RNA interference in the Colorado potato beetle, *Leptinotarsa decemlineata*: Identification of key contributors

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ABSTRACT

RNA interference (RNAi) is a useful reverse genetics tool for investigation of gene function as well as for practical applications in many fields including medicine and agriculture. RNAi works very well in coleopteran insects including the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*. We used a cell line (Lepd-SL1) developed from CPB to identify genes that play key roles in RNAi. We screened 50 genes with potential functions in RNAi by exposing Lepd-SL1 cells to dsRNA targeting one of the potential RNAi pathway genes followed by incubation with dsRNA targeting inhibitor of apoptosis (*IAP*, silencing of this gene induces apoptosis). Out of 50 genes tested, silencing of 29 genes showed an effect on RNAi. Silencing of five genes (*Argonaute-1*, *Argonaute-2a*, *Argonaute-2b*, *Aubergine* and *V-ATPase 16 kDa subunit 1*, *Vha16*) blocked RNAi suggesting that these genes are essential for functioning of RNAi in Lepd-SL1 cells. Interestingly, *Argonaute-1* and *Aubergine* are known to function in miRNA and piRNA pathway respectively are also critical to siRNA pathway. Using ³²P labeled dsRNA, we showed that these miRNA and piRNA Argonautes but not *Argonaute-2* is required for processing of dsRNA to siRNA. Transfection of pIZT/V5 constructs containing these five genes into Sf9 cells (the cells where RNAi does not work well) showed that expression of all genes tested, except the *Argonaute-2a*, improved RNAi in these cells. Results from *Vha16* gene silencing and bafilomycin-A1 treatment suggest that endosomal escape plays an important role in dsRNA-mediated RNAi in Lepd-SL1 cells.

Keywords: Argonaute, Aubergine, RNAi, dsRNA, Lepd-SL1

INTRODUCTION

RNA interference (RNAi) is a useful reverse genetics tool for investigating of gene function (Fire et al., 1998). Moreover, RNAi also has potential practical applications in many fields including medicine and agriculture (Andersen et al., 2009; Barnard et al., 2012; Gaur and Rossi, 2006; Kim and Rossi, 2008; Lares et al., 2010; Morris and Rossi, 2006; Palli, 2014; Reidhaar-Olson and Rondinone, 2009; Tripp and Tompkins, 2009). Due to its potential as a therapeutic agent and insecticide, many studies were conducted to elucidate RNAi mechanism in model organisms ranging from nematodes to insects (Buhler et al., 2007; Flamand and Duchaine, 2012; Grishok, 2005; Holen, 2005; Huvenne and Smagghe, 2010; Tijsterman and Plasterk, 2004).

The overall process of dsRNA-mediated silencing includes dsRNA uptake, intracellular dsRNA transport, dsRNA processing to siRNA, RISC complex formation, binding and digestion of target mRNA. To induce RNAi, dsRNA needs to enter the cell, in *Caenorhabditis elegans*, a dsRNA-selective transmembrane channel protein known as systemic RNA interference deficient-1 (sid1) mediates dsRNA entry (Shih and Hunter, 2011; Winston et al., 2002). However, whether or not sid1-like proteins are responsible for dsRNA transport into insect cells is not known yet. Clathrin-mediated and scavenger receptor-mediated endocytosis are shown to be involved in dsRNA uptake in insect cells (Huvenne and Smagghe, 2010; Saleh et al., 2006; Xiao et al., 2015). It is likely that insects utilize endocytosis-mediated pathways where endosomes carry dsRNA and release them by acidification into cytoplasm before they reach the lysosomes. Therefore, the importance of the endosomal acidification and trafficking has been investigated, and specific genes involved in these pathways have been identified, and their function has been verified by various pharmacological reagents that inhibit endosomal escape (Saleh et al., 2006;

Xiao et al., 2015). After released from the endosomes, the dsRNA are digested by RNase III-type Dicer2 enzymes producing short interfering RNAs (siRNAs) that are approximately 21~23 nucleotides in length (Moazed, 2009). Subsequently, siRNAs are loaded onto RNA-induced silencing complexes (siRISC) containing Argonaute-2, which initiates the gene silencing by endonucleolytic cleavage in a sequence-specific manner. It is thought that one of the reasons for efficient RNAi in coleopterans is the duplication of core RNAi pathway genes (e.g. Argonaute-2) in *Tribolium castaneum* (Guo et al., 2015; Tomoyasu et al., 2008). Recently, duplications of Argonaute-2 and Dicer-2 have been identified in CPB, but their function in RNAi was not elucidated (Guo et al., 2015).

Screening of 50 genes (selected based their similarity to the proteins with a probable function in RNAi pathway) in RNAi of RNAi assay in Lepd-SL1 cells identified 29 genes that blocked RNAi in these cells. Out of these 29 genes, knockdown of five genes (*Argonaute-1*, *Argonaute-2a*, *Argonaute-2b*, *Aubergine* and *vacuolar H⁺ ATPase 16 kDa subunit 1*) blocked RNAi significantly. Also, expression of these five genes in Sf9 cells improved RNAi in these cells. Using ³²P labeled dsRNA, we showed that Argonaute-1 and Aubergine that are known to be associated with miRNA and piRNA pathways are required for dsRNA to siRNA processing.

MATERIALS AND METHODS

Cell culture

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 in EX-CELL 420 (Sigma–Aldrich) supplemented with 10% FBS (Seradigm Fetal Bovine Sera)

and incubated at 27 °C. Sf9 cells are derived from IPLB-Sf21-AE, an established cell line originally isolated from *Spodoptera frugiperda* ovaries. This cell line was maintained in Sf-900™ II SFM and incubated at 27 °C.

Total RNA extraction, PCR and Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the CPB cells using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH) and treated with DNase I (Ambion Inc., Austin, TX). cDNAs were synthesized with M-MLV reverse transcriptase (Clontech Laboratories) followed by DNase I treatment. PCR amplifications were conducted in 20 µl reactions with 5 µM primers each, 10 µl of 2x Taq premix (Promega) and 20 ng of template was used for PCR. PCR conditions were 94 °C for 30 s, followed by 35 cycles of 94 °C for 30s, 55 °C for 30 s and 68 °C for 1 min, finishing with an extension step at 68 °C for 10 min. PCR products were purified using the QIAquick PCR purification kit (QIAGEN) and gel extraction kit (QIAGEN). The purified PCR product was used as a template to synthesize dsRNA.

qRT-PCR was performed in Applied Biosystems StepOnePlus™ Real-Time PCR System (Life technologies™, Carlsbad, CA). Each qRT-PCR reaction (10µl final volume) contained 5 µl FastStart SYBR Green Master (Roche Diagnostics, Indianapolis, IN), 1.0µl of cDNA, 3.6 µl ddH₂O, and 0.6µl each of forward and reverse gene specific primers. An initial incubation of 95°C for 3min, followed by 40 cycles of 95°C for 10s, 55°C for 60s settings were used. A fluorescence reading determined the extension of amplification at the end of each cycle. Quantitative mRNA measurements were performed in triplicate and normalized to an internal control of ribosomal protein 4 (RP49) mRNA for Lepd-SL1 cells and RP10 for Sf9. For the induction assay, both cell lines were seeded 4 x 10⁵ cells /well in the 12 well plate. Next day, 1

µg of dsGFP was soaked, and cells were collected at 0, 30 min and 60 min time points. The cells were collected with 1ml TRI reagent and performed qRT-PCR.

dsRNA synthesis and labeling

DsRNA synthesis and labeling were performed as described previously (Shukla et al., 2016). Briefly, primers containing T7 polymerase promoter (TAATACGACTCACTATAGGG) at the 5' end of both the forward primer and the reverse primer were used to amplify the 350-500bp amplicons (Table 1S). The PCR product was used as a template for dsRNA synthesis using the Ambion MEGAscript transcription kit (Ambion, Austin, TX). Double-stranded RNA was purified using a Phenol/chloroform extraction followed by ethanol precipitation and dissolved in nuclease-free water. The quality of dsRNA was checked by running on an agarose gel, and the concentration was measured using NanoDrop2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The ³²P UTP-labelled dsGFP were prepared by using the Ambion MEGAscript transcription kit according to manufacturer instructions. 2 µl each of 10X buffer, ATP, GTP, CTP, 0.1 µl of UTP, 8.4 µl of ³²P UTP, 1.5 µl template (250 ng/µl) and 2 µl of T7 enzyme were mixed and incubated the reaction at 37 °C for 16 hrs. The input template DNA was digested with Turbo DNase (Ambion, USA) at 37 °C for one hour and ³²P UTP-labelled dsRNA was purified using PCR purification kit (Qiagen, USA) and eluted using nuclease-free water and stored at -20 °C for further use. The incorporated radioactivity in dsGFP (1 µl) was measured using scintillation counter.

RNAi of RNAi Assay

CPB cells were seeded with 5 x 10⁴ cells / well in 96-well plate. On the following day 500 ng of dsRNA targeting one of the target genes was added to the medium. At 24 hr after

addition of first dsRNA, 500 ng of a second dsRNA targeting inhibitor of apoptosis (IAP) gene was added to the medium. dsRNA targeting GFP gene was used as a control. At 24 hr after addition of second dsRNA the cells were observed under a microscope. The medium was replaced and the number of living cells in each treatment was determined using CCK, a Cell Counting Kit-8 (Dojindo, Japan). 10 µl of CCK-8 solution was added to each well and cells were incubated with CCK for 2~3 hrs. The absorbance was measured at 450 nm using a microplate reader. Cell survival rate was calculated using the equation: $[(\text{Gene O.D.} - \text{IAP O.D.}) / (\text{GFP O.D.} - \text{IAP O.D.})] \times 100 (\%)$ (Fig. 2).

Functional verification of Ago1, Ago2a, Ago2b, Aub and Vha16 proteins in Sf9 cells

Coding sequences of CPB Ago1, Ago2a, Ago2b, Aub, and Vha16 were cloned into the expression vector, pIZT/V5-His. Sf9 cells were cultured in 250 µl of Sf-900 II SFM at a concentration of 1.0×10^5 cells/well of 48-well plate (Costar, Corning, USA) and incubated at 28 °C overnight. 0.25 µl of Cellfectin II® (Gibco Invitrogen, USA) was mixed with 0.5 µg of total DNA (0.25 µg luciferase construct + 0.25 µg expression construct) for transfection of DNA into Sf9 cells. DsLuc (1 µg) was added to the medium at four hours post-transfection. Cells were washed with 1X PBS at 48 hr post-transfection and 100 µl of lysis buffer was added to each well, and the plate was placed on a shaker for 30 minutes to facilitate the cell lysis. 20 µl of cell lysate was used for measuring luciferase activity, and 20 µl of lysate was used to determine the protein concentration using Bradford's assay. The luciferase activity was measured using Fluoroskan Ascent FI machine (Thermo LabSystems). Three independent transfections of three replicates for each treatment were performed.

Role of Group A proteins in processing of dsRNA in Lepd-SL1 cells

Lepd-SL1 cells were used to determine the role of Group A proteins in processing of dsRNA. One day prior to the experiment, 1×10^6 cells/well were seeded in a six-well plate and 1 μ g of dsRNA was added to the medium for knocking down Group A genes (Shukla et al., 2016). At two days after addition of dsRNA, the medium was replaced with fresh medium containing 1.6 million counts of 32 P UTP-labeled dsGFP. At 16 hr after addition of labeled dsGFP, the cells were harvested, washed with 1xPBS buffer for five times and total RNA was isolated using TRI Reagent® RT (Molecular Research Center, Inc., USA). Total RNA (2000 CPM) samples were run on 20% polyacrylamide 8M urea gels using 1xTBE buffer. Gels were washed, fixed (10% methanol and ethanol) and dried in a gel drier. Dried gels were exposed overnight on a phosphorImager screen, and the screen was scanned in a phosphorImager (Typhoon 9500, GE Healthcare Life Sciences, USA).

Expression of Ago1, Aub and Vha16

To prepare a standard curve, Standard DNA dilutions were generated by 10-fold serial dilutions of the PCR amplified products. Each dilution was performed in duplicate. A standard curve was generated by plotting Ct values against relative log copy number. The copy number of the template was calculated using the formula:

$$\text{Copy number} = [\text{amount (ng)} * 6.022 \times 10^{23}] / [\text{length (bp)} * 1 \times 10^9 * 650]$$

The coefficient of determination (R²) and the equation of regression curve (y) were calculated.

No template controls accompanied each set of reactions to monitor for the presence of contamination

Inhibitor Studies

Bafilomycin-A1 (Baf-A1) was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). After pre-incubation of 10 nM Baf-A1 for one hour, 1 µg of dsIAP was added to the medium. Two types of controls were performed: dsIAP treatment without inhibitor treatment and pretreatment of inhibitor without dsIAP treatment. Apoptosis level was checked under a microscope.

Statistical analysis

For testing the cell survival rate and knockdown efficiency, Student's t-test was used to compare the cell survival rate and gene expression difference between control and treatment groups. A p-value of 0.05 or less between groups was considered as a significantly different.

RESULTS

Identification of genes coding for proteins required for RNAi

RNAi works very well and systemic in the Colorado Potato beetle (Zhu et al., 2011). To study RNAi mechanism in this insect, we identified 50 CPB genes coding for proteins with potential functions in RNAi pathway using the sequences of RNAi genes previously identified in other insects including *Drosophila melanogaster* (Saleh et al., 2009; Saleh et al., 2006), *T. castaneum* (Tomoyasu et al., 2008) and *L. decemlineata* (Swevers et al., 2013) as well as the nematode, *C. elegans*. Out of 50 genes used in this study, 28 of them were previously identified in a transcriptome sequencing study (Swevers et al., 2013) we identified additional 22 genes by searching CPB genome (<https://i5k.nal.usda.gov/webapp/blast/>). Inhibitor of apoptosis (IAP) protein protects Lepd-SL1 cells from apoptosis, knockdown of the gene coding for this protein causes apoptosis resulting in a reduction in the survival of these cells. Cell Counting Kit-8 (Dojindo), CCK-8 which determines the number of living cells was used to quantify the effect of

IAP knockdown. We hypothesized that silencing of an essential RNAi gene in these cells would block IAP knockdown and hence cause apoptosis in cells exposed to dsIAP. Preliminary results verified our hypothesis and led to the development of RNAi of RNAi assay outlined in Figure 1. In this assay, the cells are exposed to dsRNA targeting one of the genes predicted to play a role in RNAi (we will call these genes as RNAi genes from here on) for 24 hr followed by exposure of these cells to dsIAP for an additional 24 hr. Then the phenotypes and cell survival are determined. The double-stranded green fluorescent protein (dsGFP) is used as a control. The cell survival and statistical significance were calculated comparing treatments and controls. The absorbance readings in cells exposed to dsGFP followed by dsIAP and the absorbance readings of cells exposed to dsGFP followed by dsGFP were used as a positive and a negative control, respectively. Based on the phenotypes and the statistical significance, the 50 genes tested were divided into three groups (Fig.2, Table 1). Group A cell survival levels are not significantly different from the cell survival levels in dsGFP-dsGFP negative control (Fig.2). Group B cell survival levels are significantly different from cell survival levels in positive (dsGFP followed by dsIAP) and negative (dsGFP followed by dsGFP) controls (Fig.2). Group C cell survival levels are not significantly different from cell survival levels in positive control. In other words, the knockdown of a gene in Group A blocks RNAi response and hence no knockdown of IAP in cells exposed to dsIAP therefore these cells did not undergo apoptosis and survived well similar to the negative control cells. Knockdown of a gene in B group resulted in a partial block in RNAi and partial knockdown of IAP in cells exposed to dsIAP and hence intermediate levels of cell survival when compared to negative and positive controls.

Group A

Knockdown of *Ago1*, *Ago2a*, *Ago2b*, *Aub* and *Vha16* resulted in an outstanding block in RNAi and protection of cells exposed to dsIAP from apoptosis. Among these five genes, knockdown of *Ago2b* caused a complete block of RNAi, none of the cells exposed to dsIAP died and all cells survived (Fig. 3A). Knockdown of *Ago2a* also showed a significant block in RNAi but not as strong as the response observed after *Ago2b* knockdown. *Ago1* and *Aub* are known to be involved in miRNA and piRNA pathway, respectively. Interestingly, knockdown of these two genes also blocked RNAi in Lepd-SL1 cells. Also, silencing of the gene coding for *Vha16*, vacuolar H⁺ ATPase 16kD subunit 1 known to be involved acidification of endosomes also blocked RNAi and apoptosis in these cells (Fig. 3A).

Group B

Two *dicer-2* genes are present in the CPB genome; these two genes contain distinct sequences, but both genes contain typical core Dicer domains. Knockdown of both of these genes partially blocked RNAi, and the cell survival is significantly different from both positive and negative controls. Interestingly, knockdown of *Dicer-1*, *Loquacious*, *Drosha*, and *Pasha* which are known to function in miRNA pathway also partially blocked RNAi in these cells. Knocking down the genes coding for proteins involved in endocytosis including HPS4, FBX011, and SC-R2, Epn-1, Chc and Rsd-3 also partially blocked RNAi in these cells. Two *sid1*-like genes were found in the genome and exposure of Lepd-SL1 cells to dsRNA targeting these genes partially blocked RNAi. Endosomal trafficking genes, *Rab7* and *Arf72*, are known to be involved in endosomal trafficking from endosomes to lysosomes. These genes contained dsRNA binding motifs and knocking down these genes in Lepd-SL1 caused a partial block in RNAi. Knockdown of genes coding for Armitage and Neuron-Specific Staufien and some of the clathrin-mediated receptors including sortlin-like receptor and transferrin receptor 3 and innexin2, a well-known gap junction

gene, also partially blocked RNAi. Cells treated with dsFBX, dsDrosha or dsR2D2 followed by dsIAP showed intermediate levels of apoptosis (Fig.3B, Table 1).

Group C

Unlike the other core RNAi factors that are associated with Group A and B, depletion of Ago3 has no influence on RNAi. Also knockdown of genes coding for other piRNA factors, such as Maelstrom, PRMT5 and Armitage-like showed no effect on RNAi. Knockdown of a different subunit VhaSFD Vacuolar H [+-]ATPase SFD subunit, many genes coding for RNA binding proteins and RISC proteins including Belle, Transline, TudorSN, Maelstrom, 68helicase, Armitage-like, Gawky, Clp-1, HEN1, and Staufien showed no effect on RNAi. Incubation of these cells with dsRNA targeting Nibbler, SDN-1, and SDN-3 caused no effect on RNAi response (Table 1). Cells treated with dsAP50 or VhsSFD followed by dsIAP that showed levels of apoptosis similar to the cells treated with dsIAP are shown in Figure 3C.

Verification of knockdown of selected genes

Since 21 of the 50 genes tested showed no effect on RNAi response in these cells. To check if these genes are knockdown well in these cells, the knockdown efficiency of each gene was determined. All genes showed a significant reduction in mRNA levels in dsRNA exposed cells when compared to their levels in the dsGFP exposed cells (Fig.1S) suggesting that there is efficient knockdown of these genes in cells exposed to dsRNA.

Verification of function of Group A proteins in Sf9 cells

RNAi does not work very well in Sf9 cells, a clonal isolate of *Spodoptera frugiperda* Sf21 cells (Shukla et al. 2016). To determine if the expression of genes identified as essential for RNAi Lepd-SL1 cells could improve RNAi response in Sf9 cells, coding regions of Group A genes

were cloned into pIZT/V5-His vector. These constructs were co-transfected with pIZT/V5_Luciferase construct into Sf9 cells. The transfected cells were exposed to 1 µg of dsLuc targeting the luciferase gene (Fig. 4). At 48 hr after transfection, the luciferase activity was measured, and the protein concentrations were determined. Expression of genes coding for CPB Ago1, Ago2b, Aub, and Vha16 reduced the expression of the luciferase gene by 30-65% in Sf9 cells exposed to dsLuc when compared to the control cells transfected with pIZT/V5 vector alone (Empty vector). These data suggest that *Ago1*, *Ago2b*, *Aub* and *Vha16* expression improved RNAi in Sf9 cells.

Confirmation of function of Group A proteins in dsRNA processing using ³²P labeled GFP dsRNA

To verify whether any of the five Group A proteins are involved in dsRNA processing, the Lepd-SL1 cells were exposed to dsAgo1, dsAgo2a, dsAgo2b, dsAub or dsVha16. At 48 hr after exposure to dsRNA, the cells were exposed to ³²P labeled dsGFP for 24 hr. The total RNA was extracted and loaded into a Urea-polyacrylamide gel. As shown in Figure 5, expression of Ago1 and Aub and Vha16 caused a significant decrease in the levels of siRNA produced when compared to that in control cells exposed to dsGFP. In contrast, expression of Ago2a and Ago2b did not affect processing to dsRNA to siRNA. These data suggest that not only Ago2a and Ago2b but also Ago1, Aub, and Vha16 participate in dsRNA to siRNA processing in Lepd-SL1 cells.

Comparison of expression and dsRNA induction of Group A genes between Lepd-SL1 and Sf9 cells

We investigated differences in expression levels of Group A genes between Lepd-SL1 and Sf9 cells. Two *Ago2* genes (*Ago2a* and *Ago2b*) have been identified in the Lepd-SL1 cells. Whereas only one *Ago2* gene has been identified in Sf9 cells. Because of the discrepancy in gene number, comparison of expression levels of *Ago2* is not straightforward, therefore; we focused on comparing the expression levels of the rest of the three Group A genes *Ago1*, *Aub*, and *Vha16*. As shown in Figure 6A, higher levels of mRNA of all three genes were detected in Lepd-SL1 cells when compared to their expression in Sf9 cells. Then we compared dsRNA induction response of Group A genes in Lepd-SL1 and Sf9 cells. *Aubergine* was induced in 30 min after exposure of Lepd-SL1 to dsRNA (Fig.6B). The *Vha16* was induced in 30 min after exposure of Lepd-SL1 to dsRNA (Fig.6B). In contrast, none of the five genes tested were induced in Sf9 cells exposed to dsRNA targeting these genes (Fig. 6B). These data suggest that higher expression levels of RNAi genes and their induction by dsRNA may contribute to RNAi efficacy on Lepd-SL1 cells.

V-ATPase inhibitor bafilomycin-A1 blocks RNAi in Lepd-SL1 cells

RNAi experiments showed that *Vha16* is required for RNAi. *Vha16* is a multi-subunit transmembrane complex and shown to regulate the activity of V-ATPase proton pump acidification of endosomes. To determine whether or not block in RNAi in *Vha16* knockdown cells is due to a block in acidification of endosomes preventing the escape of dsRNA-treated Lepd-SL1 cells with V-ATPase inhibitor, bafilomycin-A1 and checked the dsIAP effect on these cells. As shown in Figure 7A, treatment of these cells with bafilomycin-A1 blocked RNAi and no apoptosis was detected after exposure to dsIAP. Also, treatment of Lepd-SL1 cells with bafilomycin-A1 caused a significant reduction in the processing of labeled dsRNA to siRNA

(Fig. 7B). These data suggest that endosomal acidification which leads to dsRNA released from endosomes is a major step in successful RNAi in Lepd-SL1 cells.

DISCUSSION

One of the main findings of this paper is the discovery that not only Argonaute-2 but also Argonaute-1 and Aubergine which are normally associated with miRNA and piRNA pathways are required for RNAi function in Lepd-SL1 cells. We used the RNAi of RNAi approach to identify five genes including *Ago1*, *Ago2a*, *Ago2b*, *Aub* and *Vha16* that are required for the functioning of RNAi these cells. We also identified 24 additional genes that partially block RNAi when their expression levels are reduced by exposing cells to dsRNA targeting these genes.

Ago1 and *Aub* are identified as miRNA and piRNA pathway proteins, respectively. This study investigated the contribution of *Ago1* and *Aub* in dsRNA-mediated RNAi pathway using several different methods. Knockdown of *Ago1* and *Aub* blocked RNAi and prevented apoptosis in Lepd-SL1 cells exposed to dsIAP (Figs 2&3). Expression of *Ago1* and *Aub* genes in RNAi refractive Sf9 cells improved RNAi in these cells (Fig.4). Also, knockdown of *Ago1* or *Aub* gene in Lepd-SL1 cells caused a decrease in processing of dsRNA to siRNA (Fig. 5). Both these genes are expressed at higher levels in Lepd-SL1 cells where RNAi works well when compared to their expression in Sf9 cells where RNAi does not work well (Fig.6). Taken together, these data suggest that both *Ago1* and *Aub* play important roles in dsRNA-mediated RNAi in Lepd-SL1 cells. Previous studies in Sf21 cells (Ghosh et al., 2014) and in *T. castaneum* (Tomoyasu et al., 2008) suggested that not only core siRNA pathway genes but also miRNA and piRNA pathways are required for dsRNA-triggered RNAi. In *D. melanogaster*, DmAgo1 mutant is defective for RNAi during embryonic stage suggesting that *Ago1* is necessary for RNAi in this insect

(Williams and Rubin, 2002). A similar result has been shown with *Aub*, where a mutation to *Aub* inhibits RNAi activity in oocyte maturation (Kennerdell et al., 2002). In silkworm Bm5 cells, the requirement of Ago1, Ago2, and Ago3 for successful RNAi has been reported (Kolliopoulou and Swevers, 2013). Since Ago1 and Aub depletion reduces dsRNA to siRNA processing in Lepd-SL1 cells, it is possible that these proteins are required for RNAi steps before Dicer processing of dsRNA to siRNA. These steps could include intracellular transport of dsRNA and/or recruitment of dicer enzymes. Further studies are required to define the precise function of Ago1 and Aub in dsRNA pathway.

Besides Argonautes, Vha16 is identified as the protein required for RNAi in Lepd-SL1 cells. Vha16, a multi-subunit transmembrane complex, plays an important role in regulating the activity of V-ATPase proton pump acidification of endosomes (Nishi and Forgac, 2002). In our studies, knockdown of Vha16 blocked RNAi in Lepd-SL1 cells (Group A gene, Fig. 2 and Table 1). The role of Vha16 in RNAi was reported previously based on the studies in *Drosophila* S2 cells (Saleh et al., 2006) and CPB (Cappelle et al., 2016). In *T. castaneum* the involvement of another transmembrane protein, VhaSFD, in RNAi has been reported recently (Xiao et al., 2015). Interestingly, knockdown of CPB VhaSFD did not result in a significant decrease in RNAi response of Lepd-SL1 cells (Group C gene, Fig. 2 and Table 1). Studies on the conversion of dsRNA to siRNA in Vha16 knockdown Lepd-SL1 cells showed a decrease in dsRNA to siRNA in these cells suggesting that Vha16 plays an important role in intracellular transport of dsRNA before Dicer action. Moreover, treatment of Lepd-SL1 cells with bafilomycin-A1, a V-ATPase inhibitor, blocked RNAi as well as processing of dsRNA to siRNA (Fig.7). Similar results have been reported in *T. castaneum* where pretreatment of bafilomycin-A1 caused a significant decrease in the knockdown of TcLgl, lethal giant larvae (Xiao et al., 2015). We also identified

two other genes ADP-ribosylation factor-like 1 and Rab7 that are known to function in the endosomal pathway and showed that knockdown of these genes resulted in a partial block in RNAi in our assay. ADP-ribosylation factor-like 1 similar to Arf72 in *Drosophila*, belongs to the small GTPase superfamily and functions in vesicle budding and transport between specific organelles in the membrane-trafficking pathway (Lee et al., 2011). Rab7, a member of small GTPases that are involved in the maturation of endosomes and autophagosomes known to be a multifunctional regulator of autophagy and endocytosis (Hytinen et al., 2013). Taken together, these data suggest that Vha16 is required for endosomal acidification, and escape of dsRNA and these steps are critical for the intracellular transport of dsRNA to the cytoplasm where Dicers can initiate their action.

For dsRNA to be processed by Dicers and Argonautes, dsRNAs need to be translocated to the cytoplasm using one of the transport pathways including the transmembrane channel-mediated uptake and endocytosis-mediated uptake (Huvenne and Smagghe, 2010). Experiments using pharmacological inhibitors (Chlorpromazine) and an RNAi of RNAi showed that clathrin-mediated endocytosis plays a major role in cellular uptake of dsRNA in *T. castaneum* (Xiao et al., 2015). In our studies, depletion of key components of clathrin pathway such as Epn-1, Chc and Rsd-3, TRF3 and SLR-induced delay in dsIAP phenotype due to the reduction in internalization of dsRNA through clathrin-mediated endocytosis (Table. 1). Internalization of dsRNA into *Drosophila* S2 cells is mediated by scavenger receptors that belong to the family of proteins involved in endocytosis of certain polyanionic ligands and antiviral immunity induced systemic RNAi (Saleh et al., 2006; Ulvila et al., 2006). Scavenger receptors (SR-CI) and Eater play a role in the uptake of dsRNA in *Drosophila* cells; a combination of SR-CI and Eater RNAi caused 90% decrease in the internalization of dsRNA when compared to that in control cells

(Saleh et al., 2006). In the CPB genome database, we identified one scavenger receptor, which is similar to *Drosophila* Scavenger receptor class C, type II (CG8856). Knockdown of CPB Sc-R2 partially blocked RNAi in Lepd-SL1 cells (Group B gene, Fig. 2 and Table 1).

In *C. elegans* systemic RNAi defective (*sid*) proteins mediate dsRNA uptake (Shih and Hunter, 2011; Winston et al., 2002). A number of *sid1*-like genes have been identified in insects including *T. castaneum*. However, their function in uptake of dsRNA has not been shown yet. The number of *sid1*-like genes identified in insect genomes is also variable. For example, no *sid1*-like gene was found in *Drosophila* genome, but three *sid1*-like genes were identified in *T. castaneum* (Tomoyasu et al., 2008). In this study, we found two *sid1*-like genes in *L. decemlineata*: *SIL-A* and *SIL-C* that are homologous to *T. castaneum* *sid1*-like protein A and C. Knockdown of CPB *SIL-A* or *SIL-C* resulted in a partial block in RNAi Lepd-SL1 cells (Group b gene, Fig. 2 and Table 1). A recent study in CPB also showed the requirement of CPB *SIL-A* and *SIL-C* for successful RNAi in this insect (Cappelle et al., 2016). It is still not clear on how insect *sid1*-like proteins function in RNAi. Additional studies on localization and function of these proteins are necessary to determine whether insect *sid1*-like protein has a conserved function as *C.elegans* *sid1* protein.

Duplication of *Dicer-2* is also an unusual characteristic in CPB; both *Dicer-2a* and *Dicer-2b* knockdown partially blocked RNAi in Lepd-SL1 cells. This is the first species to be documented with a duplication of *Dicer-2* genes, which could explain why CPB is more sensitive to RNAi compared to other species. A recent paper on CPB reported duplication of *Dicer-2* in CPB, but the function of these identified duplicated genes was not studied (Guo et al., 2015). Interestingly, knockdown in *Dicer-1* also caused a partial block in RNAi in Lepd-SL1 cells. In *Drosophila* *Dicer 1* and *Dicer2* play distinct roles in siRNA/miRNA silencing pathways,

Dicer 1 is also a component of siRNA-dependent RISC activation complex and is required for assembly of this complex that includes Dicer-2 (Lee et al., 2004). Involvement of miRNA and piRNA pathway genes in dsRNA-mediated RNAi pathway interesting but needs further investigation to define the precise functions of these proteins in dsRNA-mediated RNAi pathway.

Since soaking of dsRNA induces a RNAi response in Lepd-SL1 cells, we were able to develop high throughput “RNAi of RNAi” assay using these cells. CPB cell line (Lepd-SL1) is an outstanding model to investigate players in RNAi pathway. We were able to screen 50 candidates genes identify 29 key players in RNAi using this system. Identification of a significant role for Ago1, Aub and Dicer 1, the proteins are known to function in miRNA and piRNA pathways, in siRNA pathway as well as an important role for Vhs16-mediated endosomal acidification and escape in successful RNAi are highlights of this study (Fig.8). Work in progress in our laboratory and other will begin to identify precise functions of these proteins in siRNA-mediated RNAi.

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FIGURE LEGENDS

Figure 1. RNAi of RNAi Assay. Outline of RNAi of RNAi assay developed and used to screen 50 genes with a potential function in RNAi. We used a cell line (Lepd-SL1) developed from CPB to identify genes that play key roles in RNAi. Lepd-SL1 cells were exposed to dsRNA targeting one of the potential RNAi pathway genes followed by incubation with dsRNA targeting inhibitor of apoptosis (IAP, silencing of this gene induces apoptosis). Phenotypes were observed under an inverted microscope. Cell Counting Kit-8 (CCK-8 from Dojindo) was used to estimate the cell viability and survival rate was calculated. Controls included exposing cells to dsGFP followed by dsIAP (most cells showed apoptosis phenotype) and dsGFP followed by dsGFP (no apoptosis was detected).

Figure 2. Summary of results from screening 50 genes. (A) The formula used to calculate cell survival rate. (B) Summary of results, the student's t-test was performed to compare the significant differences in the absorbance of the samples against the positive and negative controls. Based on the statistical analyses, the 50 genes tested were classified into three groups. Group A (survival rate is significantly different from that of cells exposed to dsGFP followed by dsIAP but not that of cells exposed to dsGFP followed by dsGFP), Group B (significantly different from dsGFP and dsIAP exposed cell survival rate as well as dsGFP followed by dsGFP exposed cell survival rate) and Group C (not significantly different from dsGFP followed by dsIAP control cell survival rate).

Figure 3. Phenotypes observed in cells treated with dsRNA.

- A. Lepd-SL1 cells were exposed to dsGFP for 24 hr; a fresh aliquot of dsGFP was added and incubated for an additional 24 hr. Lepd-SL1 were exposed to dsAgo1, dsAub, dsAgo2a, dsAgo2b or dsVha16 for 24 hr followed by treatment with dsIAP for an additional 24 hr. Cells were photographed at 24 hr after second dsRNA treatment. Scale Bar:50um.
- B. Lepd-SL1 cells were treated with dsFBX, dsDrosha or dsR2D2 followed by dsIAP.
- C. Lepd-SL1 cells were treated with dsIAP, dsAP50 or dsVhaSFD followed by dsIAP.

Figure 4. Effect of expression of Group A genes on the RNAi in Sf9 cells.

- A. Schematic drawing of the procedure for transient transfection of pIZT/V5 constructs expressing CPB genes coding for Argonautes and Vha16 into Sf9 cells. 10,000 Sf9 cells per well were seeded in 48-well plates. Next day pIZT/V5 CPB gene constructs along with pIZT/V5 Luciferase construct were co-transfected into cells using Cellfectin. Four hours post-transfection, 1 µg of dsLuc was added to the medium. At 48 hr after exposure to dsLuc, the luciferase activity and protein concentration were determined. The protein content of the cells was used to normalize the luciferase activity.
- B. Expression of genes coding for CPB Argonaute 1 (Ago1), Argonaute 2B (Ago2b), Aubergine (Aub), and Vha16 reduced expression of the luciferase gene by 30-65% in Sf9 cells exposed to dsLuc when compared to the control cells transfected with pIZT/V5 vector (Vector). The percent expression of the luciferase gene in each treatment was calculated by comparing its expression in control cells transfected with pIZT/V5 Luc and empty vector. The bars show Mean+S.D (n=3). *=significantly different at a p-value ≤ 0.05 .

Figure 5. Influence of Group A genes on the processing of dsRNA in CPB cells. To identify stages of RNAi pathway where Group A genes are required, Lepd-SL1 cells were exposed to

dsRNA targeting one of the Group A genes for 48 hr. Then the cells were exposed to ^{32}P labeled GFP dsRNA for 16 hr. Total RNA was isolated and resolved on 20% polyacrylamide 8M urea gels using 1xTBE buffer. Gels were washed, fixed and dried. Dried gels were exposed overnight on a phosphorImager screen, and the screen was scanned in a phosphorImager. The numbers at the bottom of the gel show the percent conversion of dsRNA to siRNA when compared to conversion in the control cells set as 100.

Figure 6. Comparison of expression and dsRNA induction of Group A genes between Lepd-SL1 and Sf9 cells.

- A.** Comparison of mRNA levels Ago1, Aub and Vha16 genes between Lepd-SL1 and Sf9 cells. Total RNA isolated from Lepd-SL1 and Sf9 cells were used in qRT-PCR to determine Ago1, Aub and Vha16 mRNA levels. The bars show Mean+S.D (n=3). *=significantly different at a p-value ≤ 0.05 .
- B.** mRNA levels of Ago1, Ago2a, Ago2b, Aub and Vha16 in Lepd-SL1 and Sf9 cells exposed to respective dsRNAs. Lepd-SL1 and Sf9 cells were exposed to respective dsRNAs for 30 or 60 min. Total RNA was isolated and used in qRT-PCR to determine relative mRNA levels. Ribosomal protein 4 (RP49) for Lepd-SL1 cells and RP10 for Sf9 were used as internal controls. The bars show Mean+S.D (n=3). *=significantly different at a p-value ≤ 0.05 .

Figure 7. Bafilomycin-A1 (Baf-A1) blocks RNAi in Lepd-SL1 cells.

- A.** 10 nM Baf-A1 in DMSO or DMSO were added to Lepd-SL1 cells. At 1 hr after addition of the inhibitor, 1 μg of dsIAP dsRNA was added to the cells. The cells were photographed at 24 hr after addition of dsRNA. Apoptosis level was checked under a microscope.
- B.** 10 nM Baf-A1 in DMSO or DMSO were added to Lepd-SL1 cells. At 1 hr after addition of the inhibitor, the cells were exposed to ^{32}P labeled GFP dsRNA for 16 hr. Total RNA was isolated

and resolved on 20% polyacrylamide 8M urea gels using 1xTBE buffer. Gels were washed, fixed and dried. Dried gels were exposed overnight on a phosphorImager screen, and the screen was scanned in a phosphorImager. The numbers at the bottom of the gel show the percent conversion of dsRNA to siRNA.

Figure 8. Schematic drawing on the overall processes of dsRNA-mediated silencing in

Lepd-SL1: DsRNA uptake, intracellular transport, and processing. Receptor mediated and clathrin-mediated endocytosis are the major dsRNA uptake pathways. Intracellular transport likely includes endosomes. The results from the function of Vha16 gene and the inhibitor experiments suggest that acidification of endosomes is an important for dsRNA release into the cytoplasm. Duplication of core RNAi may help Lepd-SL1 cells to process dsRNA efficiently.

Table 1. 50 genes tested in RNAi of RNAi assay classified into three groups

Gene Group		Gene	Survival Rate (%)	Full Name	Effective Group
Core RNAi machinery	siRNA	Ago2b	99	Argonaute2b	A
		Ago2a	81	Argonaute2a	A
		Dicer2a	70	Dicer2a	B
		R2D2	35	R2D2	B
		Dicer2b	19	Dicer2b	B
	miRNA	Ago1	83	Argonaute1	A
		Pasha	38	partner of Drosha, DGCR8	B
		Loqs	35	loquacious	B
		Dicer1	33	Dicer1	B
		Drosha	24	Drosha Ribonuclease Type III	B
	piRNA	Aub/Piwi	72	Aubergine/PIWI	A
		Ago3	5	Argonaute3	C
dsRNA uptake	Clathrin Mediated Transport	Epn-1	28	polyubiquitin-selective clathrin-associated sorting protein	B
		Chc	26	Clathrin Heavy chain	B
		Rsd-3	16	Tc_epsin1-like protein	B
		AP50	9	Adaptor Protein 50	C
	Receptor Mediated Transport	TRF3	56	Transferrin Receptor Like protein 3	B
		SC-R2	40	Scavenger Receptor II	B
		SLR	33	Sortilin Like Receptor	B
		Innexin2	18	Gap Junction protein	B
		LDL	8	low density lipoprotein Receptor	C
		TRF2	0	Transferrin Receptor Like protein 2	C
	<i>C.elegans</i>	Sil-C	40	<i>C. elegans</i> Sid1 Like Protein C	B
		Sil-A	22	<i>C. elegans</i> Sid1 Like Protein A	B
Intracellular Transport	Proton Transport	Vha16	79	Vacuolar H ⁺ ATPase 16kD subunit 1	A
		VhaSFD	11	Vacuolar H ⁺ -ATPase SFD subunit	C
	Endosome	Rab7	30	Small Rab GTPases	B
		Arf72	17	ADP-ribosylation factor 72	B
	Lysosome	Light	10	Light (Lysosomal Transport)	C
	Exosome	Idlcp	0	Idlcp (Exocytosis)	C
Lipid Metabolism		SapoR	47	Saposin Receptor	B
Nucleases		SDN-3	0	Small RNA degrading nuclease 3	C
		SDN-1	0	Small RNA degrading nuclease 1	C
Antiviral RNAi		CG4562	45	Peptidase S10	B
		Ars2	0	Arsenate Resistance Protein2	C
		egghead	0	beta 1,4-mannosyltransferase	C
RISC factors		FBX011	61	Ubiquitin-protein transferase	B
		HPS4	46	Hermansky-Pudlak Syndrome 4 protein	B
		Armitage	29	RNA helicase Armitage	B
		NSS	21	Neuron-Specific Staufen	B
		Belle	16	Drosophila DEAD-box protein	C
		68helicase	14	68helicase	C
		Gawky	9	Gawky, GW180	C
		Maelstrom	7	a Drosophila spindle-class gene	C
		Transline	4	RNA-binding proteins	C
		PRMT	0	protein arginine methyltransferase 1	C
		TudorN	0	Human Tudor staphylococcal nuclease	C
		Clp-1	0	Clp1 cleavage complex I	C
		Armi-like	0	Armitage-Like Protein	C
Others		RdRp	0	RNA-dependent RNA polymerase	C

RNAi of RNAi Assay

(A)
$$\frac{(\text{Gene O.D.} - \text{dsIAP O.D.})}{(\text{dsGFP O.D.} - \text{dsIAP O.D.})} \times 100 (\%) = \text{Cell Survival Rate } (\%)$$

(A)

(B)

E.E. : Early Endosome
L.E. : Late Endosome
Baf A1: Bafilomycin A1