ARC Syndrome-linked Vps33B protein is required for inflammatory endosomal maturation and signal termination

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Supplemental Information

A. Supplementary Experimental Procedures.

Q-RT-PCR

For the measurement of AMPs, RNAs were isolated from injected flies and AMP RNA expression was compared to the transcript encoding ribosomal protein 49 (RP49) by qRT-PCR as described (Akbar et al., 2011). RNA expression in macrophages was measured in comparison to Histone 2A with primers listed in Supplemental Table I using qRP-PCR. qRT-PCR was performed using the Applied Biosystem kit (DNA-free, High capacity cDNA reverse transcription kit, Fast SYBR Green master mix) and using 7500 Fast Real-Time PCR System.

Western Blotting

For western blots measuring signaling components, macrophages were stimulated with or without 1 µg/mL LPS, for the indicated times, washed with cold PBS (pH-7.4), and lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% TWEEN 20) containing Protease Inhibitor Mixture (Roche Cat No.11836170001), 1 mM sodium orthovandate, and 20 mM glycerol 2-phosphate. Equivalent amounts of protein (10 µg) from cell lysates were heat denatured in 1X Laemmli sample buffer and western blotting was performed following standard procedure. Primary antibodies were purchased from Cell Signaling Technology for total and phospho-specific p38 1:1000 # 9211 & 9212), ERK (1:1000 # 9101 & 9102) and JNK (1:1000 # 9251 & 9252) kinases. IR-labeled secondary antibodies (IRDye-800CW or -680RD) were purchased from LI-COR detected and quantified using an ODYSSEY infrared imaging system (LI-COR).

Immunofluorescence Staining

To study endocytosis of AcLDL (Alexa Fluor® 488 AcLDL Invitrogen, #L-23380), cells were incubated with 20 µg/ml of labeled AcLDL in serum-free RPMI-1640 medium for 2 min at 37°C. After incubation, cells were washed 4 times, and chased in absence of ligand for various times as indicated. To study Tf recycling, cell were incubated with 20 µg/ml of labeled Tf (Alexa Fluor®

568, Invitrogen Cat. No. T-23365) for 2 min at 37°C. Unbound Tf was removed by washing. Cells were then chased for different time periods at 37°C. Alexa Fluor® 647 labeled cholera toxin B subunit (Invitrogen, #C-34778) was used at 1 µg/ml only or with Tf and chased for indicated times. To visualize LPS trafficking, macrophages were incubated with LPS-Alexa594 (Thermofisher, L-23353).

Macrophage lysosomes were labeled with lysotracker or loaded with dextran-Alexa Fluor594 (MW-10, 000; 150 µg/ml; Invitrogen, Cat. No. D22913) overnight, followed by a 4-h chase in fresh RPMI-1640 medium.

Phagocytosis of *E. coli* into *Drosophila* hemocytes or mouse macrophages was measured as described (Akbar et al., 2011). In short, GPF-expressing *E. coli* (at MOI of 8) were allowed to adhere to cells for 20 min at 4°C, before being phagocytosed and chased for the indicated times. Cells were counterstained with Phalloidin-Alexa594 and internal bacteria detected at the indicated chase times were quantified.

To follow trafficking of PGRP-LC, isolated fat bodies expressing PGRP-LC-GFP from the endogenous PGRP-LC promoter (Neyen et al., 2012) were cultured in S2 cell medium with or without PGN-BS (10 µg/ml), fixed with 4% paraformaldehyde and stained with anti-GFP-antibodies (1:1000, Invitrogen, A11120) as described (Nandi et al., 2014).

Cytokine ELISA

Supernatants from cultured stimulated and unstimulated Vps33B-silenced and control iBMDC were collected at different time points within 0-24 h time period. Paired antibody ELISAs were performed as described (Hu et al., 2011). Capture/detection antibodies were purchased from BD Bioscience and the pairs were as follows: IL-6 (#554543, 554402); IL-12 (#551219,554476); TNF-α (# 557516, 554415).

For stimulation of macrophages with LPS-coated beads, Fluoresbrite Carboxy YG 4.5mm latex beads (Polysciences) were incubated with LPS (30 µg/ml) for 16 hours at 4°C. Unbound LPS was removed by washing the latex beads 10 times with large volumes of sterile endotoxin-free PBS. After phagocytosis of LPS-coated beads, cells were incubated for 6 hours for cytokine assays (West et al., 2011).

Flow Cytometry

To measure endocytosed TLR4, Vps33B silenced and control iBMDMs were first incubated with the anti-TLR4-PE antibody (Biolegend #145404) for 30 min, followed by stimulation with LPS (1µg/ml) for the indicated time. Cells were washed three times with PBS and surface fluorescence was quenched with trypan blue and intracellular fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences).

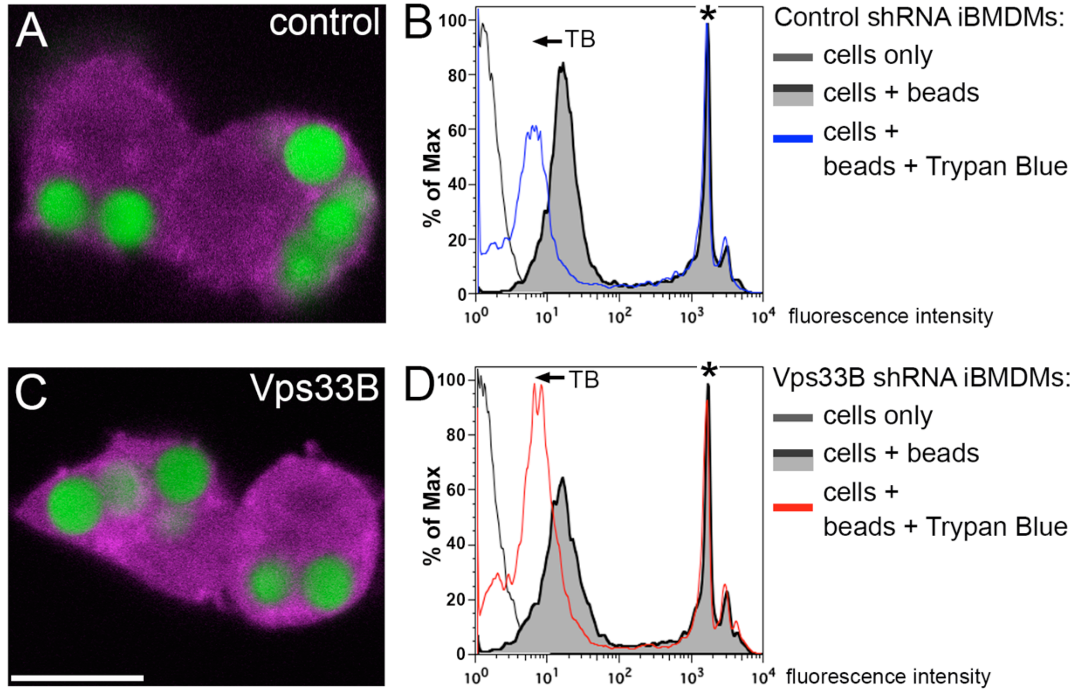
To measure fate of phagocytosed *E. coli* by follow cytometry, overnight GFP *E. coli* culture was harvested by centrifugation and washed three times with PBS. The bacteria were resuspended in media and incubated at MOI of 8 with macrophages for 20 min. Cells were washed three times with PBS and resuspended in complete media. After the indicated chase time, cells were washed with PBS, surface GFP was quenched with trypan blue, and intracellular fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences).

For measuring p38 MAP kinase activation by phospho-flow cytometry, control and VPS33B-silenced iBMDMs were stimulated with the different concentrations (1 or 10 ng/ml) of LPS for indicated times. After stimulation cells were fixed directly with 4% PFA (final concentration of 1.6%). Then cells were washed twice with FACS buffer and permeabilized with cold 100% methanol (stored at -20^{0}C) for 30 min. Cells were washed twice with FACS buffer and incubated with AF-647 mouse anti-p38 MAPK antibody (BD Biosciences: pT180/pY182 #612595) diluted in FACS buffer for 1h at room temperature. Cells were washed three times with FACS buffer and intracellular fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Statistical methods

Statistical significance was determined in Prism (GraphPad) using log-rank for survival assays and one-way ANOVA for multiple comparisons, followed by Tukey's test. Colocalization of TLR4 with different Rabs or dextran was calculated in Prism from normalized integrated intensities measured in ImageJ. All bar graphs resulting from these comparisons show mean ± standard deviation. P-Values smaller than 0.05 are considered significant and values are indicated as * (<0.05), ** (<0.01), *** (<0.001). Statistical significance of differences between survival curves were determined by Log-Rank (Mantel-Cox) test.

B. Supplemental Figures



Supplemental Figure S1. Vps33B silencing does not impair phagocytosis.

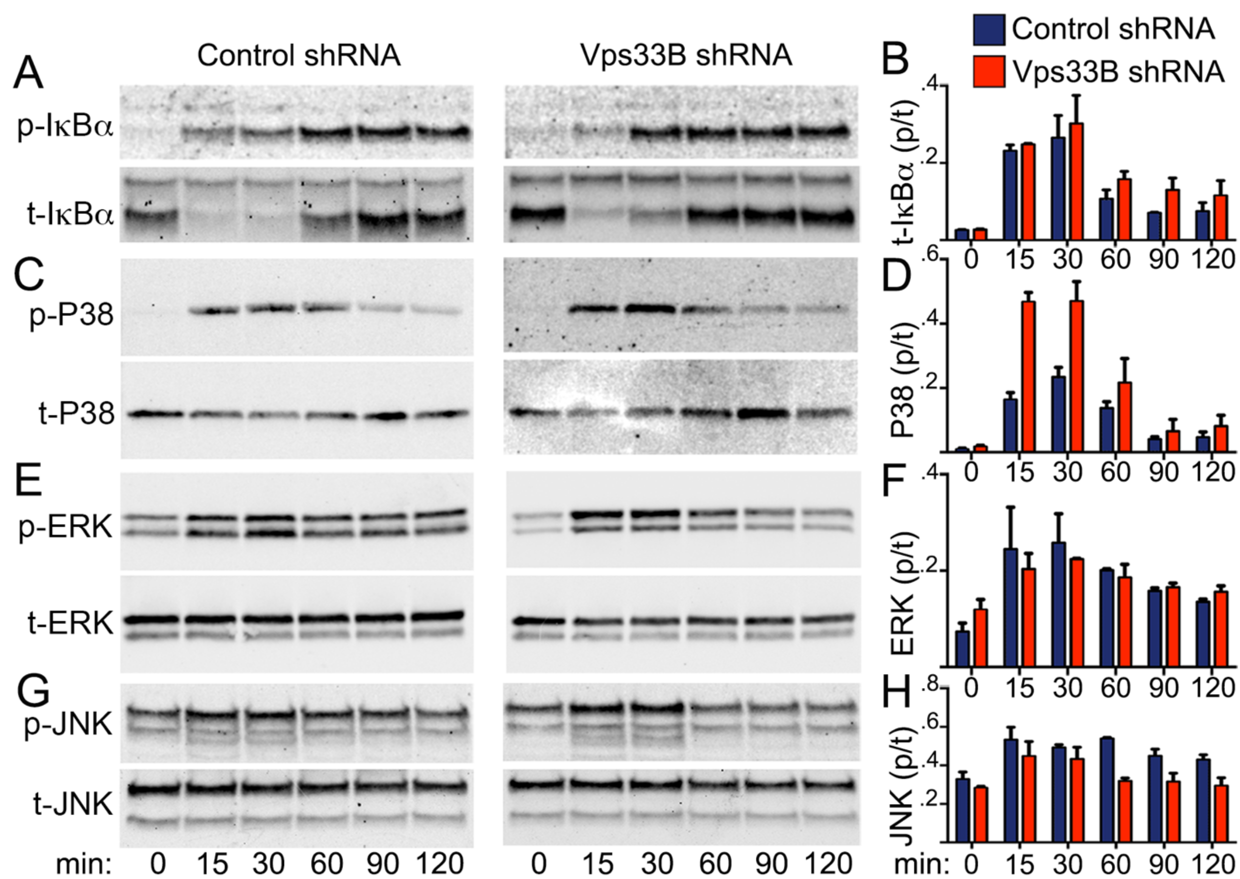
Fluorescent latex beads were used to measure phagocytosis by macrophages expressing control (A,B) or Vps33B shRNA (C,D),

A,C) Micrographs depict latex beads (green) that were phagocytosed into control (A) or Vps33B deficient (C) macrophages that were visualized using Alexa594-Phalloidin (magenta).

B,D) Flow cytometry-based quantification of bead fluorescence associated with cells did not detect a difference in the amount of bead phagocytosis. To distinguish between extracellularly attached beads and phagocytosed internal beads (*) fluorescence of extracellular beads was quenched with Trypan Blue (Quenching-based loss of fluorescence from external beads is indicated by the TB-labeled arrows).

Scale bar in C is 10 µm in A, C.

Data in this figure are representative of at least 2-3 independent experiments. This supplemental figure supports main text figure 1.

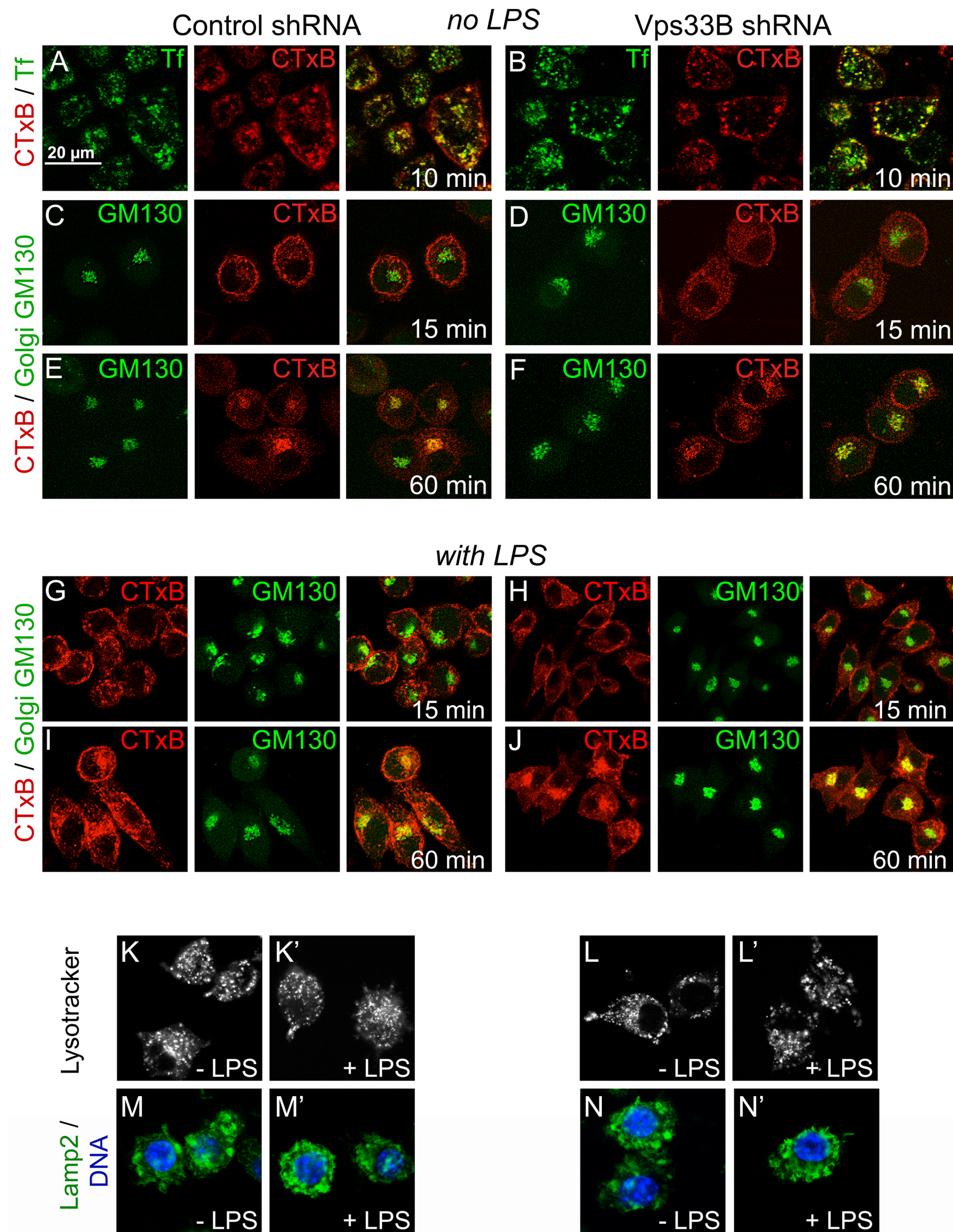


Supplemental Figure S2. Effects of Vps33 silencing on TLR4 signaling

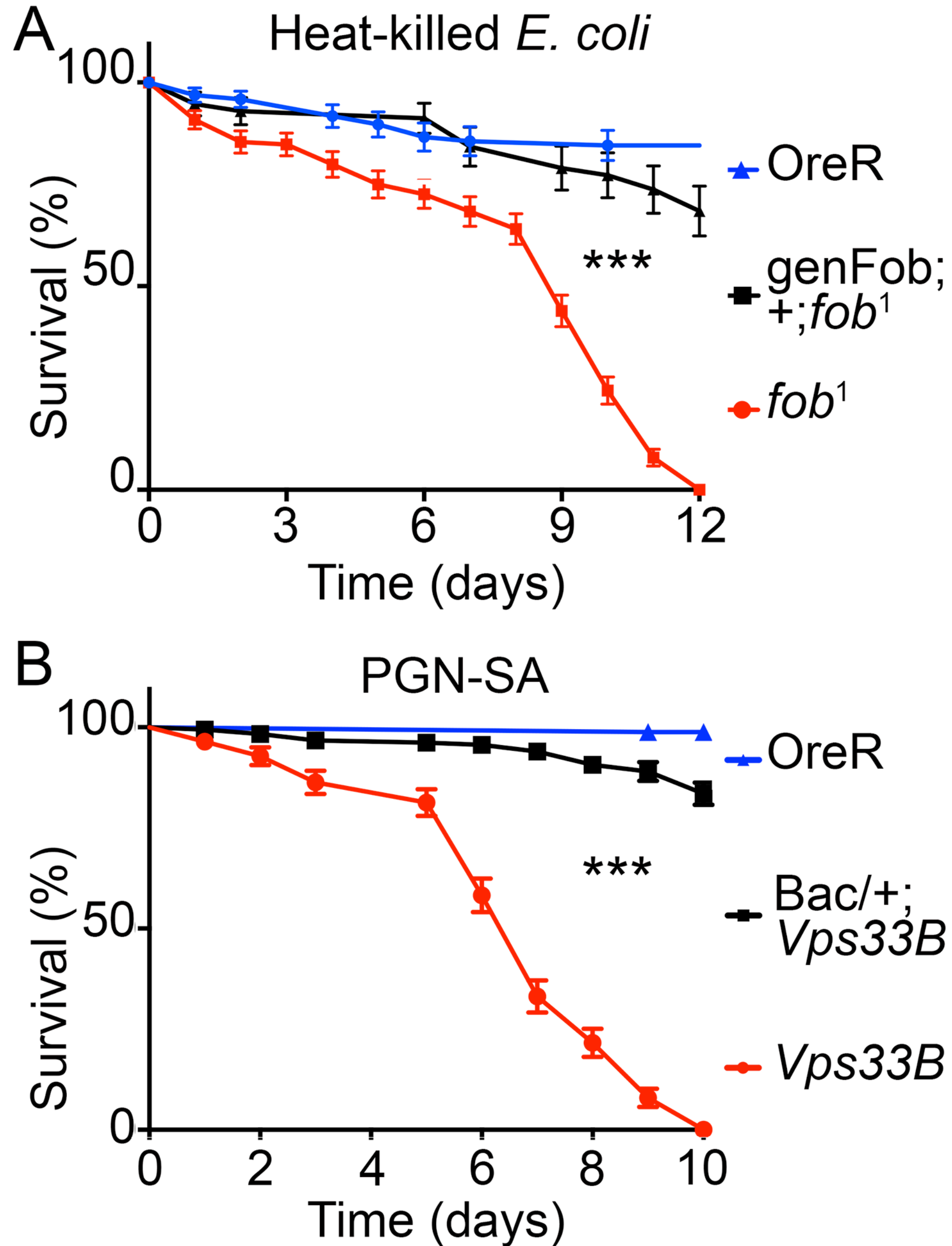
A-H) Control or Vps33B-silenced macrophages were exposed to LPS (1μg/ml) for the indicated times and the amounts of indicated total and phosphorylated proteins were detected by Western blots (A,C,E,G) and for each, the ratio of phosphorylated to total protein quantified (B,D,F,H).

A, B) IκBα, C,C',D) p38 MAP kinase, E ,F) ERK1 kinase, G, H) JNK kinase.

Data here are representative of 3 independent experiments. This supplemental figure supports main text figure 3.



Supplemental Figure S3. Vps33B is not required for retrograde transport of Cholera Toxin A-J) CTxB uptake and retrograde transport in control (A,C,E,G,I) or Vps33 silenced (B,D,F,H,J) macrophages. A,B) At early time points (10 min), CTxB (red) colocalizes with Tf (green) in endosomes. C-J) At later time points (15 and 60 min) CtxB (red) accumulated in the Golgi complex marked with GM130 (green), regardless of whether cells were untreated (C-F) or pretreated for 30 min with 1 µg/ml with LPS (G-J). K-N) Lysotracker (K,L) or LAMP2 (M,N) staining was not altered between control (K,M) and Vps33B silenced (L,N) macrophages, regardless of LPS pretreatment (for 30 min with 1 µg/ml when indicated). Data in this figure are representative of 2 independent experiments. This supplemental figure supports main text figure 6.



Supplemental Figure S4. Absence of Fob and Vps33B causes death in flies exposed to non-pathogenic bacteria or bacterial ligands

Diagrams show the fraction of surviving flies with indicated genotypes after injection with heat-killed *E. coli* (A) or PGN-SA (B).

Genotypes: A) OreR, w^{1118}; fob^{1}, w^{1118}; *genFob/+ ; fob*^{1} B) OreR, w^{1118}; $Vps33B^{5.2}$, w^{1118}; BAC$^{CH322\text{-}65H17}$/+; $Vps33B^{5.20}$. (***: $p<0.001$, log-rank). Data are representative of 3 independent experiments. This supplemental figure supports main text figure 7.

Supplemental Table I. Primers and shRNA sequences for quantitative RT-PCR and gene silencing in mouse macrophages.

Primers for qRT-PCR in iBMDMs (Main Figure 2)		
H2A fw	5’-TTGCAGCTTGCTATACGTGGAGATG-3’	
H2A rv	5’-TGTTGTCCTTTCTTCCCGATCAGC-3’	
IFN-β fw	5’-TCCGAGCAGAGATCTTCAGGAA-3’	
IFN-β rv	5’-TGCAACCACCACTCATTCTGAG-3’	
Vps33B-1 fw	5’-GCATGGAACTGCCAGAATTT-3’	
Vps33B-1 rv	5’-CCTGTTTCAGTCCCTTGAGC-3’	
Vps33B-2 fw	5’-ATCGGTCCCTGAAAACACAG-3’	
Vps33B-2 rv	5’-CCCAAGAACACCACCAAGAT-3’	
Control fw	5’-AGCAACTGCTGAGGATCCTT-3’	
Control rv	5’-ACCATTACACCCTGCTGGAG-3’	
MISSION shRNA (Sigma-Aldrich) sequences against Vps33B (Main Figure 1)		**clone ID**
shRNA #1	CCGGCGAATTGCTAACGTCTCTATCCTCGAGGA TAGAGACGTTAGCAATTCGTTTTTG	NM_178070 .4-480s21c1
shRNA #2	CCGGCCAGAGATTGGACACATCTTTCTCGAGAA AGATGTGTCCAATCTCTGGTTTTTG	NM_178070 .2-998s1c1
shRNA #3	CCGGCGCAATGAGCACTTCTCCAATCTCGAGAT TGGAGAAGTGCTCATTGCGTTTTTG	NM_178070 .2-1199s1c1
shRNA #4	CCGGGCTGGGAACTTTGGAACTAATCTCGAGAT TAGTTCCAAAGTTCCCAGCTTTTTG	NM_178070 .2-2353s1c1
shRNA #5	CCGGGCTGCTAAACTGCAGTGAGTTCTCGAGAA CTCACTGCAGTTTAGCAGCTTTTTG	NM_178070 .2-1957s1c1
Primers for amplification and sequencing of Vps33B$^{5.20}$ deletion (Main Figure 1)		
Vps33B fw	5’-TTGGGATTGGAATCACCTTC-3’	
Vps33B rv	5’-TTGTTATTCCCCGGAAACTG-3’	
Primers for quantification of AMP expression (Main Figure 2)		
Cecropin fw	5’-TCTTCGTTTTCGTCGCTCTC-3’	
Cecropin rev	5’-ACATTGGCGGCTTGTTGAG-3’	
AttacinA fw	5’-TGCAGAACACAAGCATCCTAA-3’	
AttacinA rev	5’-TAAGGAACCTCCGAGCACCT-3’	
Cecropin fw	5’-TCTTCGTTTTCGTCGCTCTC-3’	
Cecropin rev	5’-ACATTGGCGGCTTGTTGAG-3’	
RP49 fw	5’-ATCGGTTACGGATCGAACAA-3’	
RP49 rev	5’-GACAATCTCCTTGCGCTTCT-3’	