Systemic RNAi Relies on the Endomembrane System in *Caenorhabditis elegans*

YANI ZHAO

The membrane system of a eukaryotic cell is a large and complex system handling the transport, exchange and degradation of many kinds of material. Recent research shows that double-stranded RNA (dsRNA) mediated gene silencing (RNA interference) is a membrane related process. After long dsRNA is processed to small interfering RNA (siRNA) by Dicer, the guide strand and passenger strand are separated in the RNA induced silencing complex (RISC) by Argonaute. The process of loading siRNA into RISC has been suggested to occur at the rough Endoplasmic Reticulum (rER). The components of RISC also associate with late endosomes/multivesicular bodies (MVBs). Furthermore, disturbing the balance between late endosomes/MVBs and lysosomes has been shown to affect the efficiency of silencing.

We use the nematode Caenorhabditis elegans as our model organism to study two questions: how does membrane transport affect RNAi and spreading of RNAi from the recipient cells to other tissues (systemic RNAi); and how does RNA transport contribute to the multigenerational silencing induced by dsRNA (RNAi inheritance)? Using SID-5, a protein required for efficient systemic RNAi, as bait in a yeast two-hybrid (Y2H) screen, we got 32 SID-5 interacting candidate proteins. Two of these are the SNARE protein SEC-22 and the putative RNA binding protein C12D8.1. In two additional Y2H screens, we found that SID-5 interacts with multiple syntaxin SNAREs, including SYX-6, whereas SEC-22 only interacts with SYX-6. SNAREs usually function in vesicle fusion processes. We found the two SNARE proteins SEC-22 and SYX-6 to be negative regulators of RNAi and to localize to late endosomes/MVBs. In addition, loss of sid-5 leads to an endosome maturation defect. Finally, we found that the putative RNA binding protein C12D8.1 negatively regulates RNAi inheritance and that C12D8.1 mutant animals show impaired RNAi upon targeting a new gene. Taken together, the results presented in this thesis provide us with more evidence for the connection of the membrane transport system and RNAi. The identification of a putative negative regulator of RNAi inheritance further enriches this research field.

Keywords: Systemic RNAi, RNAi inheritance, late endosomes/MVBs, SID-5, SEC-22, SYX-6, C12D8.1

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Abbreviations

AGO: Argonaute

dsRNA: double-stranded RNA

CUP: Coelomocyte Uptake Defective

ER: Endoplasmic Reticulum

GFP: Green fluorescent Protein

HRDE: Heritable RNAi Defective

ILV: Intralumenal Vesicle

miRNA: microRNA

mRNA: messenger RNA

MVB: Multivesicular Body

piRNA: PIWI-associated RNA

RdRP: RNA-Dependent RNA Polymerase

rER: Rough Endoplasmic Reticulum

RISC: RNA-Induced Silencing Complex

RME: Receptor Mediated Endocytosis

RNAi: RNA Interference

RSD: RNAi Spreading Defective

SID: Systemic RNAi Defective

siRNA: Short/small interfering RNA

SNARE: Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor

NRDE: Nuclear RNAi Defective

Y2H: Yeast two-hybrid
Introduction

The membrane system is the main machinery for handling different kinds of material from its origin to its destination in a eukaryotic cell. The rough Endoplasmic Reticulum (rER) serves as the platform for mRNA translation to proteins by ribosomes, Golgi is where post-translational modification of proteins happens, endosomes are the sorting centers that determine the destination of cargo, and lysosomes break down material destined for degradation (Farquhar, 1981; Mellman, 1996; Palade, 1955; Settembre et al., 2013). In recent years, more and more evidence has connected membrane compartments with small RNA regulation. microRNA and small interfering RNA (siRNA) mediated translational inhibition or mRNA degradation are two types of small RNA regulation (Ghildiyal, M; Zamore, 2009). For years, small RNA regulation has been believed to be a cytoplasmic process. However, it has been shown that rER is an important site for siRNA-mediated silencing (RNA interference, RNAi) in human cells (Stalder et al., 2013) and that core factors mediating silencing localize to the sorting center late endosomes (Gibbings et al., 2009). In some organisms, siRNA-induced gene silencing can spread from the recipient cell to other tissues (systemic RNAi), and in some cases the silencing information can be inherited by progeny (Fire et al., 1998; Winston et al., 2002). Recent studies show that most of the factors taking part in the transport process are membrane-associated (Hinas et al., 2012; Imae et al., 2016; Winston et al., 2002, 2007). Together, this indicates that the membrane system plays an important role in RNAi as well as RNA transport. However, the connection among the factors affecting these processes is still not clear.

My thesis is mainly about searching for proteins that could function in RNA transport. I studied the effect of these proteins on RNAi, their subcellular localization, and how they affect the membrane system. For the protein that has RNAi inheritance related function, we studied how it can affect the RNAi inheritance. The results in my thesis connect the membrane system with systemic RNAi even more, and also provide more evidence that RNAi inheritance relies on the transport system.
Vesicle Trafficking, the Major Transport System in Cells

Overview of Vesicle Trafficking

The vesicle trafficking system is a complex and orderly system that contains several pathways and many factors (Figure 1)(Scott et al., 2014). Through vesicle trafficking, cells take up and exchange material including nutrients, macromolecules, proteins, RNAs, and signal substances. Vesicles are mainly derived from endocytosis, the biosynthetic secretory pathway, and autophagy. Endocytosis is the process by which cells take up extracellular materials (Mellman, 1996). By a critical series of selection starting in the early endosome, and later on in late endosome/multivesicular bodies (LE/MVBs) during maturation of endosomes, cargo is either degraded by LE/MVBs and lysosome fusion, or sent out through the exocytosis pathway (Mellman, 1996). The biosynthetic secretory pathway refers to the process of proteins synthesized on the endoplasmic reticulum (ER) traveling to Golgi for different kinds of post-translational modifications (PTMs) and transported to plasma membrane to either become resident proteins or sent out (Nickel and Wieland, 1998). Autophagy is a cell self-cleaning process to eliminate proteins and useless organelles by engulfment and fusion with the lysosome (Mizushima and Komatsu, 2011).

Different Pathways, Similar Factors

The functions of all of the pathways mentioned above differ a lot, however the processes also share many features. The transport vesicle first buds from a membrane, then needs to be tethered and docked with the organelle or vesicle it is aiming for, and eventually fuse with it and release the content. Factors that facilitate vesicle budding, tethering/docking, and fusion are Ras-associated binding (Rab) GTPases, membrane tethers, and SNAREs (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors), respectively (Schwartz et al., 2007; Zhi et al., 2014). Rab GTPases are a family of proteins that function in inducing vesicle budding by promoting cargo and coat protein selection, enable vesicle transport along the cytoskeleton by recruiting actin- or microtubule-based motor protein complexes (MPCs) and help to recruit membrane tethers and SNAREs for vesicle fusion (Schwartz et al., 2007). Membrane tethers are complexes that function in the initial docking process of a vesicle to its target membrane, and are also required for efficient SNARE assembly (Zhi et al., 2014). SNARE proteins are a large protein family that is conserved in all eukaryotes. They associate with the vesicle and target membranes, and mediate membrane fusion by forming a zipper-like complex and zipping up the two membranes (Ungar and Hughson, 2003). The three factors (Rab GTPases, tethers, and SNAREs) function in a manner that can keep the fidelity of the endomembrane
transport (Scott et al., 2014). Different Rab GTPases associate with specific types of vesicles and recruit different membrane tethers to ensure transport and docking accuracy (Cabrera and Ungermann, 2010). With the assistance of membrane tethers, SNAREs bring the appropriate membranes of vesicle and target together. The co-operation among all factors ensures order in the busy endomembrane transport system.

Figure 1. Vesicle transport and sorting. The cell engulfs extracellular material via endocytosis. The receptors are recycled to the plasma membrane. The ESCRT complex promotes the formation of multivesicular bodies and sorts the material to degradation by lysosome or to exocytosis. Compounds synthesized in the cell are also exported via the exocytosis pathway. Compounds are also transported to the cell’s organelles, and the transport proteins and receptors are recycled to Golgi. In the autophagy process, isolation membrane engulfs the cell compartments to form an autophagosome. The autophagosome then fuses with lysosome to degrade the contents.
Endocytosis

Cells are able to take up extracellular fluid, particles and macromolecules via vesicles that undergo inward budding from the plasma membrane. This process is called endocytosis (Mellman, 1996). Clathrin-mediated endocytosis includes a series of steps requiring cell surface receptors, the coat protein clathrin, several adaptors and many different accessory proteins (Popova et al., 2013). The initiation of clathrin-mediated endocytosis is the binding of macromolecules to receptors on the cell surface at clathrin pits (Ehrlich et al., 2004; Perrais and Merrifield, 2005). The cargo sequestration is dependent on the Rab GTPase Rab5 (McLauchlan et al., 1998). During membrane binding and clathrin polymerization, a jar like structure forms on the plasma membrane (Kirchhausen, 2009). The scission process is mediated by the large GTPase dynamin (Bashkirov et al., 2008). Dynamin forms a helical collar at the neck of the budding vesicle, and pinches off the vesicle from the membrane when the bulbous structure is around 100 nm in diameter (Traub, 2011). The clathrin molecules are released from the vesicle and recycled to the plasma membrane to start a new round of initiation of endocytosis (reviewed in (Yarden and Tarcic, 2013))

Clathrin-independent endocytosis is a general name for endocytosis pathways that do not rely on clathrin. Some of the pathways function constitutively, while some require a certain trigger (Mayor et al., 2014). Caveolar endocytosis is a type of clathrin-independent endocytosis. Instead of clathrin, caveolin proteins function as the coat to mediate internalization of specific proteins (Ludwig et al., 2013). The vesicles budding inwards through caveolar-mediated endocytosis range from 50 nm to 80 nm in diameter (Barber, 1966). In addition to taking up extracellular material, caveolar endocytosis also regulates the caveolae density on the plasma membrane (Parton and del Pozo, 2013).

Examples of endocytic processes involving larger vesicles are phagocytosis (vesicles >0.25 µm in diameter) and macropinocytosis (vesicles ranging from 0.15 to 5.0 µm in diameter). In multicellular organisms, only certain cell types, such as white blood cells in mammals, can perform phagocytosis (Rosales and Uribe-Querol, 2017). The process is used to take up large particles such as bacteria, cell debris and parasites to eliminate them and protect the organism (Rosales and Uribe-Querol, 2017). Macropinocytosis is a non-specific endocytic pathway to take up solutes from extracellular fluid (Peng et al., 2017).

Endocytosis is the first step by which cells accept information from the outside. The newly formed vesicles, except for phagosomes, then fuse with early endosomes and start a journey inside the cell (see section on endosome maturation below).
Biosynthetic-Secretory Pathway

Proteins are synthesized on the rough endoplasmic reticulum (ER) (Lerner, 2003; Palade, 1955). Once correctly folded, membrane proteins and secretion proteins are packed in COPII vesicles and sent to the Golgi apparatus for further post-translational modifications. COPII coat vesicles can concentrate cargo and promote the formation of vesicles on the ER membrane for further transport towards the cis-Golgi apparatus (Barlowe et al., 1994). The recruitment of COPII coat requires the active form of a small GTPase with GTP bound (Nakano et al., 1988). After fusion with the ER-Golgi intermediate compartment, the COPII coat is disassembled (Hanna et al., 2017). The cargo protein then enters the Golgi apparatus from the cis-Golgi cisterna (Szul and Sztul, 2011). Various kinds of enzymes conducting post-translational modifications (PTMs), such as phosphorylation, glycosylation, and sulfation, are located in the Golgi apparatus (Rabouille et al., 1995). The cargo protein matures with the addition of different PTMs as it is transported through the Golgi apparatus (referred to as anterograde transport). When the protein is mature, it reaches the trans-Golgi network (TGN) and is shipped off to different destinations. Secretion proteins are transported in vesicles to the plasma membrane and secreted in a constitutive or regulated secretion pathway (Anderson, 2006). The retrograde transport takes the opposite route, which starts from trans- to cis- Golgi, and then all the way to ER. This process is mediated by COPI-coated vesicles (Beck et al., 2009). The COPI vesicles contain escaped ER resident proteins and Golgi enzymes (Beck et al., 2009). A recent study shows that COPI is essential for Golgi cisternae maturation, and affects Golgi dynamics (Ishii et al., 2016).

Endosome Maturation: Cargo on the Way

The early endosome is a pleomorphic sorting compartment. It is the first stop for endocytic vesicles and TGN-derived vesicles containing cargo destined for endosomes and lysosomes, for example digestive enzymes (Scott et al., 2014). Being sorted in the early endosome, cargos are destined to plasma membrane for recycling, retrograde transport to the TGN, or to late endosomes/multivesicular bodies (LE/MVBs) for further fusion either to the plasma membrane for secretion or to lysosomes for degradation (Scott et al., 2014).

Functioning as the first sorting center, the early endosome has a highly dynamic morphology to fulfill the requirement of handling material coming from endocytosis or TGN (Gruenberg et al., 1989). In the same early endosome, thin tubular extensions (~ 60 nm diameter) and large vesicular or multivesicular (~ 400 nm diameter) sub-domains can co-exist (Gruenberg and Stenmark, 2004). Acidity is one of the factors that segregate different cargoes. In the tubular extensions, where receptors are recycled, pH increases from 6.2 to ~6.5, causing receptors from the plasma membrane to release
their ligands (Mayor et al., 1993). The receptors then recycle to the plasma membrane via one of two different small GTPase mediated pathways: the Rab4 dependent fast and direct pathway, or the Rab11 dependent indirect pathway through recycling vesicles (Sönnichsen et al., 2000). In the multivesicular appearance part which will eventually bud off and mature to MVBs, the pH can be even lower at ~4.5 (Mayor et al., 1993; Mellman, 1996).

For the maturation of early endosome to late endosome, the multi-unit endosomal sorting complex required for transport (ESCRT) plays a major sorting role. The four ESCRT complexes (-0, -I, -II, -III) and the Vps4 complex work sequentially at the inward vesicle-budding site on the endosome membrane (Hurley, 2008). The first sorting step in animals and fungi starts with ESCRT-0 (Raiborg et al., 2001). It then recruits ESCRT-I from the cytoplasm by protein-protein interaction (Bache et al., 2004; Katzmann et al., 2003; Lu et al., 2003). On the membrane-facing side, ESCRT-I can bind two ubiquitin moieties; on the other side, it binds to ESCRT-II (Kostelansky et al., 2006). These two complexes can promote inward budding of vesicles from the endosome membrane. All the first three complexes can bind to ubiquitinated proteins (Henne et al., 2011). Ubiquitinated proteins have a modification with a single or a chain of ubiquitins. The process is called ubiquitination and is catalyzed by the ubiquitin ligase (Shields and Piper, 2011). It is the main signal for protein degradation via the proteasome or lysosome (Pickart and Eddins, 2004). The process of how ESCRT-0, -I, and II cooperate is not completely clear. They can sort cargo into MVBs by transferring the cargo in a certain order like on a conveyor belt, or they can bind to different cargoes and segregate these to a certain region on the membrane (Schuh and Audhya, 2014). The ESCRT-III filament and Vps4 complexes are the most conserved complexes compared to the others, and research shows that the ESCRT-III filament plays a key role in the membrane scission process. Different subunits of ESCRT-III assemble temporarily on the endosome membrane (Alonso Y Adell and Teis, 2011). By recruiting deubiquitinase, it recycles ubiquitins from cargo, sequesters cargo and finishes the budding process from the endosome membrane (Alonso Y Adell and Teis, 2011). Vps4 is a mechanoenzyme, which disassembles the ESCRT-III subunits and releases them back into the cytoplasm for the next round of vesicle budding on the endosome membrane (Babst et al., 1997). When the large vesicle part matures, it pinches off from the early endosome as free multivesicular bodies and is transported to fuse with other late endosomes (Scott et al., 2014). During this process, GTPase Rab5 is exchanged for Rab7, which is also required for fusion of late endosomes to lysosomes (Rink et al., 2005; Vanlandingham and Ceresa, 2009).

Late endosomes, as the second sorting center, sort cargo to different destinations. In mammalian cells, it also has a dynamic and complex structure containing tubular cisternal elements and multivesicular regions (Griffiths et al., 1988). Late endosomes contain newly synthesized lysosome proteins and
cargo aimed for degradation (Scott et al., 2014). They fuse with lysosomes in a concurrent and kissing manner, but not in a complete fusion process (Bright et al., 2005). The cargo is degraded in lysosomes.

Late endosomes also sort cargo for recycling, such as cholesterol to the plasma membrane and ER, and mannose 6-phosphate receptors to the TGN. For recycling cholesterol, after uncoupling from the low-density lipoprotein (LDL) receptor in the early endosome, the free LDL particles are transported to late endosomes (Möbius et al., 2003). Acidic lipase cholesterylester hydrolyses the LDL particles and the free cholesterol can be recycled (Goldstein et al., 1985). Recycling of mannose 6-phosphate receptor is mediated by Rab9 (Díaz and Pfeffer, 1998; Reddy et al., 2006). Mannose 6-phosphate activates acid hydrolase protein simultaneously during the transport to lysosomes (Settembre et al., 2013). In the late endosome, the mannose 6-phosphate and its receptor are decoupled in the low pH environment (Schmid et al., 1989). The receptor is recycled by retrograde transport to the TGN (Braulke and Bonifacino, 2009).

**Autophagy**

Autophagy is a process whereby cells degrade organelles or proteins via the lysosome. There are three types of autophagy in mammalian cells: macroautophagy, micro-autophagy, and chaperone-mediated autophagy (CMA) (Glick et al., 2010). In mammalian cells, lysosomes are the final destination for all kinds of autophagy. Macro- and micro-autophagy can both engulf large cell organelles for lysosomal degradation (Glick et al., 2010). However in the macro-autophagy process, cells engulf the content in a double-membrane bounded vesicle called autophagosome, which then fuses with lysosomes forming the autolysosome, and degrades the contents by acidic lysosome hydrolysis (Glick et al., 2010). On the other hand, micro-autophagy is a process where cytoplasmic material is engulfed directly into lysosomes (Parzych and Klionsky, 2014). In chaperone-mediated autophagy (CMA), the target protein is recognized and delivered to the lysosome membrane by cytosolic chaperons and unfolded before being transported across the membrane (Susmita Kaushik and Ana Maria Cuervo, 2012).

The best-characterized pathway is macro-autophagy, which includes formation of the autophagosome vesicle structure. There are several steps in this process: formation of phagophore, target organelle or protein segregation, and fusion with lysosome for degradation (Parzych and Klionsky, 2014). The membrane forming the phagophore is derived from ER (Axe et al., 2008). The formation of the phagophore requires cooperation of several autophagy-related (Atg) proteins, such as Atg5 and Atg12 (Ohsumi, 2001). The conjugation of Atg5 and Atg12 is required for the curvature of the phagophore as it is growing (Kirkin et al., 2009). Atg8 is recruited during phagophore growth (Nakatogawa et al., 2007). Its function is cargo selection.
and hemifusion of the membrane (Nakatogawa et al., 2007). Once the autophagosome is formed, it fuses with lysosomes for degradation.

Exocytosis

Exocytosis refers to the process of sending out material from a cell by fusion of a vesicle with the plasma membrane. In general, there are two types of exocytosis in multicellular organisms, Ca\(^{2+}\)-independent exocytosis and Ca\(^{2+}\)-dependent exocytosis (Alberts et al., 2002). Ca\(^{2+}\)-independent exocytosis is also known as constitutive secretion (Alberts et al., 2002). The constitutive secretion vesicles that are derived from the TGN and fuse with the plasma membrane contain membrane proteins, lipids, and soluble proteins, such as proteoglycans and glycoproteins, building blocks of the extracellular matrix (Alberts et al., 2002). All cells use this pathway to restore cell membrane material and maintain the extracellular surroundings. In certain secretory cells, the Ca\(^{2+}\)-dependent pathway, which is also known as regulated secretion, controls the switch for the release of the content from secretory vesicles. The binding of signal and receptor initiates the increase of Ca\(^{2+}\) concentration as well as the action potential (Knight and Baker, 1982). Inside the cell, Ca\(^{2+}\) ions bind to a special sensor and when the voltage-gated Ca\(^{2+}\) channel opens because of the high potential, the vesicle fuses with the plasma membrane and releases the content (Alberts et al., 2002). Hormones, peptides, and neurotransmitters are chemically modified in the TGN, packed in secretory vesicles, and released following the regulatory pathway. Usually, the content of secretion needs to be released speedily and accurately, especially for neurotransmitters at the synapse, so vesicles do not fuse with plasma membrane completely but in a “kiss and run” manner (Rutter and Tsuboi, 2004). Ca\(^{2+}\)-dependent secretion is one pathway by which intraluminal vesicles (ILVs) in MVBs are secreted as exosomes into the extracellular environment (Scott et al., 2014). Exosomes are a population of small vesicles around 50 nm in diameter, secreted by many kinds of cells in vivo and in vitro (Théry et al., 2002) Researchers observed that in cultured human lymphoblastoid cells, MVBs with high levels of cholesterol are mainly engaged in secretion, while the ones poor in cholesterol are mainly targeted for degradation (Möbius et al., 2002). However, how the ILVs/MVBs are selected for secretion is still not clear.

SNAREs, Zip Them Up

In all processes described above, SNARE proteins can form a trans-SNARE complex to promote homotypic and heterotypic fusion of membranes and organelles (Chen and Scheller, 2001). They are small and abundant proteins with a SNARE motif, and associate with membranes. The SNAREs were originally classified into t-SNAREs and v-SNAREs depending on their locations, with t- abbreviating target and v- abbreviating vesicle, respectively
(Söllner et al., 1993). However, there are situations in which t-SNAREs and v-SNAREs are sitting on the same type of vesicles, such as during a homotypic fusion process. Another classification method was developed, classifying SNAREs as Q-SNAREs and R-SNAREs. This classification depends on if they donate a glutamine (Q) or arginine (R) residue to form the zero ionic layer in the assembled core SNARE complex (Ahn, 1998). During vesicle fusion, typically three Q-SNARE motifs and one R-SNARE motif from two membranes/organelles form a four-helix bundle core SNARE complex (Ahn, 1998). As the bundle becomes tighter, which is called trans-configuration, the distance between the two membranes decreases and the membranes fuse with each other (Chen and Scheller, 2001). The bundle formed by the two types of SNAREs changes conformation to cis-SNARE complex and is dis-assembled by recruitment of the AAA ATPase NSF (N-ethylmaleimide-sensitive factor) (Müller et al., 1999, 2002). In this way, SNAREs can be recycled. In mammalian cells, there are more than 30 SNAREs (Chen and Scheller, 2001). Different SNARE sets function in different processes of membrane fusion.

Recent studies show that the function of SNAREs is not only fusogenic; by pairing with other SNAREs in a non-canonical way, they can function in a different process. Specifically, Sec22b, a SNARE with its canonical fusogenic role in ER-to-Golgi transport, can also pair with plasma membrane SNARE Syntaxin 1 to position the ER membrane close to the plasma membrane and mediate plasma membrane expansion in neuronal cells (Petkovic et al., 2014). Additional functions of SNARE proteins are still under discovery.

**Small RNA Regulation**

**Overview of Small Non-Coding RNA Regulation**

In the world of RNA, mRNAs are templates for protein translation, whereas RNAs that do not code for proteins are non-coding RNAs (ncRNAs). ncRNAs cannot be translated to protein, however they play important roles in many aspects. The first discovered ncRNAs were ribosomal RNA (rRNA) and transfer RNA (tRNA) (Cech and Steitz, 2014). With the research of RNA biology digging in, more and more ncRNAs have appeared on the list. ncRNAs function in many different ways: rRNA functions in protein synthesis; tRNA transports amino acids for the same process; other ncRNAs function in regulation of gene expression. However the studies on regulatory ncRNAs are not complete and the functions of most of the ncRNAs are still unknown(Mattick and Makunin, 2006).

Among the best studied ncRNAs are the small (20-30 nt) ncRNA families of microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). In animals, microRNAs are endogenously tran-
scribed small RNAs that function in regulation of mRNA abundance and translation by usually non-perfect base pairing to its target mRNAs (Ghildiyal, M; Zamore, 2009). They act in many biological pathways to control gene expression. siRNAs can be processed from synthesized long double stranded RNAs (dsRNAs) *in vitro* or expressed endogenously (Ghildiyal, M; Zamore, 2009). The siRNA can induce specific gene silencing, so called RNA interference (RNAi) (Fire et al., 1998). The complementarity between siRNA and its target mRNA is specific. Since they can be designed perfectly complementary to their targets, siRNAs are broadly used as a tool to control gene expression in many aspects, such as functional studies of genes and gene therapy discovery. piRNAs are a more recently discovered small ncRNA species enriched in germ cells (Batista et al., 2008). The classical function of piRNAs is repression of transposons in germ cells (Weick et al., 2014). In addition, recent studies show that piRNAs can target endogenous mRNAs and cause mRNA deadenylation followed by degradation in *Drosophila*, mouse, and *C. elegans* (Bagijn et al., 2012; Gou et al., 2014; Lee et al., 2012; Rouget et al., 2010).

**miRNA and siRNA: Biogenesis and Regulatory Pathway**

In animal cells, miRNAs are transcribed endogenously by RNA polymerase II to long primary (pri)-miRNA forming one or more hairpin structures, which is/are processed to precursor (pre)-miRNA by ribonuclease III Drosha (Lee et al., 2003, 2004; Zhou et al., 2007). The pre-miRNA is shuttled from the nucleus to the cytoplasm, where the RNase III-like enzyme Dicer matures it by cutting the hairpin to 20-25 bp miRNA (Bernstein et al., 2001). The mature miRNA is separated into guide strand and passenger strand by the RNA-induced Silencing Complex (RISC) (Bernstein et al., 2001; Elbashir et al., 2001). The guide strand stays bound to RISC for the silencing process. Usually the miRISC has two core components: Argonaute (Ago) and GW182. Ago proteins bind to miRNAs directly. They separate the two miRNA strands and use the guide miRNAs to find their complementary mRNAs, causing inhibition of translation and/or mRNA degradation (Mollory and Vaucheret, 2010). GW182 is a 182 kDa protein family that contains several glycine/tryptophan repeats (Lian et al., 2009). It has been shown to be necessary for the silencing process by binding to Argonaute proteins and recruiting deadenylase complexes for target silencing (Braun et al., 2011). Repression of either GW182 or Ago leads to a decrease in silencing efficiency (Yao et al., 2013). Homologs of GW182 are found in various species including *Drosophila* and *C. elegans*, but have so far not been found in non-metazoan eukaryotes (Yao et al., 2013). Usually, one miRNA can base pair perfectly or imperfectly to several mRNAs to regulate expression of multiple genes.
In contrast to miRNAs, siRNAs are formed from perfectly base paired dsRNA - either artificial dsRNA introduced into host cells or dsRNA transcribed endogenously (Ghildiyal, M; Zamore, 2009). When long dsRNA serves as a trigger, Dicer cleaves it into 20-25 bp siRNAs (Ghildiyal, M; Zamore, 2009). After RISC loading, the passenger strand is removed and the guide strand stays bound to RISC. If the siRNA is perfectly complementary to its target mRNA, the Ago protein in RISC cleaves the target mRNA and induces gene silencing (Ghildiyal, M; Zamore, 2009). In some organisms, such as C. elegans, plants and fission yeast, a special RNA polymerase, RNA dependent RNA polymerase (RdRP), amplifies secondary siRNAs using target mRNA as template (Sijen et al., 2001). The secondary siRNAs enhance the efficiency of RNAi. An overview of the miRNA and siRNA pathways is shown in Figure 2 (Ghildiyal, M; Zamore, 2009).

RNAi Inheritance

Gene silencing mediated by dsRNA that can persist for multiple generations is termed RNAi inheritance (Fire et al., 1998; Grishok et al., 2000). Early studies in C. elegans showed that following injection of dsRNA targeting soma expressed genes, both the parental worms (P0) and their progeny (F1) are affected, but the effect does not persist to the F2 generation (Fire et al., 1998). Further experiments showed that the silencing in the F1 generation is mainly triggered by the injected dsRNA, since knocking out the factors processing dsRNAs to siRNAs in parents does not affect the RNAi efficiency in heterozygous F1 animals (Tabara et al., 1999). This could be because the initial dsRNAs are distributed by the zygotes to every cell, and the dsRNA is processed to siRNA where its target mRNA is expressed (Grishok, 2013). Another study showed that the effect of dsRNA targeting germline-expressed genes could be observed until the F4 generation (Alcazar et al., 2008). This longer term RNAi inheritance requires the factors that process dsRNA to siRNA and the production of secondary siRNAs in the P0 animals indicating that it is a different process compared to persistence of RNAi in soma (Grishok et al., 2000). Genetic analysis showed that trimethylation of histone H3 at Lys9 (H3K9me3), a marker for gene repression, at the corresponding locus exists in both RNAi persistence and long-term inheritance (Burton et al., 2011; Gu et al., 2012). In the fission yeast Schizosaccharomyces pombe, H3K9 methylation via the histone methyltransferase Clr4 requires the RNAi machinery (Hall, 2002; Volpe, 2002). H3K9me3 can recruit RNAi silencing factors to induce RNAi (Motamedi et al., 2004). Thus, both ncRNAs and H3K9 methylation are required to maintain stable states of nuclear gene silencing in S. pombe (Martienssen and Moazed, 2015). In a recent genetic screen in C. elegans, researchers found that SET-32, which is
Figure 2. miRNA regulation and siRNA regulation. Top: miRNA pathway. RNA polymerase II amplifies pri-miRNA from its DNA template. In animals, Drosha processes pri-miRNA to pre-miRNA, which is exported to the cytoplasm. Dicer processes pre-miRNA to miRNA duplex. As RISC binds, the passenger strand is degraded and the guide strand with RISC induces translational inhibition and/or mRNA degradation. Bottom: siRNA pathway. Dicer processes dsRNA precursors to siRNA duplexes. Upon RISC binding, the passenger strand is degraded and the guide strand with RISC induces mRNA degradation in the cytoplasm or enters the nucleus to induce nuclear RNAi. Dashed box: some organisms, e.g. C. elegans can synthesize secondary siRNA by RdRP.

possibly a H3K9 methyltransferase, is required for the accumulation of H3K9me3 at the siRNA targeted sites on the genome (Spracklin et al., 2017). This indicates that H3K9 trimethylation induced by siRNA is an important step for RNAi inheritance (Spracklin et al., 2017). Although the epigenetic
modification are all dependent on NRDE proteins (Nuclei RNAi Defective) for transportation of silencing information and locating the target locus, a specific germ line nucleus-expressed protein named HRDE-1 (Heritable RNAi Defective 1) is required for inheritance of the silencing signal (Ashe et al., 2012; Buckley et al., 2012). Disturbing the function of HRDE-1 leads to abnormal germ cell formation and function (Buckley et al., 2012). The RNAi inheritance machinery may play a role in maintaining germ cells by transmitting selected small RNAs to progeny (Spracklin et al., 2017). However, the machinery behind it has not been clearly understood.

When RNAs Meet the Membrane System

Initially, people considered the RNA silencing process as taking place in cytoplasmic, non-membrane-associated areas. However, more recently, people found that the rough ER serves as the site of translation of most proteins and is also the site of miRNA- and siRNA-mediated gene silencing (Lerner, 2003; Li et al., 2013; Stalder et al., 2013). In Arabidopsis, the main miRNA Argonaute atAgo1 was found to partially co-localize with rough ER (Brodersen and Sakvarelidze-achard, 2012; Li et al., 2013). Drosophila studies showed that miRISC is associated with polysomes and co-sediments with ER, indicating the function of ER in the silencing process (Wu et al., 2013). For siRNA mediated silencing, a study from human cells showed that most of loaded hsAgo2 associates with ER (Stalder et al., 2013).

The connection between the small RNA regulation process and rough ER indicates the possibility of participation of other membrane compartments in small RNA processing and transport. Indeed, as an important part of the transport and sorting system, late endosomes/MVBs were found to be a possible site for RISC assembly and disassembly (Gibbings et al., 2009). The study showed that the two core factors in RISC, GW182 and AGO2, colocalize with MVBS in vivo (Gibbings et al., 2009). In Drosophila, when the formation of MVBS is blocked by knock down of ESCRT proteins, small RNA induced gene silencing is impaired and GW-bodies are lost (Gibbings et al., 2009). Conversely, blocking the MVB to lysosome fusion results in accumulation of GW-bodies and enhanced silencing effect (Lee et al., 2009). It has been shown that exosomes derived from MVBS contain miRNA, mRNA and repressible-mRNAs (Gibbings et al., 2009). However, how RNAs are sorted into MVBS is still poorly understood. Analysis of protein in exosomes showed that GW182 and AGO2 are present, and the level of GW182 is much higher than that of AGO2 (Gibbings et al., 2009; Kosaka et al., 2010). It indicates that the GW182 may be the factor that mediates the sorting of RNA into ILVs in late endosomes/MVBs (Kosaka et al., 2010).
Systemic RNAi

More evidence connecting the cell membrane transport system with small RNA comes from the study of systemic RNAi in *C. elegans*. To induce gene silencing in *C. elegans* by RNAi is convenient, since no matter where or how one introduces dsRNA, the silencing signal can reach the target cell and induce gene silencing (systemic RNAi). In the laboratory, one can induce systemic RNAi by feeding *C. elegans* with bacteria expressing dsRNA, soaking *C. elegans* in a solution that contains dsRNA, injecting the dsRNA into *C. elegans*, and even expressing the dsRNA endogenously (Fire et al., 1998; Tabara et al., 1998; Timmons et al., 2001; Winston et al., 2002). From the Systemic RNAi Defective forward genetics screen (sid), several proteins required for transport of silencing information were found (Winston et al., 2002). Most of the proteins are membrane associated. SID-1 is a multipass transmembrane protein that functions in taking up extracellular long dsRNAs (Winston et al., 2002). SID-2 sits on the lumenal membrane of the intestine and imports dsRNA from the environment, likely in an endocytic process (McEwan et al., 2012; Winston et al., 2007). SID-3 is a tyrosine kinase required for the import of silencing information (Jose et al., 2012). SID-5 has been suggested to function in export of silencing information since expression of SID-5 in the intestine, which is the first barrier for environmental dsRNA to enter the worms, can rescue the reduced RNAi in body-wall muscle of sid-5 mutant animals (Hinas et al., 2012). However the detailed function is still unclear. SID-5 is a 67 amino acid single transmembrane protein expressed in most tissues in *C. elegans* (Hinas et al., 2012). Immunohistochemistry showed that SID-5 forms small punctae associated with late endosomes/MVBs in *C. elegans* intestinal cells (Hinas et al., 2012). In another screen, rsd-2, rsd-3, and rsd-6 were identified as RNAi Spreading Defective (rsd) genes (Tijsterman et al., 2004). In a recent report, rsd-3 was found to be important for import of silencing RNAs (Imae et al., 2016). However, the function of rsd-2 and rsd-6 appears related to secondary siRNA amplification and germ line cell immortality rather than systemic RNAi (Sakaguchi et al., 2014). The entire picture of how RNA is taken up and exported by cells is still under discovery (Figure 3) (Hinas et al., 2012; Imae et al., 2016; Jose et al., 2012; McEwan et al., 2012; Winston et al., 2002, 2007).
Figure 3. Possible model of RNA transport in *C. elegans*. Environmental dsRNA is imported into intestinal cells with assistance of SID-2. The imported RNAi signal is sorted into late endosomes/MVBs. With the assistance of SID-5, the RNAi signal is likely exported to the extracellular space. SID-1 facilitates the uptake and/or release of extracellular RNAi signal. SID-3 and RSD-3 are also involved in the import process of extracellular RNAi signal. The imported RNAi signal then silences the target.
Caenorhabditis elegans, a Nice Model Organism

An Overview of Caenorhabditis elegans

*Caenorhabditis elegans* is a nematode (roundworm) species that feeds on microbes. It is a transparent free-living animal around 1 mm long (Figure 4) (Corsi et al., 2015). The natural living environment for *C. elegans* is temperate soil all over the world, especially in rotting matters, such as fruit (Félix and Braendle, 2010). *C. elegans* is easily grown in laboratory environment on agar petri dishes with *Escherichia coli* (*E. coli*) as the most common food source (Corsi et al., 2015). The self-fertilizing hermaphrodite is the major population in a group of *C. elegans* (Anderson et al., 2010). Males are born at a low frequency (Anderson et al., 2010). Treatment of hermaphrodites with high temperature leads to generation of male progeny at a higher frequency (Anderson et al., 2010). By mating the two sexes, it is easy to transfer a mutation or transgene between strains (Brenner, 1974).

![Figure 4. Caenorhabditis elegans. Top: Bright field image of a C. elegans hermaphrodite. Bottom: Anatomical diagram of a C. elegans hermaphrodite.](image)

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Even though *C. elegans* is small, it has simple tissues and organs constructed by around 1000 somatic cells (959 cells for hermaphrodites and 1031 cells for males), such as muscle, nervous system, reproduction system, epidermis and gastrointestinal tract (Corsi et al., 2015). In addition, the transparent body makes it convenient to be observed under microscopes. Researchers cannot only observe the behavior change in a mutant worm but also see how the organs differ. Furthermore, *in vivo* expressed proteins with fluorescent tags can be clearly seen by microscopy in a living worm (Corsi et al., 2015).

The generation time of *C. elegans* is 3-4 days, with a lifespan of approximately 2-3 weeks. There are several stages of *C. elegans* development: embryonic stage, four larval stages (L1-L4) and adulthood (Figure 5)(Corsi et al., 2015; Jorgensen and Mango, 2002). During the transition between each stage, it sheds the old cuticle. The L1 larvae can enter a dauer stage with a lifespan up to month when the living conditions become harsh, such as during food limitation. The larva recovers to the L4 stage in approximately two days when the living conditions become suitable (Byerly et al., 1976).

*Figure 5.* Life cycle of a *C. elegans* hermaphrodite at 25°C. Adapted from Jorgensen and Mango (2002).
The genome of C. elegans has also been studied extensively. It has five pairs of autosomes and one pair of sex chromosomes (Jorgensen and Mango, 2002). The genome size is around 100 Mb, and there are over 20,000 protein-coding genes, of which many have homologs in other organisms (Shaye and Greenwald, 2011). When compared with the human genome, about a third of the C. elegans genes are conserved (Shaye and Greenwald, 2011). The C. elegans Genetic Center (CGC) stores and distributes many worm strains, for example carrying mutations that researchers may be interested in (Nigon and Felix, 2017). Because worms are easy to cultivate under laboratory conditions and their life cycle is short, studying gene function in worms is a fast way to provide preliminary data for functional studies in higher animals.

C. elegans as a Model Organism in the Study of RNA Transport

The membrane transport pathway in C. elegans has been well studied. Even though there are still a lot of questions that need to be answered, C. elegans is quite convenient as a model to study trafficking related topics. First, the C. elegans genome contain many membrane transport related genes shared with humans (Gallegos et al., 2012; Pereira-Leal and Seabra, 2001; Sato et al., 2011). Second, strains with markers of most of the membrane related compartments are available from CGC and individual researchers. Third, several forward and reverse genetic screens for factors that function in the membrane transport pathway have been carried out in C. elegans. One of these is the Receptor Mediated Endocytosis (RME) screen searching for defects in oocyte uptake of intestine secreted yolk protein (Balklava et al., 2007; Grant and Hirsh, 1999). Using this assay, 11 genes were identified in a forward genetics approach, and hundreds of gene candidates were identified by a genome wide RNAi screen (Balklava et al., 2007; Grant and Hirsh, 1999). Another classic screen is the Coelomocyte Uptake Defective (CUP) screen (Fares and Greenwald, 2001). The coelomocyte is a type of cell that continuously and non-specifically takes up fluid from the worm body cavity. The CUP screen identified mutants showing a defective uptake of GFP from body cavity. By using this screen, 14 genes important for the endocytosis were identified, including three genes overlapping with the RME screen (Fares and Greenwald, 2001). Other tissues of C. elegans are also being used to study membrane transport, such as intestinal cells. Gut granules are intestine-specific vesicles containing birefringent material, which have been hypothesized to be lysosome-related organelles (Clokey and Jacobson, 1986; Hermann et al., 2005). A screen named gut granule loss (glo) was set up searching for mutants defective in gut granule biogenesis (Hermann et al., 2005). Except for the genes already identified by other screens, they found a set of four genes that functions in biogenesis of gut granules (Hermann et al., 2005). The platform of membrane transport is building up gradually which made us choose C. elegans as our model organism. In the world of RNA, C.
*elegans* is used broadly to study function of RNAs. The very first miRNA *lin-4* was found in *C. elegans*, opening the door for functional studies of small ncRNA in gene regulation in 1993 (Lee et al., 1993; Wightman et al., 1993). RNAi was initially characterized and defined in *C. elegans* (Fire et al., 1998), because of which Andrew Z. Fire and Craig C. Mello got the Nobel Prize in 2006. Their discovery of systemic RNAi showed researchers a tool to deliver artificial dsRNA to its target conveniently and opened up for subsequent findings on RNA transport proteins such as SID-1 (Winston et al. 2002). Taken together, *C. elegans* is a highly suitable model organism to study RNA transport.
Present Investigation

We use *C. elegans* as our model organism to study intracellular/cell autonomous RNAi and intercellular transport of RNA in animals. In *C. elegans*, SID-5 is a factor important for systemic RNAi, possibly functioning in export of silencing information (Hinas et al., 2012). The small transmembrane protein is expressed in almost all types of cells in the worm. In intestinal cells, SID-5 associate with late endosomes (Hinas et al., 2012). To study the detailed function of SID-5, thereby characterizing a part of the RNA transport pathway, our research started from searching for proteins that interact with SID-5 by a yeast two-hybrid (Y2H) screen modified for membrane proteins.

Identification of SID-5 Interacting Proteins by Membrane Y2H Approach (Papers I, II and III)

SID-5 is predicted to be a single-span transmembrane protein, and we therefore used the split-ubiquitin yeast two-hybrid (Y2H) system modified for detecting the interaction between a membrane bait protein and membrane proteins/cytoplasmic proteins (Staglarj et al., 1998), (Figure 6). In a classic Y2H screen, a transcription factor is split into two halves, which are fused to the bait and prey, respectively. When bait and prey interact, the two halves of transcription factor get close enough to reunite and the transcription of reporter gene(s) is started (Coates and Hall, 2003). However localizing a membrane protein to the nucleus could lead to conformation changes and other problems. The split-ubiquitin method differs from the classical Y2H method in that the ubiquitin protein is split into two halves and fused to the bait and prey instead of the two halves of transcription factor. The entire transcription factor is fused to one of the ubiquitin halves (Staglarj et al., 1998). Our bait, SID-5, was fused to the C terminal half of the split ubiquitin protein (Cub), in turn fused with the entire transcription factor (LexA-VP16). A *C. elegans* cDNA library, as the prey, was fused to the other half of the ubiquitin (Nub). The bait and prey were co-expressed in NMY51 yeast cells. Interaction of the SID-5 bait construct with a prey protein from the cDNA library leads to the reconstitution of the ubiquitin protein, which is then recognized by ubiquitin specific proteases (UBPs). UBPs degrade the reconstituted ubiquitin protein, releasing the LexA-VP16 transcription fac-
tor, which moves to the nucleus and activates transcription of the reporter genes. This results in yeast colony growth on selective medium. The reporter genes used in the system are two auxotrophic growth markers, HIS3 and ADE2, and lacZ. The first two auxotrophic markers enable yeast cells containing two interacting proteins to grow on defined minimal medium lacking histidine and adenine. The lacZ gene encodes β-galactosidase, causing colonies with a positive interaction to turn blue in a β-galactosidase test.

Figure 6. Split-ubiquitin membrane Y2H system (DUAL System) Bait construction: SID-5 was fused to the Cub-LexA-VP16 (C terminal half of ubiquitin fused to the LexA-VP16 transcription factor). Prey construction: a cDNA library was fused to the Nub (N terminal half of ubiquitin). Interaction of bait and prey when co-expressed in yeast NMY51 cells leads to reconstitution of the ubiquitin protein, which is then recognized by ubiquitin specific proteases (UBPs). The UBPs degrade the ubiquitin and release the transcription factor, which then activates transcription of the reporter genes. Left, no interaction. Middle, interaction between SID-5 and another transmembrane protein. Right, interaction between SID-5 and a cytosolic protein.

The screen gave us 533 colonies, of which 192 colonies were tested for false positives by re-transforming the bait and extracted prey plasmids into NMY51. After sequencing the plasmids extracted from the 148 colonies that still exhibited a positive interaction of SID-5 bait and prey in the re-transformation test, 32 were found to contain a piece of or full-length C. elegans genes (Table 1). Even though we performed re-transformation and tested the β-galactosidase activity, the yeast two-hybrid screen can still give us false positives. In our study, to reduce the risk of candidates being false positives, we also assay the RNAi efficiency upon mutation or RNAi knock down of the corresponding genes. In that way, we can at least know if the
function of the candidates is related to RNAi. If so, the candidates may be more likely to interact with SID-5.

Table 1. Proteins interacting with SID-5 in the Y2H screen

<table>
<thead>
<tr>
<th>ER-associated proteins (known or predicted)</th>
<th>Conserved in human</th>
<th>Not conserved in human</th>
</tr>
</thead>
<tbody>
<tr>
<td>R05D11.5, UBC-6 (Ub-conjugating enzyme), SEC-22* (SNARE), ODR-4, NBET-1 (SNARE), Y43F8C.7</td>
<td>F17E9.5, NSPA-3*, SUP-10, T04G9.7, T21C12.8, Y110A7A.2, M02B1.4, ZK688.11, C08F11.11</td>
<td></td>
</tr>
<tr>
<td>Soluble proteins</td>
<td>CKR-3 (CDK5RAP3-like), RPIA-1 (ribose 5-phosphate isomerase), T05F1.11 (thioredoxin-like), SKP-1 (SKI-interacting), EFT-3/EEF-1A.1 (elongation factor), DNJ-22 (DnaJ, chaperone), C12D8.1* (RNA-binding), KDP-1 (KASH domain)</td>
<td></td>
</tr>
</tbody>
</table>

*RNAi phenotype

The Conserved SNARE SEC-22 Localizes to Late Endosomes and Negatively Regulates RNA Interference in *Caenorhabditis elegans* (Paper I)

In recent years, more and more evidence has connected RNA transport with membrane compartments. It has been shown that rough ER is an important site for siRNA mediated silencing in human cells (Stalder et al., 2013). Endosomes, as a big sorting center in cells, can also affect the RNAi efficiency. Disturbing the balance between late endosomes/multivesicular bodies (MVBs) and lysosome can affect RNAi efficiency significantly (Gibbings et al., 2009; Lee et al., 2009). In Paper I, the SNARE protein SEC-22 was identified as a SID-5 interacting protein. Sequencing of the corresponding Y2H clone showed that it was the 44 aa at the C terminal synaptobrevin domain of SEC-22, including the entire transmembrane part, that showed interaction with SID-5. In our RNAi tests, SEC-22 functions as a negative regulator of RNAi. Despite the central role of this protein family in the membrane trafficking system, it was the first time showing that a SNARE protein can function to regulate RNAi.
SEC-22 Negatively Regulates RNAi Mainly Cell-Autonomously

We fed sec-22 mutant worms and wild type worms with bacteria expressing dsRNA against *C. elegans* target genes. By comparing the phenotype of mutant and wild type worms, we can know whether SEC-22 affects RNAi. In this assay, the sec-22(ok3053) deletion mutant showed an enhanced RNAi phenotype when we performed feeding RNAi targeting epidermis-expressed *dpy-13* gene, body wall muscle-expressed *unc-22* gene and intestine-expressed *act-5* gene. The enhanced RNAi could also be observed when feeding RNAi targeting a body wall muscle-expressed GFP transgene. The enhanced RNAi phenotype was confirmed using a mutant carrying an independent mutant allele, sec-22 (gk887451) which substitutes glutamic acid 2 for lysine. The enhanced RNAi effect could be partially rescued by an extrachromosomal array carrying mCherry::SEC-22, which was used to investigate the localization of SEC-22 (see below). Furthermore, overexpression of SEC-22 in body wall muscle cells in wildtype worms led to reduce RNAi efficiency. Together, these results show that SEC-22 negatively regulates RNAi. The enhanced *unc-22* RNAi phenotype could be rescued by a body wall muscle-expressed SEC-22 extrachromosomal array, but was only partially rescued by a intestine-expressed SEC-22 extrachromosomal array, indicating that SEC-22 mainly functions in cell-autonomous RNAi.

**In vivo** Localization of mCherry::SEC-22

To study the *in vivo* localization of SEC-22, we constructed an mCherry::SEC-22 extrachromosomal array. The rescuing mCherry::SEC-22 is expressed in pharynx, intestine, body wall muscle, tail, vulva, spermatheca, and coelomocytes in *C. elegans*. Since the localization of mCherry::SEC-22 appeared concentrated to vesicle structures, we introduced it into strains expressing various GFP fusion proteins known to localize to specific membrane compartments (Chen et al., 2006; Kang et al., 2007; Treusch et al., 2004). We observed mCherry::SEC-22 localizing significantly to late endosomes marked with GFP::RAB-7 and LMP-1::GFP, but not with the LysoTracker stained acidified lysosomes, nor with early endosome marker (GFP::RAB-5), Golgi marker (Mans::GFP), autophagosome marker (LGG-1::GFP), or recycling endosome marker (GFP::RAB-11).

Loss of SEC-22 Affects Endosome Maturation or Formation

Since mCherry::SEC-22 displayed late endosome/MVB localization, we wanted to ask what would happen to this important sorting center if we knock out sec-22? We therefore investigated the morphology of late endosomes/MVBs marked with LMP-1::GFP in worms carrying the sec-22 deletion allele. We found that in the sec-22(-) strain, LMP-1::GFP positive vesicles were enlarged. The abnormal appearance of LMP-1::GFP marked vesi-
cles indicates that the late endosome/MVBs may not be properly acidified and degraded by lysosomes.

Function of SEC-22 in RNAi Process is SID-5 Dependent
We also wanted to study the relationship between SEC-22 and SID-5 in the RNAi pathway. We first studied SID-5 localization by immunohistochemistry in LMP-1::GFP worms carrying the sec-22(-) allele. SID-5 punctae were detected around the enlarged LMP-1::GFP marked vesicles, showing that localization of SID-5 is not affected by loss of sec-22. We next constructed a sec-22(-) sid-5(-) double mutant worm and carried out feeding RNAi to see if there was any difference in RNAi efficiency among wild type worms, the single mutant worms and the double mutant. This experiment showed no significant difference between the phenotype of the double mutant and sid-5(-) single mutant worms. Thus, SEC-22 functions in a SID-5 dependent manner in the RNAi process. SEC-22 may function in degradation of vesicles that SID-5 localizes to or inhibit SID-5, thus decreasing RNAi efficiency.

RNA Transport Protein SID-5 Interacts with Multiple SNAREs and Affects Membrane Trafficking in C. elegans Intestinal Cells (Paper II)
We identified the SNARE protein SEC-22 as a negative regulator of RNAi in Paper I and wanted to study the function of SID-5 and SEC-22 in RNAi, RNA transport, and membrane trafficking, in more detail. From two additional membrane Y2H screens, we found that SID-5 interacts with multiple syntaxin SNARE proteins, whereas SEC-22 only interacts with SYX-6. syx-6 mutants display enhanced RNAi phenotype indicating that the function of SYX-6 and SEC-22 is in the same route of vesicle transport. Furthermore, we found that loss of SID-5 leads to late endosome maturation defects.

Sid-5 Interacts with Multiple Syntaxin SNARE Proteins in Membrane Y2H Assay, While SEC-22 Only Interacts with SYX-6
In the previous membrane Y2H screen (Paper I), we identified the R-SNARE SEC-22 as one of the SID-5 interacting protein candidates. R-SNAREs usually form a trans-SNARE complex with certain Q-SNAREs, including syntaxins, on other membranes. We therefore wanted to identify the interacting syntaxins of SEC-22. Meanwhile we wanted to test if SID-5 can also interact with syntaxins. So we performed two membrane Y2H assays using SEC-22 and SID-5 as baits. For the preys, we cloned nine of the ten C. elegans syntaxins, all except for SYX-16. SEC-22 showed a strong
interaction with SYX-6 in the assay. SID-5 showed interaction with several syntaxins; SYX-1, SYX-3, SYX-4, SYX-6, SYX-7 and SYX-17.

SID-5 and SEC-22 Affect Endosome Maturation or Formation
Since mCherry::SEC-22 and SID-5 have late endosome/MVB localization, we wanted to ask what would happen to this important sorting center if we knock out sec-22 or sid-5? We investigated the localization and morphology of late endosomes/MVBs marked with GFP::RAB-7 and LMP-1::GFP in worms carrying sec-22(-) or sid-5(-) mutant alleles, respectively. Both in the sec-22(-) and sid-5(-) mutants, LMP-1::GFP positive vesicles were enlarged indicating the lysosome could not function properly. In the sid-5(-) mutant, the enlarged vesicles are more concentrated to the basolateral membrane. For GFP::RAB-7, in wild type worms, the majority localizes to the limiting membrane of late endosomes, uniformly sized vesicles, and less as small punctae near apical/luminal membrane. In the sec-22(-) mutant, the GFP::RAB-7 positive vesicles are larger than in wild type worms. Furthermore, the localization of the GFP::RAB-7 is more internalized in the vesicle rather than sitting on the limiting membrane. It indicates that the fusion of late endosomes/MVBs with lysosome may be affected. On the other hand, the sid-5(-) mutant displayed the opposite pattern of GFP::RAB-7 positive vesicles compared to that in wild type worms. In the sid-5(-) mutant, most of the GFP::RAB-7 positive vesicles are small punctae near the apical/luminal membrane, and a small amount localizes to the limiting membrane of late endosomes. A possible explanation for this phenotype is that the homotypic fusion of late endosomes/MVBs is affected in the sid-5(-) mutant.
	syx-6 Negatively Regulates RNAi
To test if syx-6 affects RNAi, we performed feeding RNAi on two different syx-6 mutants. The syx-6(tm4733) deletion mutant displayed an enhanced RNAi phenotype following RNAi against the intestine-expressed act-5 gene. An independent mutant, syx-6(gk436540), which has a single nucleotide change that substitutes glutamine 64 for an amber stop (UGA) codon, also displayed the enhanced RNAi phenotype in the same experiment, additionally, it showed the enhanced RNAi phenotype in a dpy-13 (epidermis) RNAi experiment.

mCherry::SYX-6 Localizes to Late Endosomes and Reticulate Structures
To study the subcellular localization of SYX-6, we constructed an intestine-expressed mCherry::SYX-6 extrachromosomal array. We then assayed the colocalization of mCherry::SYX-6 with GFP::RAB-7 and LMP-1::GFP,
showing that mCherry::SYX-6 has a similar localization pattern as mCherry::SEC-22 in intestinal cells. In addition to late endosomes/MVBs, mCherry::SYX-6/LMP-1::GFP positive structures displayed a reticulate pattern, probably cytoskeletal fibers. mCherry::SYX-6 also colocalized with GFP::RAB-7, but much less than with LMP-1::GFP. Furthermore, mCherry::SYX-6 showed almost no overlap with LysoTracker Green stained vesicles. mCherry::SYX-6 positive foci could often be found near Golgi mini stacks labeled by α-mannosidase::GFP, indicating it may be recycled from late endosomes/MVBs or participate in the transport between Golgi and late endosomes/MVBs.

A Putative RNA-Binding Protein C12D8.1 Negatively Regulates Inheritance of RNAi in Caenorhabditis elegans (Paper III)

In C. elegans, worms can pass silencing information to their progeny, and the effect can last for a few generations (Fire et al., 1998; Grishok et al., 2000). In addition to RNA transport proteins, the process needs factors that shuttle cytoplasmic silencing information to the nucleus and the cooperation of the factors that function in the nuclear RNAi pathway (Grishok et al., 2000). In a sequential RNAi test, we found that RNAi against C12D8.1 or either of two other genes results in a reduced RNAi phenotype. However, C12D8.1 mutants showed an enhanced RNAi phenotype. In the follow up tests, the phenotype of C12D8.1 mutants indicated that it affects RNAi inheritance, possibly by interfering with the uptake of new environmental RNA by the progeny.

A Sequential RNAi Screen Identifies C12D8.1 As a Regulator of RNAi

To test the effect on RNAi efficiency of more candidates from the SID-5 Y2H screen, we performed a sequential RNAi screen designed to be sensitive both for enhanced and reduced RNAi. The sequential RNAi screen contains three steps. The first step is silencing the gene of interest. We fed L4 larval stage worms (P0) expressing GFP in body wall muscle cells with bacteria expressing dsRNA targeting a gene of interest. The second step is silencing GFP expressed in body wall muscle cells in the offspring of P0s. When the (F1) offspring reached L4 larval stage, we moved them to another petri dish with bacteria expressing dsRNA targeting the GFP transgene. The third step is quantification of number of body wall muscle cells still expressing GFP. The worms we use in the quantification are young adult F2 worms. Based on the set up, the screen cannot only detect the core genes involved in the RNAi machinery, but also genes that function in environmental RNAi.
uptake, transport, and inheritance. After quantification, RNAi knock down of C12D8.1, nspa-3, and T02G5.12/mct-5 showed reduced silencing phenotype. C12D8.1 encodes a putative RNA-binding protein; nspa-3 encodes a 34 aa nematode-specific protein of unknown function; and T02G5.12/mct-5 encodes a homolog of human monocarboxylate transporter 14. Among the three candidates, we chose to start with the putative RNA-binding protein C12D8.1.

C12D8.1 encodes a putative RNA-binding protein. It has three paralogs and its closest mammalian homolog is human far-upstream element binding protein 1 (FBP1/FUBP1) (Zhang and Chen, 2012). C12D8.1 is predicted to have four RNA or ssDNA-binding K homology (KH) domains and a conserved C terminal domain of unknown function, DUF1897 (Sonnhammer et al., 1997). It has three annotated coding sequences, C12D8.1a, b, c (WormBase web site, http://www.wormbase.org, release WS258, 2 April 2017). C12D8.1a and c are conserved in C. briggsae and translation of C12D8.1a is supported by protein mass spectrometry (WormBase web site, http://www.wormbase.org, release WS258, 2 April 2017). The C12D8.1c isoform is identical to amino acids 42-589 of the C12D8.1a isoform. The C12D8.1a isoform has a 41 aa N-terminal flexible extension compared to c.

A C12D8.1::mCherry Fusion is Broadly Expressed and Localizes to Both Nucleus and Cytoplasm

To investigate the expression pattern of C12D8.1, we constructed a transgenic C12D8.1::mCherry translational fusion driven by C12D8.1 upstream sequence. Since the transgene can give rise to all three isoforms, the localization of C12D8.1::mCherry could represent its expression pattern, but not specific for a certain isoform. We found C12D8.1::mCherry to be expressed in many type of cells in C. elegans such as pharynx, body wall muscle, intestine, seam cells, epidermis, and neurons. It has both nuclear and cytoplasmic localization. In the cytoplasm, C12D8.1::mCherry localizes to perinuclear foci. In embryos, it localizes to the nucleus, sometimes forming nuclear foci. Furthermore, by using cNLSMapper (Kosugi et al., 2009), we predicted the highly disordered N-terminal sequence in C12D8.1a isoform to localize to both the nucleus and cytoplasm. The result indicates that C12D8.1a is present in both nucleus and cytoplasm and that C12D8.1c exists in the cytoplasm.

C12D8.1 Mutants Display Enhanced RNAi Phenotype

To further study the effect of C12D8.1 on RNAi, we fed C12D8.1 mutant worms with bacteria expressing dsRNA targeting GFP expressed in body wall muscle, endogenous body wall muscle-expressed gene unc-22 and epidermis expressed gene dpy-7, respectively. The C12D8.1 mutant showed an
enhanced RNAi phenotype when the targets were in the body wall muscle, but no significant difference when the target was in the epidermis. The enhanced RNAi phenotype against body wall muscle-expressed GFP was confirmed in an experiment using another, independent, mutant strain. The enhanced RNAi phenotype is the opposite to our observation of reduced RNAi when using worms with \textit{C12D8.1} knocked down by RNAi as subjects in feeding RNAi test.

The Enhanced RNAi Phenotype in \textit{C12D8.1} Mutant is Due to Enhanced Inheritance of Silencing Signal

To determine if inheritance is the reason why worms that carry a mutation in \textit{C12D8.1} display a different RNAi efficiency phenotype compared to worms with \textit{C12D8.1} silenced by RNAi, we did RNAi feeding with different strategies. In the RNAi feeding performed within one generation, which removes the effect of inheritance, \textit{C12D8.1} mutant worms did not show any change in RNAi phenotype. We then checked the number of RNAi affected progeny, fed with bacteria containing empty vector, from the \textit{C12D8.1} mutant parental worms, that had been fed bacteria expressing dsRNA with a target. Here, \textit{C12D8.1} mutant worms showed an enhanced response to inherited RNAi. With a similar setup but feeding the second generation of worms with bacteria expressing dsRNA with a different target, we found the \textit{C12D8.1} mutant to be less sensitive to the second generation RNAi. We also fed the worms with bacteria expressing dsRNA against a different target in the same gene region, showing that wild type worms are less susceptible to a second RNAi treatment. Taken together, \textit{C12D8.1} likely affects RNAi efficiency by interfering with RNAi inheritance.

Other SID-5 Interacting Candidate Proteins From Y2H Screen (unpublished data)

In addition to SEC-22 (Papers I and II) and \textit{C12D8.1} (Paper III), several candidates in the list are waiting to be studied. Most of the genes from our Y2H screen are not very well classified or with unknown functions in \textit{C. elegans}, but functions of the homologs in other organisms are interesting. For example, \textit{D1022.1} (\textit{ubc-6}) encodes an E2 ubiquitin conjugation enzyme, the human homolog of which is involved in ER-associated protein degradation localized to the cytosolic side of ER (Jones et al., 2001). \textit{T05F1.11} encodes a protein containing an F-box motif which could function as a part of E3 ubiquitin protein ligase complex (Kipreos and Pagano, 2000). In a previous study, SID-5 was found to form cytoplasmic punctate that associate with late endosomes (Hinas et al., 2012). However, additional subcellular locations of SID-5 have not yet been defined. In our Y2H screen, UBC-6 and
SEC-22 are not the only ER-associated proteins; NBET-1, ODR-4, R05D11.5, and Y43F8C.7 are all putative ER-associated proteins. Like SEC-22, NBET-1 is also a SNARE protein, the yeast homolog of which binds to Sec23/Sec24 complex and functions in ER-Golgi transport (Ortiz et al., 2014). ODR-4 is a chemosensory neuron-expressed protein, which facilitates odorant receptor localization (Chen et al., 2014; Dwyer et al., 1998). R05D11.5 and Y43F8C.7 are two putative ER-associated proteins with unknown function. Even though we do not know how SID-5 functions with these proteins, the localization indicates that SID-5 could also localize to ER membrane, although this hypothesis needs more evidence. Other interesting candidates include a nuclear envelope protein KDP-1 required for meiotic progression in gonad and normal cell cycle progression in embryo (Bank and Gruenbaum, 2011). The functions behind the candidate genes are not limited to the RNAi process. For most of them we have not done any research, thus the authenticity of the interaction needs to be tested. If the interactions turn out to be true, SID-5 may not only contribute to RNA transport, but may take part in other cellular pathways. Alternatively, the candidates may have hidden roles in RNAi.
Conclusions and Future Perspectives

In Papers I and II, we identified the SNARE proteins SEC-22 and SYX-6 as interacting with SID-5, a protein required in systemic RNAi, in Y2H screens. We also found that SID-5 interacts with multiple syntaxin SNARE proteins whereas SEC-22 only interacts with SYX-6 in our Y2H. In the localization investigation, both mCherry::SEC-22 and mCherry::SYX-6 associate with late endosomes/MVB markers. Furthermore, the mCherry::SYX-6 display a reticulate pattern and the positive foci are near Golgi mini stacks. Loss of sid-5 or sec-22 leads to late endosomes/MVB maturation defects. All our results connect RNA transport tightly with the membrane transport system, but the details on how they cooperate are still unknown. Through RNAi knock down of some of the candidates from the SID-5 Y2H screen followed by a feeding RNAi assay we showed that the putative RNA binding C12D8.1 affects RNAi (Paper III). The opposite RNAi phenotypes - enhanced and reduced, respectively, showed by the C12D8.1 mutant and RNAi knock down attracted our attention. The mCherry::C12D8.1 fusion protein localizes both in the cytoplasm and the nucleus. After carrying out feeding RNAi experiments with different strategies, we found that C12D8.1 can affect RNAi inheritance in C. elegans. However, we have not yet figured out the pathway in which C12D8.1 functions. Here I present my hypotheses as well as the follow up research that could be done.

SID-5, SNAREs, RNAi and Vesicles (Paper I and II)

We started our research with SID-5, the small transmembrane protein required for efficient systemic RNAi (Hinas et al., 2012). Through Y2H screens, using SID-5 as bait and C. elegans cDNA library as preys, we got several candidates including the R-SNARE proteins SEC-22. Generally, in the membrane fusion step of intracellular transport of vesicles, R- and Q-SNAREs form a four-helix bundle complex, keeping the two membrane structures together to promote fusion. Aiming to figure out the pathway of intracellular transport of RNAi silencing signals, we wanted to identify the Q-SNAREs that interact with SEC-22. In the other two Y2H assays we did in Paper II, we used the Q-SNARE family syntaxins as preys and SID-5 and SEC-22 as baits, respectively. From the two tests, we identified multiple SNAREs that interact with SID-5, and only SYX-6 as interacting with SEC-
22. There are no sequence similarities among the six syntaxins that interact with SID-5, and we therefore assume that the interaction may happen through SNARE motifs, linker and/or transmembrane domain.

How Does SID-5 Function with SEC-22 and SYX-6?

Through the data we have until now, we cannot say the interaction happens directly between SID-5 and SEC-22 or SID-5 and SYX-6. But results from our subsequent experiments indicate that the interaction could happen. First of all, both sec-22 and syx-6 mutants showed an enhanced RNAi phenotype, which indicates that the two proteins participate in the RNAi regulation process. In an additional study, expressing sec-22 in worm intestinal cells could partially but significantly restore the enhanced RNAi phenotype in body wall muscles. But expressing sec-22 in body wall muscle can completely rescue the enhanced RNAi phenotype targeting the same tissue. This indicates SEC-22 may function more in cell autonomous RNAi than in transport through intestinal cells. Second, in a previous study, sid-5 mutants showed a reduced RNAi phenotype (Hinas et al., 2012). Expressing sid-5 in C. elegans intestine can restore the reduced RNAi phenotype in body wall muscle of sid-5 mutant animals, which indicates that SID-5 functions in systemic RNAi, probably in exporting the silencing signal (Hinas et al., 2012). The opposite RNAi phenotypes in sid-5 mutants versus the SNARE mutant strains, suggests that the function of SID-5 could be to protect the RNAi signal from degradation mediated by SEC-22 and SYX-6. In a sec-22 sid-5 double mutant strain, the worms displayed a sid-5 mutant like reduced RNAi phenotype, indicating that SID-5 is required for the function of SEC-22 in RNAi. We have not yet tested the RNAi phenotype in a sid-5 syx-6 double mutant worm. Combined with the results that SEC-22 interacts with SYX-6 in a Y2H screen, we assume that such a double mutant would also show a sid-5 like RNAi phenotype.

Furthermore, mCherry::SYX-6 positive foci were often found near Golgi and almost completely colocalized with late endosomes/MVBs and lysosome marker LMP-1::GFP but not with acidified lysosomes (Chotard et al., 2010; Treusch et al., 2004). This indicates that SYX-6 may function in vesicle transport between Golgi and endosomes. LMP-1::GFP/mCherry::SYX-6 also showed a reticulate pattern, which could represent the vesicles under transport along the cytoskeleton or ER tubules that associate with cytoskeleton. mCherry::SEC-22 localizes to late endosomes but also showed a reticular localization pattern (Zhao, Holmgren and Hinas, unpublished). For the interaction between SEC-22 and SYX-6, a hypothesis would be that SYX-6 and SEC-22 may form a trans-SNARE complex to promote the fusion of vesicles derived from Golgi with late endosomes. In sec-22 mutant worms, the late endosome/MVB fusion with lysosome may be affected as the worms have both enlarged GFP::RAB-7 and LMP-1::GFP marked late endosomes/MVBs. Normally, GFP::RAB-7 localizes to the limiting membrane of
late endosomes/MVBs. But in the sec-22 mutant worm, GFP::RAB-7 is internalized in the late endosomes/MVBs. This indicates that the vesicles are not acidified since GFP is reduced in acidic environment (Nicot et al., 2006). Knock down of syx-6 has been shown to lead to enlarged LMP-1::GFP marked lysosomes in coelomocytes (Luo et al., 2011). Taken together, this indicates that the function of SEC-22 and SYX-6 could be related to fusion between late endosomes/MVBs and lysosomes. Linked to the RNAi transport, SEC-22 and SYX-6 could promote the fusion between RNA-containing late endosomes/MVBs and lysosomes, and degrade the silencing information. Alternatively, because of the reticular pattern they showed, they could also be involved in a non-fusogenic complex that form ER-endosome contact sites without membrane fusion (Petkovic et al., 2014; Phillips and Voeltz, 2015). The SID-5 localization showed that, in addition to the small punctae that surround late endosomes/MVBs, it also localizes to other unidentified compartments which could be transport vesicles derived from Golgi to endosomes, or vesicles recycling certain proteins or lipids back to Golgi or ER. Together with the feeding RNAi data of the sec-22 sid-5 double mutant, SID-5 may interact with SEC-22 and SYX-6, for example in retrograde transport to Golgi, thereby disturbing the fusion of late endosomes/MVBs. One possibility could be that SID-5 promotes the recycling of SYX-6 and SEC-22 when the late endosomes/MVBs contain RNAi silencing information.

**Late Endosomes/MVBs Play Important Role in Small RNA Regulation**

Recently, more and more research link late endosomes/MVBs with small RNA regulation. It has been shown that late endosomes/MVBs are likely the site for RISC assembly and turn over (Gibbings et al., 2009). Inhibition of late endosome/MVB maturation to lysosome increases the RNAi efficiency (Lee et al., 2009). In a recent study, *C. elegans* RSD-3 was found to localize both to Golgi and endosomes and to be required for import of RNAi silencing signals (Imae et al., 2016). Its mammalian ortholog interacts with SNAREs and functions in retrograde endosome-Golgi transport (Imae et al., 2016). In our study, depletion of SEC-22 induced enlarged late endosomes/MVBs and increased RNAi response while depletion of SID-5 likely inhibits maturation of early endosome to late endosomes/MVBs and reduced RNAi response. This indicates that the function of late endosomes/MVBs may not be limited to be the place of RISC turn over. As sorting centers in cells, late endosomes/MVBs could also play a role in exporting the RNAi silencing signal. It has been shown that human exosomes derived from late endosomes/MVBs contain small RNA as well as GW182 (Gibbings et al., 2009). Furthermore, enhancing the level of ILV formation can increase the amount of miRNA in exosomes (Kosaka et al., 2010). However, the machinery sorting small RNA into ILVs is poorly understood. Could SID-5 be a
factor that functions in recognition of RNAi silencing signals or vesicles containing silencing information? Since feeding RNAi on the sec-22 sid-5 double mutant showed a sid-5 like reduced RNAi phenotype, it is possible that SID-5 recognizes the late endosomes/MVBs containing RNAi silencing signals and inhibit the fusion of late endosomes/MVBs with lysosomes. But how and why SID-5 moves to late endosomes is unknown. The localization data showed that SID-5 forms small punctae around late endosomes/MVBs (Hinas et al., 2012; Zhao et al., 2017) It gave rise to a hypothesis that SID-5 may function with RNA binding proteins that recognize the imported RNAi silencing information and transport it to late endosomes/MVBs. After handing over the silencing information to late endosomes/MVBs, SID-5 may stay there and function in other steps. However, in sid-5 mutant animals, late endosomes/MVBs maturation were affected, indicating that SID-5 may function in a more general way. The relation between RNAi sorting in late endosomes/MVBs and SID-5 recognition of RNA-containing late endosomes/MVBs would be an interesting topic to study.

C12D8.1 and RNAi Inheritance (Paper III)
Different Tissues Could Have Different RNAi Response
In Paper III, we found that C12D8.1 could function as a negative regulator of RNAi inheritance. In our feeding RNAi test in two generations, the C12D8.1 mutant worms showed enhanced RNAi response to the target gene when compared to wildtype worms. The C12D8.1 F2 progeny retains the RNAi phenotype even if they are fed with bacteria that do not express dsRNA with a target while the wildtype F2 worms have almost no response to the RNAi trigger taken up by the P0 animals. An interesting phenotype that we noted when performing RNAi against different targets is that depending on the tissue, the response of C12D8.1 worms to RNAi is different. The C12D8.1 worms are sensitive to RNAi when the target is in body wall muscle, but have almost similar response to wildtype worms when the target is in hypodermal cells. Different genes are silenced differently, and different tissues and cells have different ability to handle RNAi silencing signals. Some targets are mainly silenced by mRNA degradation, while others are silenced by nuclear RNAi dependent transcriptional and cotranscriptional effects (Grishok et al., 2005). However, in our experiments, we only checked two genes expressing in different tissues. Except for the tissue specificity, in which stage the gene is expressed is also likely an important determinant of RNAi efficiency.
Function of C12D8.1 in RNAi Inheritance

RNAi silencing information can be transported between somatic cells and be inherited for a few generations (Fire et al., 1998; Grishok et al., 2000). It was later shown that the bottle-neck number of generations that can keep the inherited RNAi is until F4 (Alcazar et al., 2008). After determining that C12D8.1 could negatively regulate inheritance of RNAi silencing, we wanted to find out more about how C12D8.1 functions in inheritance. By construction of a C12D8.1::mCherry transgene, we detected the fusion protein both in nuclear and perinuclear structures. C12D8.1 is a putative RNA binding protein and shares features with other RNA granule proteins, thus the perinuclear structures could be RNA granules. However, this still needs to be investigated. By carrying out feeding RNAi tests, we observed that the mutant worms are less sensitive to the second RNAi treatment when inheriting an RNAi signal, but have slightly enhanced RNAi sensitivity to the second RNAi treatment if the experiment is performed within one generation. Wildtype worms, on the other hand, showed a lower sensitivity to the second RNAi treatment under both conditions. The results indicate that C12D8.1 mutant worms, unlike the wildtype worms, could not reach the saturation stage after a strong RNAi induction, or just reach it for a short period. One explanation could be that in C12D8.1 mutant worms, the imported RNAi silencing signals can rapidly be exported to other tissues, for example germline to cause inheritance of RNAi. As the newly introduced RNAi triggers do not have any competitors that occupy RNAi machinery factors, the worms show a slightly enhanced RNAi sensitivity. However in the progeny, which already carries the previous RNAi signal, the second RNAi signal needs to compete for limiting RNAi machinery factors and the worms show a reduced RNAi sensitivity to the second RNAi treatment. In wildtype worms, the first RNAi treatment could be trapped from being exported, which at the same time will occupy more RNAi machinery factors. Thus, the response to the second RNAi treatment within one generation in wildtype worms is slightly reduced. From the inheritance point of view, the wildtype worms may clear the inherited RNAi signal efficiently, so that it will not affect the response to the second RNAi treatment too much. Taken together, C12D8.1 could function to keep the RNAi silencing signal, perhaps in RNA granules, from being sent to the germline. An alternative explanation would be that the germline increases the uptake ability in C12D8.1 mutants. We did not detect mCherry::C12D8.1 expression in germline, but this could be due to transgene silencing in germline cells (Kelly et al., 1997).

Transport of Silencing Signal to Germline

The method we used to test the RNAi inheritance is feeding RNAi. In this method, the first tissue that gets contact with the RNAi signal is the intestine, which is far away from where the inheritance happens. How the RNAi si-
lencing signal is transported to the germline is still not completely solved. One recent paper showed that SID-1, the protein required for import of the RNAi silencing signals (Winston et al., 2002), is required for maternal extracellular transport of RNAi silencing signal into germline (Wang and Hunter, 2017). In the absence of SID-1, the RNAi silencing signal is transported to germline along with yolk (Marré et al., 2016; Wang and Hunter, 2017). The authors also tested if any of the other SID proteins are required for the RNAi inheritance, and found SID-5 as an important factor that functions with SID-1 in initializing the silencing in the embryo (Wang and Hunter, 2017). Deletion of sid-5 leads to no response to the inherited RNAi silencing signal in the embryos (Wang and Hunter, 2017). The authors interpreted this as SID-5 functioning together with SID-1 after the late endosomes/MVBs sorting step and mainly for the import process of the RNAi silencing signal. In addition, they noted that the imported RNAi silencing information appears to rapidly go through late endosomes labeled by GFP::RAB-7 (Wang and Hunter, 2017). After the sorting step, the imported RNAi silencing signal was detected in an unknown structure waiting for processing toward the RNAi silencing pathway (Wang and Hunter, 2017). Since SID-5 has previously been reported to form small punctae around the GFP::RAB-7 labeled late endosomes/MVBs (Hinas et al., 2012), it is possible that SID-5 participates also in the sorting step in the process, which is earlier then that in their hypothesis.

In Paper III, we got C12D8.1 from the Y2H screen using SID-5 as bait. We have not yet proven the in vivo interaction between SID-5 and C12D8.1 by another method. If C12D8.1, as in our hypothesis above, keeps RNAi silencing information in RNA granules, less RNAi signal will be available to enter RNAi machinery and for the transport system factors, such as SID-5, to promote RNAi inheritance. It would be interesting to see the phenotype of a C12D8.1 sid-5 mutant. If our hypothesis is true, we assume that the RNAi inheritance would decrease, since without SID-5, RNAi silencing signals may not be sorted properly. On the other hand, the RNAi signal cannot be kept in RNA granules by C12D8.1, and may therefore lead to an increased cell autonomous RNAi response.

SID-5 and Other Candidates from Y2H Screen (unpublished data)

Including SEC-22, and C12D8.1, we have a list of 32 SID-5 potential interact candidates. In the list there are putative ER-associated proteins, other membrane associated proteins, and some soluble proteins. Except for 14 candidates, all the other candidates are conserved in humans. Although the interactions need to be confirmed by further experiments, it indicates that SID-5 could function in other membrane related processes. Alternatively, those candidate proteins have hidden functions in RNAi or RNA transport.
Furthermore, because several of the candidates are putative ER proteins, the possible localization of SID-5 could also include ER. *sid-5* mutant worms appear healthy, even though part of the endosomes cannot mature properly. This indicates that SID-5 may not take part in important cell processes that determine the life of *C. elegans*. It is possible that SID-5 is specific for RNA transport, for example if the affected endosomes contain small RNAs. However, this hypothesis still needs a lot of work to be tested.

In my thesis, we showed that two SNARE proteins that interact with systemic RNAi protein SID-5 in Y2H possibly modulate the destination of late endosomes/MVBs to exocytosis or lysosome degradation. The findings connect the endomembrane system more with systemic RNAi. The story of C12D8.1 in inheritance RNAi indicate the export of soma RNAi silencing signal to germline, where the inheritance starts, is a dynamic process and can be controlled probably by C12D8.1 together with an RNAi export protein, possibly SID-5. However, there are still many more questions awaiting an answer. Understanding the function of SID-5 may not only help us to know the transport pathway of RNAi signal, but also to get a better understanding of the entire membrane system.
Svensk Sammanfattning


På senare år har forskare upptäckt att membransystemet inte bara har betydelse för transport av olika molekyler utan också för genreglering, t.ex. medierad av små RNA-molekyler. De vanligaste typerna av sådana små regulatoriska RNA är mikroRNA (miRNA) och små interfererande RNA (siRNA). När en gen uttrycks transkriberas DNA till mRNA-molekyler som sedan transporteras till cellplasman där de translateras till funktionella proteiner. miRNA och siRNA kan binda till mRNA genom basparning och därmed hämma translationen eller inducera nedbrytning av mRNA. Ribosomer som translaterar mRNA till protein sitter på det korniga endoplasmatiska nätverket (ER), en av cellens många membranstrukturer. Det har visat sig att även miRNA- och siRNA-medierad genreglering sker vid ER. En annan membranstruktur, det viktiga sorteringscentret sena endosomer/multivesikulära kroppar (MVB) har dessutom föreslagits sortera in små RNA i sina inre vesiklar, antingen för degradering eller exocytos (export ut ur cellen).

siRNA-medierad genreglering kallas också RNA-interferens (RNAi). Naturliga eller artificiella dubbelsträngade RNA (dsRNA) tas upp av celler och processas till korta siRNA. Desa siRNA kan sedan binda till komplementära mRNA-molekyler och leda till specifik nedreglering av genuttryck-


Vår forskning har ännu bara upptäckt toppen av det isberg som utgörs av hur membrantransportsystemet är sammanfogat med systemisk RNAi och ärtlig RNAi. Gällande systemisk RNAi behövs mycket mer forskning för att studera interaktionerna mellan *SID*-5, SEC-22 och SYX-6 *in vivo* och hur dessa tre proteiner underlättar transport av vesiklar med RNAi-information. Vad gäller ärtlig RNAi behöver vi förutom att undersöka interaktionen mellan *SID*-5 och C12D8.1 även samla mer information om hur C12D8.1 minskar nedärvningen av RNAi. Dessutom väntar många andra *SID*-5-interagerande proteiner från jäst-två-hybridexperimentet på att studeras.
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