Flag) antibody (upper panel). Expression of TM9-GFP fusion proteins was assessed by Western blotting of fly lysates with anti-GFP antibody (α GFP; bottom panel). Genotypes are as follows: $w^{1118};daGal4/+$ (daughterless-gal4), $w^{1118};daGal4/+;PGRP-LCx-Flag\#16B, TM9SF2-GFP$ and $w^{1118};daGal4/+;PGRP-LCx-Flag\#16B, TM9SF4-GFP$.

cells. TM9SF2-GFP and TM9SF4-GFP proteins localized both in intracellular round structures and at the cell membrane (fig. 2a–d). Similar staining was observed for PGRP-LC-V5, with about half of the cells showing mainly membrane-bound PGRP-LC (fig. 2e–g) and the second half presenting both membrane-bound and cytosolic staining (fig. 2f, h). These experiments revealed a close co-localization of each TM9SF protein with PGRP-LC (fig. 2i–l). We conclude from these observations that TM9SF2 and TM9SF4 co-localize with PGRP-LC in both intracellular and plasma membrane compartments.

TM9SF2 and TM9SF4 Interact with PGRP-LC

To assess whether TM9SF4 and TM9SF2 interact with PGRP-LC, S2 cells were co-transfected with either TM9SF2-GFP or TM9SF4-GFP and PGRP-LC-V5. Cell lysates were immunoprecipitated with anti-GFP or anti-V5 antibodies, and the immunoprecipitated product was analysed by Western blotting with anti-V5 or anti-GFP antibodies, respectively. In both cases, we observed co-immunoprecipitation of each of the TM9 proteins with PGRP-LC (fig. 3a). Deletion of the entire PGRP-LC cytoplasmic domain (PGRP-LC- Δ 1–263) disrupted the inter-

action between PGRP-LC and the TM9 proteins, whereas deletion of only half of the cytoplasmic domain (PGRP-LC- Δ 1–144) still allowed the interaction with both TM9 proteins (fig. 3b). Thus, amino acids 144–263 of the PGRP-LC cytoplasmic domain are required for the interaction of PGRP-LC with TM9SF2 and TM9SF4. To investigate if this interaction occurs *in vivo*, we expressed each TM9SF-GFP protein in combination with PGRP-LC-Flag in transgenic flies. Indeed, PGRP-LC co-immunoprecipitated from fly extracts with both TM9SF-GFP proteins (fig. 3c). These experiments thus demonstrate that TM9 proteins interact with PGRP-LC.

TM9SF4 Is Required for PGRP-LC Localization at the Plasma Membrane

To test whether TM9SF2 and/or TM9SF4 regulate PGRP-LC subcellular localization, we depleted S2 cells of either *TM9SF2* or *TM9SF4* by dsRNA interference-mediated gene silencing [15] (online suppl. fig. 2). Silencing *TM9SF2* had no significant effect on the subcellular localization of PGRP-LC-V5, whereas silencing *TM9SF4* provoked a significant loss of PGRP-LC-V5 at the cell surface (fig. 4a–c). Co-silencing both *TM9SF2* and *TM9SF4* resulted in a similar loss of PGRP-LC-V5 from the cell membrane to silencing *TM9SF4* alone, suggesting no significant contribution of TM9SF2 to PGRP-LC membrane localization or stabilization (fig. 4d). Monitoring cells displaying PGRP-LC at the cell membrane or not confirmed that silencing *TM9SF4* resulted in a strong reduction in the proportion of cells displaying PGRP-LC-V5 at the plasma membrane or both at the plasma membrane and in the cytoplasm compared to cells displaying only cytoplasmic PGRP-LC, namely from 75% in control cells to 15% in the silenced cells (fig. 4e). By comparison, expression of another phagocytosis receptor, NimC1 [9], was unchanged in cells in which *TM9SF2*, *TM9SF4* or both were silenced (online suppl. fig. 3). To quantify the amount of plasma membrane-associated PGRP-LC, we expressed a PGRP-LC-V5 construct in which the V5 tag was fused to the C terminus of the protein, which is on the extracellular face of the plasma membrane, and we stained the cells without permeabilizing them. Hence, only the plasma membrane PGRP-LC-V5 was accessible to the primary antibody. FACS quantification of stained cells revealed a significant reduction in the proportion of fluorescent cells (63%) in *TM9SF4*-silenced cells compared to control cells (93%; fig. 4f). Negative control cells treated with a PGRP-LC-silencing dsRNA showed, as expected, a drastic reduction of fluorescent cells (14%; fig. 4f). Loss of PGRP-LC staining at the cell surface was not the result of a decrease in the total amount of cel-

lular PGRP-LC, as observed on a Western blot of cell lysate (fig. 4g). These data indicate that TM9SF4 is required for PGRP-LC localization at the plasma membrane.

TM9SF2 and TM9SF4 Prevent Inappropriate PGRP-LC Signalling

Since PGRP-LC localization is perturbed in *TM9SF4* mutant cells, activation of the Imd pathway could be affected when TM9SF4 is absent. However, *TM9SF4*¹ null mutant flies (fig. 5a) displayed no major changes in AMP gene induction in response to bacterial infection [15] (online suppl. fig. 4). Similarly, a P element insertion located in the *TM9SF2* coding sequence that reduced *TM9SF2* expression by more than 80% (fig. 5b) did not show a major change in *Dipt* or *AttA* expression in response to *E. coli* infection (online suppl. fig. 4). However, in non-infected flies we show here that *TM9SF2* mutant flies displayed a 30- and 40-fold higher level of *Dipt* and *AttA* mRNA gene expression, while *TM9SF4* mutant flies displayed a modest but significant increase of 3.6- and 4.2-fold compared to control flies (fig. 5c, d). We then used dsRNA-mediated gene silencing in S2 cells and measured the activation of the Imd pathway by using an *AttA-Luc* [33] reporter gene in the presence of heat-killed *E. coli* in the culture media. Actually, silencing *TM9SF4* or *TM9SF2* or both genes had no significant effect on activation of the *AttA-Luc* reporter gene in response to *E. coli* (fig. 5e). By contrast, in S2 cells transfected with a pAcPGRP-LC expression construct, silencing *TM9SF2* or *TM9SF4* enhanced the PGRP-LC-mediated *AttA-Luc* gene induction, with a particularly strong increase in the case of *TM9SF2*-silenced cells (fig. 5f). Moreover, co-expressing *TM9SF2* or *TM9SF4* with PGRP-LC strongly inhibited the induction of *AttA-Luc* induced by PGRP-LC (fig. 5g) but not that induced by Imd (fig. 5h). This indicates that TM9SF2 and TM9SF4 exert a negative regulatory function at the level of PGRP-LC.

Finally, expressing PGRP-LC-Flag [27] specifically in the larval fat body was lethal when flies were raised at 25°C, and strongly perturbed the structure of the tissue by provoking cell rounding and detachment when flies were raised at 18°C to reduce the efficiency of the UAS-Gal4 expression system (online suppl. fig. 5A, B). This phenotype is in accordance with a previous report indicating that PGRP-LC overexpression induces apoptosis [34]. Co-expression of TM9SF2 and TM9SF4 together with PGRP-LC in the fat body partially rescued the fat body defects induced by PGRP-LC overexpression (online suppl. fig. 5C, D), demonstrating that both TM9SF2 and TM9SF4 counteract the deleterious activity of overexpressed PGRP-LC *in vivo*.

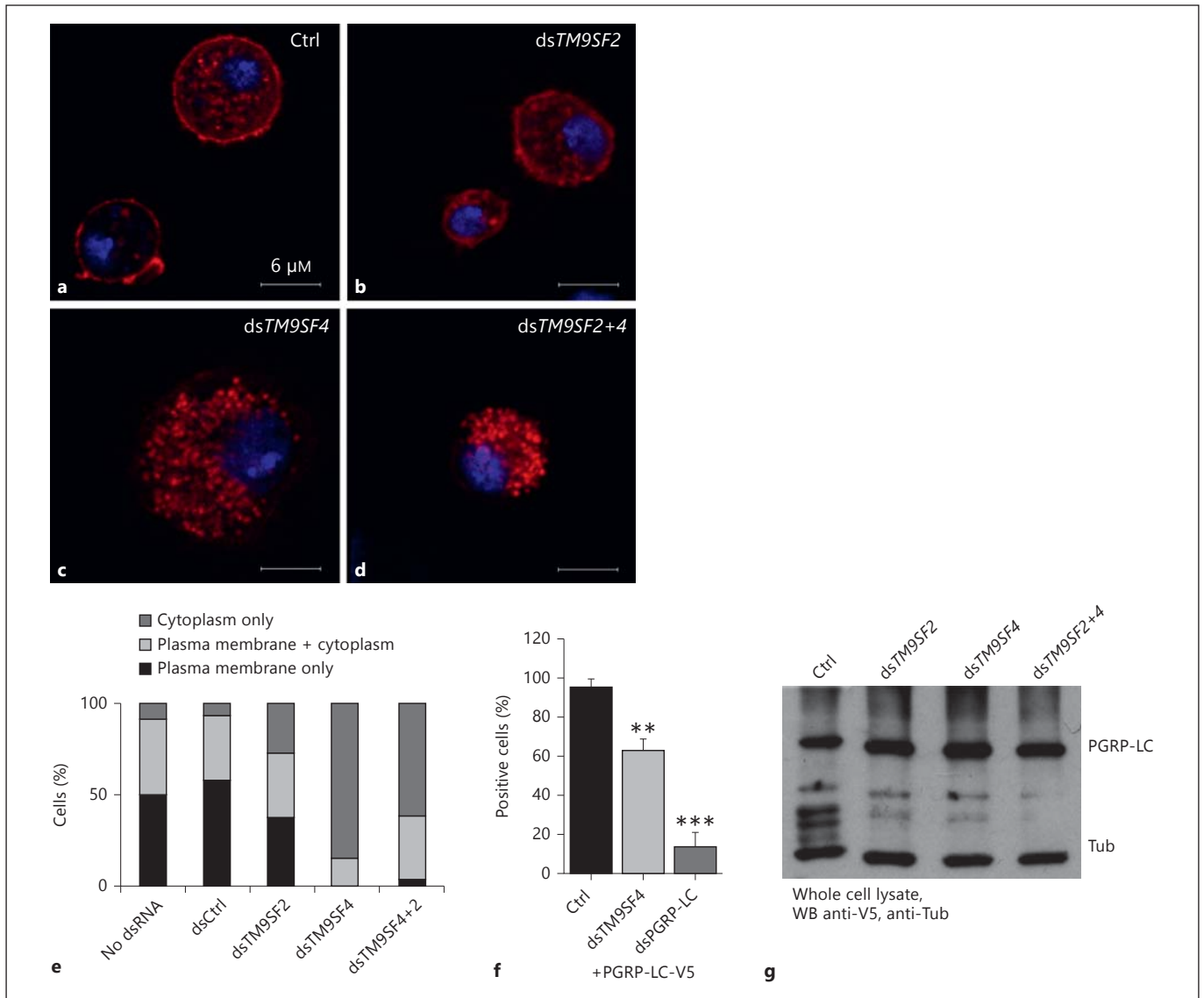


Fig. 4. TM9SF4 is required for PGRP-LC localization at the plasma membrane. **a–d** *Drosophila* S2 cells expressing PGRP-LC-V5 together with a control dsRNA (**a**) or a dsRNA to silence *TM9SF2* (**b**) or *TM9SF4* (**c**) or the two dsRNAs (**d**). PGRP-LC-V5 was stained with an anti-V5 antibody (red). Nuclei were stained with Hoechst (blue). **e** The percentage of cells expressing PGRP-LC exclusively at the cell membrane, both at the cell membrane and in the cytoplasm or only in the cytoplasm was determined from the observation of a hundred cells per condition. **f** FACS analysis of

the percentage of positive cells expressing PGRP-LC-V5 on the surface, as determined by staining unpermeabilized cells with α V5 in either control or *TM9SF4*- or *pgrp-lc*-silenced cells. FACS analysis was performed on 20,000 cells. Histograms show the percentage of fluorescent cells relative to control cells. Error bars indicate the standard deviation between 3 independent experiments. ** $p < 0.02$, *** $p < 0.01$: significant differences (t test). **g** Lysates of cells as in **a–d** were analysed by Western blotting with anti-V5 and anti-tubulin (Tub) antibodies. Ctrl = Control.

Discussion

Our results demonstrate the differential and common requirement of TM9SF2 and TM9SF4 in the control of PGRP-LC subcellular localization and signalling activity. Both TM9SF2 and TM9SF4 co-localize and interact with

PGRP-LC, a receptor which can mediate Gram-negative internalization in S2 cells [4, 15]. As we previously showed that TM9SF2 and TM9SF4 synergistically promote bacterial phagocytosis and actin cytoskeleton network reorganization [15], they possibly contribute to the internalization of Gram-negative bacteria by PGRP-LC through

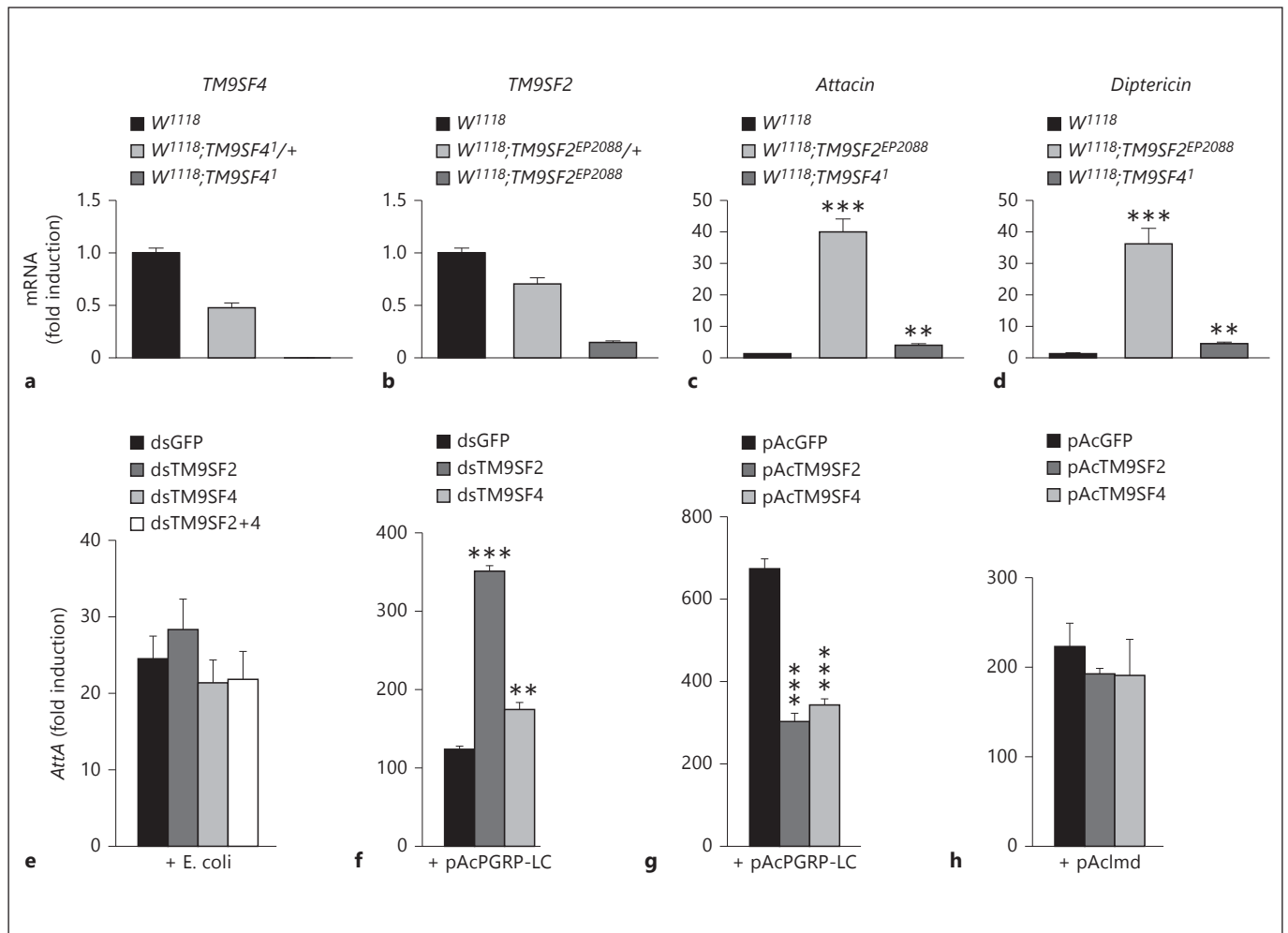


Fig. 5. TM9SF2 and TM9SF4 down-regulate unstimulated PGRP-LC. **a-d** Quantitative analysis of mRNA levels of *TM9SF4* (**a**), *TM9SF2* (**b**) and the AMP genes *AttA* (**c**) and *Dipt* (**d**) by real-time quantitative PCR of fly mRNA in control (*w¹¹¹⁸*), heterozygous (*w¹¹¹⁸;TM9SF4^{1/+}* or *w¹¹¹⁸;TM9SF2^{EP2088/+}*) or homozygous mutant flies (*w¹¹¹⁸;TM9SF4¹* or *w¹¹¹⁸;TM9SF2^{EP2088}*) as indicated. **e-h** S2 cells were co-transfected with the *pAttA-Luc-Firefly* reporter gene and *pAc-Luc-Renilla* normalizer to monitor activation of the Imd pathway. Histograms represent the fold induction compared

to control cells transfected with empty pAc vector. **e, f** Imd pathway signalling was induced by heat-killed *E. coli* (**e**) or by expressing *pAc-PGRP-LC-V5* (**f**) in control cells (dsGFP) or *TM9SF2* (dsTM9SF2)- and/or *TM9SF4* (dsTM9SF4)-silenced cells. **g, h** S2 cells were transfected with *pAc-PGRP-LC-V5* (**g**) or *pAc-Imd-V5* (**h**) and either *pAc-GFP* (control), *pAc-TM9SF2-GFP* or *pAc-TM9SF4-GFP* constructs as indicated. Error bars indicate the standard deviation between technical triplicates. ** $p < 0.005$, *** $p < 0.0001$: significant differences (t test).

their direct interaction with this receptor, thus coupling bacterial recognition with cytoskeleton reorganization. In addition, TM9SF4 is non-redundantly required for PGRP-LC localization at the cell membrane; specific loss of PGRP-LC at the plasma membrane in *TM9SF4* knock-down mutant cells thus likely accounts for the previously observed non-redundant function of TM9SF4 in Gram-negative bacterial internalization [15].

PGRP-LC is essentially known for its signalling activity, which enables Imd pathway activation in various cell

types and tissues. The observation that PGRP-LC is weakly present at the cell membrane in *TM9SF4*-silenced cells while these cells still retain normal signalling activity indicates that in the absence of TM9SF4, PGRP-LC may signal from the cytoplasmic compartment. Alternatively, the amount of remaining PGRP-LC at the plasma cell membrane may be sufficient to ensure signalling.

Similarly to S2 cells, we could not detect major changes in *AttA* and *Dipt* antimicrobial gene expression in either *TM9SF4* or *TM9SF2* mutant flies. This suggests that

TM9 proteins are not required for the up- or down-regulation of PGRP-LC activation by peptidoglycan. Alternatively, redundancy between TM9 proteins may mask their function. Indeed, we observed that the *TM9SF2/TM9SF4* double mutant is lethal at the embryonic stage, while *TM9SF4^l* null mutant flies are normally viable and *TM9SF2^{EP2088}* are poorly viable. Finally, we still have no information on the putative role of the third TM9 protein, TM9SF3 (CG10590). The question of the contribution of TM9 proteins to Imd pathway activation in response to infection should therefore be addressed in the future with conditional double or triple knock-out mutant flies.

Interestingly, *TM9SF2* mutant flies and, to a lesser extent, *TM9SF4* null mutant flies displayed moderate but significant constitutive activation of *AttA* and *Dipt* genes – two main targets of the Imd pathway. Therefore, TM9 proteins may act as negative regulators of the unstimulated PGRP-LC receptor, preventing inappropriate activation of the Imd pathway in the absence of infection. Indeed, complementary experiments in S2 cells showed that both TM9SF2 and TM9SF4 counteract PGRP-LC signalling activity when the receptor was overexpressed in either S2 cells or fly tissues; TM9 protein expression notably rescued the fat body phenotypic defects induced by PGRP-LC overexpression and inhibited PGRP-LC-dependent (but not Imd-dependent) AMP gene activation in S2 cells. Moreover, silencing either *TM9SF2* or *TM9SF4* greatly enhanced the activation of the Imd pathway mediated by PGRP-LC overexpression in S2 cells. In these conditions, PGRP-LC likely mediates signal activation by auto-activation through self-oligomerization, independently of peptidoglycan binding and stimulation.

Thus, our results suggest that TM9SF2 and TM9SF4 prevent inappropriate PGRP-LC signalling in the cytoplasm and/or at the plasma membrane by counteracting auto-activation of the receptor that possibly occurs through self-oligomerization.

In conclusion, our results together with previous studies show that this widely conserved family of TM9 proteins plays an essential function in membrane receptor trafficking and regulation of signalling activity, putatively exerting a chaperone function both in the intracytoplasmic and the cell plasma membrane compartment. They also demonstrate the differential requirement of these two TM9 proteins; TM9SF2 seems to be mostly required to prevent inappropriate signalling from the unstimulated receptor while TM9SF4 has a major role in PGRP-LC subcellular localization at the plasma membrane.

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