Published in final edited form as:
Insect Biochem Mol Biol. 2012 August ; 42(8): 545–556. doi:10.1016/j.ibmb.2012.04.002.

Drosophila melanogaster NPC2 Proteins Bind Bacterial Cell Wall Components and May Function in Immune Signal Pathways

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Abstract

ML (MD-2 (myeloid differentiation factor 2)-related Lipid-recognition) is a conserved domain identified in MD-2, MD-1, NPC2 (Niemann-Pick disease type C2), and mite major allergen protein from animals, plants, and fungi. Vertebrate members of the ML family proteins, such as NPC2 and MD-2, play important roles in lipid metabolism and immune signaling pathway. MD-2 is an essential co-receptor in the lipopolysaccharide (LPS)/Toll-like receptor 4 (TLR4) signaling pathway. Insects contain multiple *ML* genes, arbitrarily named *md-2*- or *npc2*-like genes. However, whether insect *ML* genes have functions similar to vertebrate *md*-2 is unknown. In *Drosophila melanogaster*, there are eight *npc2* genes (*npc2a-h*), and they can be further divided into three subgroups based on the numbers of cysteine residues (6, 7 and 8 Cys) in the mature proteins. The purpose of this study is to investigate whether any *Drosophila npc2* genes may have functions in immune signaling pathways. We chose *npc2a*, *npc2e* and *npc2h* genes representing the three subgroups for this study. We showed that recombinant NPC2a, NPC2e and NPC2h not only bound to LPS and lipid A, but also bound to peptidoglycan (PG) and lipoteichoic acid (LTA), a property that has not been reported previously for vertebrate NPC2 or MD-2. More importantly, we showed that over-expression of NPC2a and NPC2e activated *diptericin* promoter reporter in S2 cells stimulated by PG, suggesting that NPC2e and NPC2a may play a role in the immune deficiency (Imd) pathway. This is the first *in vitro* study about NPC2 proteins in innate immunity of *D. melanogaster*.

Keywords

MD-2; NPC2; *diptericin*; Imd; signaling pathway; peptidoglycan

1. Introduction

Innate immune system is the first line of defense against non-self microbial infection. Host immune responses are initiated when microbial components are recognized by pattern recognition receptors (PRRs) (Peri et al., 2010). Several classes of PRRs have been identified, including Toll-like receptors, C-type lectin receptors, NOD (nucleotide-binding oligomerization domain protein)-like receptors, peptidoglycan recognition proteins

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(PGRPs), and Gram-negative binding proteins (GNBPs) (Royet, 2004; Akira et al., 2006; Cherry and Silverman, 2006; Pal and Wu, 2009; Kawai and Akira, 2011). These PRRs can recognize conserved microbial components, known as pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acid (LTA), and fungal β-1, 3-glucans (Royet, 2004), which are present in pathogens but not in hosts.

Microbial components can activate humoral and cellular immune responses in insects, such as phagocytosis, nodule formation, encapsulation, synthesis of antimicrobial peptides (AMPs), and activation of the prophenoloxidase system (Lavine and Strand, 2002; Hoffmann, 2003; Lemaitre and Hoffmann, 2007; Cerenius et al., 2008; Marmaras and Lampropoulou, 2009; Ganesan et al., 2011). In *Drosophila melanogaster*, expression of AMP genes is regulated by the Toll and immune deficiency (Imd) pathways (Lemaitre et al., 1995, Lemaitre et al., 1996; De Gregorio et al., 2002). The Toll pathway is activated by fungi and Lys-type peptidoglycan (PG) of Gram-positive bacteria, while the Imd pathway is activated by *meso*-diaminopimelic acid (DAP)-type PG of Gram-negative bacteria and some *Bacilli* species (Kaneko et al., 2004; Leulier et al., 2003; Lemaitre and Hoffmann, 2007). In *Bombyx mori*, a cytokine signaling pathway also plays an important role in humoral and cellular immune responses (Ishii et al., 2008; Ishii et al., 2010b). Different microbial components can be recognized by a variety of PRRs, and some of the PRRs may serve as co-receptors or modulators in the innate immune signaling pathways.

ML (MD-2 related Lipid-recognition) is a conserved domain, which has been identified in MD-2 (myeloid differentiation factor 2), MD-1, NPC2 (Niemann-Pick disease type C2), mite major allergen protein, and some other proteins from animals, plants, and fungi (Inohara and Nunez, 2002). The ML domain is about 150 amino acids with two pairs of conserved cysteine residues, and more than one hundred ML family proteins have been identified so far. Members of the ML family proteins, such as NPC2 and MD-2, play important roles in lipid metabolism and innate immune signaling pathway (Inohara and Nunez, 2002; Bryant et al., 2010). In humans, MD-2 and Toll-like receptor 4 (TLR4) can form a heterodimer to recognize LPS and activate the NF-κB signaling pathway (Shimazu et al., 1999; Park et al., 2009). Among the three ancillary proteins (LBP (LPS-binding protein), CD14 and MD-2) in the TLR4-LPS signaling pathway, only MD-2 is absolutely required for LPS/TLR4 activation (Visintin et al., 2006).

In insects, multiple *ML* genes, arbitrarily named *md-2*- or *npc2*-like genes in different species, have been identified. For example, there are thirteen *md*-2-like genes (*AgMDL*1–13) in *Anopheles gambiae*, fifteen *npc2*-like genes in *Aedes aegypti*, eight *md*-2-like genes in *Tribolium castaneum*, and eight *npc2*-like genes in *D. melanogaster*. Since only one *npc2* gene is present in yeast, worm, mouse and human, and one *md*-2 gene in mouse and human, these insect *ML* genes may not all be *md*-2- or *npc2*-like genes. Some of the insect *ML* genes may be homologous to vertebrate *npc2* gene, while others may be homologous to vertebrate *md*-2 gene. For example, *Drosophila npc2a* and *npc2b* have functions similar to vertebrate *npc2* in sterol homeostasis and steroid biosynthesis (Huang et al., 2007). *An. gambiae AgMDL*1 participates in immune response against *Plasmodium falciparum* (Dong et al., 2006), the tobacco hornworm *Manduca sexta* ML (*Ms*ML-1) protein can bind to LPS and may be involved in LPS-induced signaling (Ao et al., 2008), and the European hard tick *Ixodes ricinus* genes encoding ML-domain containing protein (IrML) and Der-p2 (*Dermatophagoides pteronyssinus*) allergen-like protein may take part in tick immune response and defense reactions, and/or lipid metabolism (Horackova et al., 2010a; 2010b). A shrimp ML protein (LvML) can also bind LPS and its transcription level is up-regulated by LPS challenge (Liao et al., 2011). Thus, *Ag*MDL1 and *Ms*ML-1 may have functions in

innate immunity similar to vertebrate MD-2. However, whether insect *ML* genes have functions in innate immune signaling pathways remain to be determined.

Drosophila genome encodes two NPC1 proteins (NPC1a and NPC1b) and eight NPC2 proteins. *Drosophila npc1* gene is required for molting (Huang et al., 2005), intracellular trafficking of sterols (Fluegel et al., 2006), neuronal function and viability (Phillips et al., 2008), and spermatogenesis (Wang et al., 2011). Among the eight *Drosophila* NPC2 proteins, NPC2a is most similar to human NPC2 (36% identity), and *npc2a* and *npc2b* genes play redundant roles in sterol homeostasis and ecdysteriod biosynthesis (Huang et al., 2007). But functions of the other six *Drosophila npc2* genes (*npc2c-h*) have not been studied.

Drosophila npc2 genes can be further divided into three subgroups depending on the numbers of cysteine residues (6, 7 or 8 Cys) in the amino acid sequences. NPC2a, 2b, 2c and 2f have six cysteine residues, NPC2d and 2e contain seven cysteine residues, and NPC2g and 2h have eight cysteine residues. The purpose of this study is to investigate whether any *Drosophila* npc2 genes may have functions in immune signaling pathways. We chose NPC2a, NPC2e and NPC2h, which represent each of the three subgroups, respectively, in this study to compare their binding properties and functions since the numbers of cysteine residues and formation of disulfide bonds are important in the structures and functions of vertebrate NPC2 and MD-2 proteins (Friedland et al., 2003; Kim et al., 2007). In addition, NPC2a is homologous to vertebrate NPC2 in functions, NPC2e has seven cysteine residues similar to vertebrate MD-2, and formation of the disulfide bonds and the free cysteine residue in MD-2 are important for ligand binding and signal transduction (Keestra and van Putten, 2008; Mancek-Keber et al., 2009). *Ag*MDL1 and *Ms*ML-1, the two insect proteins that have functions in innate immunity, contain eight cysteine residues (Dong et al., 2006; Ao et al., 2008). Our *in vitro* assay showed that the three recombinant *Drosophila* NPC2 proteins not only bound to LPS and lipid A, but also bound to LTA and PG, a property that has not been reported previously for vertebrate NPC2 or MD-2 protein. More importantly, over-expression of NPC2e and NPC2a proteins activated *diptericin* promoter reporter in S2 cells stimulated by DAP-type and Lys-type peptidoglycans, respectively, suggesting that NPC2 proteins may play a role in the Imd pathway. Over-expression of NPC2a protein also suppressed *drosomycin* promoter activity in S2 cells activated by *Bacillus subtilis* PG (DAP-type PG). This is the first report about *in vitro* functions of *Drosophila* NPC2 proteins in innate immunity. Our results suggest that some *Drosophila* NPC2 proteins, and maybe some other insect ML family proteins, may function as co-receptors or PRRs for different ligands to modulate innate immune signal pathways.

2. Materials and methods

2.1 D. melanogaster strain and total RNA isolation

D. melanogaster (wild-type yellow strain) flies were kindly provided by Dr. Erika Geisbrecht, School of Biological Sciences at University of Missouri-Kansas City. These flies were raised in artificial diets at 25°C and used for developmental expression of *npc2* genes. *D. melanogaster* Schneider S2 cells were purchased from American Type Culture Collection ATCC). Total RNAs were extracted from these flies at different developmental stages embryos, three larval stages, pupae, male and female adults (4-day old)) and S2 cells with TRI reagent (Sigma) following the manufacturer's instruction.

D. melanogaster (wild-type Oregon^R (Or^R) strain) flies were kindly provided by Dr. Qisheng Song, Division of Plant Sciences, College of Agriculture, Food and Natural Resources, University of Missouri at Columbia. These flies were reared on artificial blue diet (Fisher Scientific) at room temperature for bacterial infection experiments. *Escherichia coli* DH5α and *Staphylococcus aureus* were cultured overnight in LB broth at 37°C,

collected by brief centrifugation, washed with phosphate-buffered saline (PBS) (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH=7.4), and then resuspended in PBS. For infection experiments in *Drosophila* larvae, newly hatched third instar larvae were fed diet containing *E. coli* or *S. aureus* (by mixing equal volume of diet and OD_{600}=1.0 bacterial cells in PBS) at room temperature. Larvae were then collected at 0, 2, 6, 12, and 24h after feeding for total RNA extraction, and each group contained at least 15 larvae. For infection experiments in adult flies, *E. coli* culture was diluted to OD_{600}=0.8 (Jensen, 2007), and *S. aureus* culture was diluted to OD_{600}=0.2 (Nehme, 2011). Female and male adult flies (2–4 days old) were pricked at the dorsal thorax with glass needles dipped in the diluted *E. coli* or *S. aureus* culture, or PBS (control). Flies were collected at 0, 2, 6 and 12h after infection for total RNA extraction. Each group contained at least 12 female or male flies. We did not include RNA samples from 24h post-infection since ~50% female and almost all male flies died at 24h after *S. aureus* infection. These experiments were repeated at least three times.

2.2 Sequence and data analyses

Eight *Drosophila npc2* genes were obtained from *D. melanogaster* genome at NCBI (http://www.ncbi.nlm.nih.gov/). Primers (Table 1) were designed to amplify cDNA sequences, which were verified by DNA sequencing. Protein sequences were deduced from cDNA sequences. Multiple sequence alignments were performed by ClustalW (http://www.ch.embnet.org/software/ClustalW.html). A phylogenetic tree of the ML family proteins from some insect species was constructed by MEGA 5.05 software. Figures were made with the GraphPad Prism software (GraphPad, CA, USA) with one representative set of data. Significance of difference was determined by an unpaired t-test or by one way ANOVA followed by a Tukey's multiple comparison test using the same software (GraphPad, CA, USA).

2.3 Expression and purification of three recombinant NPC2 fusion proteins

Total RNA from adult flies was reverse transcribed to the first stand cDNA, which was used as the template for the following PCR reactions. Primers npc2a-pET32a-F and npc2a-pET32a-R, npc2e-pET32a-F and npc2e-pET32a-R, and npc2h-pET32a-F and npc2h-pET32a-R (Table 1) were used to amplify *npc2a*, *npc2e* and *npc2h* cDNA sequences, respectively (encoding mature NPC2a, NPC2e and NPC2h proteins). PCR conditions were: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 45 s, followed by 1 cycle of 72°C for 10 min.

Then the three DNA fragments were purified and digested with *Eco*R I/*Not* I, purified again, and ligated into the *Eco*R I/*Not* I sites of the pET32a vector (Novagen). Recombinant NPC2a-pET32a, NPC2e-pET32a and NPC2h-pET32a fusion proteins, as well as the thioredoxin (TRX)-tag (as a control protein from empty pET32a vector) were expressed in *E. coli* BL21 (DE3) cells after isopropyl-1-thio-β-D-galactopyranosid (IPTG, 1 mM) induction at 16°C overnight. All three recombinant NPC2 fusion proteins and the control TRX were partially soluble, thus soluble fractions were used to purify recombinant proteins by nickel-nitrilotriacetic acid (Ni-NTA) resins (Qiagen) under native conditions following the manufacturer's instructions. Purified recombinant proteins were collected and dialyzed with Tris buffer (25 mM Tris-HCl, 100 mM NaCl, pH 7.0) containing 5% glycerol, and stored at 4°C until use. Purified proteins were analyzed by Western blot analysis using mouse monoclonal anti-polyHistidine antibody (Sigma) as the primary antibody.

2.4 Developmental and induced expression of npc2 genes by quantitative real-time PCR

Total RNAs from the embryos, three stages of larvae, pupae, adults (4-day old females and males), S2 cells, and immunized third instar larvae, female and male adult flies (after

infection with *E. coli* and *S. aureus*) were digested by RQ1 RNase-Free DNase (Promega) and then reverse transcribed to the first strand cDNAs, which were diluted 50-fold and used as the templates for quantitative real-time PCR. Primers npc2a-RT-F and npc2a-RT-R, npc2e-RT-F and npc2e-RT-R, npc2h-RT-F and npc2h-RT-R, dpt-RT-F and dpt-RT-R, drs-RT-F and drs-RT-R, rp49F and rp49R (Table 1) were used for *npc2a*, *npc2e*, *npc2h*, *diptericin*, *drosomycin*, and *rp49* (ribosomal protein 49 gene as an internal control), respectively. The 20μl reaction mixture contains 10 μl 2×RT^2 SYBR Green/ROX qPCR Master Mix (SABiosciences, QIAGEN), 2 μl diluted cDNA template, 4 μl forward primer (1 pmole/μl), and 4 μl reverse primer (1 pmole/μl). Real-time PCR reaction conditions were: 1 cycle of 95°C for 10min, 40 cycles of 95°C for 15s and 60°C for 1min. Real-time PCR was performed in an AB7000 real-time PCR instrument (Applied Biosystems). After relative quantitative PCR, the dissociation curve analysis was performed. Real-time PCR results were analyzed using a comparative method ($2^{-\Delta\Delta Ct}$) and output with sequence detection software (SDS-7000 software, Applied Biosystems). Real-time PCR experiments were repeated with 3 different biological samples, and each real-time PCR was repeated 3 times.

2.5 Ligand binding assay

To test binding ability of NPC2 proteins to different bacterial components, plate ELISA assays were performed. The following ligands were used: smooth lipopolysaccharide (LPS) from *Salmonella enteric*, *E. coli* 055:B5, *E. coli* 0111:B4; rough mutants of LPS from *E. coli* EH100 (Ra mutant), *E. coli* F583 (Rd mutant), *E. coli* J5 (Rc mutant), and *S. enterica* serotype minnesota Re 595 (Re mutant), lipid A monophosphoryl from *E. coli* F583 (Rd mutant), lipid A diphosphoryl from *E. coli* F583 (Rd mutant) (all from Sigma-Aldrich); TLRgrade lipoteichoic acid (LTA) and peptidoglycan (PG) from *B. subtilis* and *S. aureus*, TLRgrade LPS and PG from *E. coli* K12 (all from Invivogen).

Wells of flat-bottom 96-well microtiter plates (Nunc MaxiSorp, eBioscience) were coated with 2 μg/well of each ligand (50 μl/well of 40 μg/ml in water) as described previously (Yu and Kanost, 2000; Yu et al., 2005). Purified recombinant NPC2a, NPC2e, NPC2h fusion proteins and the control TRX protein were diluted in binding buffer (50 mM Tris-HCl, 50 mM NaCl, pH 8.0) containing 0.1 mg/ml BSA to 100 nM and added to each well of the coated plates (50 μl/well) and incubated at room temperature for 3 h. Plates were washed with binding buffer four times (each for 5 min), and mouse monoclonal anti-polyHistidine antibody (Sigma, 1:3000 dilution in binding buffer containing 0.1 mg/ml BSA) was added (100 μl/well) and incubated at 37°C for 2 h. Then plates were washed again, and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, 1:2000 dilution in binding buffer containing 0.1 mg/ml BSA) was added (100 μl/well) and incubated at 37°C for another 2 h. At last, *p*-nitro-phenyl phosphate (1 mg/ml in 10 mM diethanolamine, 0.5 mM $MgCl_2$) was added (50 μl/well) to the plates and incubated for 20 min at room temperature. Absorbance at 405 nm of each well was measured by a plate reader (BioTek PowerWave XS Spectrophotometer) and collected. Specific binding was calculated by subtracting the total binding of the control TRX from the total binding of NPC2 proteins. These binding assays were repeated at least 3 times.

For competitive binding assays, NPC2 proteins were diluted to 100nM and pre-incubated with increasing amounts of competitors (free LPS-0111:B4, lipid A diphosphoryl, LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA and PG-SA) at room temperature for 1h with gentle rotation. The mixtures were centrifuged briefly to remove any precipitates and the supernatants were added to the plates coated with different microbial components. Binding assays were then performed essentially the same as described above. These binding assays were repeated at least 3 times.

2.6 Over-expression of NPC2a, NPC2e and NPC2h in S2 cells

cDNA sequences encoding mature proteins of NPC2a, NPC2e, NPC2h and a C-type lectin control protein from *An. gambiae* (CTL4, Genbank accession number: XP_315348) were amplified and ligated into a modified pMT/Bip/Flag vector (modified from the original pMT/Bip/V5-HisA vector (Invitrogen) by addition of a Flag-tag to the N-terminus and removal of the V5-His tag, Zhong X and Yu XQ, unpublished results). The first strand cDNA from adult flies was used as the template for PCR amplification. Primers pMT-ctl4-NotIF and pMT-ctl4-PmeIR, pMT-npc2a-NotIF and pMT-npc2a-PmeIR, pMT-npc2e-NotIF and pMT-npc2e-PmeIR, pMT-npc2h-NotIF and pMT-npc2h-PmeIR (Table 1) were used to amplify *ctl4*, *npc2a*, *npc2e* and *npc2h* cDNA sequences, respectively.

Then the four cDNA fragments were digested with *Not* I and *Pme* I, purified, and ligated into the *Not* I/*Pme* I site of the modified pMT/Bip/Flag vector. Recombinant plasmids were used to transfect *Drosophila* S2 cells using Gencarrier-2 TM DNA transfection reagent (Epoch Biolabs) in serum free insect cell culture media (Hyclone SEX, Thermo Scientific). After overnight transfection, serum free cell culture media were discarded, fresh complete cell culture media (Hyclone TNM-FH, Thermo Scientific) containing 1/10 volume Standard fetal bovine serum (Hyclone), 1/100 volume L-Glutamine and Penicillin-Streptomycin (Sigma)) were added, and then $CuSO_4$ (final concentration of 500μM) was added to induce recombinant protein expression. After 48h protein expression, recombinant proteins were verified by Western blot analysis. In brief, 10 μl cell culture media and cell lysates were separated on 15% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane (Bio-Rad). The membrane was incubated in blocking buffer (100 mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1%Tween-20, 5% BSA) for 1 h, monoclonal mouse anti-FLAG® M2 antibody (Sigma, 1:3000 dilution in blocking buffer) was added as the primary antibody, and alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Sigma, 1:10000 dilution in blocking buffer) was used as secondary antibody for Western blot analysis. Finally, AP conjugate substrate kit Bio-Rad) was used to develop color reaction.

2.7 Dual luciferase activity assay in S2 cells

Recombinant pMT-*ctl4*, pMT-*npc2a*, pMT-*npc2e*, pMT-*npc2h* or empty pMT plasmid 6 μg each), along with pGL-*drosomysin* or pGL-*diptericin* reporter vector (3 μg each), and 0.3μg pRL-TK vector (wild type *Renilla* luciferase control reporter vector) were used to co-transfect S2 cells (in 6-well plates) using Gencarrier-2 TM DNA transfection reagent Epoch Biolabs). After overnight transfection, cell culture media were replaced with fresh complete media, and $CuSO_4$ was added to induce protein expression. Then after protein expression for 48h, TLRgrade LPS-K12 and PG-K12 from *E. coli* K12, LTA-BS and PG-BS from *B. subtilis*, and LTA-SA and PG-SA from *S. aureus* (Invivogen) were added to final concentrations of 0.5 and 5 μg/ml, respectively, incubated for 24h, and activities of the firefly and sea pansy luciferases were measured sequentially by the GloMax®-Multi detection system Promega). At the same time, cell culture media and cell lysates were also collected for Western blot analysis to verify expression of recombinant proteins. These transfection experiments were repeated at least three times.

3. Results

3.1 Sequence analysis of Drosophila NPC2 proteins

There are eight *npc2*-like genes in *D. melanogaster*, encoding proteins with a signal peptide and a single ML domain (MD-2 related Lipid-recognition domain). Therefore, these NPC2 proteins are members of the ML family proteins. Based on the numbers of cysteine residues in the mature peptide sequences, the eight NPC2 proteins can be further divided into three subgroups: subgroup one includes NPC2a, 2b, 2c and 2f with 6 cysteines, subgroup two

contains NPC2d and 2e with 7 cysteines, and subgroup three includes NPC2g and 2h (isoform A) with eight cysteines. NPC2h has two isoforms, A and B, and isoform B is almost identical to isoform A except for a deletion of 27 residues close to the C-terminus and one residue difference prior to the beginning of the deletion. Thus NPC2h isoform B contains 7 cysteines. We use NPC2h to represent isoform A in this study. Multiple sequence alignment showed that six cysteine residues and three amino acids (Tyr, Pro and Asp) are conserved in all eight NPC2 proteins (Fig. 1, asterisks and filled triangles). Among *Drosophila* NPC2 proteins, NPC2a, 2b, 2c and 2f (subgroup 1) shares ~20–31.5% identify, NPC2d and 2e (subgroup 2) share 37% identity, while NPC2g and 2h (subgroup 3) share 78% identify. *Drosophila* NPC2 proteins share only 15–20% identity to human MD-2.

A phylogenetic tree was constructed with NPC2 proteins from *D. melanogaster* and ML family proteins from some other insect species (*An. gambiae*, *Ae. aegypti*, *T. castaneum*, *B. mori*, *M. sexta*, and *I. ricinus*) to study the evolutionary relationship of the insect ML family proteins. The phylogenetic tree showed that insect ML family proteins clustered into four groups (Fig. 2). The eight *Drosophila* NPC2 proteins clustered into 3 different groups: NPC2c, 2d and 2e clustered in group 1, NPC2g and 2h clustered in group 3 with *Ag*MDL1, *Ms*ML-1 and tick ML protein, NPC2a, 2b and 2f clustered in group 4 (Fig. 2).

3.2 Developmental and induced expressions of npc2a, npc2e and npc2h

We chose NPC2a, NPC2e and NPC2h, which contain 6, 7 and 8 cysteine residues, respectively, and represent the three different groups of *Drosophila* NPC2 proteins (Fig. 2), to compare their binding properties and functions. Developmental expression profiles of the eight *npc2* genes were different based on the high throughput expression data from the flybase (http://flybase.org/). The *npc2a* transcript was at a very high level in the embryos (0–4h and 14–16h), at a moderate high to high level in larvae, at a very high (peak) level in prepupae (new to 12h), at a very high level in pupae, and at a high level in both adult males and females (5 day old, same for the followings). The *npc2e* expression was at a very low to moderate level in the embryos, at a moderate high, high to very high level (peak) in larvae, at a very low level in pupae, at a low level in adult males, and at a moderate high level in the adult females. The *npc2h* mRNA was at a very low to low level in the embryos, at a moderate to moderate high level in larvae and pupae, at a very high (peak) level in adult males, and at a moderate high level in adult females.

We performed quantitative real-time PCR to confirm the developmental expression profiles of *npc2a*, *npc2e* and *npc2h* (Fig. 3A–C). The *npc2a* transcript was expressed at a high level in the embryos (2044 folds, relative expression, same for the followings), and its mRNA level dropped to a low level in the first larval stage (238 folds), then increased in the second and third larval stages (617 and 1156 folds, respectively), peaked in the pupae (5017 folds), and remained at high levels in both adult males (3209 folds) and females (2999 folds) (Fig. 3A). The *npc2e* mRNA was expressed at low levels in the embryos, pupae and both adult males and females (15, 2, 21 and 35 folds, respectively), but at higher levels in the larval stages (115, 233 and 163 folds in L1, L2 and L3 stages, respectively) (Fig. 3B). The *npc2h* transcript was expressed at a low level in the embryos (6 folds), and its expression level increased during development from larvae (16, 53 and 72 folds in L1, L2 and L3 stages, respectively) to pupae (136 folds) to adult females (153 folds), but at a significantly higher level in adult males (5861 folds) (Fig. 3C). Our quantitative PCR results were consistent with those of high throughput data from the flybase (see above). In S2 cells, *npc2a* was expressed at a high level (1716 folds), *npc2e* was expressed at a very low level (1 fold), and *npc2h* was expressed at a low level (35 folds) (Fig. 3A–C).

We also determined induced expression of *npc2a*, *2e* and *2h* genes in *D. melanogaster* larvae and adult flies after immune challenge with Gram-negative *E. coli* and Gram-positive *S.*

aureus. Real-time PCR results showed that, the two positive antimicrobial peptide genes *drosomycin* and *diptericin* were induced by *S. aureus* and *E. coli*, respectively, in larvae (Fig. 3D and H), female (Fig. 4A and B) and male (Fig. 4C and D) flies at 12h after bacterial infection, indicating that both *S. aureus* and *E. coli* can activate genes in *Drosophila* larvae and adult flies.

For the three *npc2* genes in the larvae at 12h post-infection, *npc2a* was almost not induced by *S. aureus* (~1.3 folds) and was induced by *E. coli* to a low level (~2.2 folds) (Fig. 3E), *npc2e* was significantly induced by *S. aureus* (~7.7 fold) and *E. coli* (~20.7 fold) (Fig. 3F), and *npc2h* was induced to low levels by *S. aureus* (~3.3 fold) and *E. coli* (~5 fold) (Fig. 3G). In adult flies, expression of the three *npc2* genes was not induced by *S. aureus* or *E. coli* compared to the PBS control group (Fig. 4E–J), although low levels of induction by *S. aureus* were observed for *npc2e* in female (~1.6 fold) (Fig. 4F) and male (~2.6 fold) (Fig. 4I) at 6h post-infection, for *npc2a* in male (~2.4 fold) (Fig. 4H) and *npc2h* in male (~2.1 fold) (Fig. 4J) at 2h post-infection. In fact, expression of *npc2e* and *npc2h* in female was inhibited by *E. coli* compared to the PBS control group (Fig. 4F and G).

3.3 NPC2 proteins bind to LPS and lipid A

The three NPC2 proteins were expressed in pET32a vector as thioredoxin (TRX) fusion proteins, and empty pET32a vector was also expressed as a control TRX protein. The recombinant proteins were purified to homogeneity as analyzed by SDS-PAGE (Fig. 5A), and these proteins could be recognized by monoclonal mouse anti-polyhistidine antibody (Fig. 5B).

To test whether NPC2a, 2e and 2h proteins can bind to LPS and lipid A, plate ELISA assays were performed using smooth LPS from *S. enteric* and *E. coli*, rough mutants of LPS (Ra-, Rc-, Rd- and Re-LPS), monophosphoryl and diphosphoryl lipid A as ligands. Bacterial LPS is composed of three moieties: the lipid A, the O-specific antigen, and the core carbohydrate (Raetz, 1990; Yu and Kanost, 2002). Our ELISA assay results showed that all three NPC2 proteins bound to smooth and rough mutants of LPS, monophosphoryl and diphosphoryl lipid A (Fig. 5D), suggesting that the three NPC2 proteins could bind to LPS and lipid A. More NPC2 proteins bound to smooth LPS and lipid A than to rough mutants of LPS, and least amount of NPC2 proteins bound to Re-LPS (Fig. 5D). Among the three NPC2 proteins, more NPC2h than NPC2a and NPC2e bound to some LPS and lipid A, and least amount of NPC2e bound to most ligands (Fig. 5D). For NPC2a and NPC2h, more proteins bound to lipid A than to rough mutants of LPS, and similar amounts of proteins bound to lipid A and smooth LPS (Fig. 5D), suggesting that NPC2a and NPC2h may mainly bind to the lipid A moiety of LPS. For NPC2e, less protein bound to diphosphoryl lipid A and rough mutants of LPS than to smooth LPS (Fig. 5D), suggesting that NPC2e may bind to both the O-specific antigen and lipid A moieties.

To confirm binding of NPC2 proteins to LPS and lipid A, a competitive binding assay was performed (Fig. 5E and F). The binding results showed that with increasing amounts of free LPS and lipid A, binding of NPC2 proteins to LPS (Fig. 5E) and lipid A (Fig. 5F) decreased significantly. The binding curve also showed that more NPC2h than NPC2a and NPC2e bound to LPS and lipid A, and similar amounts of NPC2a and NPC2e bound to LPS (Fig. 5E and F).

3.4 NPC2 proteins bind to LPS, peptidoglycan (PG) and lipoteichoic acid (LTA)

Although vertebrate MD-2 is an essential co-receptor for LPS in the TLR4-LPS signaling pathway (Shimazu et al., 1999; Viriyakosol et al., 2001), there has been no report about binding of MD-2 to other bacterial components such as peptidoglycan (PG) and lipoteichoic

acid (LTA). In order to test whether insect ML family proteins can function as co-receptors for different bacterial components, plate ELISA assay was performed using different bacterial components (LPS-K12 and PG-K12 from *E. coli* K12, LTA-BS and PG-BS from *B. subtilis*, LTA-SA and PG-SA from *S. aureus*). The binding results showed that all three *Drosophila* NPC2 proteins bound to LPS, PG and LTA (Fig. 6A). There was no significant difference among the three proteins in binding to PG and LTA, but significantly more NPC2h than NPC2a and NPC2e bound to LPS-K12 (Fig. 6A).

To confirm binding of the NPC2 proteins to bacterial components, competitive binding assays were also performed. With increasing amounts of free LPS-K12, LTA-BS and LTA-SA, binding of the three NPC2 proteins to the three bacterial components decreased significantly (Fig. 6B–D). The binding curve also showed that more NPC2h than NPC2a and NPC2e bound to LPS-K12 (Fig. 6B), and more NPC2a than NPC2e and NPC2h bound to LTA-SA (Fig. 6D). Increasing amounts of free PG-K12 and PG-BS also decreased binding of the three NPC2 proteins to PG (Fig. 6E and F), but the competition was less effective for free PG than for free LPS or LTA. Free PG-SA did not compete for binding of the three NPC2 proteins to immobilized PG-SA (data not shown).

3.5 Over-expression of NPC2 proteins activate diptericin in S2 cells stimulated by peptidoglycans

To test whether *Drosophila* NPC2 proteins have a function in signaling pathways to stimulate antimicrobial peptide genes, the three NPC2 proteins were over-expressed in S2 cells, and the activity of *drosomycin*- and *diptericin*-luciferase reporters was determined after PG, LPS or LTA stimulation. We first determined expression of the three *Drosophila* NPC2 proteins and the control CTL4 protein, a C-type lectin from *An. gambiae*, in S2 cells by Western blot analysis. The results showed that majority of the three NPC2 proteins and CTL4 was secreted into cell culture media (Fig. 5C). Compared the expression levels of the four proteins, NPC2a and NPC2h were expressed at high levels, and NPC2e was expressed at a lower level, while CTL4 was expressed at a relatively high level (Fig. 5C).

We then test whether over-expression of the three NPC2 proteins can activate *drosomycin* or *diptericin* promoter in S2 cells stimulated by LPS, PG or LTA from *E. coli*, *B. subtilis* and *S. aureus*. Dual luciferase assay showed that over-expression of the three NPC2 proteins did not alter the activity of *drosomycin* luciferase reporter in S2 cells stimulated by LPS-K12, PG-K12, LTA-BS, PG-BS (except for NPC2a), LTA-SA or PG-SA compared to over-expression of CTL4 or the pMT control group (Fig. 7A–C). In fact, over-expression of NPC2a significantly decreased the activity of *drosomycin* promoter activated by PG-BS (DAP-type PG) (Fig. 7B). Over-expression of NPC2 proteins also did not increase the activity of *diptericin* luciferase reporter stimulated by LPS-K12, LTA-BS or LTA-SA compared to CTL4 or the pMT control group (Fig. 7D–F). However, over-expression of NPC2e significantly increased *diptericin* luciferase activity in S2 cells stimulated by PG-K12 (Fig. 7D) and PG-BS (Fig. 7E), which are both DAP-type PGs, and over-expression of NPC2a significantly activated *diptericin* promoter stimulated by PG-SA (Fig. 7F), a Lys-type PG. These results suggest that NPC2 proteins may function as co-receptors for peptidoglycans in the Imd pathway to activate *diptericin* expression.

4. Discussion

4.1 Vertebrate MD-2 and other members of the ML family proteins

MD-2 was first identified as an essential protein for TLR4/LPS signaling in 1999 (Shimazu et al., 1999). Human MD-2 can form a complex with TLR4, and together they play an important role in LPS induced inflammatory responses (Saitoh, 2009). Vertebrate ML

family proteins can be divided into four groups: MD-2 and MD-1, Niemann-Pick type C2 (NPC2), mite major allergen protein, and GM2 activator protein (GM2A) (Inohara and Nunez, 2002). Members of the ML family proteins contain an MD-2-related Lipid-recognition (ML) domain, which is involved in lipid recognition or immune signaling. MD-1 is an MD-2 homolog, and it shares about 20% identity with MD-2. MD-1 forms a complex with radioprotective 105 (RP105) (Nagai et al., 2005; Yoon et al., 2011). RP105/MD-1 complex is a B-cell stimulator, but in antigen-presenting cells, RP105/MD-1 negatively regulates LPS response by direct interaction with the TLR4/MD2 complex (Nagai et al., 2005; Tsuneyoshi et al., 2005; Yoon et al., 2011). Niemann-Pick type C disease (NPC disease) is an autosomal recessive lipid storage disorder caused by defective egress of cholesterol from lysosomes due to mutations in one of the two genes, *npc1* or *npc2* (Naureckiene et al., 2000; Vanier, 2010). The *npc1* gene encodes a transmembrane protein, while *npc2* gene encodes secreted NPC2 protein, which is a member of the ML family (Inohara and Nunez, 2002; Vanier, 2010). The GM2 activator protein (GM2A) is a lysosomal transfer protein that can bind lipid (Wright et al., 2003).

4.2 Insect ML family proteins

Multiple *ML* genes have been identified in insects with known genome sequences, and they are called either *md-2*- or *npc2*-like genes in different species. For example, there are eight *npc2*-like genes in *D. melanogaster*, thirteen *md-2*-like genes in *An. gambiae*, fifteen *npc2*-like genes in *Ae. aegypti*, eight *md-2*-like genes in *T. castaneum*, and at least four *md-2*-like genes in *B. mori*. Some insect *ML* family genes were also identified by cDNA cloning (Ao et al., 2008; Horackova et al., 2010b). Insect *ML* genes encode ML proteins with 6, 7 and 8 cysteine residues, with most insect ML family proteins contain 6 cysteines, which are similar to vertebrate MD-1, NPC2 and mite major allergen proteins, and some insect ML members contain 8 cysteines, which are similar to GM2 activator protein (Xie et al., 1992). Only a few insect ML family proteins (*Drosophila* NPC2d and NPC2e, *An. gambiae* EAA07746 and EAA07715) contain 7 cysteines, which are similar to vertebrate MD-2. Seven cysteines in vertebrate MD-2 proteins can form three disulfide bonds, and the remaining free cysteine, which is conserved in all MD-2 but not in MD-1 proteins, is important for LPS binding (Keestra and van Putten, 2008; Mancek-Keber et al., 2009). It has been reported that secreted MD-2 protein exists as a heterogeneous collection of disulfide-linked oligomers consisting of stable dimers (Visintin et al., 2001). However, insect ML family proteins cannot be divided into different groups like vertebrate ML proteins, since functions of most insect ML family proteins are unknown. So far, only *Drosophila npc2a* and *npc2b* genes have functions similar to vertebrate *npc2* gene in sterol homeostasis and steroid biosynthesis (Huang et al., 2007), *An. gambiae AgMDL1* is involved in anti-*Plasmodium* response (Dong et al., 2006), and *M. sexta* ML-1 (*Ms*ML-1) can bind to LPS and may be involved in LPS signaling (Ao et al., 2008). Although *An. gambiae* and *M. sexta* ML proteins have functions in innate immune responses, whether they are involved in immune signaling pathways like vertebrate MD-2 remain elusive.

4.3 Binding ability of Drosophila NPC2 proteins

Among vertebrate ML family proteins, only MD-2 is involved in LPS recognition and signaling (Tsuneyoshi et al., 2005). MD-2 is anchored with TLR4, while MD-1 is associated with RP105. MD-2 can directly bind to LPS (Viriyakosol et al., 2001). Phe residues (Phe 119 and 121) and one free Cys residue (Cysteine 133) are important for MD-2 binding to LPS, but these residues are absent in MD-1 (Mancek-Keber et al., 2009; Tsuneyoshi et al., 2005). It is not known whether MD-2 and MD-1 can bind to other bacterial components such as PG and LTA.

Among *md-2*- or *npc2*-like genes identified in insects so far, only *Ms*ML-1 protein has been purified from larval hemolymph (Ao et al., 2008). *Ms*ML-1 can bind to LPS and may be involved in LPS signaling, suggesting that insect ML family proteins may also have functions similar to vertebrate MD-2. But little is known about binding of other insect ML family proteins to LPS or other bacterial cell wall components. We expressed *Drosophila* NPC2a, 2e and 2h, which contains 6, 7 and 8 cysteine residues, respectively, in bacteria as fusion proteins and tested their binding abilities to LPS, lipid A, PG and LTA. All three *Drosophila* NPC2 proteins could bind to smooth LPS, rough mutants of LPS, lipid A, as well as to PG and LTA to some extents (Figs. 5D–F and 6). NPC2a and NPC2h may mainly bind to the lipid A moiety, while NPC2e may bind to both the lipid A and O-specific antigen moieties of LPS (Fig. 5D). Thus, insect ML family proteins such as *Drosophila* NPC2a and NPC2b may function as NPC2 proteins in lipid metabolism (Huang et al., 2007), while some other insect ML proteins, including *Ag*MDL1, *Ms*ML-1 and some *Drosophila* NPC2 proteins, may serve as PRRs or co-receptors for different bacterial ligands in innate immune responses.

4.4 Function of Drosophila NPC2 proteins in innate immune signal pathways

Drosophila Toll pathway can be activated by Lys-type PG of Gram-positive bacteria and fungal β-1, 3-glucan via PGRP-SA and Gram-negative binding proteins (GNBPs) (Michel et al., 2001; Gobert et al., 2003; Gottar et al., 2006), while the Imd pathway is activated by DAP-type PG of Gram-negative bacteria and some Gram-positive *Bacillus* species via PGRP-LC and PGRP-LE (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002; Leulier et al., 2003; Kaneko et al., 2004). Expression of AMP genes can also be activated by LPS and LTA (Tanaka et al., 2009; Rao and Yu, 2010), and melanization response can be induced by PG (Schmidt et al., 2008; Ishii et al., 2010a). However, functions of insect ML family proteins in innate immunity have not been well characterized, although *Ag*MDL-1 (Dong et al., 2006), *Ms*ML-1 (Ao et al., 2008), and *I. ricinus* ML protein (Horackova et al., 2010b) may be involved in immune responses.

In order to test whether some *Drosophila* NPC2 proteins may play a role in innate immune signaling pathways, we over-expressed three NPC2 proteins in S2 cells and all three proteins were mainly secreted into cell culture media (Fig. 5C). Over-expression of the three NPC2 proteins did not activate *drosomycin* (the Toll pathway target gene) promoter in S2 cells stimulated by LPS, LTA or PG (Fig. 7A–C, except for NPC2a with PG-BS), or *diptericin* (the Imd pathway target gene) promoter stimulated by LPS-K12, LTA-BS, or LTA-SA (Fig. 7D–F). However, over-expression of NPC2e in S2 cells activated *diptericin* promoter stimulated by PG-K12 (Fig. 7D) and PG-BS (Fig. 7E), the two DAP-type PG, while over-expression of NPC2a activated *diptericin* promoter induced by PG-SA (Fig. 7F), a Lys-type PG. Also, over-expression of NPC2a actually suppressed *drosomycin* promoter activity stimulated by PG-BS (Fig. 7B). These results suggest that NPC2a and NPC2e may serve as co-receptors for PG in the Imd pathway. *Drosomycin* is synergistically regulated by the Toll and Imd pathways (Tanji et al., 2007), and our results also suggest that Lys-type and DAP-type PGs may activate both the Toll and Imd pathways.

Among the three *npc2* genes, *npc2a* was not induced by bacteria in larvae, female and male flies (Figs. 3E, 4E and 4H), which is consistent with its main role in sterol homeostasis and steroid biosynthesis (Huang et al., 2007). But, due to its binding ability to bacterial components, NPC2a could also modulate signaling pathways by suppressing *drosomycin* or stimulating *diptericin* after binding to PG-BS (DAP-type PG) or PG-SA (Lys-type PG). Suppression of an immune response stimulated by PG may be important to the host, as some bacterial PG can over-activate melanization response resulting in production of excessive reactive oxygen species (ROS), which are lethal to the host (Ishii et al., 2010a). *Npc2e* was significantly induced by *S. aureus* and *E. coli* in larvae (Fig. 3F) and was also induced by *S.*

aureus in female and male flies (Fig. 4F and I), and NPC2e could activate *diptericin* induced by PG-K12 and PG-BS (Fig. 7D and E). Thus, NPC2e may function as a co-receptor for DAP-type PG to stimulate the Imd pathway. *Npc2h* was not induced by bacteria in larvae and adult flies (Figs. 3G, 4G and 4J) and over-expression of NPC2h in S2 cells did not activate *drosomycin* or *diptericin* (Fig. 7). We still could not rule out the possibility of NPC2h in signaling pathways, since *npc2h* transcript was expressed at a very high level in S2 cells (1716 folds) compared to *npc2e* (1 fold) and *npc2a* (35 folds) (Fig. 3A–C), thus over-expression of NPC2h may not increase its protein concentration sufficient to show difference in activation of *drosomycin* or *diptericin* promoter.

In addition to *npc2a* and *npc2e*, some of the remaining four *Drosophila npc2* genes (*npc2c*, *2d*, *2f* and *2g*) may also participate in innate immune signal pathways. Future work is to elucidate whether and how *Drosophila npc2* genes can serve as co-receptors or modulators in innate immune signal pathways *in vivo* in *D. melanogaster*.

Acknowledgments

This work was supported by National Institutes of Health Grant GM066356. We want to thank all the members in Yu lab for their help in the experiments, thank Dr. Erika Geisbrecht and Ze Liu for providing fruit flies, and Elizabeth Dobens for the fly media.

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>*Drosophila* NPC2 proteins can be divided into three subgroups based on the numbers of cysteine residues. >NPC2a, NPC2e and NPC2h, representing the three subgroups, are selected for further study. >The three NPC2 proteins can bind to lipopolysaccharide, lipid A, peptidoglycan and lipoteichoic acid. >Over-expression of NPC2a and NPC2e can stimulate *diptericin* promoter in S2 cells induced by peptidoglycan. >NPC2 proteins may serve as co-receptors for different ligands to modulate immune signaling pathways.

Fig. 1. Alignment of *D. melanogaster* NPC2 proteins

Protein sequences of *Drosophila* NPC2a to NPC2h [CG7291, CG3153, CG3934, CG12813, CG31410, CG6164, CG11314 and CG11315 (CG11315-PA and -PB for isoforms A and B)] and human MD-2 were aligned by ClustalW and six conserved cysteine residues (indicated by "*") were aligned manually. Predicted signal peptides are underlined. Three residues (Tyr, Pro and Asp) conserved in all 8 *Drosophila* NPC2 proteins (except NPC2h isoform B) are indicated by filled triangles. Extra cysteine residues in addition to the 6 conserved cysteines are boxed.

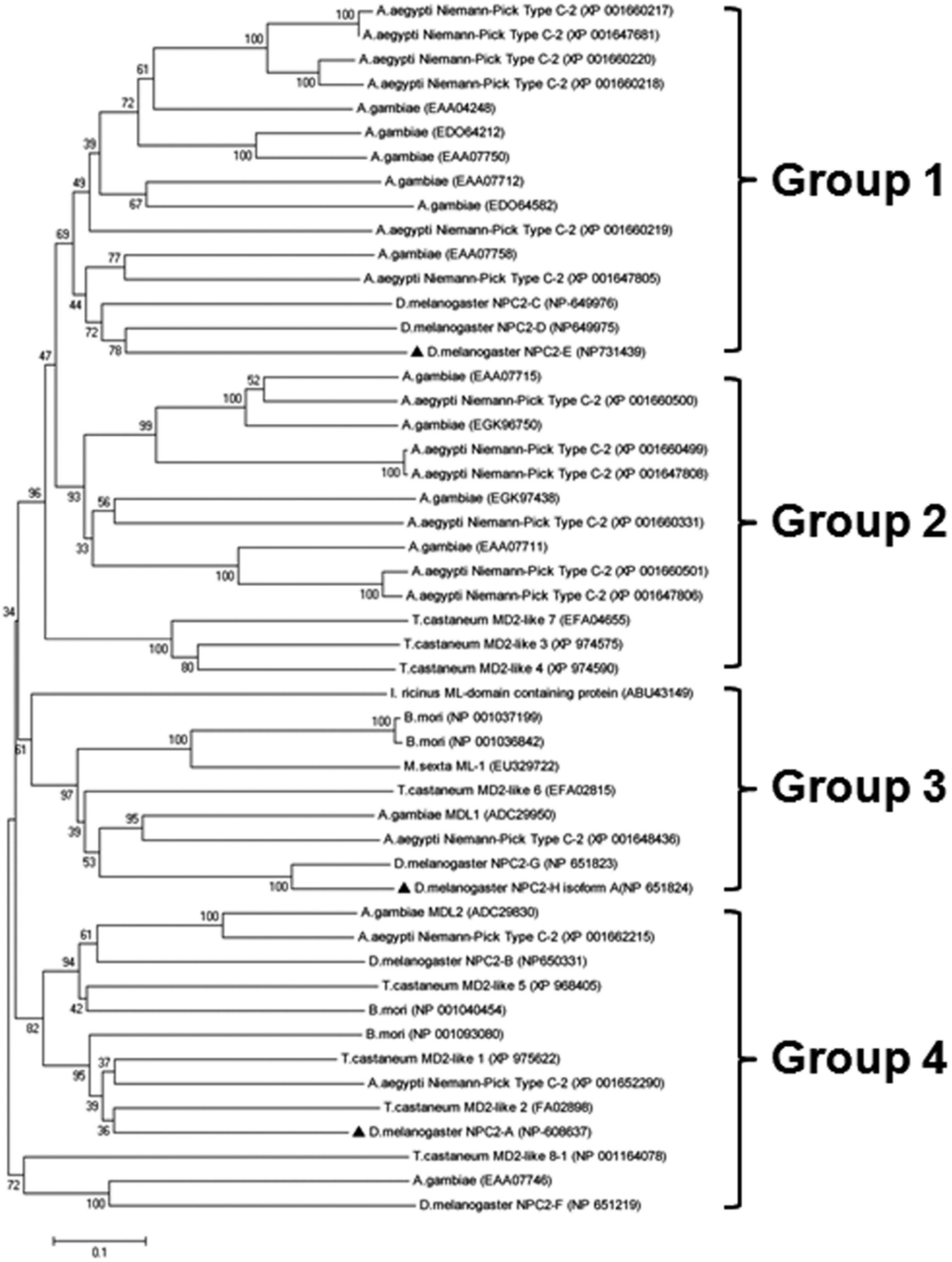

Fig. 2. Phylogenetic analysis of the insect ML family proteins

NPC2 proteins from *D. melanogaster*, MD-2 or NPC2 like proteins from *An. gambiae*, *Ae. aegypti*, *T. castaneum*, *B. mori*, *M. sexta*, and *I. ricinus* were used to construct the NJ tree by the MEGA5.05 software. The GenBank accession number for each sequence is shown in the parenthesis. The three *Drosophila* NPC2 proteins selected for this study were marked with filled triangles.

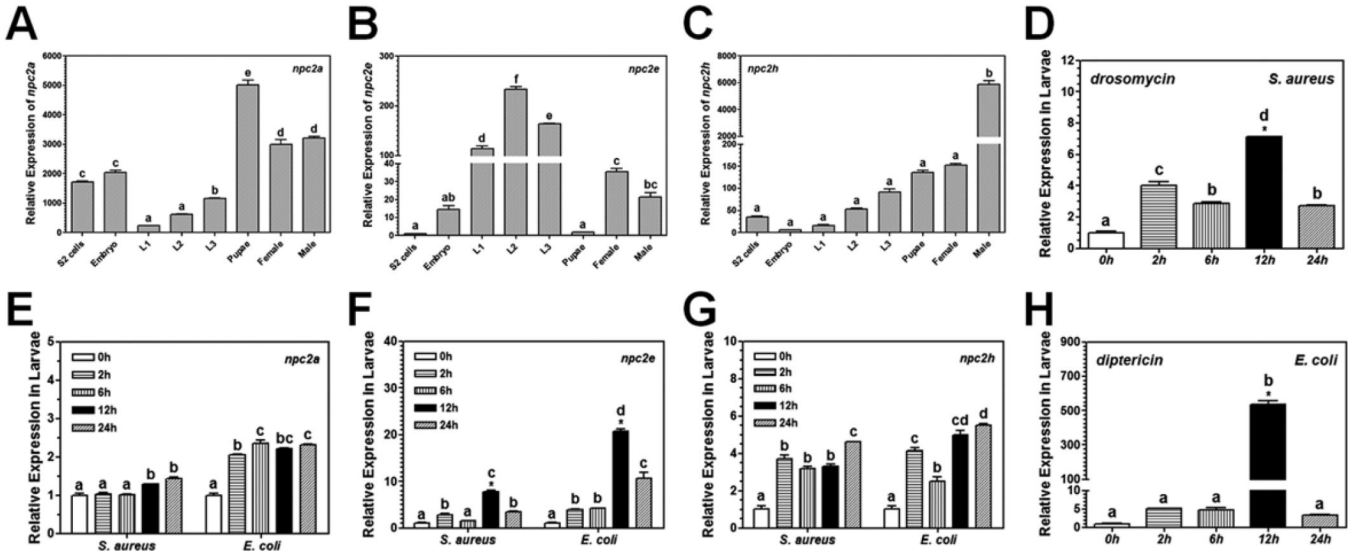

Fig. 3. Developmental expression of three *Drosophila npc2* genes and their expression in larvae after bacterial infection

Total RNAs were prepared from *Drosophila* embryos, 3 stages (L1–L3) of larvae, pupae, adult males and females (4-day old), and S2 cells, and expression of *npc2a, npc2e* and *npc2h* transcripts was determined by quantitative real-time PCR (A–C). Total RNAs were also prepared from *Drosophila* third instar larvae fed on diets containing *E. coli* or *S. aureus* for 0, 2, 6, 12 and 24h, and expression of *npc2a, npc2e*, *npc2h*, *drosomycin* and *diptericin* (two positive antimicrobial peptide genes) was also determined by quantitative real-time PCR (D–H). *Drosophila rp49* gene was used as an internal control gene. Identical letters are not significant difference ($p>0.05$), while different letters indicate significant difference ($p<0.05$) determined by one way ANOVA followed by a Tukey's multiple comparison test (for bacterial infection in panels E–G, comparison was made among different time points but not between the two bacteria).

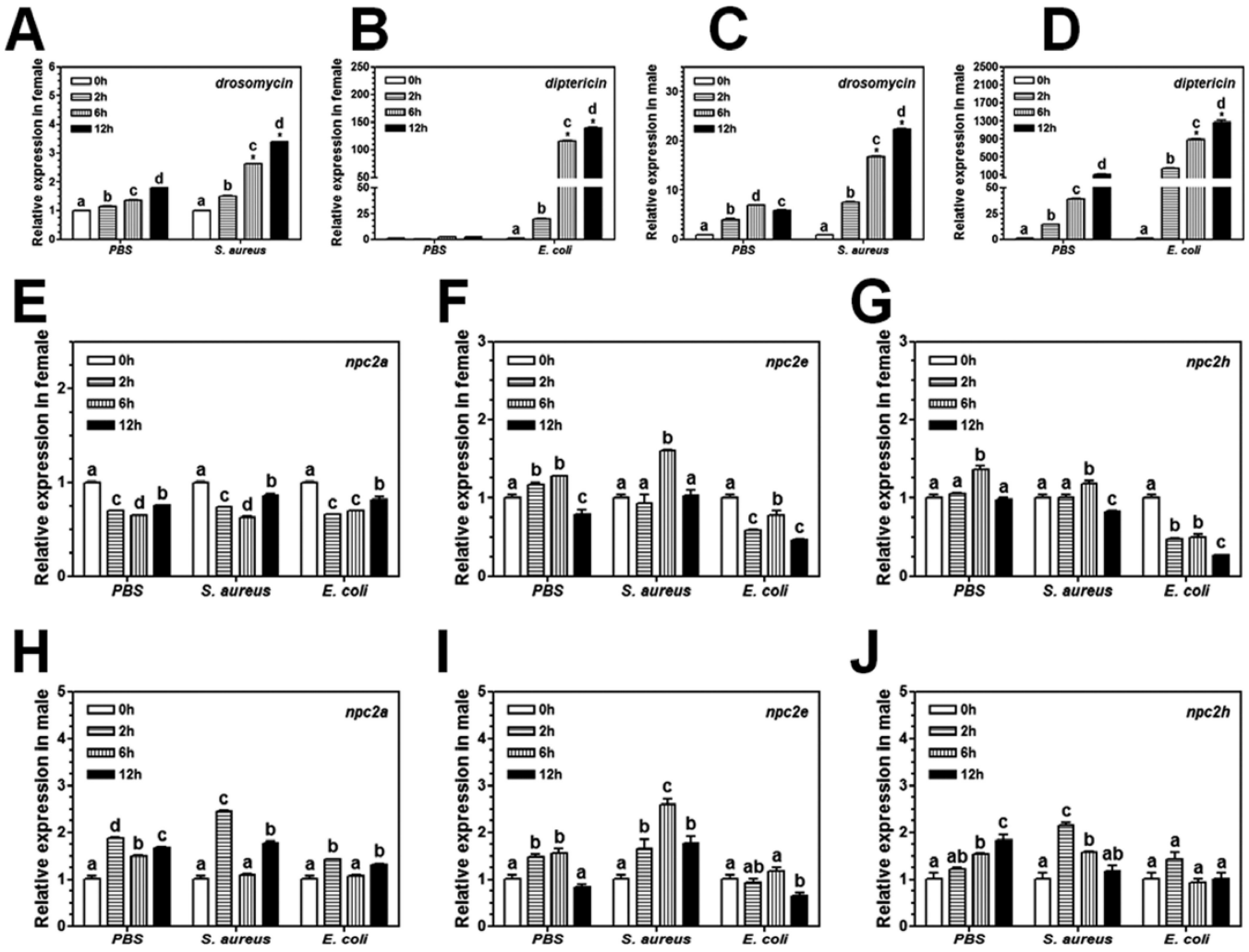

Fig. 4. Expression of three *Drosophila npc2* genes in adult flies after bacterial infection

Adult female and male flies were pricked with glass needles dipped in PBS (control), *E. coli* or *S. aureus* culture, flies were collected at 0, 2, 6 and 12h post-infection for total RNA preparation, and expression of *npc2a, npc2e*, *npc2h*, *drosomycin* and *diptericin* in these flies was determined by quantitative real-time PCR. *Drosophila rp49* gene was used as an internal control gene. Identical letters are not significant difference ($p>0.05$), while different letters indicate significant difference ($p<0.05$) determined by one way ANOVA followed by a Tukey's multiple comparison test (comparison was made among different time points within a treatment but not among different treatments).

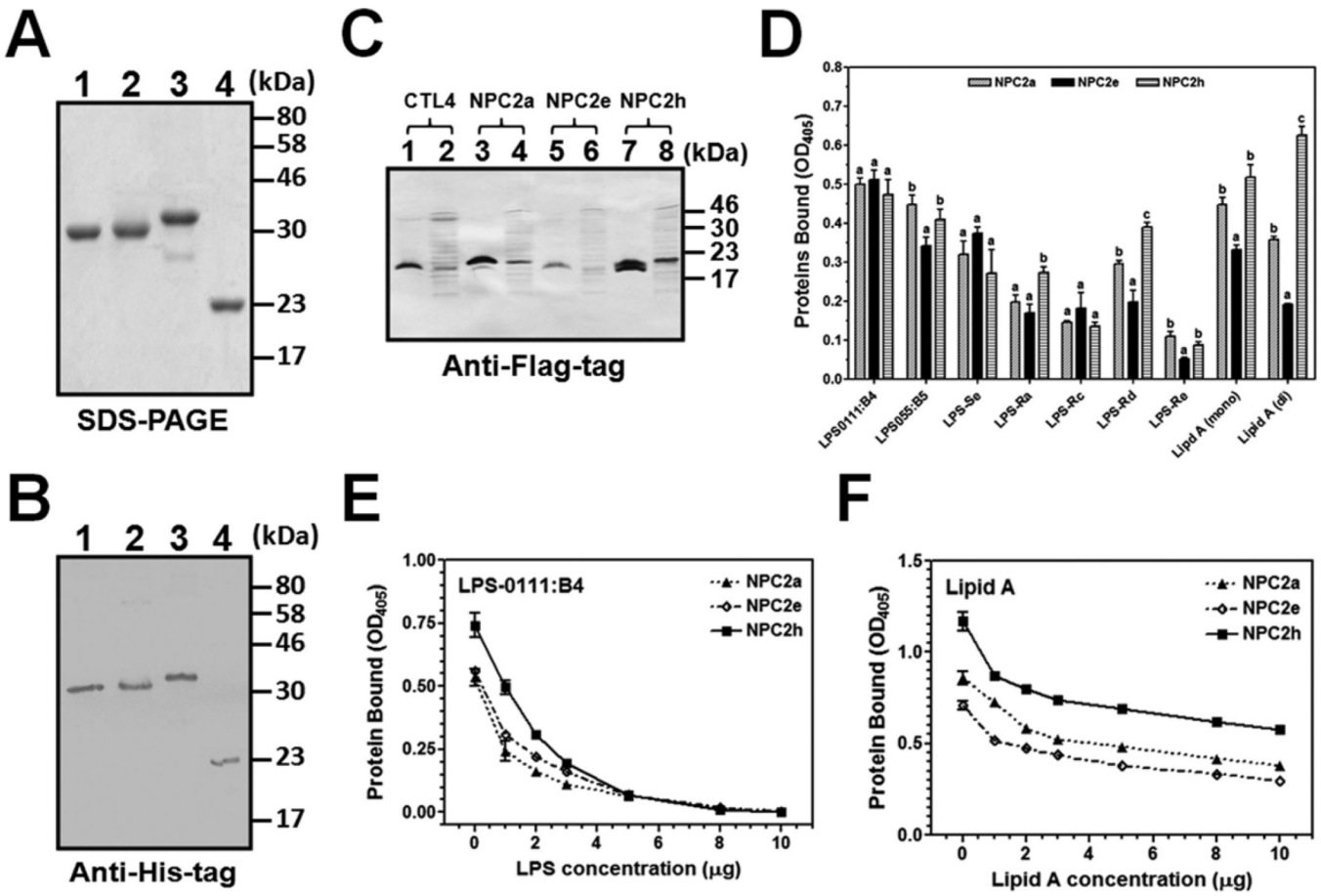

Fig. 5. NPC2a, NPC2e and NPC2h proteins can bind to LPS and Lipid A

Purified recombinant NPC2a, NPC2e, NPC2h fusion proteins and the control thioredoxin (TRX) were analyzed by SDS-PAGE (A) or Western blot (B). Proteins were separated on 15% SDS-PAGE, and then either stained with Coomassie Brilliant Blue (A) or transferred to a nitrocellulose membrane for Western blot analysis using monoclonal mouse anti-polyhistidine antibody (B). Lanes 1–4: purified recombinant NPC2a, NPC2e, NPC2h and the control TRX, respectively (2 μg each protein for the stained gel, and 50 ng each protein for Western blot). (C) Expression of NPC2a, 2e, 2h and a control protein CTL4 (a C-type lectin from *An. gambiae*) in S2 cells. Proteins were expressed for 48h, and cell culture media (lanes 1, 3, 5, and 7) and cell lysates (lanes 2, 4, 6, and 8) (10μl each) were separated on 15% SDS-PAGE and proteins were transferred to a nitrocellulose membrane for Western blot analysis using monoclonal mouse anti-FLAG® M2 antibody as described in the Materials and Methods. (D) Binding of recombinant NPC2 proteins to LPS and lipid A. Wells of 96-well fat-bottom microtiter plates were coated with smooth LPS from *E. coli* and *S. enteric* (LPS-Se), rough mutants of LPS (Ra, Rd, Rc and Re mutants), monophosphoryl and diphosphoryl lipid A. Purified NPC2a, 2e and 2h fusion proteins and the control TRX were diluted to 100nM and added to the ligand-coated plates, and plate ELISA assay was performed as described in the Materials and Methods. The figure showed specific binding of recombinant NPC2 proteins to LPS and lipid A after subtracting the total binding of the control TRX from the total binding of NPC2 proteins. Each bar represents the mean of three individual measurements ± SEM. Identical letters are not significant difference ($p>0.05$), while different letters indicate significant difference ($p<0.05$) among the three proteins to each ligand determined by one way ANOVA followed by a Tukey's multiple comparison

test. (E) and (F): competitive binding of NPC2 proteins to LPS and lipid A. Purified NPC2a, 2e and 2h fusion proteins were diluted to 100nM and pre-incubated with increasing amounts of free LPS-O111:B4 (E) or lipid A diphosphoryl (F), and the mixtures were then added to the LPS or lipid A-coated plates for the plate ELISA assays. Each point represents the mean of three individual measurements ± SEM.

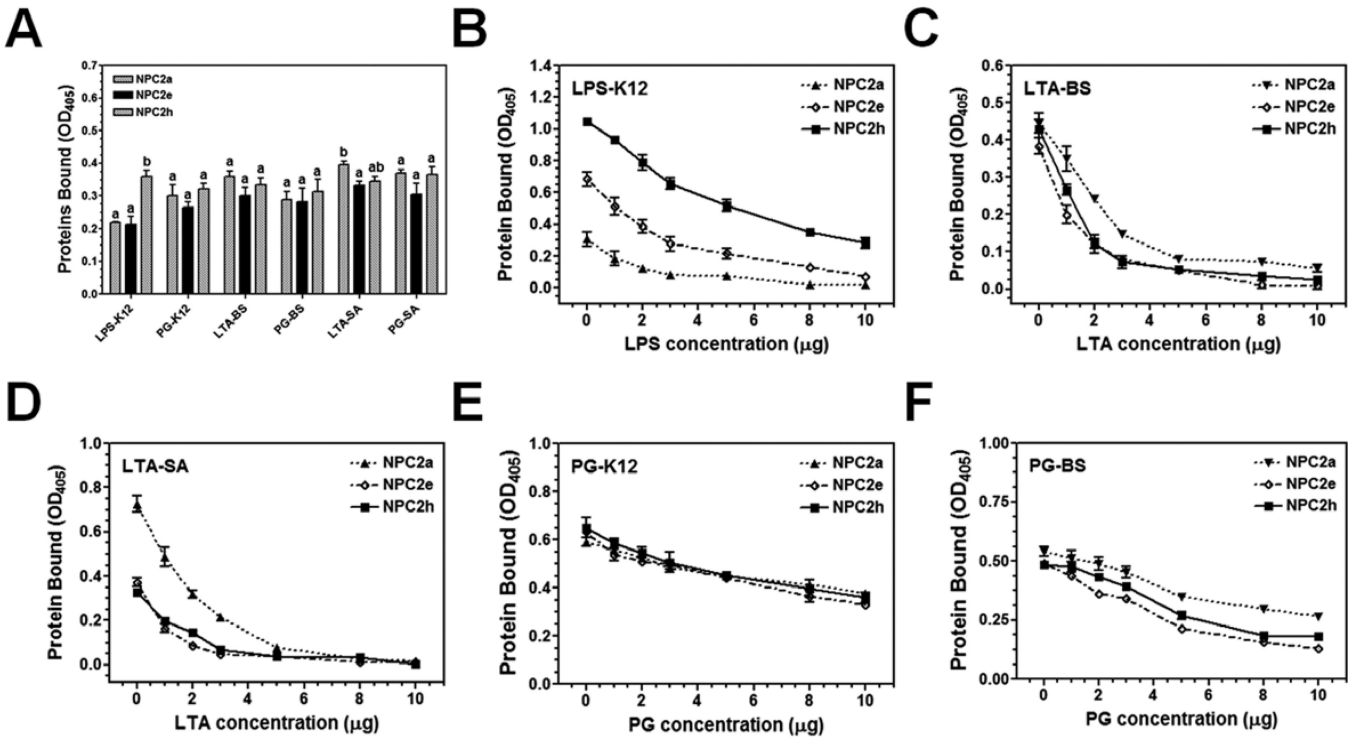

Fig. 6. NPC2a, NPC2e and NPC2h proteins bind to LPS, peptidoglycan (PG) and lipoteichoic acid (LTA)

Wells of 96-well fat-bottom microtiter plates were coated with TLRgrade *E. coli* LPS-K12 and PG-K12, *B. subtilis* LTA-BS and PG-BS, and *S. aureus* LTA-SA and PG-SA (A). Purified NPC2a, 2e and 2h fusion proteins and the control TRX were diluted to 100nM and added to the ligand-coated plates, and binding assay was performed as described in Fig. 5D and the Materials and Methods. The figure showed the specific binding of NPC2 proteins to LPS, PG and LTA after subtracting the total binding of the control TRX from the total binding of NPC2 proteins. Each bar represents the mean of three individual measurements ± SEM. Identical letters are not significant difference ($p>0.05$), while different letters indicate significant difference ($p<0.05$) among the three proteins to each ligand determined by one way ANOVA followed by a Tukey's multiple comparison test. (B) to (F): competitive binding of NPC2 proteins to LPS, LTA and PG. Purified NPC2a, 2e and 2h fusion proteins were diluted to 100nM and pre-incubated with increasing amounts of free LPS-K12 (B), LTA-BS (C), LTA-SA (D), PG-K12 (E) or PG-BS (F), and the mixtures were then added to the ligand-coated plates for the plate ELISA assays. Each point represents the mean of three individual measurements ± SEM.

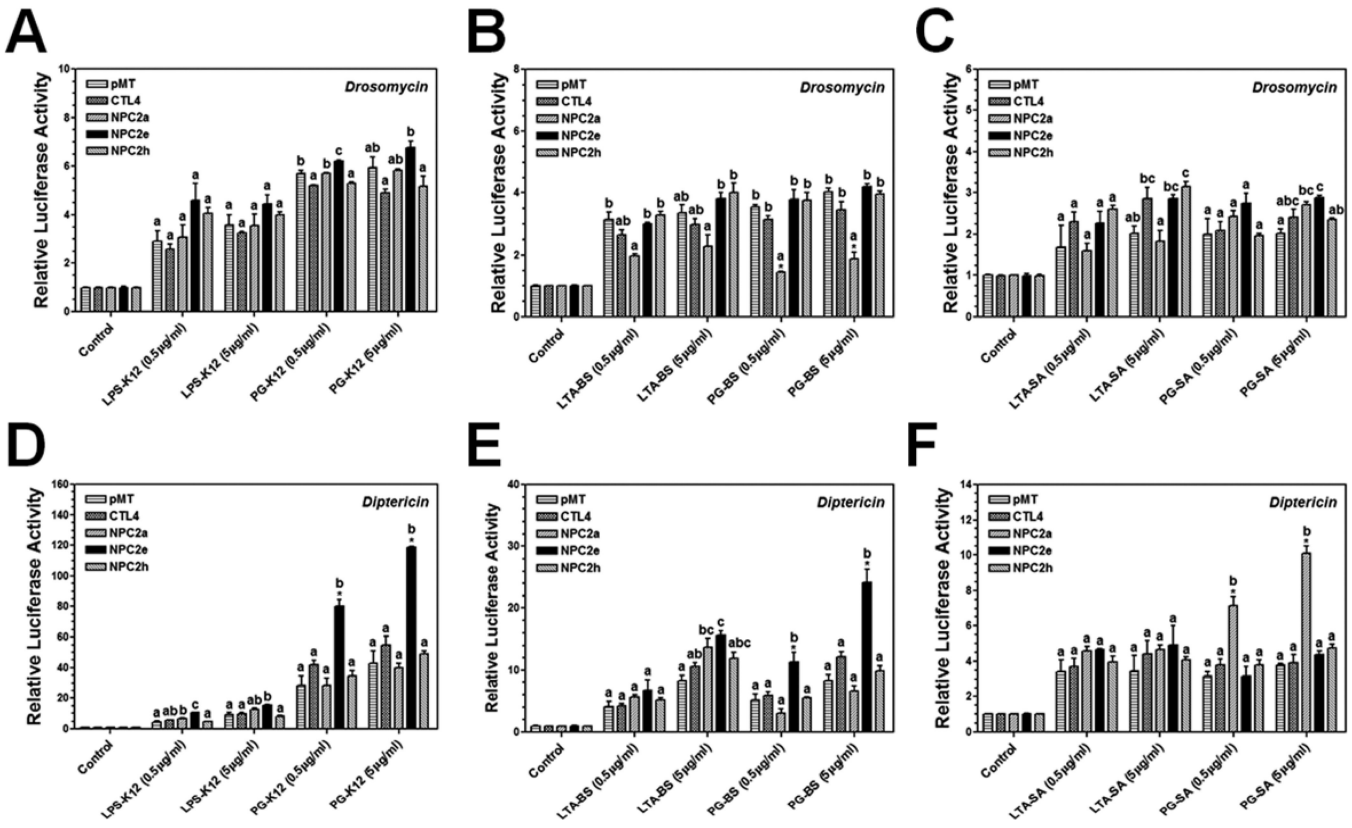

Fig. 7. Over-expression of NPC2a and NPC2e activates *diptericin* promoter in S2 cells stimulated by PG

For dual luciferase activity assays, proteins were expressed in S2 cells for 48h, and these S2 cells were then transfected with *drosomycin* (A–C) or *diptericin* (D–F) promoter luciferase reporter, and luciferase activity was determined after LPS-K12, PG-K12, LTA-BS, PG-BS, LTA-SA or PG-SA stimulation as described in the Materials and Methods. The pMT group did not have protein expression. The controls are S2 cells with protein expression for 48h (or without protein expression in the pMT group) but without LPS, LTA or PG stimulation and the relative luciferase activity of each control was set at 1. Identical letters are not significant difference ($p>0.05$), while different letters indicate significant difference ($p<0.05$) among different proteins for each stimulation determined by one way ANOVA followed by a Tukey's multiple comparison test.

Table 1

Sequences of primers used in this study

Primer	Sequence (5′–3′)	Direction
npc2a-pET32a-F	ATCGAATTCGGAGCCCTCGAGTTCAGC	Forward
npc2a-pET32a-R	ATCGCGGCCGCCTGAATTTTAGCGGGAAT	Reverse
npc2e-pET32a-F	ATCGAATTCACCAATGTCCAGCAGTGC	Forward
npc2e-pET32a-R	ATCGCGGCCGCAAGGGGGTTCGTCCAGTC	Reverse
npc2h-pET32a-F	ATCGAATTCGAGATAGTCAACTTTCAGACT	Forward
npc2h-pET32a-R	ATCGCGGCCGCGCGCACCACCTTGATGTC	Reverse
npc2a-RT-F	GTGGTCTGAAGTGCCCGTTGG	Forward
npc2a-RT-R	TGATGTCCGCTCCGTCCTGAT	Reverse
npc2e-RT-F	ACCAATGTCCAGCAGTGC	Forward
npc2e-RT-R	AAGGGGGTTCGTCCAGTC	Reverse
npc2h-RT-F	GAGATAGTCAACTTTCAGACT	Forward
npc2h-RT-R	GCG CAC CAC CTT GAT GTC	Reverse
dpt-RT-F	ATTGCCGTCGCCTTACTT	Forward
dpt-RT-R	AATTCGGAAATCTGTAGGTGTA	Reverse
drs-RT-F	GTACTTGTTCGCCCTCTTC	Forward
drs-RT-R	CCTTCGCACCAGCACTT	Reverse
rp49-F	ATGACCATCCGCCCAGCAT	Forward
rp49-R	TTCTTGGAGGAGACGCCGT	Reverse
pMT-ctl4-NotIF	AATGCGGCCGCATACAGGGCTACTACAGCGCGC	Forward
pMT-ctl4-PmeIR	AATGTTTAAACCTAATATTCACAAATAAATTG	Reverse
pMT-npc2a-NotIF	AATGCGGCCGCAGGAGCCCTCGAGTTCAGCGAT	Forward
pMT-npc2a-PmeIR	AATGTTTAAACCTACTGAATTTTAGCGGGAAT	Reverse
pMT-npc2e-NotIF	AATGCGGCCGCAACCAATGTCCAGCAGTGCAAA	Forward
pMT-npc2e-PmeIR	AATGTTTAAACTTAAAGGGGGTTCGTCCAGTC	Reverse
pMT-npc2h-NotIF	AATGCGGCCGCAGAGATAGTCAACTTTCAGACT	Forward
pMT-npc2h-PmeIR	AATGTTTAAACCTAGCGCACCACCTTGATGTC	Reverse