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Review

The mysteries of insect RNAi: A focus on dsRNA uptake and transport

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Abstract

RNA interference (RNAi) is becoming a practical tool to control insect pests. Many mysteries of how double-stranded RNA (dsRNA) is transported into, within, and between cells to generate an efficient RNAi response in insects are still to be unraveled. This review provides an overview of the evidence that supports a key role of endocytosis in the uptake of dsRNA on both cellular and tissue levels. Additionally, other components of cellular membrane transport and their impact on the efficiency of RNAi in insects are explored. It is now evident that the membrane transport and potentially dsRNA release from the endosome may comprise some of the limiting factors in insects that are recalcitrant to dsRNA. This review concludes with the apparent connection between gene products that are necessary for cellular trafficking of dsRNA and highly lethal RNAi targets.

Keywords: RNA interference, dsRNA uptake, Endocytosis, Membrane traffic, Systemic RNAi

For more than a decade, RNA interference (RNAi) has been used as a tool for understanding gene function and is being developed as a method to control insect pests. Despite its widespread use and in-depth studies of RNAi mechanisms, many questions remain regarding the nature of double-stranded RNA (dsRNA) uptake, systemic spread, differences in insect sensitivity to dsRNA (Baum et al., 2007; Belles, 2010; Bolognesi et al., 2012; Khajuria et al., 2015; Ramaseshadri et al., 2013; Li et al., 2015a; Terenius et al., 2011; Garbutt et al., 2013; Christiaens et al., 2014), and more recently, the potential to evolve resistance to dsRNA (Khajuria et al., 2018). Among the insect orders for which RNAi responses have been evaluated using dsRNA, coleopterans seem to exhibit both oral (RNAi response that can be induced by the feeding of dsRNA) and systemic RNAi responses (defined here as the spread of the RNAi effect from cell to cell) (Baum et al., 2007; Bolognesi et al., 2012; Khajuria et al., 2015; Ramaseshadri et al., 2013; Li et al., 2018). The orally-delivered RNAi response is implicitly systemic; however, the systemic RNAi response may also be induced by injection. The systemic nature of the RNAi response in Coleoptera is similar to that of *Caenorhabditis elegans*, but unlike *Drosophila melanogaster* where RNAi is cell-autonomous (RNAi response is limited to the cell which dsRNA is expressed/introduced). The cell-autonomous response in *Drosophila* has enabled tissue-specific RNAi studies that have propelled this insect to become one of the most useful animal model systems. Whereas the oral and systemic nature of RNAi in the western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) and the Colorado Potato Beetle (CPB, *Leptinotarsa decemlineata* (Say)), has facilitated the development of control methods for these pests through transgenic expression of RNA hairpins in plants and dsRNA as a foliar-applied insecticide, respectively (Baum et al., 2007; Li et al., 2015a; Hu et al., 2016; San Miguel and Scott, 2016). The study of RNAi response in insects has been complicated by the nuclease activity in the digestive systems of Coleoptera (Spit et al., 2017), Lepidoptera (Arimatsu et al., 2007; Guan et al., 2018a), Orthoptera (Spit et al., 2017; Luo et al., 2013; Wynant et al., 2014a; Song et al., 2017) and Hemiptera (Christiaens et al., 2014; Lomate and Bonning, 2016; Allen and Walker 3rd, 2012) and in the hemolymph or intracellularly in Lepidoptera (Garbutt et al., 2013; Liu et al., 2012) and Hemiptera (Christiaens et al., 2014; Cao et al., 2018). These barriers and the potential solutions have been extensively reviewed (Terenius et al., 2011; Lim et al., 2016; Joga et al., 2016; Bally et al., 2018; Christiaens and Smagghe, 2014; Kolliopoulou and Swevers, 2014; Guan et al., 2018b).

The systemic nature of RNAi in insects is supported primarily by quantitative real-time PCR (qRT-PCR) studies where high levels (> 80%) of knock-down of ubiquitously- or widely-expressed transcripts were measured when sampling whole insects for qRT-PCR (Bolognesi et al., 2012; Vélez et al., 2016;

Knorr et al., 2018). In contrast, a non-systemic or localized midgut-specific response is expected to produce partial transcript knockdown for ubiquitously-expressed genes, when measured globally. Systemic RNAi in insects has also been evidenced by the knockdown of target transcripts in tissues that are distal from the site where dsRNA is introduced. In WCR, the spread of RNAi has been documented by RNA in situ hybridization studies, where transcript knockdown is evident in structures such as distal fat bodies, which are not in direct contact with the insect's midgut (Li et al., 2018; Hu et al., 2016). A parental effect in which transcript knockdown was observed in eggs of females injected or fed with dsRNA, within the orders Coleoptera, Blattodea, Orthoptera, and Hemiptera (Khajuria et al., 2015; Shukla et al., 2016; Schroder, 2003; He et al., 2006; Cerny et al., 2005; Pueyo et al., 2008; Mito et al., 2006; Liu and Kaufman, 2004; Bucher et al., 2002), provides further evidence of the spread of RNAi effect through insect tissues. These observations lead to questions of how the RNAi effect travels through insect tissues, what are the genes involved in the process, and what is the form of this signal (dsRNA or siRNA)? In *C. elegans* and plants, the systemic spread of RNAi effect is facilitated by intracellular amplification of siRNAs; this type of RNAi response is also known as transitive RNAi. In these organisms, secondary siRNAs are generated from the mRNA target templates by RNA-dependent RNA polymerase (RdRP). However, RdRP homologs are not present in insect genomes (Gordon and Waterhouse, 2007) and there is no evidence of secondary generated siRNAs (Li et al., 2018).

The cell autonomous RNAi response as a cellular mechanism is highly conserved among eukaryotes (Ghildiyal and Zamore, 2009), while the systemic aspect of it is not (Kobayashi et al., 2012) and it seems to differ between insect orders. Systemic RNAi may involve distinct mechanisms, including uptake of dsRNA by cells from the hemolymph or the midgut and the spread of the dsRNA or siRNA molecules from cell to cell within or between tissues (Whangbo and Hunter, 2008; Timmons et al., 2003). Whether present in the midgut lumen, free-circulating within hemolymph, or released from the cells within vesicles, the systemic nature of the RNAi response is likely to depend on the cells ability to uptake dsRNA or siRNA. The importance of cellular uptake of dsRNA for RNAi response in insects has been underscored by recent observations in WCR selected for resistance to dsRNA; ex vivo midguts from RNAi-sensitive WCR robustly uptake fluorescently-labeled dsRNA, while RNAi-resistant WCR do not (Khajuria et al., 2018).

The similarities in RNAi response between coleopteran insects such as WCR, CPB, or the red flour beetle (*Tribolium castaneum*) and *C. elegans* have spurred the search for insect systemic RNA interference-deficiency (SID) orthologs. The *C. elegans* proteins SID-1, SID-2, SID-3, and SID-5, were shown to be involved in dsRNA uptake (Rocheleau, 2012). More specifically, SID-1

is a channel protein responsible for binding long dsRNA for uptake by cells and is required for the systemic RNAi response in *C. elegans* (Winston et al., 2002; Feinberg and Hunter, 2003; Shih and Hunter, 2011; Li et al., 2015b). Heterologous expression of *C. elegans* SID-1 in *Drosophila* S2 and *Bombyx mori* BmN4 cells enables dsRNA uptake (Kobayashi et al., 2012; Feinberg and Hunter, 2003; Shih and Hunter, 2011), confirming the role of this gene in uptake. *Sid-1* was also expressed in transgenic *B. mori*, but no significant enhancement of the in vivo phenotype was observed in response to injection of dsRNA into the hemocoel (Kobayashi et al., 2012). SID-2 is a single-pass transmembrane protein that is expressed in *C. elegans* gut lumen and is believed to mediate the uptake of environmental dsRNA of at least 50 bp in length via endocytosis (Winston et al., 2007; McEwan et al., 2012). However, when SID-2 from *C. elegans* was expressed in *B. mori* BmN4 cells, it did not enable an RNAi effect (Kobayashi et al., 2012). SID-3 is required for efficient uptake of dsRNA into cells in *C. elegans*; this protein is a tyrosine kinase, a homolog of activated cdc-42-associated kinase (ACK), which is known to associate with endocytic vesicles (Jose et al., 2012). Whereas SID-5 is a late endosome-associated protein that is also predicted to be a single-pass transmembrane protein (Hinas et al., 2012) that is responsible for SID-1-independent transport (Jose et al., 2009) of ingested dsRNA. SID-5 is hypothesized to mediate the release of dsRNA from the endosomes and/or facilitate its packaging into exosomes for cell-to-cell export (Hinas et al., 2012). These studies point to a particular pathway for dsRNA uptake and systemic spread in *C. elegans*, yet they also implicate a more generalized eukaryotic pathway of endocytosis in mediating RNAi response.

An absence of clear SID-2 (Tomoyasu et al., 2008; Xu and Han, 2008; Firmino et al., 2013) and SID-5 orthologs in insects suggests that the mediators of dsRNA uptake may be different across metazoa. The identification of SID-1-like (SIL) proteins that include SILA, SILB, and SILC in multiple insect species has spurred several studies aiming to characterize their function in the uptake of dsRNA. The findings of these studies have ranged from supporting evidence for SIL protein function in RNAi response in WCR and CPB (Miyata et al., 2014; Cappelle et al., 2016), to no evidence for their involvement in desert locust, *Schistocerca gregaria* and diamondback moth, *Plutella xylostella* (Luo et al., 2012; Wang et al., 2014). The interpretations of the work that provides evidence for SILA and SILC involvement in RNAi include hypotheses for partial participation and redundant pathways for dsRNA uptake (Miyata et al., 2014; Cappelle et al., 2016). Tomoyasu et al. (Tomoyasu et al., 2008) first noted that while SILA, SILB, and SILC from *T. castaneum* are the closest homologs of the *C. elegans* SID-1, however, reverse blast yields TAG-130/CHUP-1 as the closest *C. elegans* homolog to the aforementioned SIL proteins (Fig. 1). Subsequent studies confirmed that the *C. elegans* CHUP-1

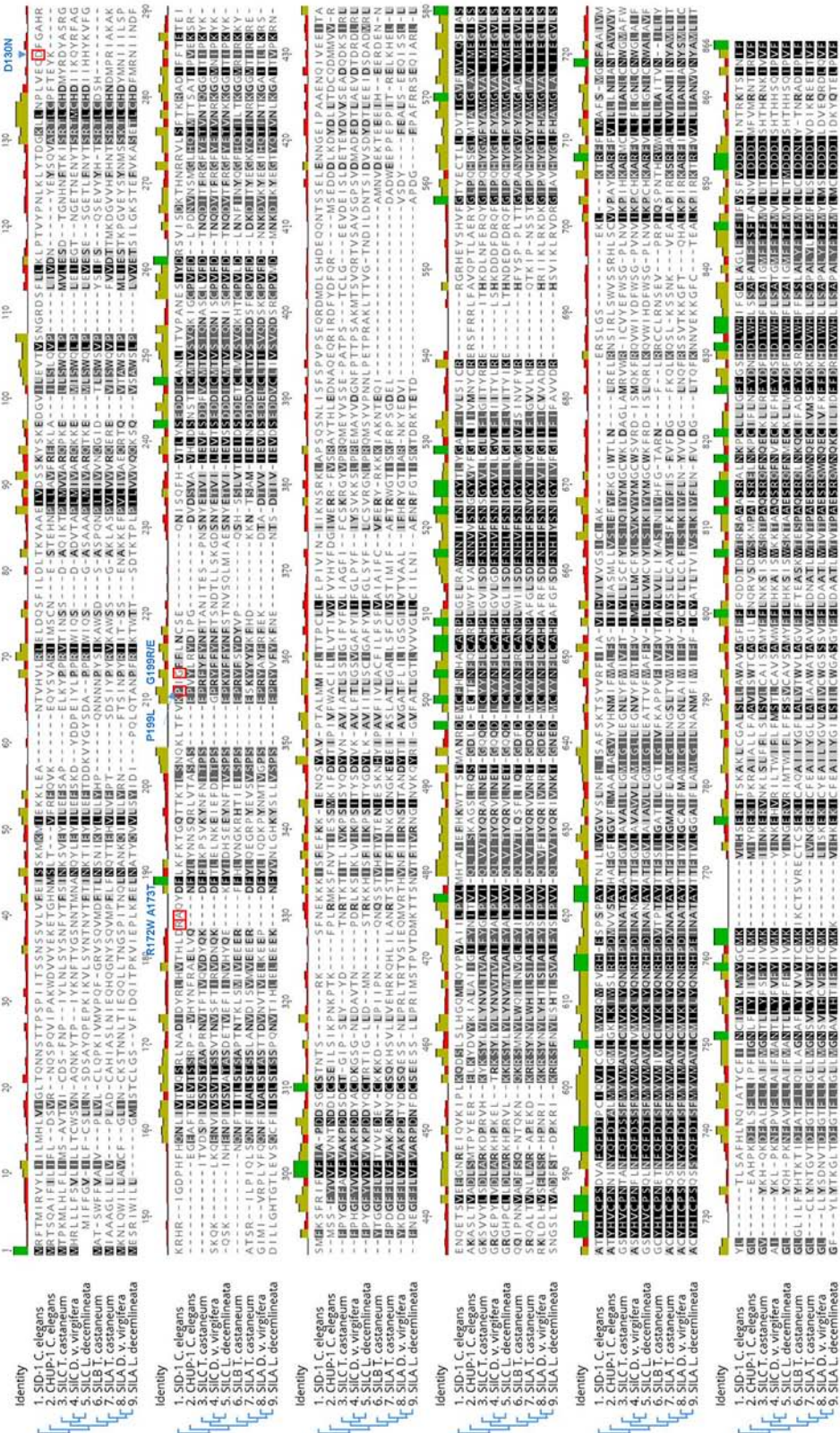


Fig. 1. Tree alignment of the *C. elegans* SID-1 and CHUP-1 with the insect SIL proteins. *C. elegans* SID-1 and CHUP-1 were aligned with *T. castaneum* SILA, SILB, and SILC, *L. decemlineata* SILA and SILC, and *D. v. virgifera* SILA and SILC. The alignment was performed in Geneious 10.0.5 using ClustalW with BLOSUM cost matrix. The amino acids are colored by similarity. Several residues that were identified by Whangbo et al. (Whangbo et al., 2017) as being important for the *C. elegans* SID-1 activity are boxed in red. The alignment shows that most these residues are not conserved with CHUP-1 or with the insect SIL proteins.

is necessary for cellular uptake of environmental cholesterol (Valdes et al., 2012), and does not support dsRNA uptake when heterologously expressed in *Drosophila* S2 cells (Whangbo et al., 2017). Other mammalian SID-related proteins, SIDT1 and SIDT2, were also shown to bind and influence cholesterol transport (SIDT1 at the plasma membrane and SIDT2 within the cell) but not dsRNA or miRNA (Mendez-Acevedo et al., 2017). Thus, it is possible that insects SIL proteins mediate cholesterol transport rather than dsRNA uptake in insects and this is consistent with the diverse SIL-associated RNAi phenotypes.

It is not clear if cholesterol is involved in dsRNA uptake or transport, yet there is evidence that blocking cholesterol transport perturbs endocytosis. In mammals, uptake of circulating cholesterol takes place by clathrin-mediated endocytosis through Low-Density Lipoprotein Receptor (LDLR) (Brown and Goldstein, 1986). Additionally, Niemann-Pick type C (NPC) proteins 1 and 2 are involved in intracellular cholesterol transport (but not necessarily uptake) in both insects (Voght et al., 2007) and mammals (Dixit et al., 2007). Not only do perturbations in clathrin-mediated endocytosis affect cholesterol transport, mutants such as NPC disrupt both receptor-mediated and bulk-phase endocytosis (Liu et al., 2007). Likewise, depletion or sequestration of cholesterol blocks clathrin-dependent endocytosis (Miwako et al., 2001; Chadda et al., 2007; Kim et al., 2017). Therefore, a blockage in cholesterol transport may decrease the rate of clathrin-mediated endocytosis and/or intracellular vesicles and thus affect the uptake of molecules such as dsRNA.

Fatty acid metabolism and the composition of the cell membrane may also be important for dsRNA uptake. Recently, a study in oriental fruit fly, *Bactrocera dorsalis*, demonstrated that fatty acid biosynthesis and metabolism pathways may influence RNAi induced by dsRNA. The ratio of the polyunsaturated fatty acids (PUFa) linoleic acid (LA) to arachidonic acid (AA) was shown to influence dsRNA uptake (Dong et al., 2017). While injection of AA restored or improved oral RNAi response in *B. dorsalis* and *D. melanogaster* (Dong et al., 2017).

Another pathway that has been implicated in RNAi in insects is clathrin-dependent endocytosis. This pathway was first associated with dsRNA uptake in S2 *Drosophila* cells (Saleh et al., 2006; Ulvila et al., 2006). These findings may have received limited initial attention from the entomology field because of the cell-autonomous nature of *Drosophila* RNAi. However, more evidence has emerged supporting the involvement of endocytic components including Clathrin heavy chain (*Chc*) (Saleh et al., 2006; Ulvila et al., 2006), Clathrin adaptor protein AP50/AP-2 μ , a subunit of the AP2 adapter complex (Saleh et al., 2006), ADP ribosylation factor-like 1 (Arf72A/Arf1) (Saleh et al., 2006), Vacuolar H⁺ ATPase 16 kDa subunit (Vha16) (Saleh et al., 2006), Vacuolar H⁺ ATPase SFD subunit (VhaSFD) (Saleh et al., 2006), and

small GTPase Rab7 (Saleh et al., 2006), in dsRNA uptake in Orthoptera and Coleoptera (Cappelle et al., 2016; Wynant et al., 2014b; Xiao et al., 2015). Saleh et al. (Saleh et al., 2006) identified dsRNA transport-associated components ranging from early endocytosis (AP50/AP-2 μ) to late endosomes (Rab7), and trans-Golgi associated (Arf72A/Arl1) proteins. Nonetheless, an in-depth analysis of the proteins and other molecules that may be associated with dsRNA uptake and transport will be vital to help identify the limiting factors in RNAi response and potential contributors to the evolution of resistance to dsRNA.

Although it is unlikely that key endocytic genes such as *Chc* may be lost or greatly suppressed in insects, it is feasible that dsRNA-specific receptors or other dsRNA-specific transport genes may be downregulated to confer resistance to the RNAi response. Two putative dsRNA receptors, class C scavenger receptor (SR-CI) and eater identified by Ulvila et al. (Ulvila et al., 2006) are particularly interesting since they point to specificity in dsRNA uptake. Further evidence of the importance of these receptors for dsRNA uptake was demonstrated in the desert locust. The use of inhibitors of the SR family, polyinosine, and dextran sulfate, blocked SRs significantly, and most importantly, inhibited an RNAi response (Kim et al., 2017; Ulvila et al., 2006; Wynant et al., 2014b). To contrast this view, Rocha et al. (Rocha et al., 2011) presented evidence that knockdown of SR-CI and eater may block *E. coli*-delivered but not naked dsRNA response in *Drosophila* S2 cells. A weak or intermediate impact of SC-R2 on RNAi response has been described in the CBP cell line Lepd-SL1 (Yoon et al., 2016); this result may be explained by the involvement of multiple receptors in mediating dsRNA uptake. The orthologs of putative scavenger dsRNA receptors have not been identified and characterized in other insect species, and it has yet to be determined if their roles are conserved between insect orders. Stabilin-1 and Stabilin-2 have recently been identified as cellular receptors that are necessary for clathrin-mediated endocytosis of Phosphorothioate (PS)-modified antisense oligonucleotides (ASOs) in human cells (Miller et al., 2016). In insects, the role of Stabilin-like fasciclin domain-containing proteins in dsRNA uptake is yet to be determined.

Visualization of dsRNA accumulating in late endosomes of lepidopteran cells is another observation that links the efficiency of RNAi response to dsRNA transport within lepidopteran cells (Shukla et al., 2016; Yoon et al., 2017). This research contrasts the fast processing of dsRNA into siRNA in Coleoptera and coleopteran cells with the accumulation of dsRNA in Lepidoptera and lepidopteran cells (Shukla et al., 2016). These studies postulate that dsRNA release from the endosome may be a limiting factor for RNAi response in lepidopterans that leads to low sensitivity to dsRNA in this order and potentially other orders. Further, these findings highlight the importance

of vesicular traffic in the RNAi response and help draw parallels to mammalian RNAi. Release from the endosome/multivesicular body (MVB) seems to be the limiting factor in the delivery of both phosphorothioate-modified antisense oligonucleotides (ASOs) and lipid nanoparticles (LNPs) loaded with siRNA in mammalian cells (Gilleron et al., 2013; Wang et al., 2017). The knowledge acquired in insects and mammals regarding dsRNA/siRNA transport might be similar. For instance, the uptake of LNPs involves both clathrin-mediated endocytosis and micropinocytosis (Gilleron et al., 2013). It is possible that micropinocytosis accounts for a portion of dsRNA uptake in insects as well, but the relationship between micropinocytosis and uptake of LNPs has not been studied in insects. Work with ASOs in mammalian cells has implicated Rab5C and early endosomal antigen 1 (EEA1) in early endosomal pathway (Miller et al., 2018) and highlighted the importance of Rab7a, lysobisphosphatidic acid (LBPA) and its key regulator, Alix, in their release from the MVB (Wang et al., 2017; Miller et al., 2018). Understanding these cellular components can inform and improve dsRNA or siRNA delivery to cells. For instance, when Miller et al. (Miller et al., 2018), introduced docosahexaenoic acid (DHA, which can be converted into LBPA) into cells, they measured increases in both LBPA and knockdown of target transcripts. MVBs and regulation of endosome traffic has been linked to RNAi response in insects as well; block of MVB formation using endosomal sorting complexes required for transport (ESCRT) mutants (*Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs)* and *Vacuolar protein sorting 25 (vps25)*) inhibits RNAi response, while block of MVB turnover with *Hermansky-Pudlak Syndrome 4 (HPS4)* mutants increases dsRNA-mediated silencing (Lee et al., 2009). It will be interesting to see if the endosome/MVB dsRNA release in insects is dependent on molecules such as LBPA and its regulators. From a different perspective, it would also be of value to identify which elements in coleopteran cells enable rapid release of dsRNA from the endosome. Such knowledge will benefit the improvement of RNAi insect pest management strategies, understanding the potential for insect resistance evolution, and efficacy in human medical fields.

In addition to transporting dsRNA or siRNA within cells, MVBs may provide means for transporting dsRNA/siRNA between cells via exosomes (Willem et al., 2002). Nanotubes have been proposed as another possibility to transport dsRNA between cells. Nanotube-like structures that contain RNAi components, including Ago2, dsRNA, and Rab7, were observed in cultured cells of *D. melanogaster* in context of viral infection, suggesting that this could be one of the mechanisms by which RNAi machinery is transported from a donor to an acceptor cell, triggering systemic antiviral immunity (Karlík et al., 2016). It is possible that these nanotube-like structures also play a role in the systemic RNAi response induced by shuttling dsRNA or siRNA molecules between insect cells.

To improve dsRNA stability, dsRNA uptake and overall RNAi response, nanoparticles and other modification, encapsulation, encapsidation and conjugation strategies have been explored. Nanoparticle-based delivery has been extensively reviewed in context of human therapy (Shajari et al., 2017; Jiang et al., 2016; Lee et al., 2013; Zhou et al., 2013). In insects, dsRNA formulation and viral encapsidation has also been explored (reviewed in (Joga et al., 2016; Yu et al., 2013; Kolliopoulou et al., 2017)); these approaches include bacterial expression (Yang and Han, 2014), liposomes (Whyard et al., 2009; Taning et al., 2016), chitosan nanoparticles (Zhang et al., 2010; Ramesh Kumar et al., 2016; Mysore et al., 2013), a fluorescent cationic core-shell nanoparticle (He et al., 2013), and guanylated polymers (Christiaens et al., 2018). It is difficult to say if nanoparticle protection or conjugation of dsRNA to delivery vehicles alters the mechanism of its uptake or intracellular trafficking. Understanding of how both naked dsRNA and the protected versions are taken up and trafficked within and between cells will greatly enhance RNAi applications for pest control.

Another conundrum in insect RNAi research is the relation between the intracellular dsRNA transport and RNAi lethality. While “RNAi-of- RNAi” approaches have been used to identify and study genes that may be involved in dsRNA uptake and RNAi response in general (Vélez et al., 2016; Tomoyasu et al., 2008; Miyata et al., 2014; Cappelle et al., 2016; Wynant et al., 2014b; Xiao et al., 2015; Yoon et al., 2016), numerous genes have been screened for lethal dsRNA response to be used as RNAi targets for pest management. Interestingly, the gene targets that are related to cellular membrane traffic seem to be some of the most lethal insect RNAi targets described. Simple reasoning argues that a block in dsRNA uptake and/or transport may self-limit an RNAi response. Hence, these genes would, in fact, be potential targets to confer RNAi resistance, yet the RNAi response for these genes seems to be robust and sustained. Therefore, the question is what is the mechanism conferring the insect mortality? We do not have an answer but hope that future work on RNAi in insects will shed more light on this puzzle. Fig. 2 depicts a parallel of the genes involved with the endocytic processing of dsRNA (Fig. 2A) and highly lethal dsRNA target genes that are also involved in membrane traffic (Fig. 2B). For example, the *D. v. virgifera Snf7* (Bolognesi et al., 2012) is being developed for commercial control of root-worm via dsRNA expression in maize (USDA-APHIS, 2013; ISAAA, International Service for the Acquisition of Agri-biotech Applications, 2014; USDA-APHIS, 2015). *Snf7/Vps32/shrub* is part of the filament-forming subunit of the endosomal sorting complexes required for transport III (ESCRT-III) complex involved in vesicle formation (Mayers and Audhya, 2012). Other ESCRT components (e. g., *Vps2* and *Vps28*) have also been identified as highly lethal via RNAi in WCR (Baum et al., 2007). The vacuolar ATPase subunits A

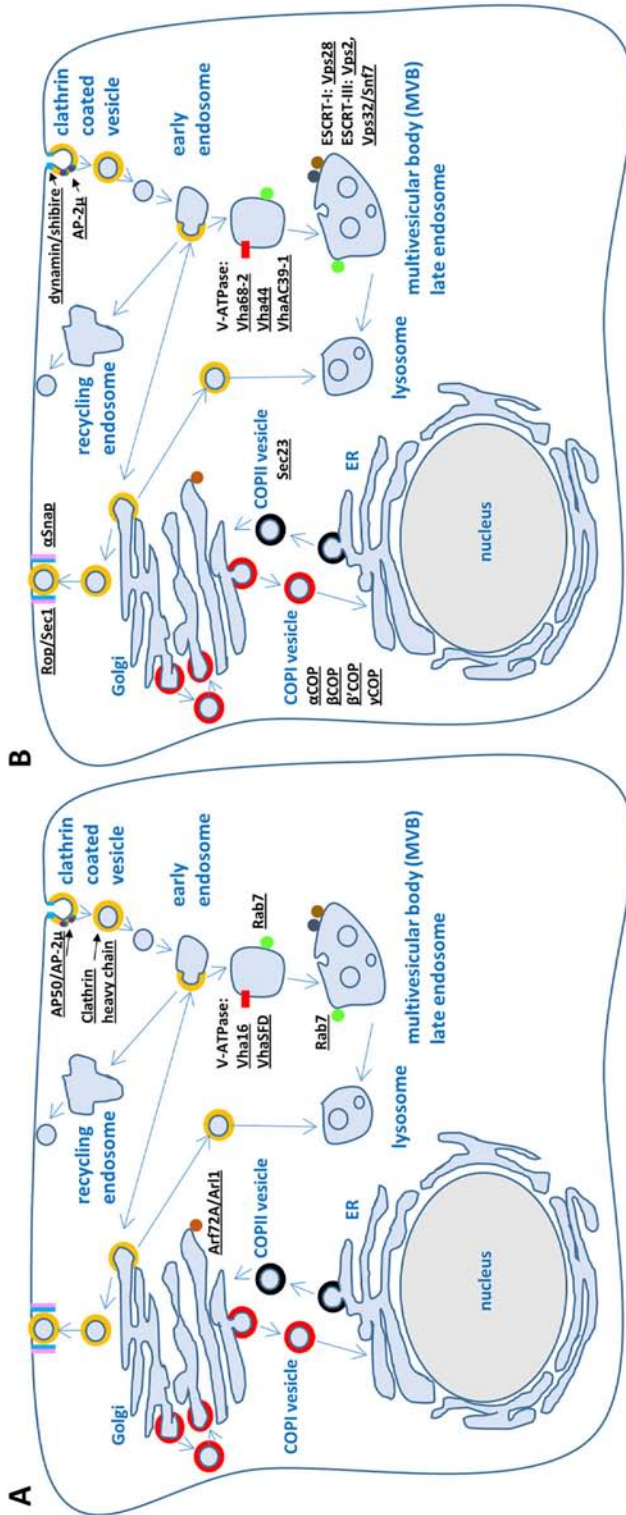


Fig. 2. Known components of dsRNA transport in insect cells and highly lethal RNAi targets that are involved in membrane transport. A. Known components of dsRNA transport in insects include Clathrin heavy chain (Cappelle et al., 2016; Saleh et al., 2006; Ulvila et al., 2006; Wynant et al., 2014b; Xiao et al., 2015), clathrin adaptor AP50/AP-2 μ (Saleh et al., 2006; Xiao et al., 2015), subunits of vacuolar H⁺ ATPase Vha16 (Cappelle et al., 2016; Saleh et al., 2006; Wynant et al., 2014b) and Vha5ED (Saleh et al., 2006; Xiao et al., 2015), small GTPase Rab7 (Saleh et al., 2006; Xiao et al., 2015), and Arf-related protein Aft72A/Arf1 (Saleh et al., 2006). B. Highly lethal insect RNAi target genes include dynamin/shibire (Ulrich et al., 2015), α subunit of the Adaptor Protein complex 2 (AP-2 α) (Prentice et al., 2017), subunits of vacuolar H⁺ ATPase Vha68-2 (Baum et al., 2007; Vélez et al., 2016; Mao et al., 2015; Ulrich et al., 2015), Vha44 (Li et al., 2015a), and VhaAC39-1 (Baum et al., 2007; Ulrich et al., 2015), ESCRT-I subunit Vps28 (Baum et al., 2007), ESCRT-I subunits Vps2 (Baum et al., 2007) and Vps32/Snf7 (Baum et al., 2007; Bolognesi et al., 2012; Prentice et al., 2017), COPI vesicle coat subunits α (Prentice et al., 2017), β (Baum et al., 2007; Mao et al., 2015; Prentice et al., 2017), β' (Baum et al., 2007; Ulrich et al., 2015), and γ (Prentice et al., 2017), COPII subunit Sec23 (Rangasamy et al., 2017), and SNARE-binding secretory protein Rop/Sec1 (Knorr et al., 2018; Ulrich et al., 2015) and α Snap (Ulrich et al., 2015).

(*Vha68-2*), C (*Vha44*), and D (*VhaAC39-1*) are also lethal RNAi targets (Baum et al., 2007; Li et al., 2015a; Vélez et al., 2016; Mao et al., 2015; Ulrich et al., 2015; Prentice et al., 2017). Further, both COPI (Baum et al., 2007; Mao et al., 2015; Ulrich et al., 2015; Prentice et al., 2017; Isoe et al., 2011) and COPII (Rangasamy et al., 2017) vesicle coat components have been identified in multiple screens for RNAi lethality. Another subunit of the clathrin AP2 adapter complex, AP-2 α , has also been identified as one of 24 highly lethal genes in sweet potato weevil (Prentice et al., 2017). Other vesicle-associated RNAi targets include dynamin/shibire, a large GTPase that catalyzes the scission of endocytic vesicles (Ulrich et al., 2015) and secretory N-ethylmaleimide-sensitive factor activating protein receptor- (SNARE-) binding proteins *Rop/Sec1* (Knorr et al., 2018; Ulrich et al., 2015) and α Soluble NSF attachment protein (α Snap) (Ulrich et al., 2015). These gene targets have been identified from multiple unrelated screens for the most lethal gene targets in insects with the goal of producing high levels of lethality at a low dsRNA dose. Another observation that suggests that RNAi-of-RNAi response may not be self-limiting is the comparison of the effects of *Dicer-1*, *Dicer-2*, *Argonaute 1* (*AGO1*), and *Argonaute 2* (*AGO2*) knockdown in WCR (Wu et al., 2017). In this study, the knockdown of known dsRNA-processing components *Dicer-2* and *AGO2* was both sustained and inconsequential for WCR survival and egg viability (Wu et al., 2017).

In conclusion, much remains that is a mystery in the mechanisms that regulate RNAi in insects. The range of RNAi sensitivity and the diversity of dsRNA-induced phenotypes across insect orders and in some cases species, argues that the studies regarding RNAi mechanisms should be performed in the species of interest and not every aspect of the RNAi response can be assumed to be conserved. A better understanding of dsRNA uptake and transport inside and between cells is critical to help us improve RNAi applications across insects and provide insights on routes to dsRNA resistance.

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