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TM9SF4 is required for *Drosophila* cellular immunity via cell adhesion and phagocytosis

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Summary

Nonaspanins are characterised by a large N-terminal extracellular domain and nine putative transmembrane domains. This evolutionarily conserved family comprises three members in *Dictyostelium discoideum* (Phg1A, Phg1B and Phg1C) and *Drosophila melanogaster*, and four in mammals (TM9SF1-TM9SF4), the function of which is essentially unknown. Genetic studies in *Dictyostelium* demonstrated that Phg1A is required for cell adhesion and phagocytosis. We created *Phg1A/TM9SF4*-null mutant flies and showed that they were sensitive to pathogenic Gram-negative, but not Gram-positive, bacteria. This increased sensitivity was not due to impaired Toll or Imd signalling, but rather to a defective cellular immune response. *TM9SF4*-null larval macrophages phagocytosed Gram-negative *E. coli* inefficiently, although Gram-positive *S. aureus* were phagocytosed normally. Mutant larvae also had a decreased wasp egg encapsulation rate, a process requiring haemocyte-dependent adhesion to parasitoids. Defective cellular immunity was coupled to morphological and adhesion defects in mutant larval haemocytes, which had an abnormal actin cytoskeleton. TM9SF4, and its closest paralogue TM9SF2, were both required for bacterial internalisation in S2 cells, where they displayed partial redundancy. Our study highlights the contribution of phagocytes to host defence in an organism possessing a complex innate immune response and suggests an evolutionarily conserved function of TM9SF4 in eukaryotic phagocytes.

Key words: Innate immunity, Macrophages, Adhesion, Phagocytosis, Cytoskeleton, Nonaspanin

Introduction

Pathogen engulfment by host phagocytic cells and their subsequent killing in the phagocytic vacuole are major events for bacterial clearance and contribute to a robust innate immunity in most multicellular organisms (Beutler, 2004). In mammals, phagocytosis is mainly achieved by neutrophils, monocytes and macrophages. These cells engage additional host defences by inducing an inflammatory response, mainly through the synthesis of Rel/NF-κB-dependent cytokines.

The unicellular phagocytic amoeba *Dictyostelium discoideum* has been used as a model organism to study and discover new genes implicated in phagocytosis (Cornillon et al., 2000). A genetic screen identified *PHG1A*, alteration of which causes a marked decrease in *Dictyostelium* adhesion to certain substrates and a strong impairment in bacterial phagocytosis and killing (Benghezal et al., 2003; Benghezal et al., 2006; Cornillon et al., 2000). *PHG1A* encodes a member of the TM9 protein family (also known as nonaspanins or TM9SF) characterised by the presence of nine transmembrane domains, and a high degree of evolutionary conservation (Chluba-de Tapia et al., 1997; Schimmoller et al., 1998). TM9 proteins were found in endosomal or lysosomal fractions in yeast (Singer-Kruger et al., 1993), *Dictyostelium* (Benghezal et al., 2003) and human cells (Bagshaw et al., 2005; Diaz et al., 1997; Schimmoller et al., 1998) where they might participate in vesicular transport (Diaz et al., 1997). More recently, TM9 proteins were implicated in lysosomal secretion in *Dictyostelium* and cell signalling in both *Dictyostelium* and yeast (Froquet et al., 2008). However, no mutant or functional data are available at the level of a metazoan organism possessing a complex immune response.

Thanks to its sophisticated immune system *Drosophila* represents a powerful host model for evaluating the contribution of phagocytic cells to host innate immunity. *Drosophila* has specialised circulating phagocytic cells derived from the haemocytic blood cell lineage (Crozatier and Meister, 2007; Williams, 2007). Plasmatocytes are the most abundant type of circulating haemocytes and represent the primary macrophages required for bacterial phagocytosis (Avet-Rochex et al., 2005; Brennan et al., 2007; Kocks et al., 2005). Upon infection by parasites, such as wasp eggs, plasmatocytes can recognise and attach to the invader. Plasmatocytes then signal to the lymph gland to promote the differentiation of another kind of haemocyte called lamellocytes (Lanot et al., 2001). These large cells attach to the plasmatocyte layer and form a hermetic capsule around the invader (Russo et al., 1996; Williams et al., 2005). In insects, plasmatocyte adhesion to wasp eggs is a crucial step for encapsulation and strongly depends on cell surface molecules such as integrins (Irving et al., 2005; Zhuang et al., 2007). Besides the cellular immune response, *Drosophila* possesses a sophisticated humoral response, which includes the synthesis of antimicrobial peptides by fat body cells under the control of the two

conserved NF-κB signalling pathways Toll and Imd (immune deficiency) (Lemaitre and Hoffmann, 2007; Ferradon et al., 2007). The Imd pathway is strongly stimulated by Gram-negative bacteria resulting in the activation of the NF-κB transcription factor Relish, which in turn activates the transcription of numerous genes, in particular the antimicrobial-peptide-encoding genes *Attacin* (*Att*), *Diptericin* (*Dipt*) and *Drosocin* (*Dro*) (Georgel et al., 1993; Lemaitre et al., 1995; Levashina et al., 1998). The Toll pathway is mainly activated by fungi or Gram-positive bacteria resulting in the expression of another set of antimicrobial peptide genes including *Drosomycin* (*Drs*) (Lemaitre et al., 1995).

In this paper, we describe the molecular characterisation of the three *Drosophila* nonaspanins and the function in innate immunity of *Phg1A/TM9SF4*, the *Drosophila* orthologue of *Dictyostelium* Phg1A and human *TM9SF4*. We created *TM9SF4*-null mutant flies and showed that their sensitivity to Gram-negative bacteria was correlated to impaired haemocyte-dependent phagocytosis. *TM9SF4* mutant larvae failed to properly encapsulate eggs from the avirulent wasp strain *Leptopilina boulardi* G486. These phenotypes are coupled to abnormal adhesion and defective cytoskeleton reorganisation in mutant plasmatocytes. Both *TM9SF4* and *TM9SF2*, its closest paralogue, were required for phagocytosis in S2 cells. Our study shows that TM9SF4 function in cell adhesion and bacterial engulfment might result from defective cytoskeleton control and that TM9SF4 plays a crucial role in cellular immunity to ensure host defence against infections.

Results

Drosophila TM9 proteins

We identified three TM9 genes in the *Drosophila* genome: CG7364 (chromosome 2L-34D), CG9318 (2L-38E) and CG10590 (3L-64D). Nonaspanins are divided into two subgroups presenting differential characteristic features in their N-terminal amino acid sequence (Benghezal et al., 2006; Sugasawa et al., 2001). Subgroup I is characterised by a shorter hydrophilic N-terminal sequence and a characteristic motif at position 50 (VGPYxNxQETY) whereas subgroup II contains a longer N-terminal domain (~280 amino acids) and a conserved sequence immediately after the signal peptide [FY(V/L)PG(V/L)AP] (Benghezal et al., 2003). Phylogenetic analysis revealed that CG10590 (*Drosophila TM9SF3*) belongs to subgroup I, along with *Dictyostelium* Phg1B and human TM9SF1 and TM9SF3. CG9318 (*Drosophila TM9SF2*) and CG7364 (*Drosophila TM9SF4*) share 48% identity in their amino acid sequence and belong to subgroup II, together with *Dictyostelium* Phg1A and human TM9SF2 and TM9SF4 (Fig. 1). The *Drosophila* TM9SF4 protein contains the FYVPGVAP consensus sequence at amino acid position 25 followed by nine conserved transmembrane domains; it is the closest homologue of *Dictyostelium* Phg1A and as such, it was previously referred to as DPhg1A (Benghezal et al., 2006). *Drosophila* TM9SF4 exhibits 46% identity with *Dictyostelium* Phg1A and 65% identity with human TM9SF4. This high degree of conservation suggests that the corresponding genes might share similar functions. TM9SF4 refers to *Drosophila* TM9SF4/Phg1A in this study.

Creating *TM9SF4*-knockout mutant flies

We created a *Drosophila* null mutant *TM9SF4*^{1} by remobilising the P{lacW}CG7364^{k07245} transposon inserted into the *TM9SF4* transcription unit (Fig. 2A). A 1.4 kb deletion was characterised by PCR analysis, which removed a portion of *TM9SF4* coding sequences including the transcription start site and the N-terminus

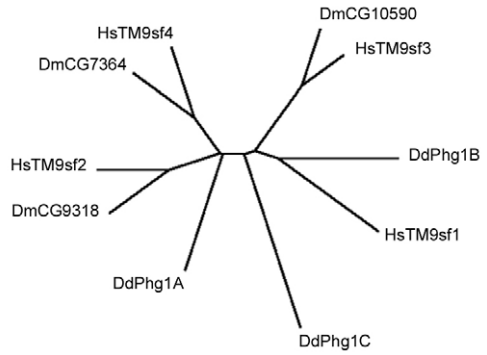

Fig. 1. The nonaspanin family in *Drosophila melanogaster*. Similarity tree of TM9 proteins in *D. melanogaster* (Dm; CG7364, CG9318, CG10590) compared with human (Hs; TM9SF1-TM9SF4) and *D. discoideum* (Dd; PHG1A, PHG1B, PHG1C).

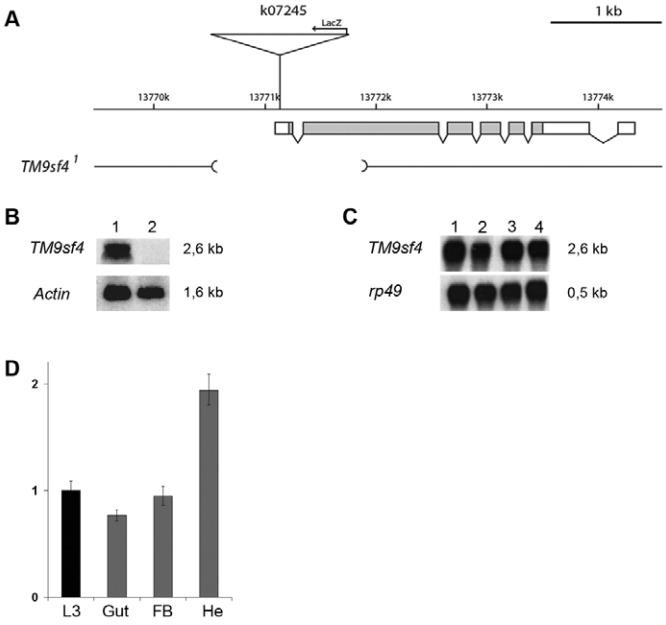

Fig. 2. *TM9SF4* gene map. (A) The *TM9SF4* gene produces one transcript of 2.6 kb which contains one coding sequence (coloured in grey). The insertion point for {lacW}CG7364^{k07245} is 94 bp upstream of the ATG translation start. One 1.4 kb deletion (*TM9SF4*^{1}) was recovered encompassing the transcription start site and the N-terminal part of the corresponding protein. (B) The deletion creates a null allele as visualised by northern analysis of *TM9SF4* transcripts in control *w*^{1118} (lane 1) compared with mutant *TM9SF4*^{1} (lane 2) flies. (C) Developmental northern blot. Lane 1, embryos; lane 2, third instar larvae; lane 3, pupae; lane 4, adult. (D) *TM9SF4* transcripts were quantified by real-time PCR from total RNAs extracted from either the whole third instar larvae (L3), the gut (Gut), the fat body (FB) or the larval circulating plasmatocytes (He). Results are mean ± s.d.

(Fig. 2A). Sequence analysis indicated that the surrounding genes were not affected by this deletion (data not shown). A revertant strain (Rev45) showed wild-type sequence following mobilisation and was selected as a control strain possessing similar genetic background as *TM9SF4*^{1}. Northern blot analysis showed that no transcripts were detectable in *TM9SF4*^{1} adult flies compared with the parental strain *w*^{1118} (Fig. 2B) or Rev45 flies (not shown). *TM9SF4* is expressed at all developmental stages (Fig. 2C);

however, *TM9SF4*^{1} flies are normally viable and fertile suggesting redundancy with other nonaspanins during development. Quantitative real-time PCR allowed for the detection of *TM9SF4* transcripts from dissected third instar larval tissues and indicated that *TM9SF4* is expressed in the main immune organs such as circulating haemocytes, fat body cells and larval gut, with the maximum expression level in haemocytes compared with the whole larvae (Fig. 2D).

TM9SF4 mutant flies have reduced resistance to Gram-negative bacteria

To assess TM9SF4 function in *Drosophila* resistance to bacterial infection, *TM9SF4* mutant flies were infected with several bacterial species by septic injury. We reported previously that *TM9SF4* (*Dphg1A*) mutant flies showed normal resistance to *Pseudomonas aeruginosa*, but reduced resistance to the Gram-negative bacteria *Klebsiella pneumoniae* a pathogen that was specifically not permissive for the growth of *PHG1A* mutant *Dictyostelium* (Benghezal et al., 2006). Here we show that *TM9SF4* sensitivity to *Klebsiella pneumoniae* was not as strong as that observed for the mutant *TAK1*^{2} which blocks activation of the Imd pathway (Rutschmann et al., 2000; Vidal et al., 2001) (Fig. 3A). We used a more physiological infection procedure consisting of oral infection by providing *P. aeruginosa* in the animal feed (Avet-Rochex et al., 2005; Avet-Rochex et al., 2007; Erickson et al., 2004; Vodovar et al., 2005). This procedure allowed detection of the significant sensitivity of *TM9SF4* mutant flies compared with control Rev45 or *w*^{1118} flies, suggesting a contribution of TM9SF4 to the intestinal resistance to *P. aeruginosa* (Fig. 3B). In addition to *K. pneumoniae* and *P. aeruginosa, TM9SF4* mutant flies were slightly sensitive to Gram-negative *Enterobacter cloacae* (Fig. 3C), whereas their resistance to nonpathogenic bacteria, such as *Escherichia coli* or *Agrobacterium tumefaciens*, was similar to that in control flies (data not shown). No difference in sensitivity was observed between *TM9SF4* mutant and control flies following infection with Gram-positive *Enterococcus faecalis*, *Staphylococcus aureus* (Fig. 3D,E) or *Micrococcus luteus* (not shown).

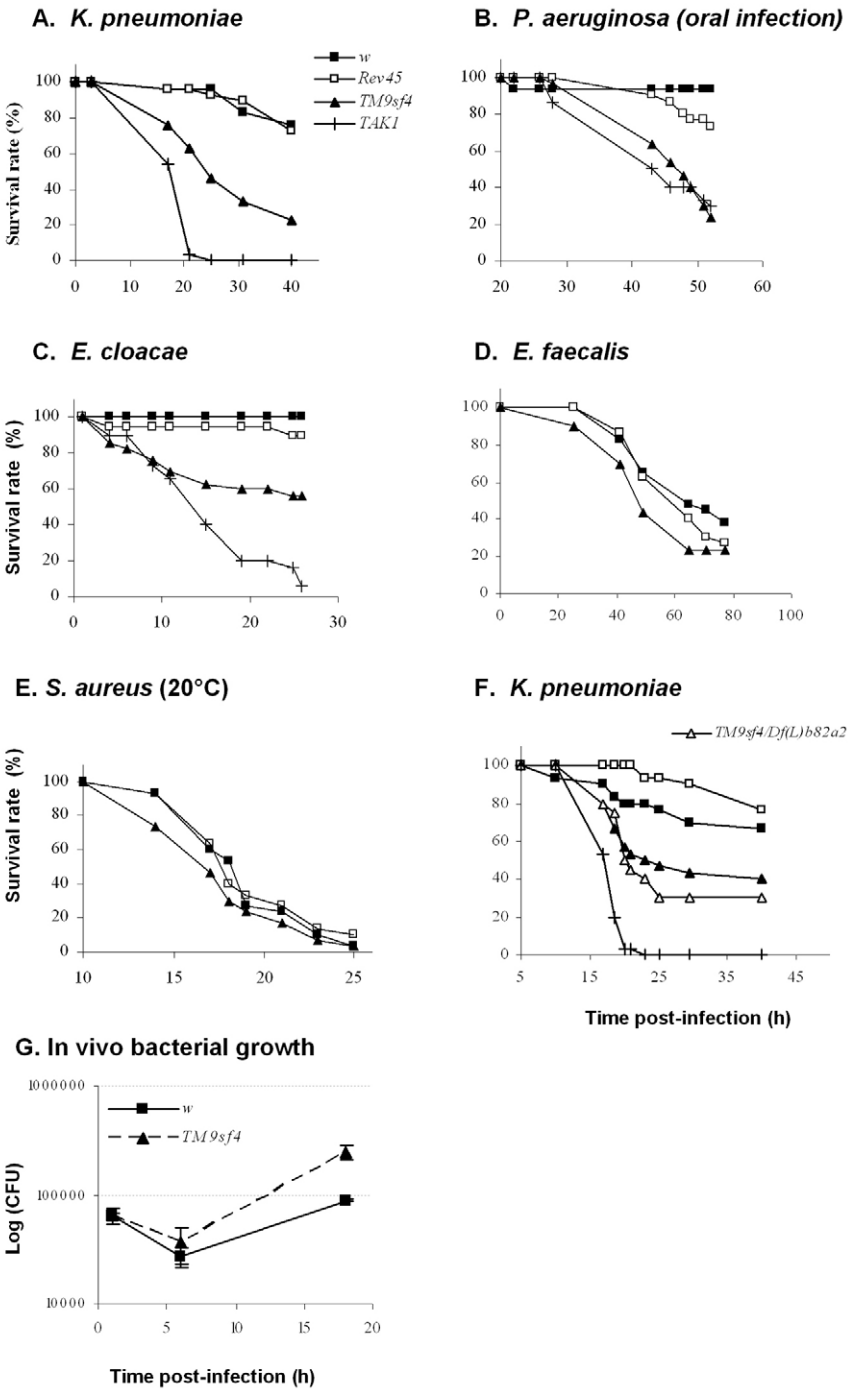

Fig. 3. Survival rate of infected *Drosophila* flies. 5- to 7-day-old males, previously raised at 25°C, were infected with indicated bacteria, either by septic injury onto the thorax with a thin needle previously dipped into the indicated bacterial solution (A,C-G) or by oral ingestion (B). Survival rate was followed at 25°C except in the case of *S. aureus* (20°C) as indicated. (A-F) Survival of *TM9SF4* mutant flies and TM9SF4/*Df(2L)b82a2* compared with control *w*^{1118} (w), Rev45 flies or *dTAK1*^{2} (TAK1) mutant flies affected in the Imd pathway. (G) The number of colony forming units (CFUs), plotted in logarithmic scale, was calculated from bacteria isolated from infected flies.

To rescue *TM9SF4*^{1} sensitivity to infections we constructed UAS-TM9SF4 transgenic flies allowing tissue-directed expression of the *TM9SF4* cDNA by various Gal4-specific driver lines (Brand et al., 1994). However, re-expressing *TM9SF4* in haemocytes either by srpGal4 (Crozatier et al. 2004), or through the more specific hmlGal4 (Goto et al., 2003) and HeGal4 (Zettervall et al., 2004) driver lines, induced pupal lethality. Vials were placed at 18°C during development, which allowed for the recovery of adults in the case of hmlGal4 (*TM9SF4*^{1};hmlGal4/UAS-TM9SF4) and HeGal4 (*TM9SF4*^{1};HeGal4/UAS-TM9SF4 flies) but not in the case of srpGal4 (srpGal4; *TM9SF4*^{1};UAS-TM9SF4/+). Surviving adult flies were placed at 25°C and infected 5 days later with *K. pneumoniae*. These flies expressed high levels of *TM9SF4* (data not shown), but were much more sensitive than Rev45 control flies and even *TM9SF4*^{1} flies, to *K. pneumoniae* infection (data not shown).

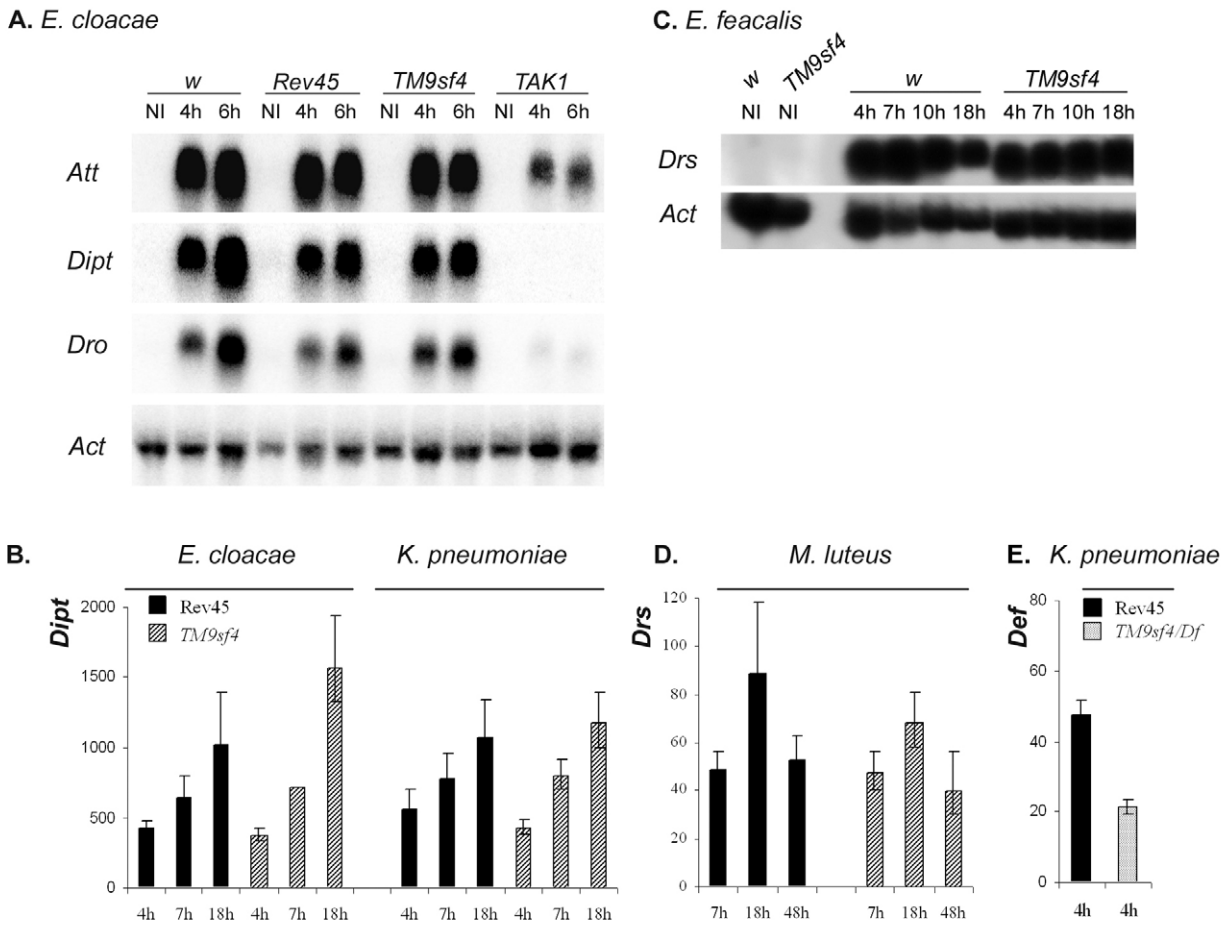

Fig. 4. Expression of genes encoding antimicrobial peptide is not affected in *TM9SF4* flies. (A,C) Expression of *Attacin* (*Att*), *Diptericin* (*Dipt*), *Drosocin* (*Dro*) and *Drosomycin* (*Drs*) as indicated, and of Actin (Act) which served as a loading control, was detected by northern blot. Expression of *Diptericin*, *Drosomycin* and *Defensin* (*Def*) by quantitative real-time PCR (B,D,E) was performed with total RNA isolated from 5- to 7-day-old flies. Control (w^{1118}, Rev45) or mutant [*TM9SF4*, *TM9SF4/Df(2L)b82a2, TAK1*^{2}] flies were sacrificed before infection (NI) or at several time points (in hours) following infection. (A) Northern analysis of antimicrobial gene expression in *E. cloacae*-infected flies. Expression level of all antimicrobial encoding is similar in *TM9SF4* mutants compared with Rev45 flies. Note that, because of different genetic background, w^{1118} flies expressed slightly higher levels of antimicrobial peptides transcripts than Rev45 flies, although both strains displayed similar resistance to infection. (B) Quantitative analysis of *Diptericin* expression level in either *E. cloacae*- or *K. pneumoniae*-infected *TM9SF4* mutant and Rev45 control flies. (C) Northern analysis of *Drosomycin* expression in *Enterococcus faecalis TM9SF4* mutant and w^{1118} (*w*) control infected flies. (D) Quantitative analysis of the expression level of *Drosomycin* in *Micrococcus luteus*-infected flies. (E) Quantitative analysis of *Defensin* expression in Rev45 and *TM9SF4/Df(2L)b82a2 transheterozygous flies* (*TM9SF4/Df*). These flies are deficient for *TM9SF4* and hemizygous for the *Defensin* locus. In B,D and E, results are expressed as the fold induction compared with non-infected flies. Post-infection times in hours are indicated below each histogram.

Increased sensitivity is probably due to the poor viability of *TM9SF4*-expressing flies. Indeed, *TM9SF4* ectopic expression might interfere with unknown signalling pathways as suggested by the observation that tissue-directed expression of *TM9SF4* induces strong morphogenesis defects (unpublished observations). Similarly, increased sensitivity was observed when Rac2 was overexpressed in the haemocyte lineage (Avet-Rochex et al., 2007). These observations indicate that expression of *TM9SF4* must be finely tuned in phagocytic cells to preserve their function. Since Rev45 flies presented a wild-type phenotype (similarly to w^{1118} control flies) (Fig. 3A-C), the sensitivity of *TM9SF4* mutant flies is unlikely to be due to a background effect. In addition, transheterozygous flies *TM9SF4*^{1}*/Df(2L)b82a2*, where the deficiency includes the *TM9SF4* gene, presented a survival phenotype to *K. pneumoniae* infections similar to that of *TM9SF4*^{1} homozygous flies (Fig. 3F).

We then observed that bacterial growth is facilitated in mutant *TM9SF4* flies infected with *K. pneumonia*. Indeed, bacterial numbers were greater in mutant *TM9SF4* flies compared with control flies at 18 hours post infection (Fig. 3G). Therefore, sensitivity of *TM9SF4*-deficient flies was observed with Gram-negative pathogenic bacteria and this sensitivity is coupled to a higher bacterial growth rate.

NF-κB-dependent immune signals are not affected in *TM9SF4*-deficient flies

Antimicrobial peptide production by fat body cells is a major mechanism contributing to bacterial clearance following fly infection and we therefore analysed whether immune signalling was affected in *TM9SF4* mutant flies. The activation of the Imd pathway was followed through the induction of *Attacin*, *Diptericin* and *Drosocin* (Georgel et al., 1993; Lemaitre et al., 1995; Levashina et al., 1998) and the activation of the Toll pathway, through the induction of *Drosomycin* (Lemaitre et al., 1996). Northern blot analysis revealed a strong induction of *Attacin*, *Diptericin* and *Drosocin* in *TM9SF4* mutant flies, similarly to control flies, following infection with *E. coli*, *E. cloacae* or *K. aerogenes* (Fig. 4A, *E. cloacae*). As expected,

a strong inhibition of antimicrobial peptide gene expression was observed in *TAK1*^{2} mutant flies (Fig. 4A). Additional quantitative real-time PCR analysis confirmed that no significant differences exist between Rev45 and *TM9SF4* mutant flies in the induction of *Diptericin* following infection by the Gram-negative bacteria *E. cloacae*, *K. pneumoniae* (Fig. 4B) or *E. coli* (not shown). Similarly, the Toll pathway was activated normally in *TM9SF4* mutants following infection by the Gram-positive bacteria *E. faecalis* (Fig. 4C) or *M. luteus* (Fig. 4D), resulting in the increased expression of *Drosomycin*. No significant changes in *TM9SF4* expression were observed in flies infected with *E. cloacae*, *K. pneumoniae* or *M. luteus*, suggesting that *TM9SF4* is not regulated at the transcriptional level by infection (data not shown).

Previous observations suggested that expression of the antimicrobial peptide encoding *Defensin* was particular in that it required normal haemocyte function (Brennan et al., 2007), raising the question whether TM9SF4, having defective haemocyte-dependent phagocytosis (see below), would be necessary for the induction of *Defensin* expression. Since genomic PCR analysis of the *TM9SF4*^{1} chromosome revealed that the *Defensin* locus was absent in this strain (data not shown), we analysed *TM9SF4/Df(2L)b82a2* transheterozygous flies. These flies strongly induced *Defensin* expression following infection by *K. pneumoniae*, reaching half the level of control flies, as expected for *Defensin* hemizygous flies (Fig. 4E). This indicates that TM9SF4 is not required for *Defensin* expression. Lack of *Defensin* expression in *TM9SF4*^{1} homozygous flies is unlikely to be the cause for increased sensitivity to Gram-negative bacteria because *TM9SF4*^{1}*/Df(2L)b82a2* transheterozygotes showed a similar sensitivity to Gram-negative bacterial infection (Fig. 3F). In addition, Defensin is essentially active against Gram-positive bacteria in vitro (Rutschmann et al., 2002) and *TM9SF4*^{1} homozygous flies resisted Gram-positive infection in the normal manner (Fig. 3D,E).

Our results demonstrate that activation of Toll and Imd immune signalling pathways by bacterial infection is not affected in *TM9SF4*-deficient flies, indicating that increased sensitivity of mutant flies to Gram-negative bacteria was not due to defective production of antimicrobial peptides.

TM9SF4 is required for haemocyte-dependent phagocytosis

In adult *Drosophila*, clusters of sessile haemocytes are present along the dorsal vessel on the anterior dorsal part of the abdomen. To assess engulfment of living bacteria by these cells, *TM9SF4* mutant and Rev45 flies were injected with GFP expressing *K. pneumoniae*. Less ingested fluorescence was observed in the clustered dorsal haemocytes in *TM9SF4* mutants compared with Rev45 flies at 3 hours post infection (arrowheads, Fig. 5A,B), suggesting that more bacteria escaped phagocytosis in mutant flies. Bacterial proliferation was detected in 20% of mutant flies as early as 5 hours post infection (arrows, Fig. 5D). In these flies, fluorescence was observed in the haemolymph and was also visualised in a drop of haemolymph bled from injured flies (not shown). This indicates that ingested bacteria do not multiply in phagocytic cells and that bacterial growth occurred extracellularly. In addition, haemocyte-associated fluorescence decreased both in Rev45 and *TM9SF4* mutant flies (arrowheads, Fig. 5D), indicating that bacteria were, most probably, properly killed by *TM9SF4* mutant haemocytes.

To quantify the phagocytosis defect of *TM9SF4* mutant haemocytes, circulating plasmatocytes from *TM9SF4* mutant or Rev45 third instar larvae were isolated and their ability to engulf fluorescent latex beads was observed. Mutant plasmatocytes displayed a reduced number of internalised fluorescent beads (Fig. 6A). We further measured the phagocytosis index of FITC-labelled latex beads or *E. coli* or *S. aureus*, as described previously (Avet-Rochex et al., 2005; Pearson et al., 2003). *TM9SF4* plasmatocytes phagocytosed latex beads and *E. coli* two times less efficiently than wild-type cells, whereas phagocytosis of *S. aureus* was unaffected (Fig. 6B). By using the srpGal4 driver line, TM9SF4 expression was mainly induced in haemocytes (Crozatier et al., 2004) in either a Rev45 or a *TM9SF4* mutant context. Rescue of the phagocytosis defect was observed in mutant plasmatocytes expressing *TM9SF4* in larvae raised at 18°C (Fig. 6B). Our results indicate that TM9SF4 is required for phagocytosis of hydrophilic particles and the Gram-negative bacteria *E. coli* by plasmatocytes, whereas it is dispensable for the internalisation of the Gram-positive bacteria *S. aureus*.

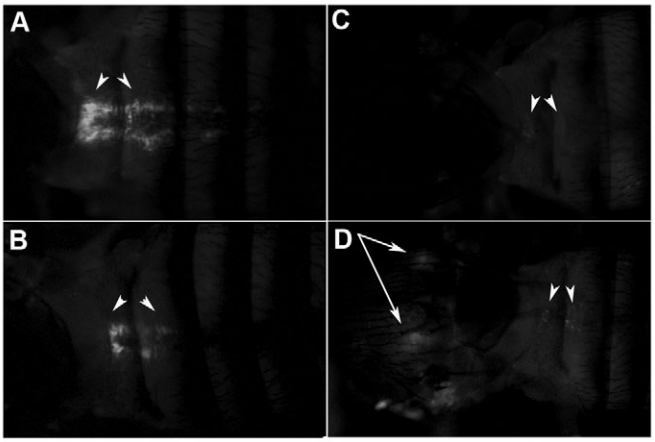

Fig. 5. In vivo engulfment of GFP-labelled *K. pneumoniae* by *Drosophila* haemocytes. Dorsal view of Rev45 (A,C) and *TM9SF4*^{1} mutant (B,D) fly abdomen injected with GFP-expressing *K. pneumoniae* at 3 hours (A,B) and 5 hours (C,D) post injection time. Arrowheads in A-D indicate the position of clustered haemocytes. Arrows in D indicate extracellular fluorescence associated with haemolymph.

TM9SF4 is required for proper encapsulation of wasp eggs

Cellular immunity in *Drosophila* plays a major role against bigger pathogens such as parasitoids. To elucidate whether *TM9SF4* is involved in the cellular immune response against parasitisation, an encapsulation assay was performed on larvae parasitised by the avirulent *Leptopilina boulardi* wasp strain G486. When the avirulent wasp strain G486 parasitises *Drosophila* larvae a darkened cellular capsule is visible in the haemoceol 30-40 hours later. At room temperature (24°C) w^{1118} or *Rev45* control larvae encapsulated the wasp eggs 88% and 79%, respectively, whereas only 48% of *TM9SF4* mutant larvae properly encapsulated and melanised foreign eggs (Fig. 6C). A stronger phenotype was observed by elevating the temperature in larvae first raised at 29°C before being parasitised. At this higher temperature, 86% of w^{1118} larvae and 76% of *Rev45* larvae still properly encapsulated and melanised the wasp egg, yet only 13% of the homozygous *TM9SF4* mutant larvae properly encapsulated the egg (Fig. 6C). From this we conclude that *TM9SF4* is necessary for haemocytes to properly encapsulate *L.boulardi* eggs.

TM9SF4 mutant macrophages display defective lamellipodia and actin organisation

The first step of phagocytosis or encapsulation requires adhesion of phagocytes to the pathogen and strong cytoskeleton

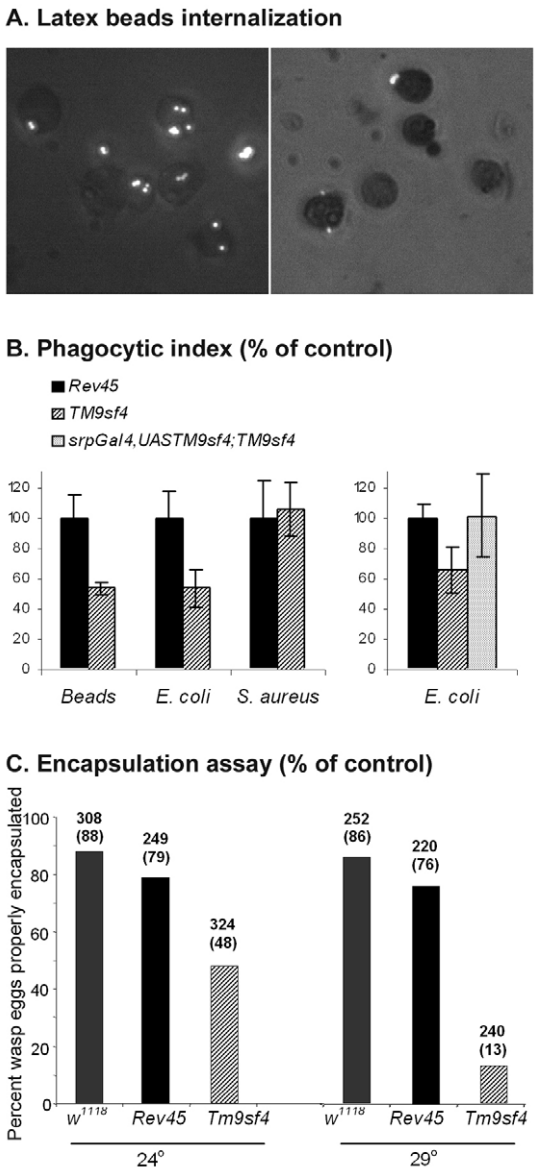

Fig. 6. *TM9SF4* mutant larval haemocytes have defective phagocytosis and encapsulation. (A) Circulating plasmatocytes were isolated from third instar larvae and incubated for 15 minutes with fluorescent latex beads. The internalisation of FITC-latex beads was observed following addition of quenching Trypan Blue solution. (B) Using the same procedure as in A, the internalisation rate of FITC-labelled beads or *E. coli* or *S. aureus*, was calculated as the number of internalised particles per haemocyte from 300-500 haemocytes. A phagocytic rate of 100% was attributed to control Rev45 cells in each experiment. The results are the mean ± s.d. of three independent experiments. A significant difference (Student's *t*-test, $P<0.03$) was found in phagocytic rate for latex beads and *E. coli*, but not *S.aureus* between Rev45 and *TM9SF4* mutant cells (left panel). Directed expression of *TM9SF4* mainly in the haemocyte lineage through the srpGal4 driver line (srpGal4/Y; *TM9SF4*^{1}; UASTM9SF4/+ larvae) partially rescued the phagocytic properties of circulating plasmatocytes (right panel) ($P<0.01$, Student's *t*-test). (In this experiment, larvae were raised at 18°C.) (C) Encapsulation rate of control (w^{1118}, Rev45) and mutant (*TM9SF4*) larvae following wasp parasitisation. The total number of parasitised larvae examined is indicated on the top of each histogram, the number in parenthesis indicates the number of larvae presenting a dark capsule. Experiments were performed at 24°C and 29°C as indicated.

rearrangements to engulf or spread on it. Circulating plasmatocytes were isolated from *Drosophila* Rev45 control or *TM9SF4* mutant larvae, and their ability to spread on glass coverslips was studied by reflection interference contrast microscopy (RICM). Unlike phase-contrast imaging (Fig. 7A,B), RICM allows visualisation of cell-substrate contact areas, which appear dark (Gingell and Owens, 1992; Pierres et al., 2003). Control Rev45 cells displayed wild-type cell-substrate contact area morphology: they spread isotropically, and a dark ring characteristic of a lamellipodium surrounded the cells after about 15-45 minutes, indicating a close contact of the cell circumference to the substrate (Fig. 7C). By contrast, *TM9SF4* plasmatocytes spread in an irregular manner and although large lamellipodium protrusions were clearly visible (Fig. 7D), the adhesive belt was absent (Fig. 7D, arrowhead) or severely disrupted. Instead, non-uniform white areas were often visible, representing portions of cells, at about 260 nm above the surface (Fig. 7D, arrow).

To further analyse their cytoskeleton organisation, circulating plasmatocytes from third instar larvae were labelled with Texas-Red-tagged phalloidin and examined by confocal microscopy. Control cells displayed a homogeneously sized surface and a round shape, as previously reported (Williams et al., 2007; Williams et al., 2006) (Fig. 7E-G). By contrast, *TM9SF4* mutant cells presented heterogeneous sizes and shapes and displayed disorganised frequently long actin spikes and punctate actin accumulation (Fig. 7H-J). Quantification of the area of the actin cytoskeleton network in close contact with the surface demonstrated that mutant cells had a 2.3-fold larger average size than control Rev45 cells. This indicates that mutant cells displayed increased spreading on the substrate (Fig. 7K). Expressing *TM9SF4* cDNA in mutant plasmatocytes partially reduced the extent of the cytoskeleton network, because these cells possessed a 1.34-fold larger average surface area compared with Rev45 cells (Fig. 7K). Our observations demonstrate that the nonaspanin TM9SF4 may control cell adhesion, cell shape and signalling to the actin cytoskeleton.

TM9SF4 and TM9SF2 contribute to bacterial phagocytosis in *Drosophila* S2 cells

Drosophila S2 cells are derived from a primary culture of late-stage embryos (Schneider, 1972). They express macrophage-specific markers, such as the two phagocytic receptors dSR-CI and Eater, and can bind and engulf particles (Kocks et al., 2005; Ramet et al., 2001). To assess functional redundancy of nonaspanins in bacterial phagocytosis, we evaluated the effect of inactivating *TM9SF4* and *TM9SF2*, either alone or in combination. Both genes are constitutively expressed in S2 cells (data not shown) and were inactivated by silencing RNAs (siRNA) as described (Clemens et al., 2000). An equivalent reduction in phagocytosis of FITC-labelled *E. coli* was observed in *TM9SF4*- or *TM9SF2*-silenced cells, presenting an internalisation rate of 53% and 57%, respectively, compared with levels in wild-type cells (Fig. 8A). This phagocytosis rate was reduced to 38% when both genes were silenced. For comparison, cells treated with siRNA targeting *PGRP-LC*, which encodes a receptor protein required for Gram-negative bacteria internalisation by S2 cells (Ramet et al., 2002), exhibited a phagocytosis rate of FITC-labelled *E. coli* of 34% compared with that in control cells (Fig. 8A). No significant inhibition of FITC-labelled *S. aureus* internalisation was observed in *TM9SF4* silenced S2 cells (Fig. 8B). By contrast, *TM9SF2* silencing provoked a reduction of the phagocytosis rate of FITC-labelled *S. aureus* to 56% that of control cells. This inhibition was significantly increased, leading to a phagocytosis rate of 32%, when *TM9SF4* was co-silenced with *TM9SF2*, suggesting that TM9SF4 contribution to *S. aureus*

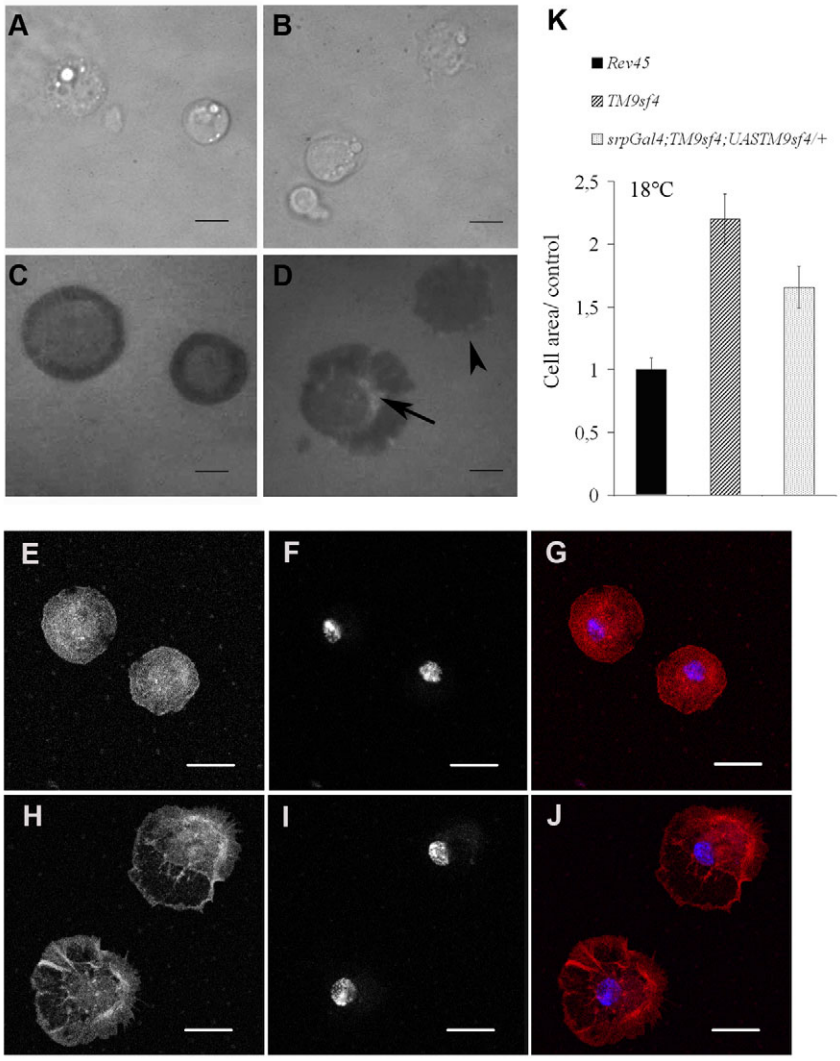

Fig. 7. Impaired lamellipodia formation and defective actin reorganisation in *TM9SF4* mutant macrophages. Circulating plasmatocytes were isolated from wild-type (A,C) or mutant *TM9SF4* (B,D) third instar larvae and allowed to spread for 15 minutes in a glass coverslip chamber. (A,B) Phase contrast. (C,D) Reflection interference contrast microscopy. Arrowhead indicates the loss of adhesive belt; arrow indicates the white area that represents more distant contacts. (E-I) Confocal analysis of actin network in isolated larval macrophages. Texas-Red-phalloidin fluorescent labelling revealed polymerised actin (E,H) and nuclei were stained with Hoechst 33258 (F,I); overlays are shown in G,J. Control cells are regularly sized and round (E-G), whereas most *TM9SF4* mutant cells have a larger area and differentiate long actin-stained filopodia (H-J). (K) The surface of the cytoskeleton network was calculated from 500-1000 cells. Mutant *TM9SF4* cells were 2.3-fold larger than Rev45 control cells ($P<0.0001$, Student's *t*-test). Cell size was partially rescued by expression of *TM9SF4* cDNA in the haemocyte lineage. A significant difference between *TM9SF4* mutant and *srpGal4; TM9SF4;UAS-TM9SF4/+* rescued plasmatocytes was found ($P<0.004$, Student's *t*-test) (larvae raised at 18°C). Scale bars: 10 µm.

engulfment was masked by redundancy with TM9SF2. Silencing Dscar, a component of the cytoskeleton required for the phagocytic process of both types of particles (Pearson et al., 2003) resulted in a strong, although not complete, reduction of phagocytosis of both FITC-labelled *E. coli* (22%) and FITC-labelled *S. aureus* (37%) (Fig. 8A,B). Our results show that the two nonaspanins TM9SF2 and TM9SF4 are both required for bacterial phagocytosis by *Drosophila* cultured cells and possess partial functional redundancy in this process.

Discussion

TM9SF4 function in cell adhesion and haemocyte-dependent phagocytosis is coupled to cytoskeleton defects

The function of the nonaspanin Phg1A in bacterial phagocytosis and cell adhesion was first demonstrated in the free-living amoeba *Dictyostelium* (Benghezal et al., 2003; Cornillon et al., 2000). Here, we show that these functions are conserved throughout evolution because *DPhg1A/TM9SF4* mutant circulating plasmatocytes isolated from *Drosophila* third instar larvae presented defective phagocytosis of latex beads and *E. coli,* two types of particles that were very poorly internalised by mutant amoeba. In addition, as observed by RICM, *Drosophila TM9SF4* mutant macrophages have a severely disrupted adhesive belt compared with control cells, which displayed a regular lamellipodium around the entire cell circumference. In *PHG1A* mutant amoeba, no cytoskeleton defects were reported (Cornillon et al., 2000). By contrast, in *Drosophila TM9SF4* mutant macrophages, defects in cell adhesion and phagocytosis were coupled with an abnormal actin cytoskeleton: whereas control cells presented a wild-type round shape, mutant macrophages presented disorganised actin staining and actin spikes. Interestingly similar actin spike phenotypes were described in S2 *Drosophila* cells following silencing of actin regulatory proteins such as Dscar (Kunda et al., 2003; Pearson et al., 2003). Our study thus indicates that TM9SF4 function in cell adhesion and phagocytosis might result from defective signalling in the control of actin reorganisation during cell attachment.

TM9SF4 requirement in host cellular immunity

When infected with pathogens, *TM9SF4* mutant flies were sensitive to the Gram-negative pathogenic bacteria but they resisted Gram-positive bacteria as well as wild-type flies did. Interestingly, *TM9SF4* mutant plasmatocytes were specifically defective for the internalisation of the Gram-negative bacteria *E. coli*, whereas no defects were observed in the internalisation of the Gram-positive bacteria *S. aureus*, putatively because of redundancy with TM9SF2 in this process (see below). Therefore, sensitivity of *TM9SF4* flies to Gram-negative bacteria might be correlated with defective phagocytosis. Supporting this hypothesis, the activation of the two NF-κB-dependent pathways, Toll and Imd, controlling the activation of the antimicrobial peptide genes by fat body cells, were not affected in *TM9SF4* mutant flies, which is consistent with the fact that these mutant flies were not as sensitive as *TAK1* mutant flies to Gram-negative pathogenic bacteria and resisted non-pathogenic strains. In an independent large siRNA screen performed in cultured *Drosophila* S2 cells, *TM9SF4* (CG7364) was also shown to be required for the internalisation of the yeast *Candida albicans* (Stroschein-Stevenson et al., 2006). In fact, we observed that *TM9SF4* mutant flies were sensitive to *C. albicans* infections (E.B.

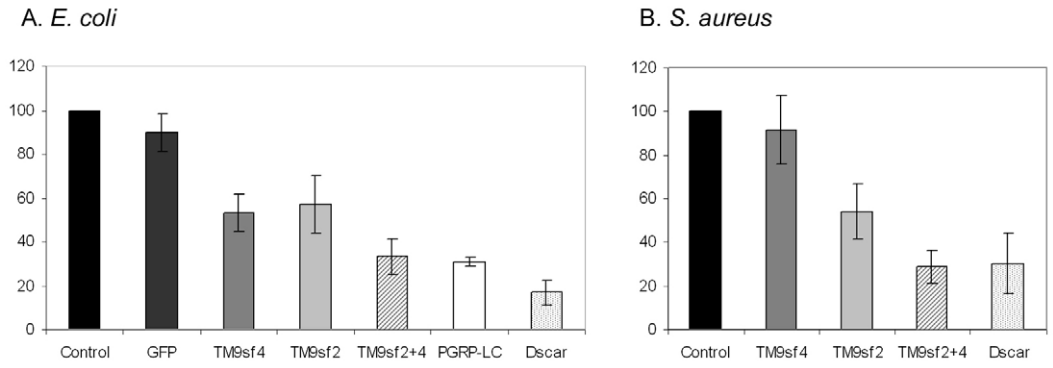

Fig. 8. TM9SF2 and TM9SF4 are required for phagocytosis in S2 cells. S2 cells were untreated (Control) or treated for 3 days with siRNA GFP (Green fluorescent protein), *TM9SF4*, *TM9SF2, PGRP-LC* or *Dscar,* either alone or in combination (TM9SF2+4), as indicated. Cells were then incubated for 10 minutes with FITC-labelled *E. coli* (A) or FITC-labelled *S. aureus* (B) and the internalised fluorescence was measured in the presence of external Trypan Blue quenching solution. The phagocytosis index was quantified as the percentage of fluorescence-positive cells multiplied by the mean fluorescence of these cells. We counted 10,000-20,000 cells from each sample. A phagocytosis index of 100% was attributed to control cells. The results presented are the mean ± s.d. of three experiments. Significant differences were observed using Student's *t*-test between: (A) TM9SF4 and control ($P<0.003$), TM9SF2 and control ($P<0.02$), PGRP-LC and control ($P<0.001$), Dscar and control ($P<0.001$); and (B) TM9SF2 and control ($P<0.001$), TM9SF2+4 and TM9SF2 ($P<0.05$), Dscar and control ($P<0.001$).

and J.P., unpublished results) suggesting that the correlation between defective phagocytosis and sensitivity to infection extends to another kind of pathogen which provokes severe infections in humans.

Finally, we showed that encapsulation of wasp eggs was partially impaired in *TM9SF4* mutant larvae and that this defect was increased at elevated temperature (29°C). Since lamellocytes differentiate and are present in the haemolymph, the adhesion defects of *TM9SF4*-null plasmatocytes might account for the reduced encapsulation rate of parasitoid eggs. Indeed, partial loss of function of the adhesion receptor β-integrin in *mys* mutant larvae is sufficient to induce temperature-sensitive encapsulation defects (Irving et al., 2005). Since $TM9SF4^{1}$ is a null allele, the temperature-sensitive phenotype of *TM9SF4* mutant flies might be an indirect consequence of increased cell adhesion defects at elevated temperature, because of loss or instability of cell surface proteins, such as integrins. In amoebae, the *PHG1A*-null mutant also displayed a temperature-sensitive phenotype (Benghezal et al., 2003). As is the case for *Dictyostelium*, removing both TM9SF2 and TM9SF4 will be required in *Drosophila* in the future to strengthen the phenotype and further elucidate the physiological causes for phagocytosis and encapsulation defects.

TM9SF2 and TM9SF4 contribute to phagocytosis in S2 cells

TM9SF4 was also required in *Drosophila* S2 cells for efficient internalisation of *E. coli*, but not *S. aureus.* By contrast, TM9SF2 was required for the internalisation of both types of bacteria in these cells, indicating that TM9SF2 and TM9SF4 are only partially redundant. When compared with silencing either *TM9SF2* or *TM9SF4* alone, silencing both *TM9SF2* and *TM9SF4* resulted in a modest enhancement of the phagocytosis defect in the case of *E. coli*, whereas a significant enhancement was observed in the case of *S. aureus*. This suggests that TM9SF4 is necessary in *S. aureus* internalisation but that this function was masked in mutant cells by redundancy with TM9SF2. Specificity towards pathogens depends on primary events such as bacterial recognition, whereas downstream events, such as cytoskeleton reorganisation, might be equally required for every type of particle. It is thus probable that the two proteins play redundant and non-redundant functions in both the intracellular mechanisms underlying phagocytosis and the mechanisms underlying bacterial recognition, such as expression of pathogen-recognition receptors (PRRs) at the cell surface. In support of this hypothesis, it was previously established in *Dictyostelium* cells that TM9 proteins are most probably not recognition molecules by themselves, but contribute to the expression of cell surface proteins (Benghezal et al., 2003). Further analysis of cell surface protein expression in single and double TM9SF2- and TM9SF4-knockout cells will be required to explore the specific involvement of TM9SF2 and TM9SF4 in cell adhesion and phagocytosis.

Materials and Methods

Stocks

Flies were raised on standard culture medium at 25°C unless indicated otherwise. The following stocks were from the *Drosophila* Bloomington Stock Center: w;P{lacW}CG7364^{k07245}/CyO and w;*Df(2L)b82a2* (BL#6072), which deletes the interval [34D1-34E5] including the *TM9SF4* locus, which we verified by RT-PCR (not shown). Mutant $dTAK1^{2}$ and Key^{1} were from Bruno Lemaitre. The lethal *l(2)k0724* mutation was not attributable to the P{lacW}CG7364^{k07245} insertion since transheterozygous flies P{lacW}CG7364^{k07245}/*Df(2L)b82a2* were semi-viable. The cryptic lethal mutation was removed by outcrossing P{lacW}CG7364^{k07245} flies over three generations with a w^{1118} strain. Remobilisation of the P{lacW}CG7364^{k07245} was performed following standard procedures (Robertson et al., 1988). Imperfect excisions were screened by PCR analysis of the sequence flanking the insertion site with forward primer: (7AVTP sens) 5′- TTAAGCCGCAAAGGAAAGGAAATGT-3′ and reverse primer (fasta as): 5′-CTAAAACGCAACGCTCACGCTCCTG-3′. One deletion was recovered among 160 independently excised lines analysed.

Survival rate experiments and bacterial growth in vivo

E. coli 1106, *A. tumefaciens*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa (PAO1)* were used as Gram-negative bacterial strains. *S. aureus* and *E. faecalis* and *M. luteus* were used as Gram-positive strains. All strains were grown on standard Luria-Broth media (LB) over night at 37°C except *A. tumefaciens* (30°C). A pellet from 50 ml overnight cultures was used to infect flies, except in the case of *P. aeruginosa* for which a exponential phase culture diluted to OD_{600}=0.4 was used (to reach an approximate multiplicity of infection of 50 bacteria per fly). For septic infection, 30 adult male flies 5-7 days old were pricked in the upper part of the thorax with a thin needle previously dipped into the bacterial pellet. Oral infections were conducted as described (Avet-Rochex et al., 2005). Results are expressed as percentage of surviving infected flies at different time points following infection. Experiments were repeated at least three times with results similar to those presented.

The multiplication of bacteria during *Drosophila* infection was quantified as described (Fauvarque et al., 2002): at each time point, 10 infected flies were harvested in Eppendorf tubes kept on ice and grounded with Teflon pestle. The homogenate was suspended in LB medium (400 μl) and centrifuged for 10 minutes at 2000 rpm. Supernatants were serially diluted in LB and spread on LB plates.

Northern blotting and RT-PCR

Total RNAs were extracted from adult flies using RNA^{plus} from QBiogene. Northern blots (15 µg RNA/lane) were probed with a ^{32}P-labelled fragment of *TM9SF4* cDNA (2619 bp) or Diptericin (1000 bp) or Drosocin (759 bp) or Drosomycin (376 bp), and with actin as an internal loading control (1239 bp). For the RT-PCR, total RNA were similarly extracted either from a pool of 10^6 S2 cells or from circulating plasmatocytes isolated from eight third instar larvae. One µg of total RNAs were submitted to a DNase digestion step [DNA-free^{TM} kit (Ambion, Inc.)] and the amplification was performed according to the manufacturer's instructions [Titanium One-Step RT-PCR kit (BD Biosciences)]. Primers were as follows: *TM9SF4* forward, 5′-GAGGTGCGGATGAACCAGCAGGTCA-3′; reverse, 5′-CTGGACATCGCG-CATGCCAAGGTAG-3′; Actin forward, 5′-ACCGCGTGCAGTTTTTCCTTCTA-3′; reverse, 5′-TATGGTTTGCTTATGCGTCGTGTA-3′. Controls were run with no reverse transcriptase. Amplified cDNA were either visualised by ethidium bromide staining on agarose gel, or transferred onto nitrocellulose by Southern blot and probed with a *TM9SF4*-specific probe.

For real-time PCR analysis, cDNAs were synthesised with AffinityScript QPCR cDNA Synthesis Kit (Stratagene). An amount of cDNA equivalent to 500 ng to 1 µg of total RNA was subjected to 40 cycles of PCR amplification consisting of a 10 second incubation at 95°C and 30 seconds at 60°C. Output was monitored using SYBR Green core reagents and the Mx3000P instrument (Stratagene). All the results were normalised to the *rpl32* RNA level. The primer sequences used were: *Diptericin* forward, 5′-ACTTTGCTGCGCAATCGCTTCTAC-3′, *Diptericin* reverse, 5′-TCCCTGAAGATTGAGTGGGTACTG-3′; *Rpl32* forward, 5′-AAGAAGTTC-CTGGTGCACAACGTG-3′; *Rpl32* reverse, 5′-AATCTCCTTGCGCTTCTTG-GAGGA-3′; *Drosomycin* forward, 5′-AAGTACTTGTTCGCCCTCTTCGCT-3′; *Drosomycin* reverse, 5′CCTTGTATCTTCCGGACAGGCAGT-3′; *Defensin* forward, 5′-TTCTTCGTTCTCGTGGCTATCGCT-3′; *Defensin* reverse, 5′ACCAGGACAT-GATCCTCTGGAATTGG-3′; *TM9SF4* forward, 5′-AGTCTCGTCCAGC-TGCAGAAATCA; *TM9SF4* reverse, 5′AGTTAGCTCACATGGCTGAGTCGT-3′.

In vivo and ex vivo phagocytosis tests on isolated third instar larval plasmatocytes

In vivo phagocytosis test was performed by injecting living GFP-expressing *K. pneumoniae* into the fly thorax. The phagocytosis of bacteria by the blood cells concentrated in the dorsal vessel was then observed using a Leica MZ FLIII dissecting microscope and photographs were taken using a numeric camera (LEICA, DC300F). For ex vivo phagocytosis tests, larval circulating plasmatocytes were obtained from eight larvae by gently ripping the larval cuticle near the posterior end and plasmatocytes were left to attach to microplaque wells for 2 minutes. Ex vivo phagocytosis monitoring was then performed essentially as described (Avet-Rochex et al., 2005; Pearson et al., 2003).

Wasp infection

The encapsulation assay was done according to published methods (Sorrentino et al., 2002). Briefly, 2 days before parasitisation, the appropriate fly strains were crossed and kept at 21-25°C. Four or five females of *L. boulardi* G486 were allowed to infest at room temperature for 2 hours, after which the *Drosophila* larvae were transferred to apple juice plates and left at room temperature for 40-42 hours. After this time, the larvae were collected, washed in PBS, and then viewed under a stereomicroscope for the presence of a dark capsule. Larvae in which no dark capsule was observed were dissected in 20 µl PBS to determine whether they had been parasitised. Larvae containing eggs from the parasitoid that had not darkened by this time were scored as non-encapsulated. Non-parasitised larvae were excluded from the count.

RICM and Phalloidin staining on isolated third instar larvae plasmatocytes

To visualise cell-substrate contact areas, haemocytes were bled from larvae into 300 µl Schneider medium (Invitrogen) and allowed to attach to a coverslip glass chamber (LAB-TEK Nalge Nunc International). Before use, the glass chamber was previously washed with 14.5 M NaOH for 5 minutes, and rinsed thoroughly with deionised water. Reflection Interference Contrast Microscopy (RICM) was performed on an inverted Olympus IX71 microscope, selecting the 546 nm peak of the episcopic mercury lamp by a combination of interference and blue-green filters and illuminating through an episcopic cube. The image of the field diaphragm was used to focus at the glass-liquid interface. Light intensity was reduced 100-fold with neutral density filters. RICM images were recorded by an intensified cooled CCD video camera (Photonic Science, UK) controlled by Image Pro Plus software (MediaCybernetics). In addition, cells were also imaged by transilluminating phase-contrast microscopy to localise the cell body. Cells extend lamellipodia after close contact with the glass substrate in about 15 minutes, which are stable for at least 45 minutes. These experiments were reproduced three times with comparable results.

To visualise filamentous actin, haemocytes were bled from larvae into 700 µl Schneider medium (Invitrogen) and allowed to attach to a CC2 glass slide (LAB-TEK Nalge Nunc International) for 1 hour. The cells were fixed at room temperature directly in medium for 5 minutes with 16% paraformaldehyde/PBS (3.7% final), washed twice for 5 minutes with PBS, then once for 5 minutes with PBST (PBS/0.1% Triton X-100), and twice for 5 minutes with PBS. The cells were then stained for 40 minutes at room temperature with Texas-Red-phalloidin (Invitrogen) diluted to a final concentration of 10 U in PBS/1% BSA. Cells were then washed twice for 5 minutes with PBS, once for 3 minutes with PBS containing Hoescht 33258 (1 µg/ml) and mounted using FluorSave (Calbiochem). Samples were analysed by confocal laser-scanning microscopy, using a Leica TCS-SP2 operating system. Texas Red and Hoechst 33258 fluorescence were excited by using the 543 nm line of a helium-neon laser and a 405 nm diode, respectively. Fluorescence emission was collected from 570-650nm for Texas Red, and from 420-480nm for Hoechst 33258.

The extent of the actin cytoskeleton network in close contact with the glass substrate was measured using the Image Pro Plus software. The background of the fluorescence images was flattened by spatial filtration (0.5 µm^{-1}) and the resulting images were segmented to determine the contours of the actin cytoskeleton network of each cell. Cells in contact with the image borders were not taken into account. For each genotype, 500-1000 cells were counted. The error on the average area was calculated as the s.d. divided by the square root of the number of cells examined. One representative experiment of three is shown.

Cell culture and FACS analysis

Drosophila S2 cells were maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated FCS (all from Invitrogen). Gene inactivation was obtained by incubating 15 µg double strand RNA (RNAi) for 72 hours at 26°C with 10^6 S2 cells cultured in six-well tissue culture plates (Clemens et al., 2000). DNA templates were generated by PCR using the primers specific for each target gene: Dscar forward, 5′-taatacgactcactatagggGCATGTGGCCCGCTCCGTGTATCAG-3′; Dscar reverse, 5′-taatacgactcactatagggCCATGGAATTCGGTGTGCGGTAGA-3′; GFP forward, 5′-taatacgactcactatagggagaccaGTATAGTTCATCCATGCCATGTG-3′; GFP reverse, 5′-taatacgactcactatagggagaccaGGAGAAGAACTTTTCACTGGATTG-3′; TM9SF4 forward, 5′-taatacgactcactatagggGGAGAGACGCAGCTATATTT-3′; TM9SF4 reverse, 5′-taatacgactcactatagggTCGCGACCCTTCATTGTT-3′; TM9SF2 forward, 5′-taatacgactcactatagggCCCACTGGAGAACGGCAAGCAATAC-3′; TM9SF2 reverse, 5′-taatacgactcactatagggCCCACTGGAGAACGGCAAGCAATAC-3′; PGRP-LC forward, 5′-taatacgactcactatagggGACCAAAGATCGGGGGAC-3′; PGRP-LC reverse, 5′-taatacgactcactatagggGCTTATCACCGAACGTCAC-3′.

Purified PCR products were used as DNA templates for in vitro transcription of RNA using the MEGAscript RNAi kit (Ambion). Flow-cytometry-based phagocytosis assays were performed as described (Ramet et al., 2002). Briefly, S2 cells were incubated for 10 minutes at 26°C with FITC-labelled particles and flow cytometry was used to analyse the ability internal fluorescence rate produced by internalised *E.coli* (K-12 strain) BioParticles FITC and *S. aureus* (Wood strain without protein A) BioParticles FITC (Molecular Probes). The samples were analysed using the CELLQuest program (Becton Dickinson).

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