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Supplementary Materials for

Double-stranded RNA binding protein, Staufen, is required for the initiation of RNAi in coleopteran insects

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Other Supplementary Materials for this manuscript include the following:

Data S1 to S3

www.pnas.org/cgi/doi/10.1073/pnas.1809381115

Data S1

The list of the genes downregulated in RNA-seq data based on the differential expression of gene expression analysis between Lepd-SL1 and Lepd-SL1RR.

Data S2

The list of the genes upregulated in RNA-seq data based on the differential expression of gene expression analysis between Lepd-SL1 and Lepd-SL1RR.

Data S3

The list of primers used in this study.

Materials and Methods

Cell culture and Generation of an apoptosis-resistant cell line

Colorado potato beetle cell line (Lepd-SL1 cells) was obtained from the Biological Control of Insects Research Laboratory (USDA-ARS, Columbia, MO). These cells were cultured at 27°C in EX-CELL 420 (Sigma–Aldrich, St. Louis, MO) medium supplemented with 10% FBS (Seradigm Fetal Bovine Sera, VWR International, Radnor, PA). Lepd-SL1 cells were seeded in 6-well plates, 5 ng of dsIAP/well was added to the medium on the first day of culture. Once the cells reached 70~80% confluency, 5 ng of dsIAP/well was added again. In subsequent rounds of selection, the concentration of dsIAP was increased gradually to reach 100 ng/well, which induced apoptosis in only 5% of cells during the final round of selection. The same concentration of dsRNA induced apoptosis in 70~80% of Lepd-SL1 cells within a day after treatment.

Total RNA extraction, PCR, Reverse-transcriptase quantitative real-time PCR (qRT-PCR) and dsRNA synthesis

Total RNA isolation, PCR amplification of dsRNA templates, qRT-PCR, and dsRNA synthesis were performed as described previously in our publications (1, 2). The shRNA and

siRNA were synthesized using primers listed in Data S3 and Megascript T7 RNA synthesis kit (Life Technologies, Carlsbad, CA). The relative mRNA levels were determined in triplicate biological samples using mRNA levels of ribosomal protein 4 (RP4) as an internal control. To determine knockdown efficiency, 200,000 cells were seeded in each well of a 12-well plate. 100 ng of dsIAP or dsGFP (control dsRNA targeting Green Fluorescent Protein gene with no detectable effect in *Leptinotarsa decemlineata*) were added to the medium. The cells were harvested, total RNA was isolated and used to measure relative IAP mRNA levels.

Internalization study of Cy3-labeled dsGFP

To label dsRNA with Cy3, Silencer siRNA labeling Kit purchased from Life Technologies (Carlsbad, CA) was used. 40 µg of lyophilized Cy3 dye was resuspended in 100 µl reconstitution solution (final stock: 400 ng/ µl). 7.5 µl of Cy®3 labeling reagent, 2.0 µl of dsRNA (5 ng), 5.0 µl of 10X labeling buffer and nuclease-free water to 40 µl were mixed and incubated at 37°C for 1 hr. Then, 5 µl of 5M NaCl (0.1 volume) and 125 µl of 100% cold ethanol were added to the samples and stored at -20°C. On the next day, the sample was centrifuged at 15,000 rpm for 20 min. The supernatant was discarded, and the pellet was washed with 175 µl of 70% ethanol and centrifuged for 5 min at 15,000 rpm. Ethanol was discarded, and the pellet was air dried for 10 min and the labeled dsRNA pellet was dissolved in 30 µl nuclease-free water. The dsRNA concentration was determined using a nanodrop spectrophotometer. Cy3-labeled dsIAP was prepared following the same method and use to check its function in Lepd-SL1 cells.

100,000 cells/well were seeded in 8-well chamber slides (Nunc Lab-Tek, ThermoFisher Scientific, Waltham, MA). On the next day, 100 ng of Cy3 labeled dsGFP was added to each well and incubated for 2 hr. The cells were washed twice with 1X PBS buffer, fixed with 4% paraformaldehyde and mounted in EverBrite mounting medium containing DAPI (Biotium,

Fremont, CA). Cells were visualized under a Leica TCS SP8 confocal microscope using DAPI, Cy3 (555-595 nm), and Bright field channels.

Processing of ^{32}P-labeled dsRNA

The dsRNA was ^{32}P-labeled following the methods described in our recent publication (1). ^{32}P-labeled shRNA and siRNA were synthesized following the same method used for dsRNA synthesis. For dsRNA processing experiments, 1.6 x 10^6 CPM (count per million) ^{32}P-labeled dsGFP was added to both Lepd-SL1 and Lepd-SL1 RR cells. After 24 hrs exposure to dsRNA, the cells were harvested, and RNA was isolated. 2000 CPM RNA was loaded on to 8M urea-16% polyacrylamide gels. The gels were dried and exposed overnight to a phosphorImager screen, and the screen was scanned in a phosphorImager (Typhoon 9500, GE Healthcare Life Sciences, Pittsburgh, PA). ImageJ program was used to determine relative levels of dsRNA and siRNA.

Comparison of transcriptomes of susceptible and resistant cells

Total RNA isolated from susceptible and resistant cells was used for the preparation of libraries as described previously (3). The libraries were sequenced using the HiSeq4000 sequencer at the Genomics Technologies Center of Duke University, NC, USA. Raw sequence data statistics are shown in Table 1S. Expression levels of mapped genes were compared using the RNA-Seq analysis tool from CLC workbench (Version 11.0, Qiagen Inc Germantown, MD). EDGE test analysis was performed using the default parameters and the genes expressed differentially between susceptible and resistant cells were identified. Differentially expressed genes were annotated using “cloud blast” feature within the “Blasto2GO plug In” in CLC Genomics Workbench.

***StauC* protein production, pull-down, and electrophoretic mobility shift assays**

Expression and purification of StauC protein and production of polyclonal antibodies were performed by GenScript on a custom contract (GenScript, Piscataway, NJ). The purified StauC protein was used for Gel-shift and the pull-down assays. 0.5 µg of StauC protein or bovine serum albumin (a negative control) in M-PER reagent (Pierce, Rockford, IL) were mixed with biotinylated dsGFP (prepared using Biotin RNA labeling mix, Roche Diagnostics Corporation, Indianapolis, IN) and incubated for an hour. Unlabeled dsGFP was used as a negative control. Biotinylated dsGFP was pulled down using the streptavidin magnetic beads (Pierce, Rockford, IL). The pulled down proteins were resolved on SDS-PAGE, transferred to a membrane. Western blot hybridization was performed using StauC antibodies. The ScanLater™ Western Blot Detection System (Molecular Devices, Sunnyvale, CA) was used to detect protein bands.

2 x 10^4 Counts per million dsGFP was incubated with 60 ng of *StauC* protein at 30°C for 30 min in 1x buffer 12 [100mM KOAc, 10mM HEPES (pH 7.4), 2 mM Mg(OAc)2 and 5 mM DTT]. After the addition of 1.2 µl 50% glycerol and 3 µl of loading dye, the reaction was resolved by non-denaturing PAGE (4%). After electrophoresis, the gels were fixed in 10% isopropanol, dried and exposed to a phosphorImager screen overnight and scanned using a phosphorImager. *L. decemlineata* embryonic extracts were prepared, and their binding to labeled dsRNA was evaluated following the methods described by Haley et al., (4)

RNAi assay

The *in vitro* RNAi assay using Lepd-SL1 cells was performed as described previously (2). For *in vivo* RNAi assay in *L. decemlineata,* 1000 ng of dsStauC, dsStau or dsGFP was injected into third instar larvae. Three days later, 25 ng of dsdIAP was fed, and mortality was recorded daily until pupation. For *in vivo* RNAi assay in *Tribolium castaneum,* 200 ng of dsStauC, dsStau or dsLuc was injected to the early stage last instar larva. At 48 hr after the first

dsRNA injection, 200 ng dsIAP was injected. The mortality was recorded until the control larvae reached the pupal stage.

Identification of *StauC* homologs and phylogenetic studies

The amino acid sequences of the mammalian, insect, and nematode dsRNA binding proteins were obtained from NCBI and i5K workspace (http://i5k.nal.usd.gov/webapp/blast/) based on sequence homology searches by running BLASTp and tBLASTn using the NCBI BLAST service (http://www.ncbi.nlm.nih.gov/) and Uniport BLAST service (http://www.uniprot.org/). The Muscle program in MEGA 7.0 was used to align the protein sequences, and the maximum likelihood analysis was performed in MEGA 7.0 with bootstrapping to estimate the reliability of phylogenetic reconstruction (1000 replicates). The SMART domain analysis (http://smart.embl-heidelberg.de/) program and conserved domain database (https://www.ncbi.nlm.nih.gov/cdd/) were used to predict the protein architecture.

Statistical analysis

Student's t-test was used to compare the gene expression difference between control and treatment groups. A *P*-value of 0.05 or less between groups was considered as a significant difference.

References

1. Shukla JN, *et al.* (2016) Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA Biol*: **13(7)**: 656-669.
2. Yoon J-S, Gurusamy D, Palli SR (2017) Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, *Spodoptera frugiperda*. *Insect Biochem Mol Biol.*. **90**: 53-60.

3. Kalsi M, Palli S.R., (2017) Cap n collar transcription factor regulates multiple genes coding for proteins involved in insecticide detoxification in the red flour beetle, *Tribolium castaneum*. *Insect Biochem Mol Biol.* **90**: 43-52.
4. Haley B, Tang G, Zamore P. D., (2003) In vitro analysis of RNA interference in *Drosophila melanogaster*. *Methods*. **30**: 330-336.
5. Yoon JS, Shukla JN, Gong ZJ, Mogilicherla K, & Palli SR (2016) RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors. *Insect Biochem Molec Biol* 78:78-88.

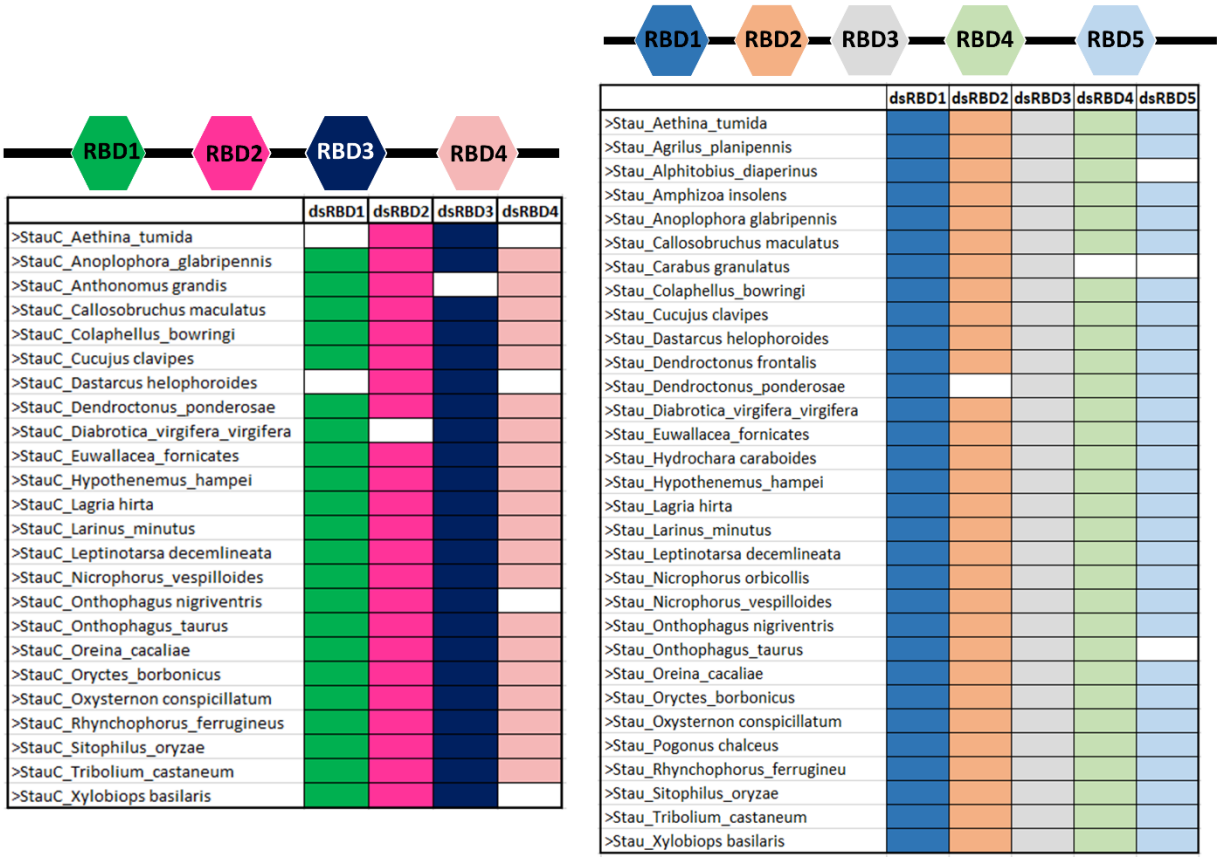


Fig. S1. Domain structures of Staufen and StaufenC identified in coleopteran insects. The panel on the left shows the domain structure of StauC, and the one on the right shows the domain structure of Stau. The SMART domain analysis (http://smart.embl-heidelberg.de/) program and conserved domain database (https://www.ncbi.nlm.nih.gov/cdd/) were used to predict domains in StauC and Stau.

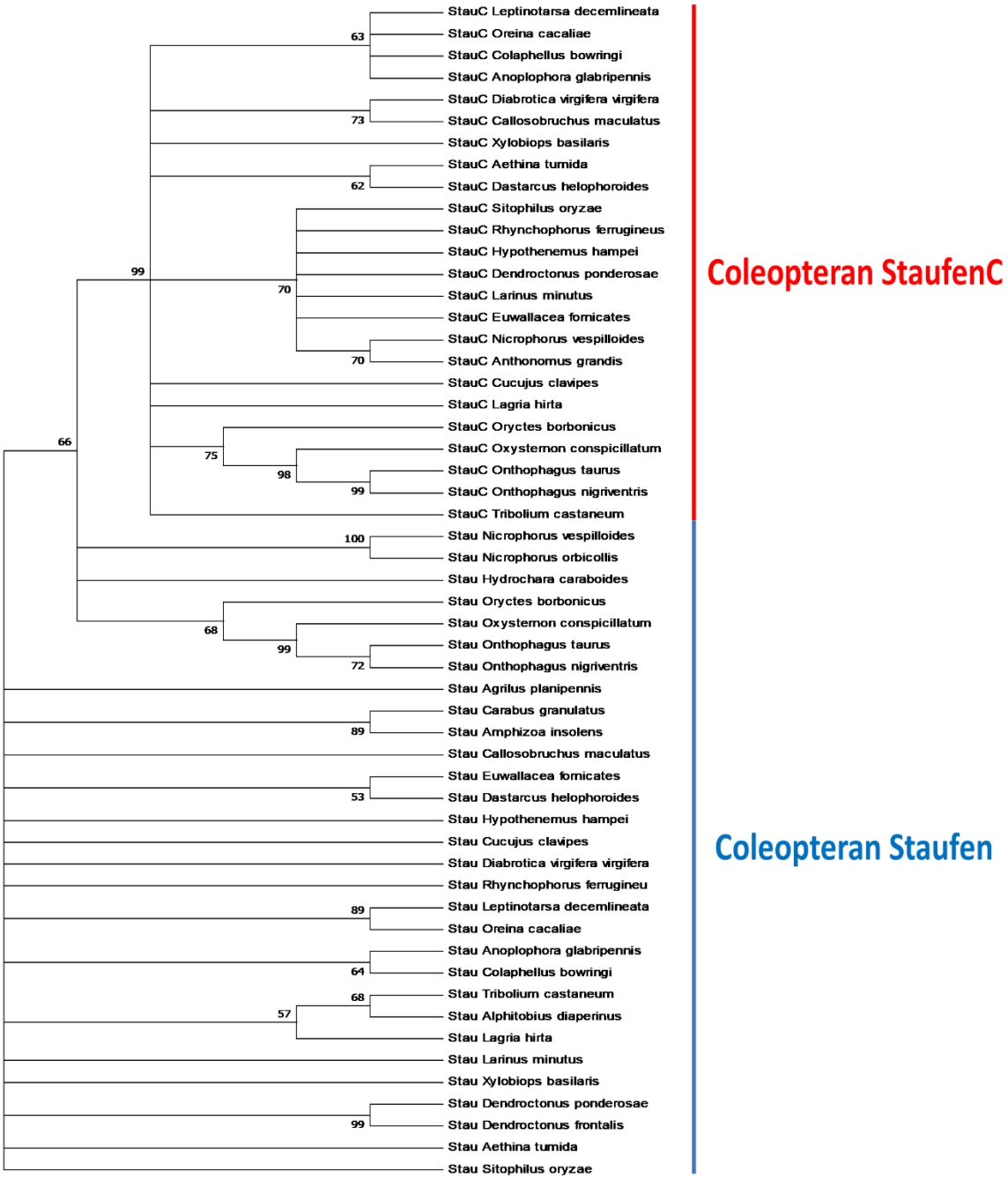


Fig. S2. Maximum likelihood phylogenetic tree of Staufen and StaufenC proteins. Staufen and StaufenC amino acid sequences were analyzed using the Maximum likelihood method.

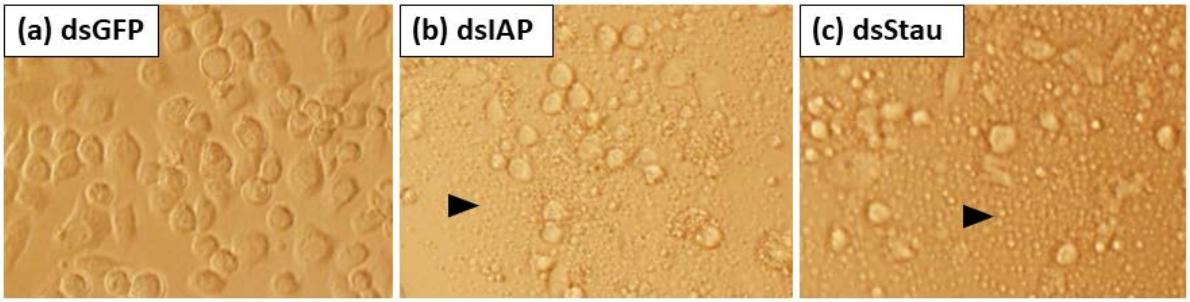


Fig. S3. Staufen is not required for RNAi response in Lepd-SL1 cells. The Lepd-SL1 cells were exposed to dsGFP followed by dsGFP (a), dsGFP followed by dsIAP (b), and dsStau followed by dsIAP (c). The photographs were taken at 48 hr after treatment with the second dsRNA. The arrow points to the apoptotic bodies seen in cells undergoing apoptosis.

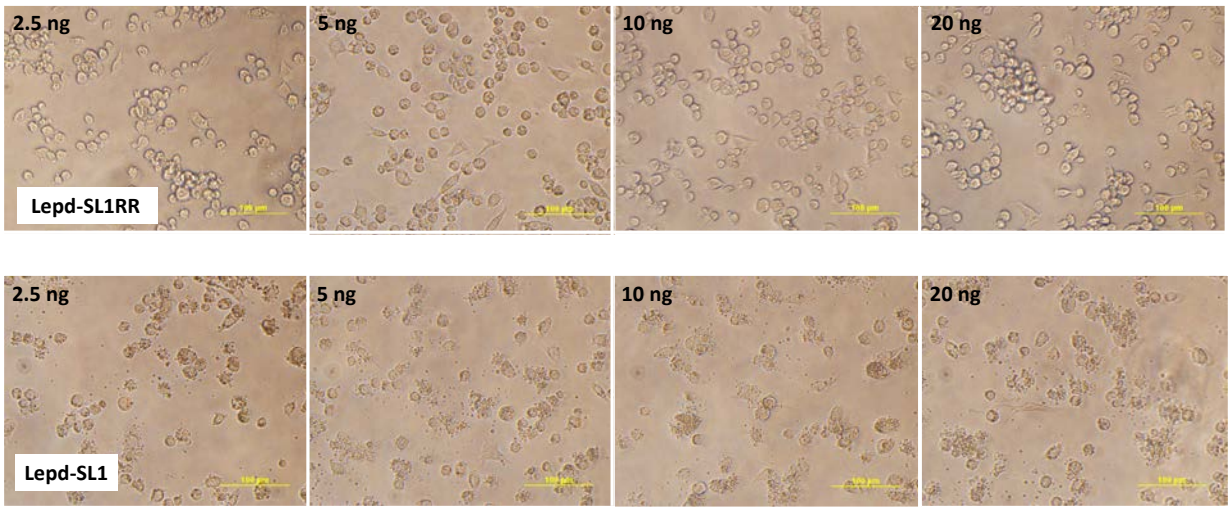


Fig. S4. Apoptosis phenotype observed after exposing Lepd-SL1 and Lepd-SL1RR (RNAi Resistant) cells to different concentrations of dsIAP. The cells were exposed to 2.5 to 20 ng of dsIAP in 100 µl medium and photographed at 24 hr after treatment with dsRNA.

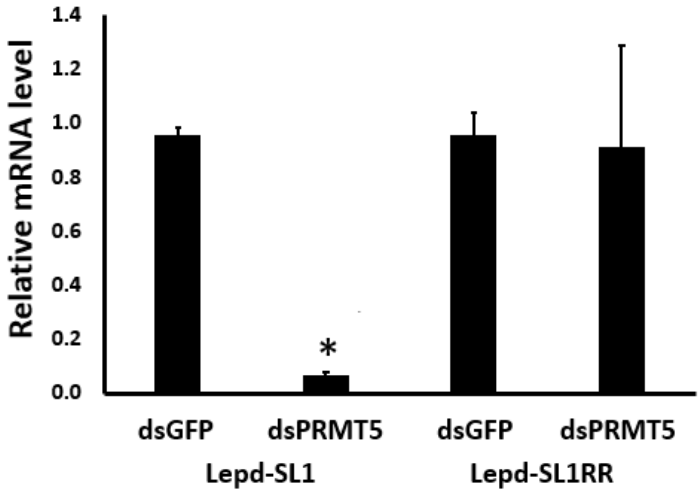


Fig. S5. Relative PRMT5 mRNA levels in Lepd-SL1 and Lepd-SL1RR cells exposed to dsPRMT5. The cells were exposed to dsPRMT5 or dsGFP (as a control). Total RNA was isolated and used to quantify relative PRMT5 mRNA levels by qRT-PCR. Ribosomal protein 4 (RP4) was used as an internal control. * significantly different from control at $P \leq 0.05$.

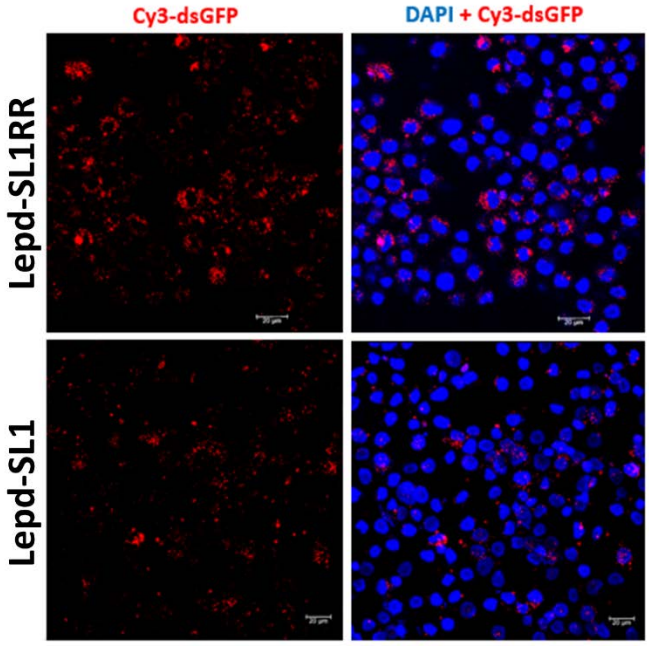


Fig. S6A. Subcellular localization of Cy3-labeled dsGFP in the Lepd-SL1 and Lepd-SL1RR cells. The cells were exposed to 100 ng Cy3-labeled dsGFP in 8-well chamber slides for 2 hr. Then the cells were fixed, mounted in DAPI containing medium and photographed using a confocal microscope.

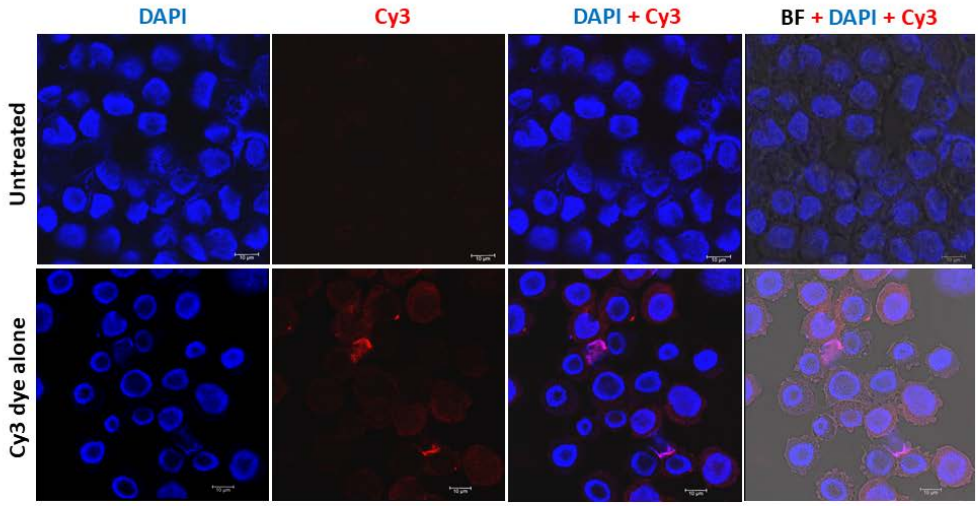


Fig. S6B. Negative controls for subcellular localization experiments. Images of Lepd-SL1 cells untreated or treated with Cy3 dye alone are shown. At 6 hr after incubation, the cells were fixed, mounted in DAPI containing medium and photographed using a confocal microscope.

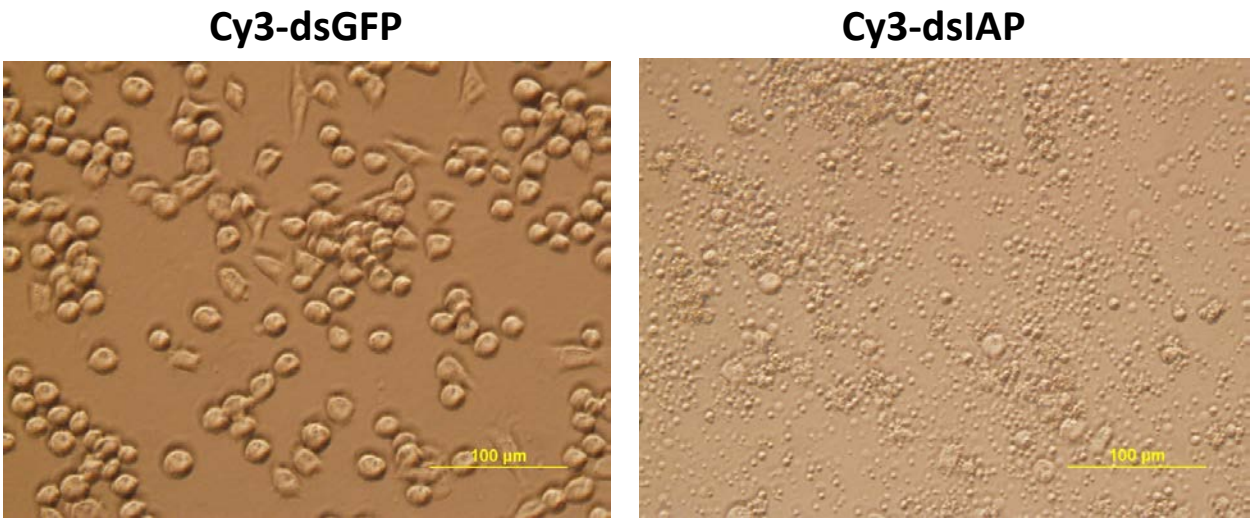
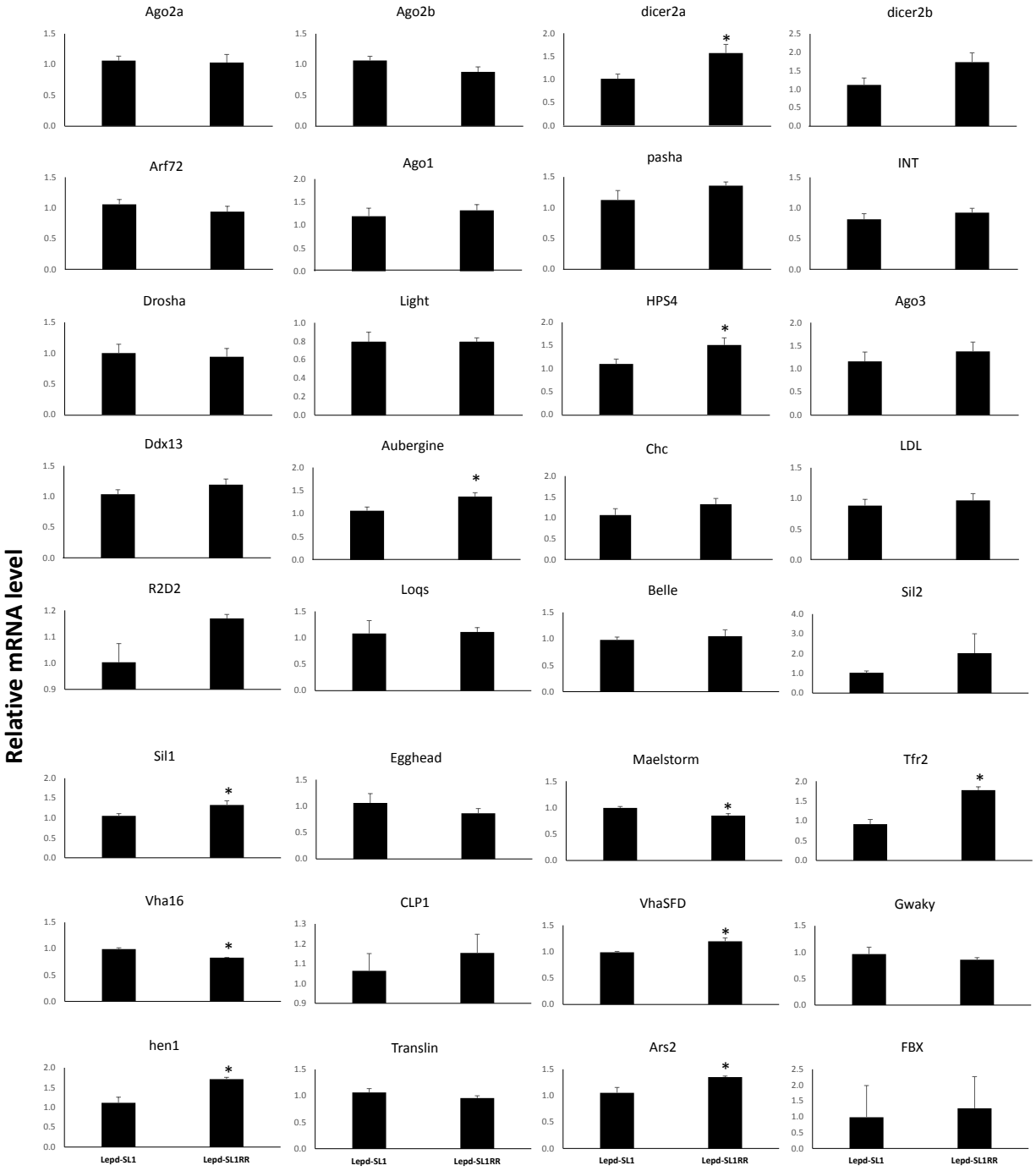


Fig. S6C. Confirmation of Cy3-labeled dsRNA function targeting IAP gene. Cy3-labeled dsGFP and dsIAP were *in vitro* synthesized and added to Lepd-SL1 cells. The cells were exposed to 100 ng of dsRNA in 100 μl medium and photographed at 24 hr after treatment with dsRNA.


Relative mRNA level
Ago2a
Ago2b
dicer2a
dicer2b
Arf72
Ago1
pasha
INT
Drosha
Light
HPS4
Ago3
Ddx13
Aubergine
Chc
LDL
R2D2
Loqs
Belle
Sil2
Sil1
Egghead
Maelstorm
Tfr2
Vha16
CLP1
VhaSFD
Gwaky
hen1
Translin
Ars2
FBX
Lepd-SL1
Lepd-SL1RR

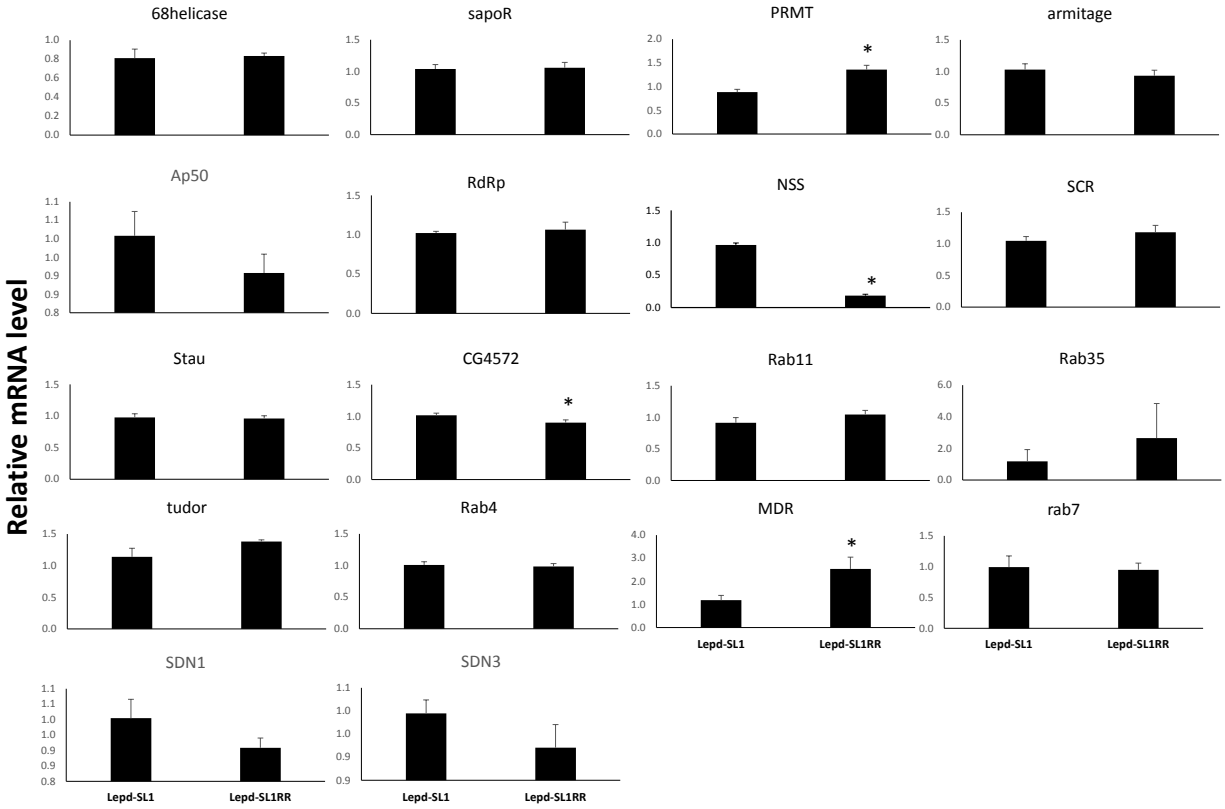


Figure S7. Relative mRNA levels of 50 RNAi genes in Lepd-SL1 and Lepd-SL1 RR cells by qRT-PCR. Total RNA was isolated and used to quantify relative mRNA levels by qRT-PCR. Ribosomal protein 4 (RP4) was used as an internal control.

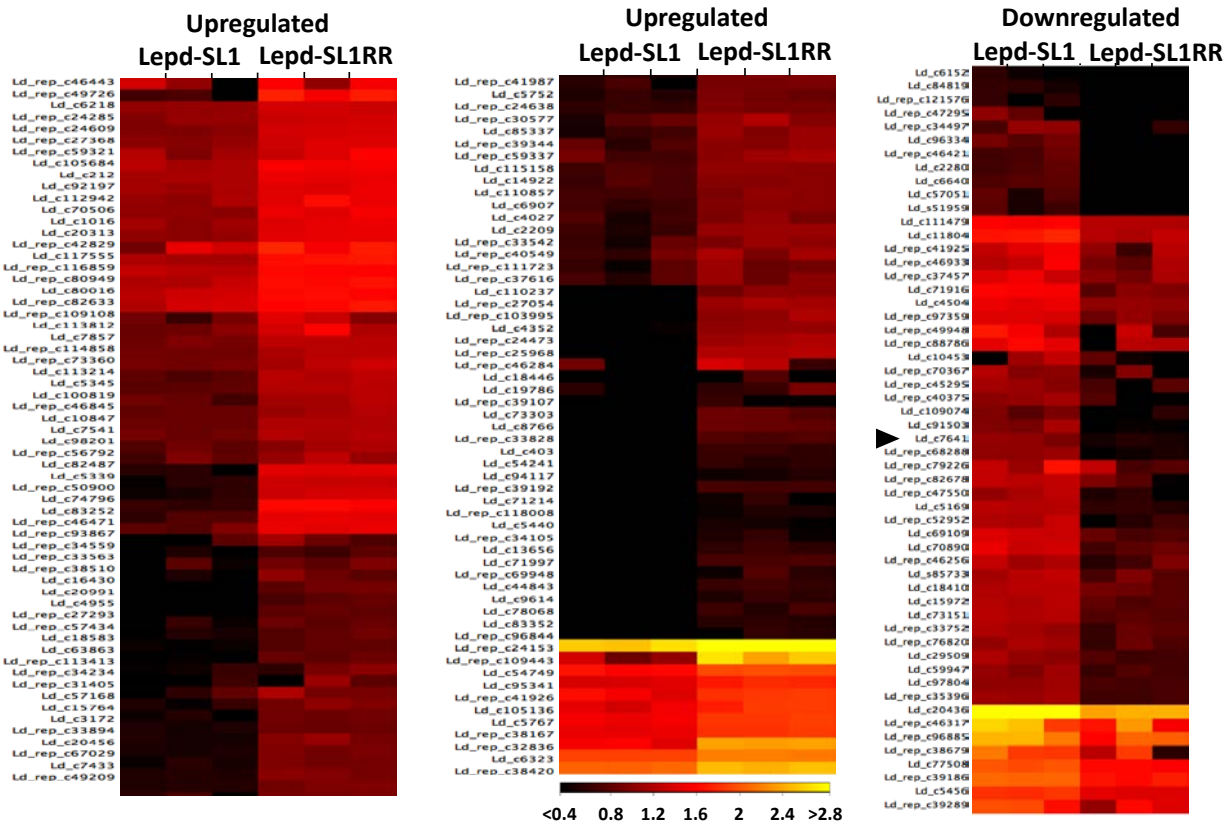


Fig. S8. Differential expression of upregulated and downregulated genes in Lepd-SL1 and Lepd-SL1RR cells shown as the heatmaps. The heatmap was generated by the CLC Genomics software version 11.0 (Qiagen Bioinformatics, USA) for genes that showed differential expression of >2 fold with a *P*-value of 0.05 between Lepd-SL1 and Lepd-SL1RR. The color key represents Log_2 transformed fold differences between Lepd-SL1 and Lepd-SL1RR. The arrow points to the *StauC* gene, Ld_c7641. The expression levels of upregulated genes (three replicates) are shown in the left two panels, and the expression levels of downregulated genes are shown in the right panel.

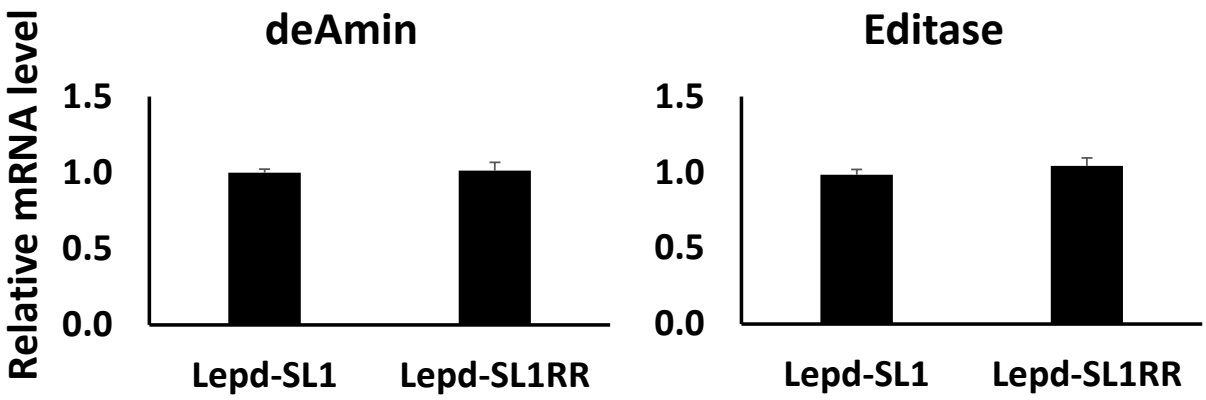


Fig. S9. Relative mRNA levels of Editase-related genes in Lepd-SL1 and Lepd-SL1 RR cells by qRT-PCR. Total RNA was isolated from the cells, and the mRNA levels were quantified using qRT-PCR.

Table S1. Summary statistics for RNA sequence obtained from Lepd-SL1 and Lepd-SL1RR

Samples	Susceptable1	Susceptable2	Susceptable3	Resistant1	Resistant2	Resistant3
Total Reads Mapped	25,361,116	29,886,461	27,119,255	26,209,881	27,119,255	25,919,852
Reads mapped	25,361,116	29,886,461	27,119,255	26,209,881	27,119,255	25,919,852
Percentage mapped	85.88	85.86	85.39	85.18	85.39	85.08

Table S2. The list of differentially expressed genes identified by RNA seq DEG analysis and verified by qRT-PCR and shown in Figs. 5B.

Feature ID	Description	Abbreviation
Ld_c2280	Unidentified	UI
Ld_c5169	PREDICTED: uncharacterized protein LOC108909080 isoform X1	LOC108909080 X1
Ld_c5456	Acetylcholine receptor subunit alpha-type acr-16	AchR alpha-acr-16
Ld_c6640	neurofilament heavy polypeptide	NHP
Ld_c46421	kynurenine alpha-aminoadipate mitochondrial-like	KAAM-like
Ld_c7641	double-stranded RNA-binding Staufen homolog 2 isoform X5	StaufenC
Ld_c4504	probable multidrug resistance-associated lethal(2)03659	MDRSL03659
Ld_rep_c33188	oligosaccharyltransferase complex subunit ostc-A	OSTC-A
Ld_rep_c68288	Unidentified	UI
Ld_c403	tyrosine- phosphatase non-receptor	PTPN
Ld_c13656	fasciclin-1 isoform X3	Fasciclin-1 X3
Ld_c8766	UNC93 isoform X2	UNC93 X2

Table S3. List differentially expressed genes tested in RNAi assay and the results are shown in shown in Fig. 5C.

Cell Survival Rate	Gene ID	Description	Abbreviation
4%	Ld_c4504	probable multidrug resistance-associated lethal(2)03659	MDRSL03659
4%	Ld_c11804	neural ectodermal development factor IMP-L2 isoform X1	NEDF IMP-L2
15%	Ld_rep_c48574	malectin	Malectin
16%	Ld_rep_c29932	dolichyl-diphosphooligosaccharide-- glycosyltransferase subunit STT3B	STT3B
104%	Ld_c7641	double-stranded RNA-binding Staufen homolog 2 isoform X5	Staufen C
10%	Ld_c91503	Catenin alpha	Catenin alpha
8%	Ld_rep_c79226	myosin heavy non-muscle	MHNM
10%	Ld_rep_c63424	T-complex 1 subunit delta	T-complex 1 delta
34%	Ld_rep_c34637	V-type proton ATPase subunit B	V-T_ATPase B
39%	Ld_rep_c97272	V-type proton ATPase subunit d	V-T_ATPase d
3%	Ld_rep_c121576	general transcription factor II-I repeat domain-containing 2-like	GTF2IRD2-like
5%	Ld_c46421	kynurenine alpha-aminoadipate mitochondrial-like	Kaam-like
11%	Ld_rep_c34497	methylosome subunit pICln	Methylosome pICln
8%	Ld_c5456	Acetylcholine receptor subunit alpha-type acr-16	AchR alpha-acr-16
6%	Ld_c73151	sodium potassium-transporting ATPase subunit beta-2-like	Na, K ATPase B2-like
11%	Ld_rep_c88786	ADP,ATP carrier -like	ADP,ATP carrier-like
12%	Ld_c77508	nicotinic acetylcholine receptor alpha 10 subunit	nAChR 10
6%	Ld_rep_c41925	clathrin light chain isoform X3	CLC

Data S1. (Separate file)

The list of the genes downregulated in RNA-seq data based on the differential expression of gene expression analysis between Lepd-SL1 and Lepd-SL1RR. This list was generated by the CLC Genomics software version 11.0 (Qiagen Bioinformatics, USA), using a differential expression of gene expression module. >2-fold difference with a p-value of 0.05 were used to filter genes.

Data S2. (Separate file)

The list of the genes upregulated in RNA-seq data based on the differential expression of gene expression analysis between Lepd-SL1 and Lepd-SL1RR. This list was generated by the CLC Genomics software version 11.0 (Qiagen Bioinformatics, USA), using the differential expression of gene expression module. >2-fold difference and a *P*-value $\leq$0.05 were used to filter genes.

Data S3. (Separate file)

The list of primers used in this study. Some of the primers used have been reported previously (5).