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A *Drosophila* ortholog of the human cylindromatosis tumor suppressor gene regulates triglyceride content and antibacterial defense

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The cylindromatosis (*CYLD*) gene is mutated in human tumors of skin appendages. It encodes a deubiquitylating enzyme (CYLD) that is a negative regulator of the NF-κB and JNK signaling pathways, in vitro. However, the tissue-specific function and regulation of CYLD in vivo are poorly understood. We established a genetically tractable animal model to initiate a systematic investigation of these issues by characterizing an ortholog of *CYLD* in *Drosophila*. *Drosophila CYLD* is broadly expressed during development and, in adult animals, is localized in the fat body, ovaries, testes, digestive tract and specific areas of the nervous system. We demonstrate that the protein product of *Drosophila CYLD* (CYLD), like its mammalian counterpart, is a deubiquitylating enzyme. Impairment of *CYLD* expression is associated with altered fat body morphology in adult flies, increased triglyceride levels and increased survival under starvation conditions. Furthermore, flies with compromised *CYLD* expression exhibited reduced resistance to bacterial infections. All mutant phenotypes described were reversible upon conditional expression of *CYLD* transgenes. Our results implicate CYLD in a broad range of functions associated with fat homeostasis and host defence in *Drosophila*.

KEY WORDS: Cylindromatosis, *Drosophila*, Fat body, Host defense, NF-kappaB

INTRODUCTION

Familial cylindromatosis is an autosomal-dominant predisposition to tumors of skin appendages called cylindromas (Bignell et al., 2000). This familial disease and the majority (57%) of sporadic cases of cylindromatosis are causally associated with defects in a single gene, named *CYLD* (Biggs et al., 1996; Biggs et al., 1995). Tumors develop in individuals who have both copies of the gene inactivated, indicating that *CYLD* is a tumor suppressor gene. *CYLD* codes for a 956 amino acid cytoplasmic protein (CYLD) with deubiquitylating activity, which has been associated genetically with its tumor-suppressing function (Borodovsky et al., 2002; Kovalenko et al., 2003; Trompouki et al., 2003). CYLD is also a negative regulator of NF-κB activation by members of the tumor necrosis factor receptor superfamily (Brummelkamp et al., 2003; Kovalenko et al., 2003; Regamey et al., 2003; Trompouki et al., 2003). The negative regulation of this pathway appears to be mediated by the ability of CYLD to induce deubiquitylation of TRAF2, TRAF6 and/or NEMO [also known as IKBKG and IKKγ – Human Gene Nomenclature Database (HUGO)]. Interestingly, mice with both *Cyld* alleles inactivated are more susceptible to chemically induced skin cancer than wild-type mice and their susceptibility is a consequence, at least in part, of increased Bcl3-dependent NF-κB activity (Ikeda and Dikic, 2006; Massoumi et al., 2006). Recent reports have indicated a broader role for CYLD in mammalian pathophysiology, consistent with its broad tissue distribution. This protein is drastically downregulated in human inflammatory bowel disease and it is required for the proper development of T lymphocytes in mice (Costello et al., 2005; Reiley et al., 2006). CYLD is significantly conserved across evolution, because putative orthologs of *CYLD* can be readily identified in a variety of organisms, including *Drosophila melanogaster* and *Caenorhabditis elegans* (Bignell et al., 2000).

The broad tissue distribution of CYLD, its implication in two complex and highly regulated signaling pathways, and the fact that multiple tissues are affected when the gene is mutated highlight the complexity of the biological roles of this protein. To facilitate the functional characterization of CYLD, we sought to establish and characterize a simpler, yet appropriately representative, animal model system. *Drosophila* was chosen for this analysis, because it is well-characterized, offers enormous advantages for genetic analysis and contains a putative ortholog of CYLD. *Drosophila* CYLD (FlyBase gene ID: CG5603) is 45% homologous to human CYLD and the two proteins have a similar domain organization (Bignell et al., 2000). Based on previous findings using human cells, we sought to validate the model by investigating the involvement of *Drosophila CYLD* in NF-κB/IKK signaling. In this context, we analyzed the potential involvement of *Drosophila CYLD* in IMD-mediated innate immunity.

In *Drosophila*, IMD (for immune deficiency) defines a signaling cascade broadly resembling the mammalian TNFR1 (also known as TNFRSF1A – HUGO) pathway (Kaneko and Silverman, 2005; Naitza and Ligoxygakis, 2004). Following microbial recognition via the *Drosophila* peptidoglycan recognition proteins (PGRPs) PGRP-LC and PGRP-LE, the signal is transduced from the cell surface to IMD, a central player of the pathway and a homolog of the mammalian TNF receptor interacting protein 1 (RIP1) (Georgel et al., 2001). In the TNFR1 pathway, RIP1 is essential for NF-κB and mitogen activated kinase (MAPK) activation (Kelliher et al., 1998; Stanger et al., 1995). In *Drosophila*, IMD transduces a signal that involves similar components. Flies deficient in IMD, dFADD (also known as BG4 – FlyBase) or dTAK1 (also known as TAK1 –

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FlyBase) are extremely sensitive to Gram-negative bacterial infections and fail to activate the relevant antimicrobial peptides (AMPs) (Leulier et al., 2002; Naitza et al., 2002; Vidal et al., 2001).

The *Drosophila* Iκβ kinase (IKK) complex (see below) acts downstream of dTAK1 in the activation of NF-κB. This complex contains two subunits identified by the *ird5* and *kenny* (*key*) mutants. The former codes for the IKKβ homolog, whereas the product of the latter shares significant homology to the regulatory subunit of the human NEMO protein (Lu et al., 2001; Rutschmann et al., 2000b; Silverman et al., 2000). Activation of the IKK complex depends on the fly homologs of the ubiquitin-conjugating enzymes UBC13 (also known as UBE2N – HUGO) and UEV1α (also known as UBE2V1 – HUGO), similarly to the activation of mammalian IKK, which depends on the mammalian proteins (Deng et al., 2000; Wang et al., 2001; Zhou et al., 2005). The *Drosophila* IKK complex phosphorylates the precursor form of the Relish protein, an NF-κB family member that can be activated by proteolytic cleavage. DREDD, a caspase-type protease, is involved in the full activation of Relish along with the *Drosophila* IKK complex (Stoven et al., 2003). Active Relish regulates directly the transcription of antimicrobial peptide genes.

In this article, we show that, like its vertebrate counterpart, *Drosophila* CYLD is a deubiquitylating enzyme that is broadly expressed in embryos and adults. Flies with impaired *CYLD* expression are sensitive to bacterial infections, a phenotype that is reversed upon restoration of *CYLD* expression. In addition, we demonstrate that *Drosophila* CYLD is biochemically and functionally associated with the IMD pathway and that it regulates fat homeostasis.

MATERIALS AND METHODS

Plasmid construction

Full-length *D. melanogaster* CYLD was amplified by PCR from *D. melanogaster* complementary (c)DNA using the primers: DMCYLDF1 (5′-GCTCTAGAATGAATTCCAAATCCGATTATGAAGC-3′) and DMCYLDR4 (5′-CTGACCTACGCCGGCGGCTCTAATGGTACATCA-TTATATCTG-3′). The resulting PCR product was digested with *Eco*RI and *Not*I, and was cloned into the corresponding sites of pCDNA3FLAG in frame with the FLAG epitope to generate pCDNA3FLAGDMCYLD (Trompouki et al., 2003). pCDNA3FLAGDMCYLD(C284S) was generated by three PCR reactions. The first PCR was performed with pCDNA3FLAGDMCYLD as the template and the primers DMCYLDF3 (5′-GGCTCGATACCACCATTACAAA-3′) and DMCYLDR5 (5′-ATC-AAGGTAGCTGGAATTATGG-3′). The second PCR was performed with the same template and the primers DMCYLDF4 (5′-CACCATAATT-CCAGCTACCTTGAT-3′) and DMCYLDR6 (5′-CCGATGAGTGAA-AGCTTTGC-3′). The third PCR was performed with the products of the previous two PCRs as template and the primers DMCYLDF3 and DMCYLDR6 (see above). The resulting PCR product was digested with *Nde*I and *Hin*dIII and cloned into the *Nde*I and *Hin*dIII sites of pCDNA3FLADDMCYLD to generate pCDNA3FLAGDMCYLD(C284S). pUAST.1DMCYLD was generated by digesting pCDNA3FLAGDMCYLD with *Kpn*I and *Xba*I and subcloning the fragment into the corresponding sites of the plasmid pUAST.1. pUAST.1DMCYLD(C284S) was generated in the same manner as pUAST.1DMCYLD. The full-length DMCYLD fragment produced from pCDNA3FLAGDMCYLD or pCDNA3FLAGDMC-YLD(C284S) by digestion with *Eco*RI and *Not*I was cloned into pGEX5X-3 (Amersham-Pharmacia Biotech) digested with *Eco*RI and *Not*I to generate glutathione-S-transferase (GST)-DmCYLD or GST-DmCYLD(C284S), respectively. GST-DmCYLD(1-317) was generated by cloning into the *Eco*RI and *Not*I sites of pGEX5X-3 a PCR fragment generated using pCDNA3FLAGDMCYLD as a template and the primers DMCYLDF1 and DMCYLDR2 (5′-GACTGGATGCGGCCGCCTGTAATTTCGAATAT-CTTGTGGGC-3′) digested with *Eco*RI and *Not*I. To generate 6×HIS- and c-MYC-tagged *Drosophila* CYLD, the *Drosophila* CYLD-RC open reading frame (ORF) was cloned into the *Eco*RI-*Xho*I site of pAc5.1/c-MYC-HIS A using the following PCR primers: AcCYLD-F: 5′-CCCGAATTCCA-AAATGATCTTAAACAACAAAAGTAAAAC-3′ and AcCYLD-R: 5′-CCCCTCGAGATGGTACATCATTATATCTGTGC-3′. Plasmid pAc5.1/c-MYC-HIS A was generated by replacing the V5 epitope of plasmid pAc5.1/V5-HIS A (Invitrogen) with a c-MYC epitope by ligating the following annealed phosphorylated oligos into *Bst*BI–*Age*I-digested pAc5.1/V5-HISA: cMyc-S, 5′-CGAAGAACAAAAACTTATTTCTGA-AGAAGATCTGA-3′ and cMyc-AS, 5′-CCGGTCAGATCTTCTTCA-GAAATAAGTTTTTGTTCTT-3′. V5 and 6×HIS C-terminal-tagged *kenny* (*key*), the full-length *key* ORF, was PCR amplified from the cDNA clone LD18356 (*Drosophila* Genomics Resource Center) using the following primers: AcKEY-F, 5′-CCCGAATTCCAAAATGTCGGACGAAGAG-TCATTC-3′ and AcKEY-R, 5′-CCCCTCGAGGTTTTTATCCAAACA-ATCGTTAACG-3′, and cloned into *Eco*RI-*Xho*I sites of the pAc5.1/V5-HIS A vector.

Generation of anti-CYLD antibody

The rabbit anti-CYLD antibody was generated by Santa Cruz Biotechnology using a GST fusion of *Drosophila* CYLD amino acids 1-317 as an antigen.

Immunohistochemical analysis of *Drosophila* embryos

Embryos from each developmental stage were dechorionated, fixed in 180 mM HEPES pH 6.9, 4 mM $MgSO_4$ and 2 mM EGTA in 4% formaldehyde and 58.8% heptane, and rinsed with methanol. After rehydration with BBT [1×phosphate-buffered saline (PBS), 0.1% Tween-20, 1% bovine serum albumin (BSA)], embryos were blocked with BBT-250 (BBT, 250 mM NaCl) containing 2% normal goat serum (NGS) for 2 hours, stained with the appropriate primary antibody (400 ng/ml) overnight at 4°C, rinsed the following day with BBT-250 and stained with secondary antibody (1:2000 dilution) for 2 hours at room temperature. Finally, the embryos were rinsed with PBT (1×PBS, 0.1% Tween-20), stained with ABC reagent (Vectastain Elite HRP kit), developed with DAB solution (1 ml PBT, 1 mg DAB, 0.1% H_2O_2) and mounted in liquid Canada Balsam (Sigma). All antibodies were diluted in BBT-250 containing 2% NGS.

Immunofluorescence analysis

Sagittal paraffin sections (8 μm) of entire adult flies were obtained and processed for immunohistochemistry as described previously (Philip et al., 2001; Skoulakis and Davis, 1996). The primary antibody was used at 5 μg/ml and the secondary antibody at 1:2000. After washing, the sections were mounted with DAKO mounting medium (DAKO Corporation). All antibodies were diluted in PBHT (20 mM NaH_2PO_4, 500 mM NaCl, 0.2% Triton X-100, pH 7.4) containing 5% NGS.

Staining with Nile red

Female flies up to 5 days old were fixed for 5 hours at 4°C in PBS containing 4% paraformaldehyde. Subsequently, they were rinsed in PBS for 10 minutes at room temperature and were incubated overnight at 4°C in PBS containing 25% sucrose. The following day, the flies were placed in OCT (Sakura Finetek Europe) and were cryosectioned in 8 μm sections. The sections were placed on gelatin-covered glass slides and rinsed for 10 minutes at room temperature in PBS. Subsequently, they were placed in Nile red solution (0.5 mg/ml Nile red in acetone) for 5 minutes at room temperature, and then they were rinsed three times with PBS and mounted with DAKO mounting medium (DAKO Corporation) for microscopy.

Deubiquitylation assay

MC1061 bacteria (*Escherichia coli*) expressing a ubiquitin-Met-β-galactosidase fusion were transformed with plasmids expressing GST, GST-CYLD(541-956), GST-CYLD or GST-CYLD(C284S). Overnight cultures of the transformants were diluted tenfold, grown for 1 hour and then incubated for 4 hours in the presence of 0.1 mM IPTG to induce the expression of GST fusion proteins. Cells (1.5 ml) were lysed in 150 μl of cracking buffer (10 mM phosphate pH 7.4, 8 M urea, 1% SDS, 1% b-mercaptoethanol) and boiled for 5 minutes. Extracts were subjected to SDS-

PAGE (6% gel) and western blotting with a rabbit anti-β-gal antibody. Expression of GST fusion proteins was detected with SDS-PAGE (8.5% gel) and western blotting using anti-GST antibody.

Analysis of *Drosophila* CYLD expression by RT-PCR

RNA was extracted from either eight male or five female flies or from the corresponding body parts using the RNA-Wiz kit (Ambion), according to manufacturer's instructions. cDNA was prepared using the Revert Aid cDNA synthesis kit (Fermentas), according to manufacturer's instructions. PCR analysis was performed using the primers DMCYLDF7 (5′-TAGAGC-CGGAGGAACCTTTAC-3′) and DMCYLDR9 (5′-GCATCTGT-TGGCTGGTAC-3′); DMCYLDF3 and DMCYLDR6; and rp49F1 (5′-GATCGTGAAGAAGCGCAC-3′) and rp49R2 (5′-CGCTCGACA-ATCTCCTTG-3′) for loading control.

Triglyceride content and starvation assay

For triglyceride (TAG)-content analysis, three groups of eight male flies per genotype, approximately 36 hours old, were homogenized in 100 μl 0.05% Tween-20, incubated at 70°C for 5 minutes and cleared twice by centrifugation at 5000 rpm (6150 ***g***) for 1 minute and 13,000 rpm (16,000 ***g***) for 3 minutes. Supernatants were analyzed for TAG content using a commercial kit (Biosis) according to manufacturer's instructions. Total protein was measured using the Bio-Rad D_C Protein Assay kit (Bio-Rad) according to manufacturer's instructions. For the starvation assay, three groups of 25 male flies per genotype, approximately 36 hours old, were starved in food-free vials with unlimited water supply at 25°C. The death rate was estimated by counting the number of flies unable to exhibit a sit-up response in 12-hour intervals (Gronke et al., 2003).

Yeast two-hybrid screening

A cDNA corresponding to the full-length ORF of *Drosophila* CYLD was cloned into the LexA DNA-binding domain vector pHybLex/Zeo (Invitrogen). The resulting plasmid, pHyb-CYLD, was transformed into the *Saccharomyces cerevisiae* strain L40 [*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS::(4lex*Aop-*HIS3*) *URA3::(8)*lexAop-*lacZ*) *GAL4*] (Invitrogen) and used as bait in a mating-based two-hybrid screen against other members of the IMD pathway (a full list of genes and PCR primer sequences is available upon request). Each pathway member was prepared by cloning their full-length cDNAs into the B42 activation-domain vector pYESTrp2 (Invitrogen) and transformed into the *S. cerevisiae* strain EGY48 (*MATα ura3 trp1 his3 6lexAop-LEU2*; Invitrogen). An interaction between JUN (bait; also known as JRA – FlyBase) and FOS (also known as KAY – FlyBase) using the abovementioned plasmids and yeast strains was employed as a positive control. Mating was performed by overlaying 1 μl of an overnight culture of L40 flies with 1 μl of EGY48 on a YPAD plate. Plates were incubated for 1-2 days at 30°C and diploids were selected. Activation of the *lacZ* reporter gene was tested according to the protocols outlined in the Hybrid Hunter handbook (Invitrogen).

S2 cell culture and transfection

Drosophila S2 cells (Invitrogen) were maintained at 25°C in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated FBS, 50 units ml^{-1} penicillin G, and 50 μg/ml streptomycin sulfate (Invitrogen). Transfection was performed using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Briefly, 7.5×10^5 cells ml^{-1} were plated in 60 mm plates 24 hours before transfection. DNA (2 μg) was used for each transfection using a 1:10 Effectene ratio. Cells were washed once in 1×PBS at 8 hours post-transfection and incubated at 25°C for 48 hours.

Co-immunoprecipitation and western blot analysis

Cell lysates were prepared from 5 ml cultures of transiently transfected cells with 2 μg each of pAcCYLD/c-Myc and pAcKEY/V5 or 2 μg of pAcKEY/V5 alone, in 100 μl RIPA buffer (150 mM NaCl, 1.0% Igepal CA-0630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) (Sigma-Aldrich), supplemented with Complete Mini Protease Inhibitor Cocktail tablets (Roche Applied Science). Lysates were incubated with 100 μl of anti-c-MYC agarose affinity gel (Sigma-Aldrich) for 1.5 hours with agitation rocking at room temperature. Immunoprecipitates were washed with CoIP buffer (900 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) six times, each for 10 minutes, followed by a final wash with RIPA buffer alone. Immunoprecipitates were eluted by boiling in 2×SDS sample buffer, separated by 13% SDS PAGE, and transferred to PVDF membranes. Blots were probed with anti-V5 antibody (1:5000 dilution; Invitrogen).

Drosophila stocks, infection experiments and northern blot analysis

The following strains were made and used in this study: *UAS-CYLD* (wild type), *UAS-CYLD/+; CYLD^{f00814};hs-Gal4/MKRS* (CYLD rescue) and four insertions from the insertional mutagenesis stocks generated by Exelixis and made available to the fly community via the Bloomington Stock Centre. These were *f00814* (*CYLD^{f00814}*), *f02494*, *d10472* and *f00135*. Df(2L)j27/CyO flies were obtained from the Bloomington Stock Center. We used a *white* isogenic strain as a wild-type control in all experiments. A *white* isogenic strain was used as a background by Exelixis in generating a series of different P element and piggyBac insertions (Thibault et al., 2004). Additional strains used were *relish* (Hedengren et al., 1999) and *dif* (Rutschmann et al., 2000a) as positive controls for Gram-negative and Gram-positive bacterial infections, respectively, and the *yolkGAL4*-driver-containing strain (Georgel et al., 2001). To rescue the *CYLD^{f00814}* mutant phenotypes, strain *UAS-CYLD/+; CYLD^{f00814};hs-Gal4/MKRS* was incubated at 37°C for 1 hour followed by recovery at 29°C for 1 hour. All strains were analyzed by PCR for the presence of the Gram-negative bacteria *Wolbachia* and were found not to be infected. Bacterial immune challenge and northern blotting was performed as previously described (Rutschmann et al., 2000a).

RESULTS

CYLD is broadly expressed during embryonic and adult stages of *Drosophila* development

Drosophila contains a putative ortholog of the mammalian tumor suppressor gene *CYLD*. Five transcripts of *Drosophila CYLD* have been identified by genomic approaches (Fig. 1A, www.ensembl.org). The expression pattern of *Drosophila CYLD* was investigated by

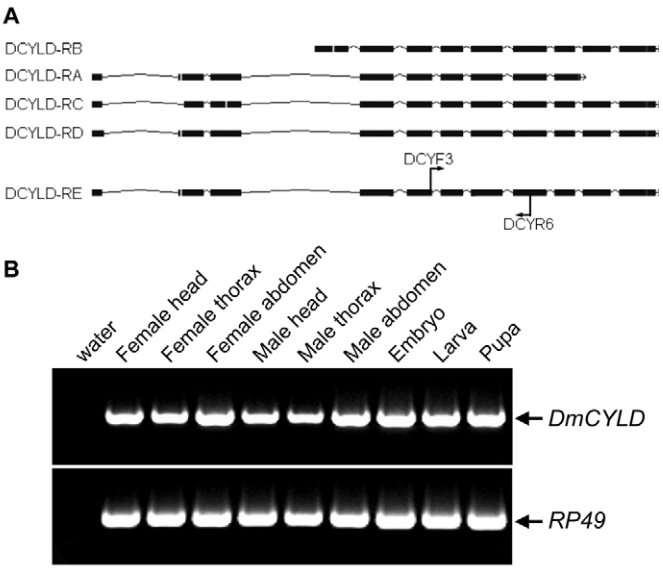

Fig. 1. Reverse transcriptase-PCR analysis of *CYLD* expression in *Drosophila melanogaster*. (**A**) Five transcripts of *Drosophila CYLD* have been described, and are shown here. The positions of the CYLD primers used in B (DCYF3 and DCYR6) are shown. (**B**) *CYLD* (*DmCYLD*) expression was detected by reverse transcriptase (RT)-PCR using the primers DMCYLDF3 (DCYF3) and DMCYLDR6 (DCYR6), and RNAs were isolated at the indicated developmental stages and in the indicated body compartments of adult *w1118* flies (upper panel). *RP49* (also known as *RpL32*) RNA was used as a control for the amount of cDNA used in the RT-PCR reaction (lower panel).

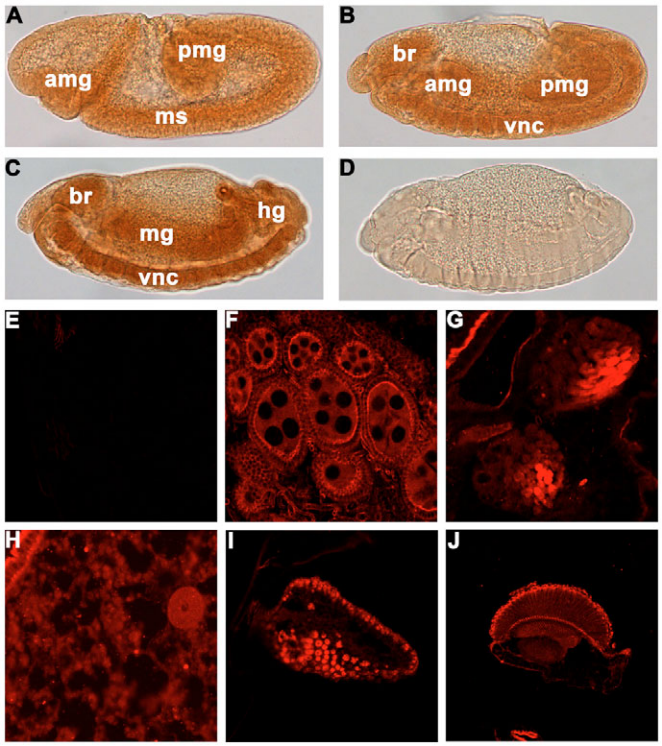

Fig. 2. Distribution of CYLD in *Drosophila* embryos and adults. (**A-D**) Detection of CYLD in embryos by immunohistochemistry. (A) At stage 7, CYLD is expressed in the anterior midgut rudiment (amg), in the posterior midgut rudiment (pmg) and in the mesoderm (ms). (B) At stage 12, CYLD is localized in the developing brain area (br), in the forming midgut (amg and pmg) and in the ventral nerve cord (vnc). (C) At stage 14, CYLD is detected at the brain (br), the ventral nerve cord (vnc), the midgut (mg) and the hindgut (hg). (D) Stage 12 embryos homozygous for the Df(2L)J21 deficiency, which deletes the *CYLD* locus, did not stain with the anti-CYLD antibody. (**E-J**) Detection of CYLD in adult tissues by immunofluorescence. (E) Negative control using a non-specific rabbit polyclonal antibody (anti-GFP) as primary antibody and the same secondary antibody used in F-J. Using the same settings as in E, endogenous CYLD levels were detected with a rabbit polyclonal anti-CYLD antibody on adult *w1118* sagittal paraffin sections (8 μm). CYLD was detected in ovaries (F), in testes (G), in the fat body (H), in the antenna (I) and in the eye (J). amg, anterior midgut rudiment; br, brain; hg, hindgut; mg, midgut; ms, mesoderm; pmg, posterior midgut rudiment; vnc, ventral nerve cord.

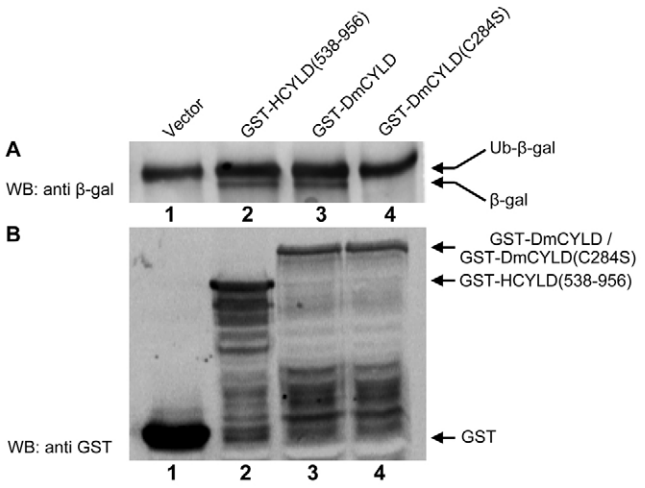

Fig. 3. *Drosophila* (Dm)CYLD is a deubiquitylating enzyme. *E. coli* expressing a ubiquitin-β-galactosidase (Ub-βgal) fusion were transformed with the indicated expression constructs for glutathione-S-transferase (GST): GST-HCYLD(538-956), GST-DmCYLD or GST-DmCYLD(C284S). (**A**) After induction of the GST-domain-containing proteins, whole extracts from an approximately equal number of bacteria were analyzed by immunoblotting with a polyclonal anti-β-galactosidase antibody. The positions of Ub-βgal and β-galactosidase (β-gal) are indicated. (**B**) The GST-domain-containing proteins were expressed at similar levels, as detected by immunoblotting of whole-cell lysates of an equal number of bacteria with polyclonal anti-GST antibody.

reverse transcriptase (RT)-PCR, and *CYLD*-specific transcripts were found in all developmental stages (embryo, larva and pupa) and in all three major adult body segments (Fig. 1B). The protein distribution pattern was then investigated using an anti-CYLD antibody that we developed (see Materials and methods). The specificity of this antibody to *Drosophila* CYLD was confirmed because embryos homozygous for the Df(2L)J21 deficiency, which deletes the 31C7-D1 chromosomal area in which *CYLD* resides, did not show any reactivity to the antibody (Fig. 2D). *Drosophila* CYLD was present throughout stage 1-5 embryos (data not shown) but, by stage 7, the protein was detected mainly in mesoderm and in developing segments of the anterior and posterior midgut (Fig. 2A). During embryonic stage 12, CYLD continued to accumulate in the developing midgut and was relatively abundant in the brain and ventral nerve cord (Fig. 2B). By embryonic stage 14, accumulation of CYLD in the brain and ventral nerve cord was notably stronger than that of earlier stages, while the protein remained abundant in the midgut and posterior intestine (Fig. 2C). Together, these data suggest a role for CYLD in the development or function of the midgut and mesodermal derivatives, as well as in the nervous system.

In adult flies, CYLD was detected in several tissues. Strong expression was detected in the cytoplasm of nurse and follicle cells in the developing oocyte (Fig. 2F), and in the testes (Fig. 2G). Weaker protein accumulation was detected in intestinal epithelial cells (data not shown) and in the fat body (Fig. 2H). In addition, CYLD accumulated strongly in the proboscis, antenna (Fig. 2I) and in the eye (Fig. 2J), whereas the protein was weakly detectable in various areas of the brain, such as the optic lobe (data not shown). The reactivity of adult tissues to the anti-CYLD antibody could be almost completely eliminated with the addition of excess antigen that was used to raise the antibody (data not shown).

Drosophila CYLD has deubiquitylating activity

The high degree of similarity between the ubiquitin-specific protease domain of human CYLD and the corresponding C-terminal region of *Drosophila* CYLD prompted an investigation into the ability of *Drosophila* CYLD to act as a deubiquitylating enzyme. For this purpose, *Drosophila* CYLD was introduced into bacteria expressing a ubiquitin-β-galactosidase (Ub-βgal) fusion, and its ability to cleave the ubiquitin moiety from the Ub-βgal fusion protein was tested. Wild-type *Drosophila* CYLD readily cleaved ubiquitin from the Ub-βgal fusion (Fig. 3, compare lanes 1 and 3). By contrast, a mutated form of *Drosophila* CYLD, containing a serine instead of a conserved cysteine in the putative catalytic domain, was unable to cleave ubiquitin from the Ub-βgal fusion (Fig. 3, compare lanes 1 and 4). As expected, human CYLD was also capable of cleaving the Ub-βgal fusion (Fig. 3,

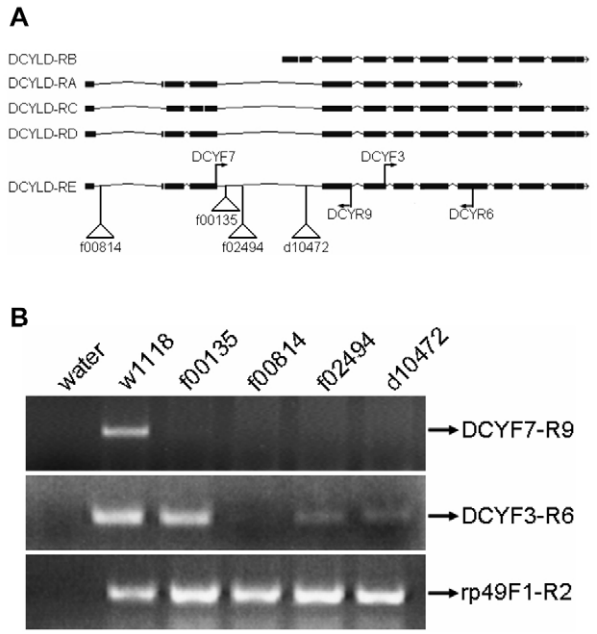

Fig. 4. Molecular characterization of P-element insertion lines. (**A**) The positions of P-element insertions analyzed and the primers that were used [DMCYLDF3 (DCYF3), DMCYLDR6 (DCYR6), DMCYLDF7 (DCYF7) and DMCYLDR9 (DCYR9)] are shown relative to the five transcripts of *Drosophila CYLD* that have been identified. (**B**) The P-element-insertion lines *f00135*, *f00814*, *f02492* and *d10472* were analyzed by reverse transcriptase (RT)-PCR for *CYLD* mRNA. The results were compared with wild-type flies (w1118). *rp49* (also known as *RpL32*) RNA was used as a control for the amount of cDNA used in the RT-PCR reaction (lower panel).

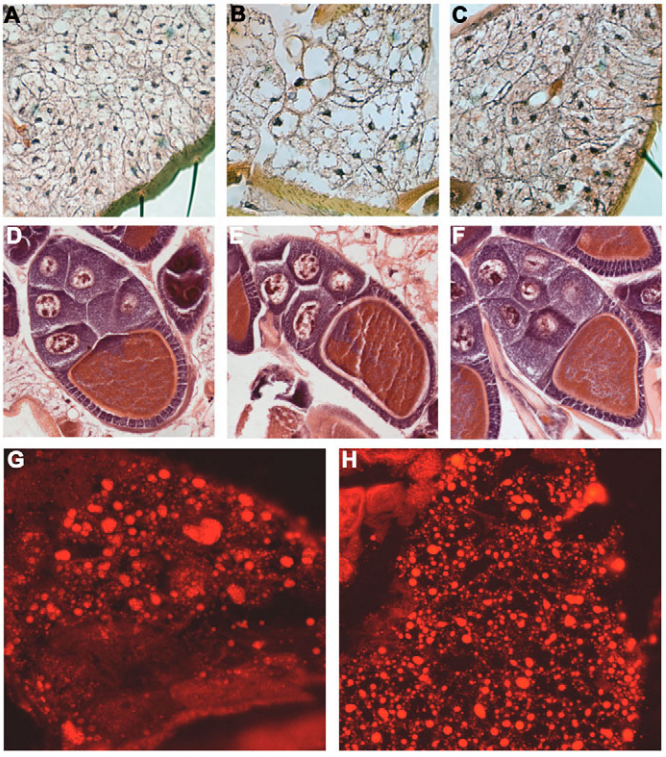

Fig. 5. Impaired *Drosophila CYLD* expression alters fat body morphology. (**A-C**) Adult female abdomens from wild-type (A), $CYLD^{f00814}$ homozygotes (B) and CYLD-rescue (C) flies were analyzed by hematoxylin and Eosin staining of paraffin sections (8 μm). (**D-F**) Oocyte morphology in wild-type (D), $CYLD^{f00814}$ homozygotes (E) and CYLD-rescue (F) flies. (**G**,**H**) Nile red staining for the presence of lipid droplets in cryosections from adult wild-type flies (G) or $CYLD^{f00814}$ homozygotes (H).

lane 2). Therefore, like its vertebrate ortholog, *Drosophila* CYLD has autonomous deubiquitylating activity that depends on the integrity of its conserved C-terminal catalytic domain.

Defective expression of *Drosophila CYLD* alters fat body morphology

In order to study the biological role of CYLD in *Drosophila*, four strains containing P-element insertions in the *CYLD* locus were obtained from the Exelixis collection. The genomic locations of all transposons were verified by PCR (data not shown). Strain *f00814*, which contains a P-element insertion in the first intron (Fig. 4A), was chosen for further analysis because this allele can be considered a strong *CYLD* hypomorph lacking detectable expression of all possible transcripts from the gene (Fig. 4B). By contrast, the insertions in intron 2 of *Drosophila CYLD* either did not appear to disrupt or disrupted incompletely the expression of *CYLD*-associated mRNAs in the *f00135*, *f02494* and *d10472* strains. Consistent with the RT-PCR analysis, staining of *f00814* homozygous-mutant embryos with anti-CYLD antibody failed to detect CYLD protein (data not shown). Therefore, this strain represents a bona fide mutant allele of the gene, henceforth called $CYLD^{f00814}$. Given the protein distribution in developing embryos, $CYLD^{f00814}$ homozygotes were surprisingly viable and did not exhibit obvious external morphological anomalies. This indicates that *Drosophila CYLD* function is either dispensable for proper embryonic development, or it is functionally redundant with another gene in the genome. However, histological analysis revealed that the majority of $CYLD^{f00814}$ adult homozygotes exhibited fat body cells of an abnormal size and morphology (Fig. 5B, Table 1). This phenotype was reversed upon inducible expression of a *CYLD* transgene under the control of the heat-shock promoter in the $CYLD^{f00814}$ mutant background (*UAS-CYLD/+;*$CYLD^{f00814}$*;hs-Gal4/MKRS*, referred to as the CYLD-rescue strain from this point on) (Fig. 5C, Table 1). The absence of CYLD did not precipitate apparent morphological defects in other tissues and organs [compare the oocyte from wild-type (Fig. 5D), *f00814* (Fig. 5E) and CYLD-rescue (Fig. 5F) flies]. Nile red staining showed that fat body cells in $CYLD^{f00814}$ adult homozygotes were able to accumulate lipids. This in turn indicated that they retained a degree of functionality despite their morphological

Table 1. Fat body morphology in wild-type and $CYLD^{f00814}$ mutant flies

	Fat body morphology		
Genotype	WT (%)	Moderate alteration (%)	Severe alteration (%)
w1118	93.33	–	–
$CYLD^{f00814}$	26.66	26.67	46.67
CYLD rescue	90.00	10.00	–

A total of 30 adult female flies of each genotype were analyzed for the morphology of the fat body. The percentage of flies with normal (WT), moderately altered or severely altered fat body is shown (see also Results and Fig. 5). In 2/30 (6.67%) *w1118* flies, the morphology of the fat body could not be determined.

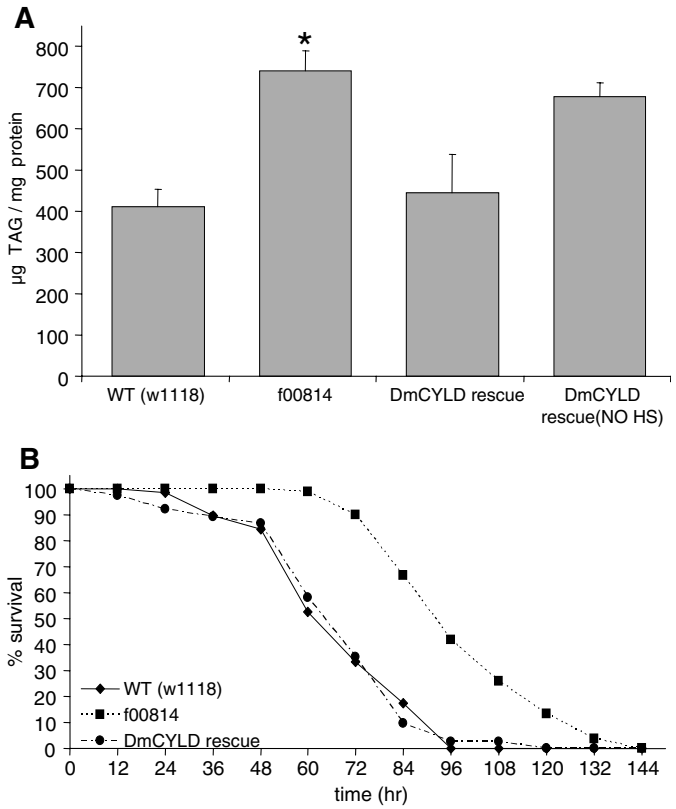

Fig. 6. Impaired *Drosophila CYLD* expression results in elevated triglyceride content and increased survival under starvation. (**A**) Triglyceride (TAG) content (expressed as µg TAG per µg protein) of 2-3-day-old male flies after hatching. The following strains were analyzed: wild type (WT) *w1118*, $CYLD^{f00814}$ homozygotes (f00814), *UAS-CYLD/Y;*$CYLD^{f00814}$*;hs-Gal4/MKRS* (DmCYLD rescue) after heat-shock induction of *UAS-CYLD* transcription and CYLD-rescue flies that were not subjected to heat-shock [DmCYLD rescue(NO HS)]. Mean values (±s.d.) from at least three independent experiments are shown. (**B**) Survival under starvation conditions of 2-3-day-old male flies. Representative data from at least three independent experiments are shown.

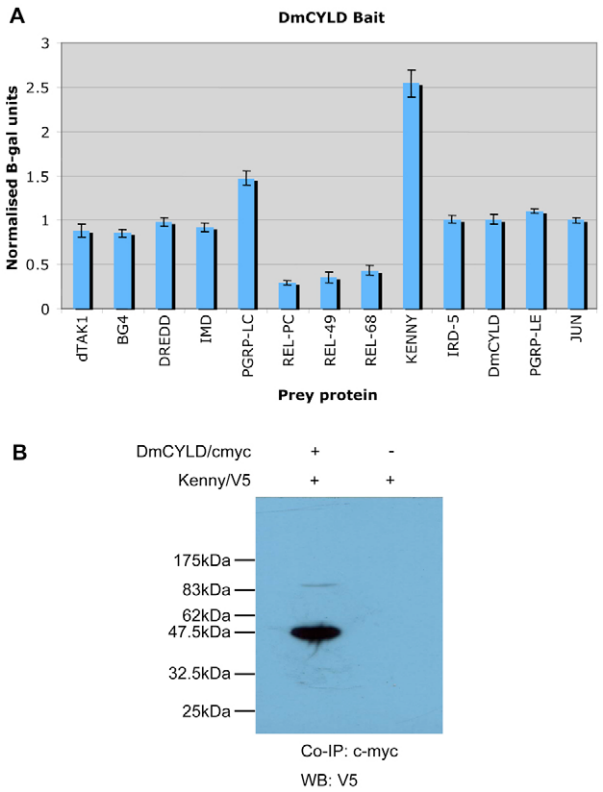

Fig. 7. *Drosophila* CYLD (DmCYLD) interacts with Kenny (dIKKγ). (**A**) Quantitative yeast two-hybrid assay with all of the components of the IMD pathway (PGRP-LC, PGRP-LE, PGRP-LA, IMD, dTAK1, dFADD, DREDD, IRD5 and Kenny). CYLD interacts specifically with Kenny, the *Drosophila* ortholog of the mammalian protein NEMO (IKKγ). (**B**) Anti-V5 western blot of anti-c-Myc-immunoprecipitated material from lysates of *Drosophila* S2 cells transfected with plasmids expressing the proteins indicated on the top of the panel.

alterations (Fig. 5G,H). A measurement of lipid droplets revealed that $CYLD^{f00814}$ adult homozygotes had significantly more but smaller lipid droplets (5.77±0.26 lipid droplets cm^{-2}) compared to the wild-type strain w1118 (3.08±0.20 lipid droplets cm^{-2}).

Defective expression of *Drosophila CYLD* is associated with increased total triglyceride content

The altered morphology of the fat body prompted an investigation into the fat content of flies with defective *CYLD* expression. The fat body is the major site of fat storage in *Drosophila*. Fat is stored primarily in the form of triglycerides (TAGs) in characteristic structures called lipid droplets. These structures consist of a single phospholipid layer that surrounds the TAGs. They are opaque and usually occupy a large portion of the cell, thus restricting the cytoplasm and nucleus to one side, and therefore contributing to the characteristic morphology of the fat body (Gronke et al., 2005). Total TAG content was found to be inversely proportional to the level of *Drosophila CYLD* expression. More specifically, total TAGs were increased by 79.8% in $CYLD^{f00814}$ adult homozygotes compared with control animals (Fig. 6A). However, $CYLD^{f00814}$ homozygotes that expressed transgenic *Drosophila CYLD* contained almost wild-type levels of TAGs (CYLD rescue, Fig. 6A). Significantly, in the absence of the heat-shock required for the expression of *Drosophila CYLD* transgenes, *UAS-CYLD/Y;*$CYLD^{f00814}$*;hs-Gal4/MKRS* flies possessed a TAG content that was not significantly different from that of $CYLD^{f00814}$ homozygotes [Fig. 6A, CYLD rescue (NO HS)]. Therefore, our data suggest that *Drosophila* CYLD is required for TAG homeostasis, a function that is most likely related to the altered morphology of the fat body in $CYLD^{f00814}$ homozygotes.

Defective expression of *Drosophila CYLD* is associated with increased resistance to nutrient starvation

The increased levels of TAGs in flies with impaired *CYLD* expression suggested that $CYLD^{f00814}$ homozygotes might be able to survive for longer periods of time under starvation conditions. For this purpose, newly emerged adults were allowed to feed normally for up to 36 hours and were then placed in vials without food but with free access to water. $CYLD^{f00814}$ homozygotes survived significantly longer without a source of nutrients in comparison with controls (Fig. 6B). Control animals and CYLD-rescue flies were dead after 4 days, whereas more than 40% of $CYLD^{f00814}$ homozygotes remained alive at the end of this time period. Heat-shock did not significantly alter the resistance to starvation of control

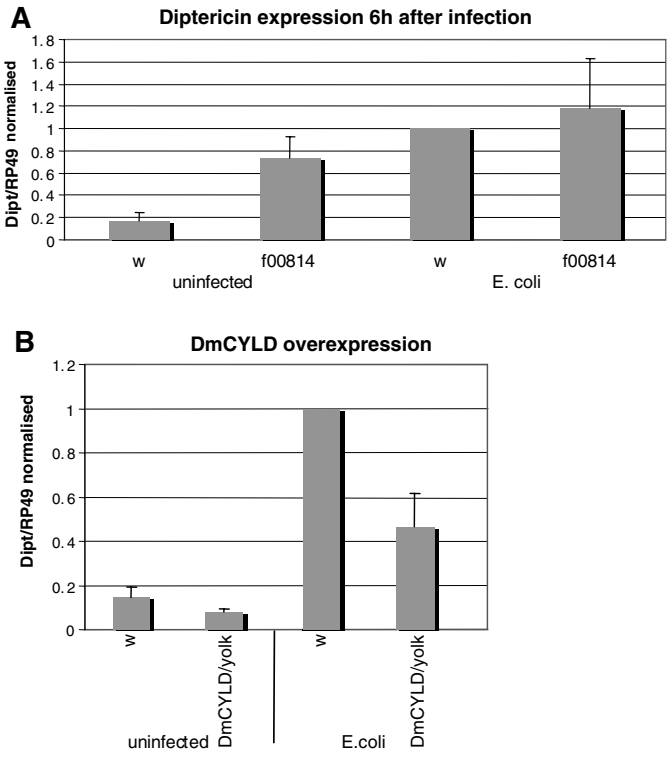

Fig. 8. Antimicrobial peptide expression in flies with impaired *Drosophila CYLD* expression. (**A**) *Diptericin* expression [expressed as a ratio of *Diptericin*:*rp49* (also known as *RpL32*) mRNA levels] in wild-type w1118 flies (w) and in $CYLD^{f00814}$ homozygotes (f00814) was determined before and after infection with *E. coli*. (**B**) *Diptericin* expression (expressed as a ratio of *Diptericin*:*rp49* mRNA levels) in wild-type w1118 (w) and flies overexpressing *CYLD* in the fat body (DmCYLD/yolk) before and after *E. coli* infection. (A,B) Mean values (±s.d.) from three independent experiments are shown.

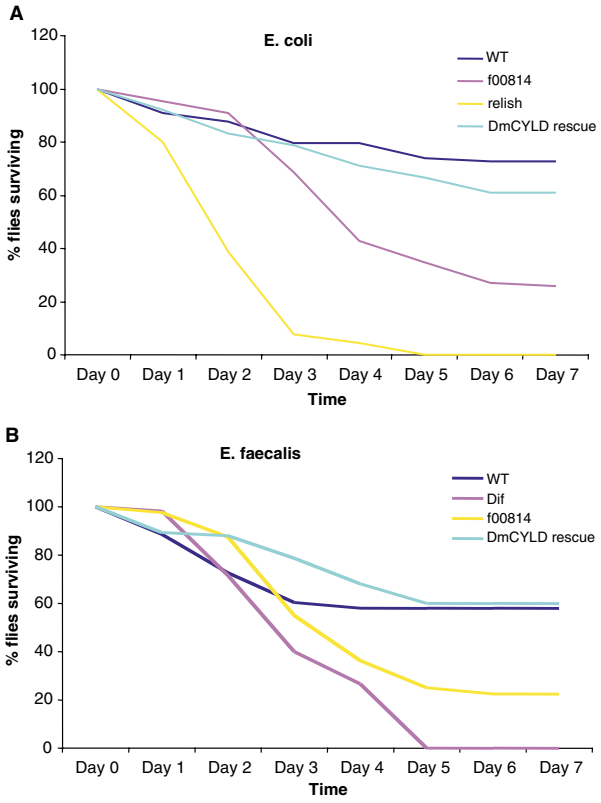

Fig. 9. A *Drosophila* strain with impaired *CYLD* expression is susceptible to infection by Gram-negative and Gram-positive bacteria. (**A**,**B**) A total of 25 flies were infected with either Gram-negative (*E. coli*; A) or Gram-positive (*E. faecalis*; B) bacteria. Survival was monitored for 7 days. Representative data from one out of four independent experiments are shown in each panel. WT, w1118 wild type; relish/Dif, positive controls; f00814: $CYLD^{f00814}$ homozygotes.

animals (data not shown). Collectively, the data suggest that reduced levels of CYLD result in an elevation in the levels of TAGs, which is the most likely cause of the observed resistance to starvation of $CYLD^{f00814}$ homozygotes.

Defective expression of *Drosophila CYLD* is associated with susceptibility to bacterial infections

Because of the deficits in fat body structure and function described above, and the known principal role of this organ in *Drosophila* host defence, we wanted to determine whether loss of *Drosophila CYLD* altered susceptibility to infections. Furthermore, this would be consistent with the given connection of mammalian CYLD with NF-κB/IKK signaling and with the function of the immune system (Brummelkamp et al., 2003; Kovalenko et al., 2003; Reiley et al., 2006; Trompouki et al., 2003).

The hallmark of the *Drosophila* systemic immune response is the synthesis and secretion by the fat body of potent AMPs. Although these responses have broad specificity, their pattern of expression depends largely on the type of invading pathogen (Naitza and Ligoxygakis, 2004). Whereas anti-fungal and anti-Gram-positive bacterial responses are controlled by the Toll signaling cascade, Gram-negative sepsis depends on the IMD pathway, the *Drosophila* homolog of mammalian TNFR signaling (Kaneko and Silverman, 2005). In human cells, CYLD interacts with NEMO and regulates the activation of NF-κB by deubiquitylating signaling molecules of TNFR-associated pathways (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). We used a quantitative yeast two-hybrid assay to investigate the ability of *Drosophila* CYLD to interact with known members of the IMD pathway in *Drosophila* (PGRP-LC, PGRP-LE, IMD, dFADD, DREDD, dTAK1, IRD5 and KENNY). We found that *Drosophila* CYLD was able to interact with KENNY, which is the *Drosophila* counterpart of the mammalian NEMO (IKKγ) protein, but not with other proteins implicated in the IMD pathway (Fig. 7A). The interaction between CYLD and KENNY was confirmed via co-immunoprecipitation in Schneider-2 (S2) cells (Fig. 7B). Next, we investigated the effect of impaired *Drosophila CYLD* expression on Toll and IMD signaling with and without infection, by measuring the expression of the AMP genes *Diptericin* (*Dipt*; *DptB*) and *Drosomycin* (*Drs*) as read-outs for IMD and Toll signaling, respectively. In uninfected flies, *Dipt* expression in mutant homozygotes was significantly higher than in wild-type flies, whereas no significant difference was observed between $CYLD^{f00814}$ homozygotes and wild-type flies upon infection with *Escherichia coli* (Fig. 8A). No significant difference in *Drs* expression was detected between mutant and wild-type flies without infection or following Gram-positive bacterial challenge (data not shown), suggesting that *Drosophila* CYLD does not regulate the Toll pathway.

In addition, we also studied the effects of a gain-of-function condition for *Drosophila CYLD*, whereby *UAS-CYLD* flies were crossed to the fat body-specific *yolk*-GAL4 driver and *Dipt* gene expression was measured (Fig. 8B). *Dipt* induction was significantly below that of wild type in infected flies.

Interestingly, following infection with Gram-positive or Gram-negative bacteria, $CYLD^{f00814}$ homozygotes exhibited a reduced rate of survival compared with wild type (Fig. 9). Flies from this strain showed reduced survival after infection by the Gram-negative bacterium *E. coli* (Fig. 9A) or by the Gram-positive bacterium *Enterococcus faecalis* (Fig. 9B), compared with wild-type or CYLD-rescued flies. Parallel experiments with the $CYLD^{f00814}$ allele over a deficiency uncovering the *Drosophila CYLD* locus produced comparable results (data not shown). This was not the case when heat-killed bacteria were used (data not shown). Notably, restoration of *Drosophila CYLD* expression in CYLD-rescue flies also restored the ability of these flies to respond to both kinds of challenges (Fig. 9, CYLD-rescue curves). Taken together, these results identify a crucial role of CYLD in anti-bacterial responses in *Drosophila*.

DISCUSSION

Drosophila melanogaster is emerging as one of the most effective tools for analyzing the function of human disease genes, including those responsible for developmental and neurological disorders, cancer, cardiovascular disease, metabolic and storage diseases, and genes required for the function of the visual, auditory and immune systems. Flies have several experimental advantages, including their rapid life cycle and the large numbers of individuals that can be generated, which make them ideal for sophisticated genetic screens (Bier, 2005).

In the present study, we characterized the expression and function of the *Drosophila* ortholog of the human tumor suppressor gene *CYLD*. Flies with impaired *Drosophila CYLD* expression showed severe susceptibility to bacterial infection. By inference from its human counterpart, which is a negative regulator of TNF/NF-κB signaling, it can be hypothesized that IMD-mediated AMP gene expression would be upregulated in *CYLD* loss-of-function mutant flies, whereas a gain-of-function condition should have the opposite effect. As expected, overexpressing *Drosophila* CYLD suppressed IMD signaling in uninfected and infected flies. In uninfected null mutants, *Dipt* expression was twice as high as in wild-type flies. Interestingly, *Dipt* levels in infected *CYLD* mutants were not significantly different from wild-type flies, whereas basal levels were elevated in comparison with wild-type controls. The most likely reason for this is that, when septic injury is used as a means of infection, the system is overloaded and *Dipt* expression reaches its maximum, such that even when an inhibitor of IMD signaling is eliminated, any further increase is not possible.

To our knowledge, this article is the first to describe a deubiquitylating enzyme implicated in the regulation of NF-κB signaling in *Drosophila*. Furthermore, the discovery that the loss of *Drosophila* CYLD leads to a concomitant increase in *Dipt* expression suggests that the IMD pathway, like its mammalian counterpart (the TNFR pathway), is activated by ubiquitylation. Our results suggest that *Drosophila* IKKγ is a likely target for this activation. Its interaction with *Drosophila* CYLD could serve as a switch to de-activate the pathway, as has been proposed for the NEMO-CYLD interaction in mammals (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). An additional IKK-regulating deubiquitylating enzyme, A20 (also known as Tnfaip3 – Mouse Genome Informatics), has been described in mice. The knock-out effect of its gene has revealed a crucial function in limiting inflammation by terminating TNFα-induced NF-κB responses (Lee et al., 2000). More-recent experiments have revealed that it is, at the same time, a deubiquitylase of K63-linked polyubiquitin chains, and it promotes K48-linked polyubiquitylation of RIP1 (Boone et al., 2004; Evans et al., 2004; Wertz et al., 2004). *Drosophila* contains an ortholog of *A20*, and P-element insertions in the 5′ untranslated region (UTR) of this gene have an immune phenotype (A.E.B. and P.L., unpublished). Systematic screening for deubiquitylating enzymes in the fly should strengthen the concept of the non-degradative function of polyubiquitin chains in NF-κB signaling.

De-regulation of the IMD pathway, as exemplified by the observed alteration in *Dipt* regulation, cannot account, however, for the distinct sensitivity of the *Drosophila CYLD* mutants to infection. Although measuring AMP gene expression is a good indication of the humoral aspect of the response, overall survival to overt infection is a more general phenomenon involving several pathways and cellular activities (Boutros et al., 2002). An intriguing explanation for the death of $CYLD^{f00814}$ homozygotes following infection could be offered by the dramatic change in fat body morphology and physiology. The fat body is the major immuno-responsive organ during *Drosophila* systemic defence and significant changes would be expected to influence survival to immune challenges. Interestingly, a recent report has identified a crucial role for murine CYLD in the development of T lymphocytes (Reiley et al., 2006). Whether higher fat levels could potentially be detrimental as well, remains to be established. A possible hypothesis could be that fly hemocytes, which are macrophage-like cells (Meister and Lagueux, 2003), are not able to engage in phagocytosis because they are overloaded with the function of internalizing excess lipids (that could presumably accumulate in the blood). In the absence of phagocytic activity, bacteria could proliferate freely and kill the host. Following the same line of thinking, mutants with high blood TAGs would also be sensitive to infection. Flies mutant for *brummer* (*bmm*), a TAG lipase homologous to human adipocyte TAG lipase, have 100% more TAGs in their blood than their wild-type counterparts (Gronke et al., 2005). Nevertheless, *bmm* flies showed a survival typical of wild-type flies when challenged with Gram-positive or Gram-negative bacteria (data not shown). This finding indicates that high levels of TAGs are not sufficient to cause immune deficiency and strengthens our hypothesis that fat body structure and function might be the principal determinant of immune deficiency in $CYLD^{f00814}$ homozygotes. Collectively, findings by Reiley et al. (Reiley et al., 2006) together with the results of this study establish a clear functional relationship between mammalian and *Drosophila* CYLD proteins in host defence.

Even though the analysis of CYLD activity in cell lines suggests that it functions as a general negative regulator of IKK and JNK activation by cleaving Ub^{Lys63} chains, cylindromatosis patients display a phenotype that is restricted to epidermal body regions. The TNFR family member ectodysplasin A receptor controls the development of epidermal appendices by activating NF-κB (Schmidt-Ullrich et al., 2001). Therefore, under physiological conditions, the role of CYLD may be restricted to specific cell types and stimulatory conditions, which points to a functional redundancy with other ubiquitin hydrolases. Similarly, our analysis showed a broad pattern of *Drosophila CYLD* expression in both embryonic and adult tissues. However, a compromise of *CYLD* expression did not appear to affect embryonic development, but rather the major effect we observed was restricted to the fat body and the physiological properties attached to it (fat storage, host defence). Again, redundancy with proteins performing similar functions in

other tissues could be envisaged (Chen and Fischer, 2000). Nevertheless, possible functional deficits in the nervous system, where *Drosophila* CYLD accumulates, were not investigated in *CYLD*^{f00814} animals, but will be the focus of future experiments. We demonstrated that impaired *Drosophila CYLD* expression is associated with increased TAG content and resistance to nutrient starvation. This result suggests that CYLD is involved in fat storage and/or metabolism in *Drosophila*, and possibly in mammals. Increased TAG content has been observed in flies with mutated insulin receptor (INR) or Chico, an INR substrate (Bohni et al., 1999; Clancy et al., 2001; Tatar et al., 2001). Therefore, it is possible that *Drosophila* CYLD is required for proper signal transduction by INR. INR is activated by neuropeptides [insulin-related peptides (ILPs)] homologous to human insulin (Brogiolo et al., 2001). We have found that the expression of *Drosophila ILP2*, the major ILP, is not affected by the decrease in CYLD levels (data not shown). This finding suggests that *Drosophila* CYLD might regulate INR signaling downstream of receptor activation. One such possible target is the transcription factor FOXO, which is activated by INR signaling (Goberdhan and Wilson, 2003). It has been shown that FOXO is activated by the JNK pathway, which itself was recently shown to be negatively regulated by CYLD in mammalian cells (Reiley et al., 2004; Wang et al., 2005). In *Drosophila*, activation of the JNK pathway contributes to oxidative stress resistance (Wang et al., 2003), and this, in conjunction with increased TAG levels observed in CYLD-deficient flies, might explain the resistance of *CYLD*^{f00814} homozygotes to nutrient starvation. Further analysis is required to determine whether *Drosophila CYLD*, like its human counterpart, is also involved in the regulation of the JNK pathway, establishing further the valuable cross-talk between flies and humans in the context of understanding disease.

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