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Connecting Systemic RNAi to the Endomembrane System in *Caenorhabditis elegans*

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ACTA
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UPSALIENSIS
UPPSALA
2017

ISSN 1651-6214
ISBN 978-91-554-9932-7
urn:nbn:se:uu:diva-320897

Dissertation presented at Uppsala University to be publicly examined in A1:111a, Uppsala Biomedicinska Centrum, Husargatan 3, Uppsala, Monday, 12 June 2017 at 13:00 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Associate Professor Peter Brodersen (Department of Biology, University of Copenhagen).

Abstract
Holmgren, B. 2017. Connecting Systemic RNAi to the Endomembrane System in *Caenorhabditis elegans*. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 1526. 61 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9932-7.

RNA interference (RNAi) is a gene regulation mechanism conserved among eukaryotes. To silence gene expression, RNAi relies on a short single-stranded guide RNA to steer the RNA-induced Silencing Complex (RISC) to mRNAs with guide strand-complementary sequences. RNAi is a highly membrane-associated process. The RISC complex is likely loaded at the rough Endoplasmic Reticulum, where it can bind to and degrade mRNAs. Components of the RISC complex also colocalize to late endosomes, and the efficiency of RNAi-mediated silencing is affected by changes in late endosome to lysosome fusion. RNAi can be systemic and inherited, effecting gene silencing in distal tissues and in the offspring.

In this thesis, the model organism *Caenorhabditis elegans* was used to identify and characterize factors connecting systemic and inherited RNAi to the endomembrane system. We identify two SNARE proteins, SEC-22 and SYX-6, that both act as negative regulators of RNAi. SNAREs are necessary for vesicle fusion. Both SEC-22 and SYX-6 localize to late endosomes, and both interact with systemic RNAi protein SID-5 in a yeast two-hybrid (Y2H) screen. We find that in addition to its function in systemic RNAi, SID-5 is required for proper maturation of late endosomes. Furthermore, we identify the putative RNA-binding protein C12D8.1 as a novel regulator of RNAi inheritance. Mutant *C12D8.1* animals will have enhanced inheritance of RNAi silencing, which negatively affects the ability of the progeny to silence new targets using RNAi. Finally, we describe a novel, object-based method for estimating significance in colocalization studies. This method helped us describe and quantify spatial relations between fluorophore-labeled proteins in situations where such analyses would otherwise be impossible.

In conclusion, the work presented here further elucidates the connection between cellular RNAi, the endomembrane system, and the outside world.

Keywords: Systemic RNAi, RNAi inheritance, Endomembrane system, Late endosome, SID-5, SEC-22, SYX-6, C12D8.1, RNA

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ISSN 1651-6214
ISBN 978-91-554-9932-7
urn:nbn:se:uu:diva-320897 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-320897)

To Felicia

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Zhao, Y., Holmgren, B., Hinas, A. (2017) The conserved SNARE SEC-22 localizes to late endosomes and negatively regulates RNA interference in *C. elegans*. *RNA*, 23(3):297–307

II Zhao, Y., Holmgren, B., Li, D., Hinas, A. RNA transport protein SID-5 interacts with multiple SNAREs and affects membrane trafficking in *C. elegans* intestinal cells. *Manuscript.*

III Holmgren, B., Bäckström, L., Zhao, Y., Hinas, A. Putative RNA-binding protein C12D8.1 negatively regulates inheritance of RNAi in *Caenorhabditis elegans. Manuscript.*

IV Holmgren, B., Hinas, A. DESCROD estimates significant colocalization by randomized object displacement. *Manuscript*.

Contents

Abbreviations

AGO	Argonaute
dsRNA	double-stranded RNA
EMS	Ethyl Methanesulfonate
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
RNAi	RNA Interference
RSD	RNAi Spreading Defective
SAGO	Secondary Argonaute
SID	Systemic RNAi Defective
siRNA	Short/Small interfering RNA
SNARE	Soluble *N*-ethylmaleimide-sensitive Factor Attachment Protein Receptor
NRDE	Nuclear RNAi Defective
WAGO	Worm Argonaute
Y2H	Yeast-2-Hybrid

Introduction

The ability to regulate gene expression is paramount for all life, for responding to environmental cues, adapt to stress factors, and guiding development, among other things. To maintain control over gene expression, regulation occurs at several levels: transcriptionally to prevent mRNA transcripts from being synthesized, co-transcriptionally to abort mRNA transcription, and post-transcriptionally to prevent translation from an mRNA transcript. Multiple levels of regulation allows for extremely fine-tuned control of expression, which is necessary not least in large and complex multicellular organisms.

Over the years, the importance of gene regulation through small RNA has become more and more recognized (Ghildiyal and Zamore 2009). In many eukaryotes, a major part of all genes are now believed to be under regulation from microRNAs (miRNAs), acting to fine-tune expression during development (Friedman et al. 2009). However, the functions of small RNAs extend beyond gene regulation. For instance, RNA interference (RNAi) through short/small interfering RNA (siRNA) can act as a defense against transposons, and in some cases as an antiviral defense (Ding 2010). RNAi can affect gene regulation at the transcriptional, co-transcriptional and post-transcriptional level (Ghildiyal and Zamore 2009). Regulation by small RNAs can in some organisms spread from the source cell to effect gene silencing in distal tissues (systemic RNAi) (Sarkies and Miska 2014). Small silencing RNAs can also be transported to germ line cells and be inherited by the progeny in some organisms (Feng and Guang 2013; Anava et al. 2015).

In my thesis, I have used the model organism *Caenorhabditis elegans* to study the RNAi factors that are important both for systemic RNAi and RNAi inheritance. I have also studied the subcellular structures that are associated with RNAi. The findings presented here increase our understanding about this important regulatory mechanism.

The Model Organism *Caenorhabditis elegans*

The work presented in this thesis has been performed in the model organism *Caenorhabditis elegans*. *C. elegans* is a free-living nematode that was first

isolated in Algiers in 1899, originally as *Rhabditis elegans* (Nigon and Félix 2017).

In the wild, *C. elegans* is found in compost heaps and around rotting fruits, where it hunts for bacteria and yeast. During development, it undergoes four larval stages, each followed by molting of its old cuticle. In periods of starvation, *C. elegans* larvae of certain stages can enter an alternative developmental pathway into long-lived and stress-resistant dauer worms. Dauer worms can travel over long distances by hitchhiking on larger and faster animals such as flies, snails, and birds. Upon reaching more promising hunting grounds, dauer worms can revert to a non-dauer state, allowing them to procreate (Kiontke and Sudhaus 2006).

Itself a self-fertilizing hermaphrodite with a low occurrence of males, *C. elegans* was used to compare the modes of reproduction of different nematode species as early as 1900. Sydney Brenner established *C. elegans* as a model organism for molecular biology research in the 1970's (Brenner 1974; Ankeny 2001). At that time, culturing of *C. elegans* in standardized liquid and solid medium had already been described (Ferris and Hieb 2015), and the first description of recessive mutant alleles had also been described in the related nematode *C. briggsae* (Nigon and Félix 2017). Determined to find a eukaryote he could work with essentially as if it was a microorganism, while still being a relevant model for other multicellular organisms, he examined a large number of different alternatives, including *Dictyostelium*, *Naeglaria*, and *Tetrahymena* (Ankeny 2001). At this time, Brenner was also interested in embryonal development and the nervous system, which made these alternatives inadequate. Eventually, he settled on *C. elegans* for a number of reasons. Free-living soil nematodes are easy to cultivate, and standardized culture media were already described. *C. elegans* also have differentiated tissue types similar to those of more complex organisms, including muscles, intestinal cells, and a simple nervous system. In addition, worms are small enough to be imaged in whole cross sections by electron microscopy, enabling detailed inspection of cells and intracellular processes within the context of a whole animal. The manageable number of somatic cells in an adult worm (959 in hermaphrodites) simplifies many whole-animal studies.

Brenner popularized *C. elegans* as a model organism for molecular biology by publishing the results of an extensive classical genetics screen using the mutagen ethyl methanesulfonate (EMS) (Brenner 1974). In this publication, he demonstrated how mutant alleles could be generated. He also described several behavioral or developmental phenotypes from genetic mutations, which could be observed by eye, and how these mutations could be mapped to genes by complementation tests and chromosomes by frequency of recombination. Genes found in this screen were named after their phenotypes in a standardized manner, along with a number e.g. *dpy-1*. Classical *C. elegans* phenotypes include Sma (small: shorter and thinner than wild type

worms), Dpy (dumpy: shorter but not thinner than wild type), and Unc (uncoordinated: various movement phenotypes such as paralysis).

As many other nematodes, *C. elegans* display invariant cell lineages, meaning the fate of each cell in the development of healthy wild-type worms does not vary between individual worms. This allowed researchers to map the complete *C. elegans* cell lineage tree (Sulston et al. 1983; Sulston and Horvitz 1977; Schnabel et al. 1997), and subsequently also the connections of the entire canonical nervous system (White et al. 1986). The function of the nervous system could then be investigated both at the genetic level, by changes in behavior from genetic mutations, and at the cellular level by laser ablation of individual neurons (Sulston and White 1980).

In addition to these advantages, several characteristics in and pertaining to *C. elegans* significantly simplifies cell and molecular biology work using the worm. The worm is completely transparent, facilitating microscopy on whole live animals. Every *C. elegans* lab uses the same strain as wild type (N2, isolated from a mushroom compost in Bristol by a student), the same well defined solid medium and the same set of food sources (usually *E. coli* strain OP50) for cultivation, enabling replication of studies and simplifying comparison of results between groups. *C. elegans* is trivial to freeze and thaw, and to transport worldwide, which has enabled the establishment of a central repository for mutant worm strains. The *C. elegans* genome is also relatively small for a metazoan (100 Mbp), simplifying cloning and sequencing among other things.

Due to its popularity as a model organism, many cell and molecular biology methods (including CRISPR-Cas9 (Dickinson and Goldstein 2016)) have now been adapted for *C. elegans,* and it has also been the subject of several large-scale projects such as whole genome sequencing (*C. elegans* Sequencing Consortium 1998) (first among multicellular organisms), neuron mapping (White et al. 1986), high-throughput *in vivo* gene expression analysis (Hunt-Newbury et al. 2007), and proteome studies (Husson et al. 2015).

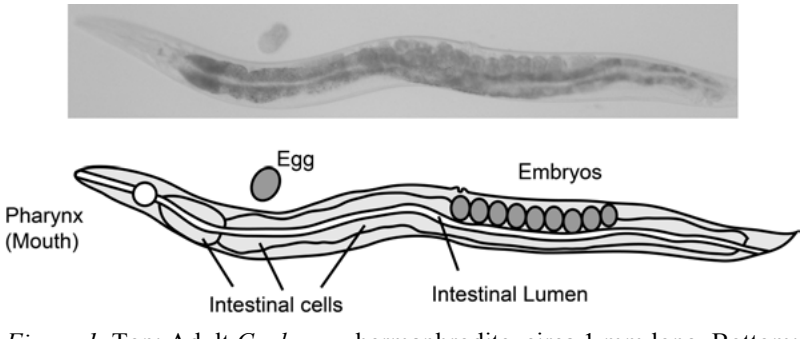

Figure 1. Top: Adult *C. elegans* hermaphrodite, circa 1 mm long. Bottom: Schematic showing pharynx, intestinal cells, intestinal lumen, and embryos.

RNA Interference and Other Small RNA Silencing Pathways

RNA interference (RNAi) is a mechanism by which short, double-stranded (ds)RNA-derived, mRNA-complementary RNA strands are used to down-regulate gene expression (Ghildiyal and Zamore 2009). RNAi is conserved among all eukaryotic branches of life, including plants and mammals, and demonstrates a broad diversity in form, capabilities, and biological function. Indeed, Argonautes (AGOs), which are core RNAi proteins, can be found also in archaea and bacteria, indicating that RNAi might predate eukaryotes. In fact, an AGO has been found to facilitate DNA-guided DNA interference in the prokaryote *Thermus thermophilus* (Swarts et al. 2014). *C. elegans* has been an important model organism for RNAi research since the beginning of the field. As such, RNAi in *C. elegans* is fairly well understood compared to that of other animals (Grishok 2013). In this thesis I will focus on *C. elegans* RNAi, but also mention when it differs from other organisms.

Gene silencing through introduction of antisense RNA has been seen in plants (Napoli et al. 1990) and *C. elegans* (Guo and Kemphues 1995) since the early 1990's. Even before then, antisense RNA was suggested and successfully demonstrated as a means to silence specific genes in mouse cells (Izant and Weintraub 1984). It was then believed that silencing occurred by the antisense RNA hybridizing to mRNA, making it unavailable for translation and degraded. However, this did not explain the fact that injection of sense RNA was just as effective as antisense RNA at inducing RNAi. It also did not explain how silencing could be inherited by the offspring in some organisms (Grishok et al. 2000). In 1998, it was proven in *C. elegans* that RNAi was not the effect of antisense RNA binding to mRNA, but rather that the mediator of RNAi was long strands of dsRNA (Fire et al. 1998). The silencing intermediate in the form of small single-stranded guide RNAs generated from the long dsRNA was identified later (Hamilton and Baulcombe 2012). Since then, several classes of small silencing RNAs have been described, each with distinct characteristics and functional roles. These classes include microRNAs (miRNA), short interfering RNAs (siRNA), and PIWI-interacting RNAs (piRNA). The following sections will go through the various small RNA pathways with an emphasis on *C. elegans.*

Core RNAi Proteins in *C. elegans*

Preceding RNAi, the triggering long dsRNA is bound by the RNA-binding protein RDE-4 and processed to short dsRNAs by the ribonuclease Dicer (Grishok 2005). The origin and characteristics of the dsRNA, and the downstream effects it has on genes, vary between the different RNAi pathways and between organisms.

RNAi-based silencing is mediated by the RNA-Induced Silencing Complex (RISC), which includes a short RNA guide strand bound to an Argonaute (AGO) protein. In all small RNA silencing pathways, AGOs are responsible for matching the guide RNA to complementary RNA transcripts and trigger silencing (Hutvagner and Simard 2008). After loading of short dsRNA, AGO separates the target-complementary guide strand from the passenger strand. This AGO may also cleave complementary mRNAs in some organisms. In addition, the RISC complex contains the helicase DRH-1, and a member of the GW182 protein family. GW182 proteins interact with directly with AGO, but additionally with mRNA-interacting poly(A)-binding proteins and deadenylases to mediate mRNA deadenylation and decapping (Yao et al. 2013). *C. elegans* has GW182 two homologs: AIN-1 and AIN-2. GW182 is historically regarded mainly as a miRNA protein, but is also required for efficient siRNA-mediated silencing.

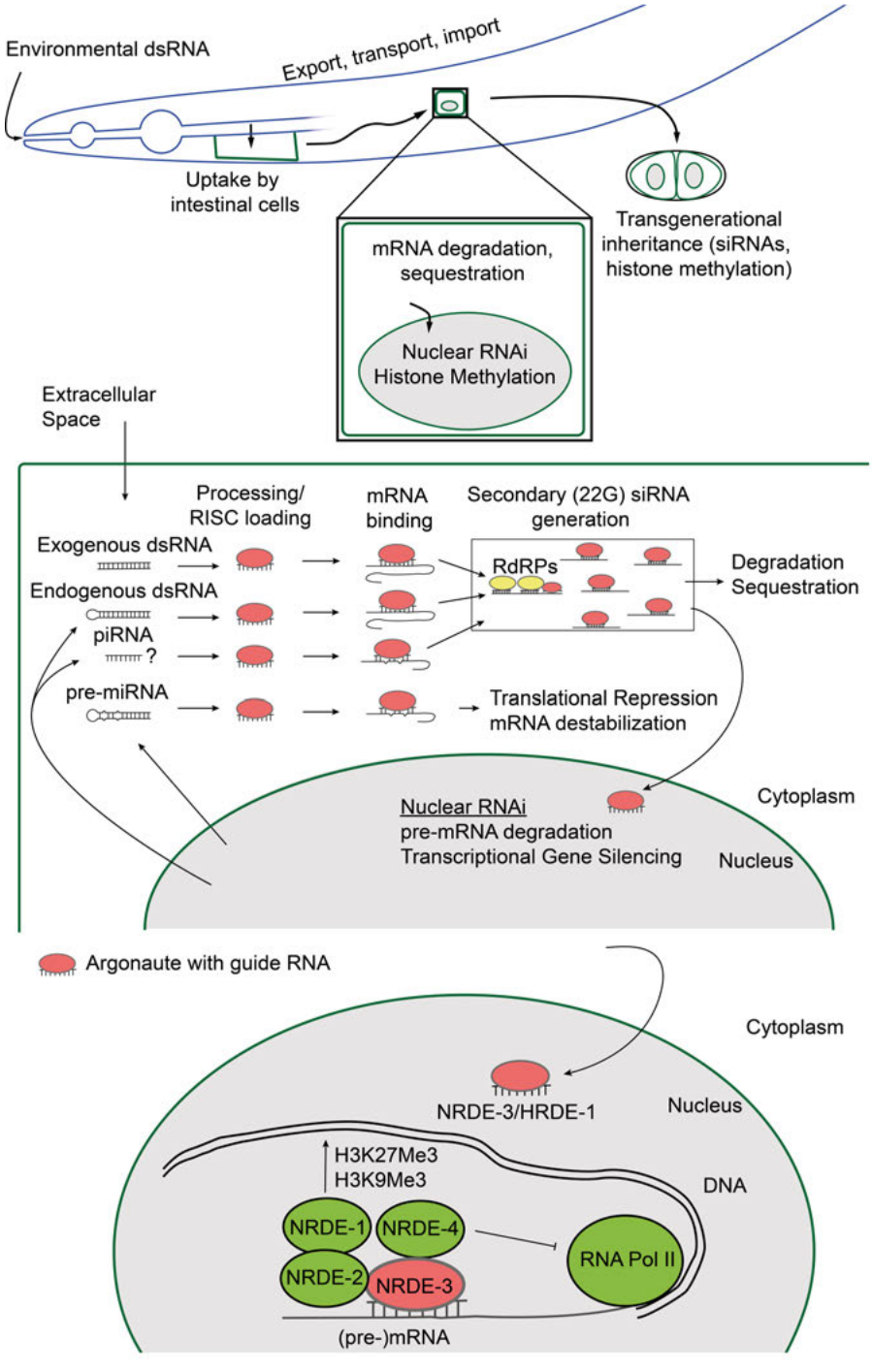
Environmental dsRNA
Export, transport, import
Uptake by intestinal cells
Transgenerational inheritance (siRNAs, histone methylation)
mRNA degradation, sequestration
Nuclear RNAi Histone Methylation
Extracellular Space
Processing/ RISC loading
mRNA binding
Secondary (22G) siRNA generation
Exogenous dsRNA
Endogenous dsRNA
piRNA
?
pre-miRNA
RdRPs
Degradation Sequestration
Translational Repression mRNA destabilization
Nuclear RNAi
pre-mRNA degradation
Transcriptional Gene Silencing
Cytoplasm
Nucleus
Argonaute with guide RNA
NRDE-3/HRDE-1
H3K27Me3
H3K9Me3
DNA
NRDE-1
NRDE-4
NRDE-2
NRDE-3
RNA Pol II
(pre-)mRNA

Figure 2. Pathways of RNAi in *C. elegans*. Top: Environmental RNAi in *C. elegans* requires uptake, export, transport, and import of RNAi triggers. Genes in somatic cells will then be silenced in somatic cells and be transported to germ line cells for inheritance. Middle: While primary RISC complexes use separate pools of AGOs depending on the small RNA pathway, *C. elegans* uses a shared pool of secondary AGO proteins for endogenous siRNA, exogenous siRNA, and piRNA. Secondary siRNAs (22G RNAs) are amplified after a primary RISC binds to a matching RNA transcript. This transcript is then used as template for 22G RNAs. For transcriptional and co-transcriptional silencing, secondary siRNAs can then be escorted into the nucleus to effect nuclear RNAi. Bottom: Nuclear AGO NRDE-3 (or HRDE-1 in germ line cells) bind to matching pre-mRNAs. NRDE-4 then blocks transcription by RNA Polymerase II, and NRDE-1 interacts with downstream factors to induce histone methylation.

Short Interfering RNA (siRNA)

Among RNAi pathways, Short (or Small) Interfering RNA (siRNA) is the most widespread and also likely the oldest form of RNAi. siRNAs are generated either from genetically encoded long dsRNAs such as transposons or repetitive elements (Piatek and Werner 2014), or from exogenous sources of dsRNA such as viruses (Ding 2010). siRNAs are around 21 nt, and silencing by siRNA generally requires perfect complementarity of the mRNA to the guide strand (Ghildiyal and Zamore 2009). The complementary mRNA can be cleaved and degraded immediately. It may also be degraded through additional proteins, and transcriptional silencing can also occur through histone methylation. Certain organisms such as *C. elegans* and *Paramecium tetraurelia* also have an amplification step, where primary siRNA binding effects production of multiple secondary siRNAs, matching the sequence of the target mRNA (Pak and Fire 2007; Carradec et al. 2015). Amplification is achieved with an RNA-dependent RNA polymerase (RdRP), using the matching mRNA as template (Sijen et al. 2007). Secondary siRNAs are then loaded into a secondary RISC with a secondary AGO.

Exogenous siRNA

In *C. elegans*, RNAi can be induced against endogenously expressed genes by introducing exogenous dsRNA (Fire et al. 1998). Exogenous RNAi is systemic, meaning it spreads from the source tissue throughout the animal to almost all cell types (Sarkies and Miska 2014), and it can also be passed along to the offspring (Grishok et al. 2000; Anava et al. 2015). The original method of introducing exogenous dsRNA to worms was by injection, but it was later shown that soaking worms with dsRNA, or simply feeding them bacteria that express dsRNA could also induce RNAi (Timmons et al. 2001). Thanks to the Ahringer library of bacterial clones, virtually any gene in *C. elegans* can now be targeted for RNAi with little effort (Kamath and Ahringer 2003).

Silencing by exogenous RNAi in *C. elegans* is highly effective, in part because of an amplification step where a large amount of secondary siRNAs is generated once a matching transcript has been found (Pak and Fire 2007). There are around 27 different AGO genes in the *C. elegans* genome (Yigit et al. 2006). Only two of these, RDE-1 and the germ line-expressed, endo-siRNA-specific ERGO-1 (Fischer et al. 2011), associate with primary (dsRNA-derived) siRNAs and can cleave the passenger strand. Instead of cleaving matching transcripts, RISC complexes loaded with a primary siRNA and the AGO RDE-1 will recruit the RdRP RRF-1 to the mRNA (Pak and Fire 2007). RRF-1 will then produce secondary siRNAs that bind to a WAGO/SAGO (Worm AGO or Secondary AGO), in a RISC complex capable of gene silencing (Yigit et al. 2006). Secondary siRNAs produced by RRF-1 are 22 nt long, with a 5' G (22G RNAs). They also have a 5' triphosphate, indicating that they are synthesized to their 22 nt length rather than cleaved from a longer strand. Moreover, secondary siRNAs cannot be used to generate tertiary siRNAs in somatic cells (Pak et al. 2012). Due to their dsRNA origins, primary siRNAs will be both sense and antisense orientation to the target gene. Secondary 22G RNAs targeting protein-coding genes are predominantly antisense however. Many WAGOs have overlapping roles in RNAi, indicated by the modest effect on RNAi efficiency of knocking out only one or a few of them(Yigit et al. 2006). In contrast, overexpression of WAGO proteins confers stronger RNAi, indicating that the total amount of secondary AGOs is a limiting factor. While AGO proteins generally have endonuclease activity for degrading the passenger strand, worm secondary AGOs are not believed to have endonucleolytic activity, and do not cleave the transcripts themselves. Rather, the ribonuclease RDE-8 is recruited to the RISC for this function (Tsai et al. 2015). Apart from inducing mRNA degradation, exogenous siRNA can also silence genes transcriptionally and co-transcriptionally, dependent on nuclear RNAi (Mao et al., 2015, see below).

Role of Exogenous siRNA in Nature

While effective and highly studied, the exact function of exo-siRNA in *C. elegans* remains somewhat controversial. A popular hypothesis is that exo-siRNA is an antiviral response, where antiviral siRNAs can spread systemically from infected cells and mount an effective response in anticipation of the virus Inheritance of antiviral RNAi would then confer a kind of inoculation against future infections in the offspring. Recently, RNAi has been shown to have a role in defense against Orsay virus infection (Sterken et al. 2014). However, whereas RNAi limited viral infection, virus-derived siRNAs were only weakly inherited, not systemic, and mutant animals lacking systemic RNAi protein SID-1 were not more affected by viral infections (Ashe et al. 2015). There are conflicting reports whether the F1 generation can be "vaccinated" by RNAi-based antiviral defense in the previous generation. It is therefore still unclear exactly what problem the elaborate *C. ele-*

gans siRNA system is the solution to. It is important to note that so far, only one virus strain has been found that can reliably infect *C. elegans* (and then only in RNAi-deficient worms). Other still unknown viruses or other pathogens might evoke an inheritable RNAi-based protection. Another suggestion is "social RNAi", where environmental RNAi facilitates spread of gene silencing between individual worms (Sarkies and Miska 2013).

C. elegans is not alone in silencing exogenous dsRNA by siRNA. In plants, antiviral defense through amplified exogenous siRNA was characterized early (Deleris et al. 2006). Exogenous dsRNA can induce primary and secondary siRNAs in the ciliate *Paramecium tetraurelia* (Carradec et al. 2015). In mammals, due to the strong interferon response against long dsRNA, the existence of functional antiviral exo-siRNA was a controversial issue for a long time. However, multipotent stem cells do not mount an effective interferon response against long dsRNA, and it has recently been shown that viral-derived siRNAs can protect suckling mice against the nodamura virus (Li et al. 2013b). Also, human Dicer protects against adenovirus infection by cleaving a virus-encoded noncoding RNA (Machitani et al. 2016). It was recently reported that *Drosophila* have circulating immune cells that mediate systemic RNAi in response to viral infection (Tassetto et al. 2017).

Endogenous siRNA

In addition to exogenous RNAi, *C. elegans* depends on several pathways of endogenous RNAi and related small RNA-based regulatory mechanisms. Endogenous small RNA silencing pathways can be subdivided into endogenous siRNA, miRNA, and piRNA. A common function of most endogenous small RNA pathways is genomic surveillance and protection from non-self genetic material, but also endogenous gene regulation during embryogenesis or stress response.

Endogenous siRNAs in *C. elegans* are expressed mainly in sperm and oocytes, but also in somatic tissues and during embryogenesis (Han et al. 2009). They are generated through the ERI pathway, and mainly target protein-coding genes (Lee et al. 2006). In particular, duplicated genes are overrepresented as targets (Fischer et al. 2011). The ERI pathway is dependent on Dicer, the RdRP RRF-3, and the exonuclease ERI-1 to generate 26 nt RNAs with a 5' G (26G RNAs) (Han et al. 2009). The 26G RNAs are then loaded in a RISC complex with AGOs ERGO-1 (gonad) or ALG-3/4 (sperm cells). Similar to exogenous siRNA, *C. elegans* endogenous siRNA undergoes an RRF-1-dependent amplification step to generate secondary 22G endo-siRNAs. The endogenous 22G RNAs share the same pool of secondary AGOs as exogenous 22G RNAs (Yigit et al. 2006).

siRNAs in other organisms are mostly generated from genomic sources of long dsRNAs, such as transposons, transcribed inverted repeats, and convergent transcripts (where one transcript may be antisense to a coding gene and

under transcriptional control) (Ghildiyal and Zamore 2009). In mouse oocytes, siRNAs can be generated from pseudogenes and used to silence a functional gene with complementarity to the source pseudogene (Tam et al. 2008; Sasidharan and Gerstein 2008). The lack of identifiable RdRP in mice and flies suggests that endogenous siRNAs in those organisms are probably not amplified (Okamura and Lai 2008). Plants can also generate siRNA from single-stranded RNA, and perform RdRP-dependent amplification of secondary siRNA (Borges and Martienssen 2015).

microRNA (miRNA)

In contrast to the ad-hoc origin of siRNA-generating dsRNAs, miRNAs are derived from miRNA genes and act as (normally negative) regulators of gene expression (Ghildiyal and Zamore 2009). Perturbed developmental timing from mutations in genes related to proper miRNA functionality, such as the miRNA-specific AGOs *alg-1* and *alg-2*, show that miRNAs are important for fine-tuning cell divisions and differentiation in *C. elegans* (Grishok et al. 2001). Families of similar miRNA genes redundantly regulate large clusters of genes, causing mutations in single miRNA genes to have only small or no observable phenotypes (Alvarez-Saavedra and Horvitz 2010). There are however exceptions to this, such as the miRNA genes *let-7* and *lin-4* (Ambros 2011).

Transcription of miRNA genes normally yield capped and polyadenylated pre-miRNA transcripts containing ~70 nt imperfect hairpin structures (Sarkies and Miska 2014). The hairpin structures are then cleaved out by the ribonuclease Drosha and further processed to mature miRNAs by Dicer, before being loaded into a miRISC complex. Except for a short seed region, the miRNA guide strand does not require perfect base-pairing with the target mRNA. In contrast to siRNA, no RdRP-dependent amplification from miRNAs is required for efficient miRNA silencing. *C. elegans* have two functionally overlapping miRNA-specific AGOs: ALG-1 and ALG-2 (Grishok 2013). miRNA-silenced transcripts are transported to RNA-rich granules (P-bodies or GW-bodies), where they are either stored in a sequestered form or decapped and degraded (Anderson and Kedersha 2009).

While the miRNA mechanism existed in the last common ancestor of Cnidaria and Bilateria (Mauri et al. 2016), many miRNA families in different eukaryote branches likely evolved independently of each other (Berezikov 2011). Unlike in metazoans, plant miRNAs are highly complementary to their target, and silencing is effected mainly through mRNA cleavage (Borges and Martienssen 2015; Arribas-Hernández et al. 2016). However, there is also wide-spread translational inhibition in *Arabidopsis* (Brodersen et al. 2008). Some plant miRNA-targeted transcripts recruit the RdRP RDR6 to generate trans-acting (ta) siRNA, which are subsequently loaded into RISC, as a kind of secondary siRNA response (Allen et al.

2005). In contrast, metazoans generally do not generate a secondary RNAi response following miRNA.

PIWI-Interacting RNA (piRNA)

In *C. elegans*, piRNAs are 21nt long with a 5'U (hence sometimes referred to as 21U RNAs), and are transcribed from around 30 000 very short piRNA genes (Batista et al. 2008). Each *C. elegans* piRNA gene encodes one piRNA. piRNAs guide the PIWI protein PRG-1 to mRNAs. Whereas siRNA relies on complete complementarity, piRNAs can effect silencing with imperfect base-pairing to the target mRNA (Lee et al. 2012). piRNA-complementary transcripts will act as template for secondary 22G RNAs by the RdRP EGO-1, which will then effect silencing of complementary regions post-transcriptionally (Bagijn et al. 2012). One effect of this amplification step is that piRNA-derived 22G RNAs will target sequences not encoded in piRNA genes.

The piRNA pathway can silence an immense number of potential transcripts; both non-self and self. The silencing effect of piRNAs in *C. elegans* is countered by the RNA activation (RNAa) of 22G RNAs associated with the AGO CSR-1 (Seth et al. 2013; de Albuquerque and Ketting 2013). Most genes not licensed by CSR-1-associated 22G RNAs will be silenced in germ line cells. It is still unclear how licensing by CSR-1 occurs for new sequences, but introducing sequences from already licensed genes into a silenced foreign transcript can cause release of piRNA-induced silencing.

C. elegans piRNA has a broad range of possible targets, coupled with a secondary amplification step that will specifically target a longer sequence of a matching transcript. In addition, tolerance to matching self targets is ensured through CSR-1 licensing. This is somewhat analogous to how the mammalian antibody-based adaptive immunity system works, except restricted to RNA transcripts. In contrast, piRNA in flies and mammals work somewhat analogously to prokaryote CRISPR-Cas systems (Iwasaki et al. 2015; Wiedenheft et al. 2012). *Drosophila* and mammal piRNAs are processed from transcripts from piRNA clusters: long (up to 180 kb) intergenic regions. piRNA clusters act as transposon traps, such that any long sequence that occurs in the piRNA cluster will be silenced by the piRNA system. Indeed, piRNA clusters contain identifiable sequences from ancient transposon insertion events. Silencing occurs through histone methylation of complementary regions, and can spread over a large genomic region beyond the transposon itself. Amplification of the piRNA response in mice and flies also differs from in *C. elegans*, employing a feed-forward (ping-pong) mechanism. In addition to transposon silencing, some protein-coding genes are under piRNA regulation in *Drosophila*. In silkworm, piRNA is required for regulating sex determination. Like in *C. elegans,* piRNA silencing can be inherited in *Drosophila*. Similar to *C. elegans*, ciliates also demonstrate

small RNA-based licensing of permitted genes to protect against piRNA silencing.

Nuclear RNAi

Small RNA-induced transcriptional and co-transcriptional silencing, as well as inheritance of silencing, is dependent on the nuclear RNAi pathway. Because of nuclear RNAi, feeding RNAi can be used to target pre-mRNAs (Burkhart et al. 2011). *C. elegans* transcribes several polycistronic pre-mRNAs, which are spliced into separate mRNAs (Zorio et al. 1994). Silencing the gene *lin-15b* will also silence *lin-15a*, located downstream of *lin-15b* in the polycistronic pre-mRNA (Bosher et al. 1999). A screen for mutants deficient specifically in pre-mRNA silencing turned up several Nuclear RNAi Deficient (NRDE) proteins (Guang et al. 2008). The AGO NRDE-3 (or, in the germ line, (Heritable RNAi Deficient) HRDE-1 (Buckley et al. 2012)) transports cytoplasmic endogenous and exogenous secondary (22G) siRNAs into the nucleus. This nuclear RISC complex will then bind to nuclear transcripts, followed by recruitment of additional factors NRDE-1, NRDE-2, and NRDE-4. RNAi-dependent transcriptional silencing is mediated by DNA methylation and trimethylation of histones, either on Lysine 9 (H3K9Me3) or Lysine 27 (H3K9Me3) (Burkhart et al. 2011; Mao et al. 2015). This epigenetic silencing can then spread over several kilobases. Nuclear co-transcriptional silencing occurs by inhibiting RNA polymerase II (Guang et al. 2010).

RNAi Inheritance

Initial studies showed that injected dsRNA induces RNAi against targets expressed in tissue types other than the one into which it is injected (systemic RNAi, see below). It was also discovered that the germ line is affected by systemic RNAi, causing silencing to be inherited by the offspring (Fire et al. 1998; Feng and Guang 2013). Further genetic studies revealed that RNAi inheritance depends on at least two different mechanisms. Through genetic crosses, it was possible to determine which RNAi genes were necessary for the parent to pass along dsRNA-induced inheritance, and for the offspring to inherit silencing.

Through one mechanism, mutant parents lacking the primary AGO RDE-1 or the RNA-binding protein RDE-4 can pass along inheritance, showing that no generation of primary siRNA is required in parent worms (Tabara et al. 1999). This indicates that the injected dsRNA itself can be inherited. This type of inheritance is only possible for one generation, however.

Through another mechanism, primary siRNA generation by RDE-1 (and interaction with RDE-4) is required in parent (P0) worms, but not in the offspring (F1) (Grishok et al. 2000). Inheritance through this mechanism spans

over several generations, becoming progressively weaker, finally becoming unable to persist. This progressive weakening was speculated to be an effect of signal diffusion (Alcazar et al. 2008). The inherited element that persists for longer than one generation was later found to be secondary siRNAs (Gu et al. 2012). Recently, it was discovered that continually inducing feeding RNAi against a second gene could prolong the duration of dsRNA-induced inheritance over several generations, indicating that the persistence of inheritance might be actively regulated rather than an effect of passive dilution (Houri-Ze'evi et al. 2016). The exact nature of this regulation is still unknown.

Both of these mechanisms of inheritance cause H3K9Me and require the nuclear RNAi pathway (see above). Expression of systemic RNAi protein (Systemic RNAi Deficiency, see below) SID-1 in the progeny is also required for inheritance (Marré et al. 2016).

Inheritance of Endogenous Small RNAs

Inheritance of small RNAs is not limited to those from exogenous sources. Gene silencing 22G RNAs downstream of the piRNA pathway – as well as the antagonistic CSR-1-associated licensing 22G RNAs – are inherited. Recently it was found that 22G RNAs generated from piRNA targets are imported to the nucleus by HRDE-1, and that this leads to production of tertiary siRNAs (Sapetschnig et al. 2015). These tertiary siRNAs enable piRNA silencing to be maintained over multiple generations; perhaps indefinitely. Starvation in *C. elegans* can affect gene regulation for multiple generations through inherited 22G RNAs (Rechavi et al. 2014). Both the gene regulation and the effecting siRNAs are dependent on nuclear RNAi in the germ line. Mild stress can also produce 22G RNAs that are inherited over several generations (Schott et al. 2014). These 22G RNAs were shown to associate with either CSR-1 or HRDE-1 to enhance or reduce gene expression, respectively.

Systemic RNAi

The systemic RNAi response to injected exogenous RNAi requires export, transport to distal cells, and import of RNAi trigger signals (Zhuang and Hunter 2012a). Environmental RNAi from dsRNA feeding or soaking also requires uptake of dsRNA from the intestinal lumen. There are two classes of systemic RNA triggers in *C. elegans*: a long dsRNA and a shorter double-stranded siRNA intermediate (Jose et al. 2011). A screen was performed for mutants with deficient systemic RNAi but functional cell-autonomous RNAi (Winston et al. 2002). Mutations were introduced in a worm strain carrying a nuclear-localized GFP expressed in body-wall muscle cells. Another GFP construct was expressed in the pharynx. In addition, a GFP-complementary RNA hairpin was also expressed in the pharynx. In worms with functional systemic RNAi, this construct silences GFP expression both in the pharynx

and (weakly) in body-wall muscle cells. To completely silence GFP expression in body-wall muscle cells, feeding RNAi against GFP was used to supplement the pharynx-expressed GFP hairpin. In cell-autonomous RNAi mutants, such as those targeting core RNAi proteins, no silencing can be seen in either pharynx or body-wall muscle cells. In worms deficient in systemic RNAi, pharyngeal GFP is silenced, but not GFP in body-wall muscle cells. Mutants deficient in environmental RNAi but not cell-autonomous RNAi were also identified.

So far, three genes have been identified as conferring systemic RNAi deficiency (SID) from this screen. Uptake of environmental dsRNA requires the short transmembrane protein SID-2, which localizes to the apical membrane of intestinal cells (Winston et al. 2007). *sid-2* mutant animals are unable to silence GFP following feeding RNAi, but systemic RNAi and cell autonomous RNAi are both unaffected. The nematode *C. briggsae*, which is a close relative of *C. elegans*, lacks a functional SID-2 analog, and is also insensitive to feeding RNAi. Expression of *C. elegans* SID-2 in *C. briggsae* is sufficient to induce sensitivity to feeding RNAi. Expression of SID-2 in *Drosophila* S2 cells is sufficient to facilitate uptake of environmental dsRNA (McEwan et al. 2012). Uptake is dependent on an acidic extracellular medium, and it is selective for dsRNA longer than 50 base pairs. SID-2 dependent environmental dsRNA uptake is likely linked to endocytosis.

SID-1 is required for efficient import of extracellular dsRNA, and is expressed in most cell types (Winston et al. 2002). A notable exception is neurons, which are also refractory to systemic RNAi. SID-1 is a large protein with 11 transmembrane domains, a 400 aa extracellular N-terminal domain and a 100 aa cytoplasmic loop between the first and second transmembrane domains (Feinberg and Hunter 2003). *sid-1* mutants displayed the strongest systemic RNAi deficiencies in the original screen. It is likely a passive facilitator of cellular bidirectional transport of long dsRNA through a membrane. While intestinal SID-2 facilitates uptake of environmental dsRNA, intestinal SID-1 is still required for feeding RNAi against genes expressed in intestinal cells (Winston et al. 2007). Despite this, tissue-specific rescue of SID-1 in body-wall muscle cells re-enables feeding RNAi against body-wall muscle genes in *sid-1* mutant animals (Jose et al. 2009). This indicates that dsRNA can still be transported through and exported from intestinal cells even in the absence of SID-1. Notably, whereas *C. elegans* neurons are normally refractory to systemic RNAi, expression of SID-1 in neurons is enough to enable silencing of neuronally expressed genes by feeding RNAi (Calixto et al. 2010). However, neurons are susceptible to feeding RNAi in *eri-1* mutant animals, where exogenous RNAi is enhanced cell-autonomously at the expense of endogenous RNAi (Kennedy et al. 2004).

Another protein required for efficient import of dsRNA is the tyrosine kinase SID-3 (Jose et al. 2012). *sid-3* mutant animals have a weaker systemic RNAi phenotype following reduced import of dsRNA, and a concomitant

stronger cell-autonomous RNAi response. Export of dsRNA does not appear affected in *sid-3* mutant animals. SID-3 is expressed in most tissue types, and localizes to cytoplasmic punctae.

The small (67 aa) single transmembrane protein SID-5 is required for efficient systemic RNAi (Hinas et al. 2012). Tissue-specific rescue of SID-5 expression in the intestinal cells can restore feeding RNAi silencing of body-wall muscle targets. SID-5 is expressed in most somatic tissue types, where it localizes to cytoplasmic foci that partially overlap with late endosomes or multivesicular bodies (MVBs, see below).

In addition to the SID genes above, another screen originally identified three previously uncharacterized genes as RNAi Spreading Defective (RSD) *rsd-2*, *rsd-3*, and *rsd-6* (Tijsterman et al. 2004). Of these, *rsd-2* and *rsd-6* are involved in secondary siRNA amplification and maintaining germ line cell immortality rather than systemic RNAi (Sakaguchi et al. 2014). *rsd-3* was recently implicated in import of silencing RNAs (Imae et al. 2016). Interestingly, RSD-3 colocalizes extensively with markers for the *trans*-Golgi network, and has an ENTH domain with affinity to membrane phosphoinositides. The ENTH domain of RSD-3 is sufficient to rescue the systemic RNAi defect in *rsd-3* mutants.

Finally, a screen specifically to find feeding RNAi mutants (Feeding defective for RNAi, FED), turned up *fed-1* and *fed-2* (Timmons et al. 2003). Except for the original identification, no further results have been published. However, *fed-1* is likely the same gene as *sid-2* (Jose and Hunter 2007).

Systemic RNAi in Other Organisms

In plants, RNAi can spread via phloem sap, but also likely to immediate neighbor cells through plasmodesmata (Molnar et al. 2010; Melnyk et al. 2011). Systemic RNAi is an important antiviral defense in plants (Deleris et al. 2006). In mammals, extracellular miRNAs and siRNAs have been found in exosomes (small extracellular vesicles, see below), where they are found with high levels of GW182, but low levels of AGOs (Gibbings et al. 2009; Valadi et al. 2007).

The *C. elegans* RNAi transport protein SID-1 is conserved in humans (Duxbury et al. 2005). Other SID proteins do not appear to have non-nematode orthologs, but the small sizes of SID-2 and SID-5 makes homology calculations difficult. It is possible that other proteins without notable sequence homology perform their functions. Indeed, the nematode *Meloidogyne incognita* takes up environmental dsRNA without a recognizable SID-2 ortholog (Maule et al. 2011).

Cross-Talk and Competition Between Pathways

The interrelatedness of RNAi pathways in *C. elegans* is reflected by the significant amount of cross-talk and competition between them. One example is between exo- and endo-siRNA, where both pathways are dependent on a pool of functionally overlapping secondary AGOs (Yigit et al. 2006). In fact, the mutant strains displaying the strongest enhanced exogenous RNAi phenotypes are the ones deficient in genes *eri-1* and *rrf-3,* required for endogenous RNAi (Lee et al. 2006). A reduced endogenous RNAi activity likely frees resources shared by both pathways, such as secondary AGOs. In endo-siRNA mutants, miRNA efficiency is also often enhanced, and induction of exo-siRNA increases expression of genes under miRNA control (Zhuang and Hunter 2012b). There is also competition in inheritance of endo-siRNA and exo-siRNA silencing. For example, continuous exposure to dsRNA over generations will reduce the level of inherited endo-siRNAs (Houri-Ze'evi et al. 2016).

In worms that have not been exposed to environmental RNAi, the majority of the exo-siRNA-specific AGO RDE-1 can be found in complex with missorted miRNAs (Corrêa et al. 2010). However, with the notable exception of miR-243, these missorted miRNAs can generally not silence expression of their targets through RDE-1.

For CSR-1 22G RNAs, uridylation by CDE-1 is required to separate them from other 22G RNA pathways (van Wolfswinkel et al. 2009). *cde-1* mutant animals have a higher amount of 22G RNAs, and more genes are down-regulated in the germ line. Unwanted crosstalk between small RNA pathways is therefore a potential problem, and uridylation of licensing 22G RNAs is likely a strategy for coping with this.

Subcellular Localization of RNAi Processes

One important characteristic of eukaryotic cells is the ability to compartmentalize processes and their products and substrates into membrane-limited vesicles and organelles. The segment of membrane-based organelles and vesicles that facilitates transport through and beyond the cell, from the endoplasmic reticulum to exosomes, is referred to as the endomembrane system (Sato et al. 2014; Mukherjee et al. 1997). While there is a great diversity in configurations of the endomembrane system among eukaryotes, among cell types in the same organism, and high flexibility in response to external effects within the same cell type, some aspects can be considered true in all or most cases. Many RNAi processes have been determined to be membrane-bound and affected by aberrant vesicle traffic (Kim et al. 2014). Here I will discuss the aspects of the endomembrane system relevant for my thesis, focusing on its relationship to RNAi in *C. elegans*.

Endoplasmic Reticulum/Golgi

As the central site for protein synthesis in eukaryotes, the rough endoplasmic reticulum (rER) is a major source of endomembrane cargo in a cell. The endoplasmic reticulum usually spans almost the entire interior of the cell and displays a characteristic reticulate pattern when visualized by ER-specific markers (Rowland et al. 2014).

Following translation, proteins that require post-translational modifications, are membrane bound, or destined for secretion or a special subcellular location, are transported from the ER to the Golgi at specific ER exit sites. Since the ER and Golgi are not topologically connected, transport between them is facilitated by vesicle traffic through the ER-Golgi intermediate compartment (Spang 2009). Topologically, the Golgi is a stack of flattened tubes or cisternae, between which vesicles ship cargo. *C. elegans* intestinal cells have several smaller Golgi stacks, in close proximity to the ER. Proteins are then sorted in the trans-Golgi network and transported to their final destinations in transport vesicles.

The rough endoplasmic reticulum has been shown to be an important site for siRNA and miRNA loading into RISC as well as subsequent mRNA silencing in mammalian cells (Stalder et al. 2013). In plants miRNA-silencing has also been linked to the rER (Li et al. 2013a). miRNA-mediated mRNA silencing at the rER seems to make sense, as it would allow for quick regulation of expression directly at the site of translation.

Endocytosis and Endosomes

Certain cells, such as the highly polarized intestinal cells, have as their major function the uptake, sorting and processing of nutrients from their apical membrane and their subsequent release through the basolateral membrane. Such cells are useful for investigating the endomembrane system (Fares and Grant 2002). By supplying intestinal cells with fluorescent or easily stainable cargo, it is possible to map and characterize different parts of the endomembrane system. Several different types of endomembrane compartments (endosomes) have been characterized from such studies, based on their protein and lipid composition as well as the order in which cargo reaches them. Endosome compartments are often spherical vesicles, but may also form tubules or other shapes. For many types of endosomes, there is a closely associated protein marker, usually a Rab GTPase, which acts as a regulator for endosome maturation through recruitment of factors necessary for the transition (Barr and Lambright 2010). By using canonical endosome marker proteins fused to a fluorophore, it is possible to map the relationships between different endosomes. Further, it is possible to find genes involved in membrane traffic by looking for membrane traffic phenotypes in genetic screens

(Grant et al. 2001). It is also possible to use these markers to determine the subcellular localization of other proteins of interest.

Internalization of small cargo from the apical membrane of the intestinal cell to the endomembrane system is achieved through endocytosis (Mukherjee et al. 1997; Fares and Grant 2002). The endocytic vesicles then either mature into or fuse with already existing early endosomes. The canonical markers for early endosome limiting membranes are the GTPase RAB-5 (Bucci et al. 1992). Endosome maturation happens through Rab conversion, where the Rab GTPase on the membrane is rapidly exchanged for a GTPase characteristic for the new endosome type (Rink et al. 2005).

Through regulated fusion and fission events, cargo is sorted in early endosomes for the appropriate destination (Jovic et al. 2010). Depending on the cargo, it can move on from early endosomes via several different pathways. Some cargo, e.g. receptors for extracellular signals, is returned to the apical cell membrane through rapid (RAB4-positive vesicles) or slow recycling (RAB11 vesicles, via the endocytic recycling compartment). Other cargo will be degraded by lysosomes via late endosomes and multivesicular bodies. Endosomal traffic can also be routed to the Golgi via the retrograde route. An example of retrograde cargo is sorting receptors that transport their ligand from the ER to endosomes before sorting back to the ER or Golgi. Retrograde transport to the Golgi can be involved with RNAi, as loss of the Golgi-associated retrograde protein (GARP) complex protein VPS-52 negatively affects the miRNA pathway (Vasquez-Rifo et al. 2013).

Late Endosomes/Multivesicular Bodies and Lysosomes

The canonical progression for cargo from early endosomes is to the late endosomes through Rab conversion from RAB-5 to RAB-7 (Rink et al. 2005). Late endosomes then fuse with pre-lysosomes or lysosomes for degradation of its content. Two complementary markers are usually used to visualize late endosomes: RAB-7 and LMP-1. A subpopulation of late endosomes lacks RAB-7, but is positive for LMP-1. Another subpopulation is RAB-7 positive but lacks LMP-1, and third population is positive for both RAB-7 and LMP-1. These three late endosome populations maintain a dynamic relationship where vesicles of each population can bud off and fuse with each other (Humphries et al. 2011).

To internalize membrane bound cargo in the late endosome limiting membrane, e.g. ubiquitylated membrane proteins, such cargo is sorted into intralumenal vesicles (ILV) (Gruenberg and Stenmark 2004). This process is dependent on the ESCRT complex. Late endosomes containing ILVs are referred to as multivesicular bodies (MVBs). Depending on the cargo of the MVBs, they can subsequently fuse either with lysosomes for cargo degradation, or the cell membrane for release of ILVs as exosomes.

Beyond the rER, late endosomes/MVBs have been pointed out as likely sites for RISC assembly and turnover. Human Ago2 and GW182 colocalize with endosomes or MVBs, likely with ILVs (Gibbings et al. 2009). Likewise, GW182 localizes close to MVBs or lysosomes in *Drosophila* (Lee et al. 2009). GW bodies, RNA granules rich in GW182 that act as sites for miRNA-mediated sequestration and decay of mRNAs, associate with late endosomes and MVBs. Furthermore, manipulating the rate of maturation from late endosomes to lysosomes affects the efficiency of RNAi. Decreased endosome-lysosome maturation enhances RNAi, and blocking the ESCRT complex from forming MVBs negatively affects RNAi. Possibly, endosomes or MVBs are necessary for Ago2 recycling to the rER through retrograde trafficking.

Exosomes

Besides the intracellular endomembrane system, there are also several kinds of extracellular membrane vesicles. Exosomes are small extracellular vesicles (30-100 nm) that are secreted from cells and can deliver cargo to distal cells. They are released to the extracellular space by MVBs fusing with the cell membrane. Exosomal cargo includes proteins, lipids and mRNA. In human exosomes, miRNA and pre-miRNA has also been detected, where miRNA occurs in the presence of GW182 but an absence of AGOs (Gibbings et al. 2009). This is in line with the observation that GW182 localizes to ILVs. Inducing endosome sorting into MVBs enhances the level of exosomal miRNAs (Kosaka et al. 2010).

Autophagosomes

Autophagosomes help maintain cellular homeostasis by engulfing and degrading cytoplasmic material (Mizushima and Komatsu 2011). Following engulfment of degradation products, autophagosomes fuse with lysosomes. Human Ago2 and Dicer are selectively degraded by autophagosomes (Gibbings et al. 2012). In *C. elegans*, decreased autophagosome function can rescue effects from impaired miRNA silencing of developmental genes (Zhang and Zhang 2013). Selective degradation of AIN-1 by autophagosomes likely regulates miRNA activity. It is therefore possible that autophagosomes also play a role in RISC turnover.

Endosome Fusion and Transport

Endosome fusion and fission are both important functions in cargo sorting. Fission happens from distinct domains of endosomal membranes, where cargo to be budded off is concentrated (Rowland et al. 2014). Endosome fusion occurs through a long-range tethering stage by special tethering fac-

tors, followed by pairing of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) proteins on both membranes (Jahn and Scheller 2006). Since energetically, lipid bilayers are very stable, fusion of two vesicle membranes is a very energy-demanding process. To overcome this, SNARE proteins on opposite membranes bind strongly to each other to assemble very stable *trans*-SNARE complexes. The conformational change of the SNAREs causes the membranes to be positioned close to each other and ultimately fuse. After fusion, the SNAREs in the *trans*-SNARE complex will occupy the same membrane, and undergo conformational change to form a *cis*-SNARE complex. After fusion, *cis*-SNARE complexes are disassembled by the ATPase NSF. SNAREs are concentrated in microdomains in membranes, and a membrane often contains several different types of SNAREs. The specificity of membrane fusion is provided in part by SNAREs, given that some SNAREs are highly selective in binding partners. Specificity is also likely provided prior to SNARE binding by tethering factors.

The role of membrane microdomains extends beyond SNARE foci and as a step preceding fission. Nanodomains and larger microdomains are transient and dynamic domains of a higher level of order than the surrounding membrane and enriched in certain lipids and proteins (Sezgin et al. 2017). The chemical properties of the microdomain lipids drive enrichment of proteins and lipids with similar properties. Microdomains have been determined important e.g. in regulating immune signaling by concentrating certain activating kinases and simultaneously excluding deactivating phosphatases (Filipp et al. 2004).

Localization and movement of endosomes is effected mainly via microtubules by kinesin and dynein motor proteins (Bonifacino and Neefjes 2017). To a lesser degree, the actin cytoskeleton may also regulate endosome movement. Specificity in transport is provided by cargo adaptor proteins. The precise control of endosome movement through the cell allows for highly regulated sorting and transport of cargo. Through cargo adaptor proteins on endosomes, interactions with the ER can regulate the movement and location of endosomes. The sites of endosome fission and fusion are also well defined at certain ER-endosome contact sites (Rowland et al. 2014).

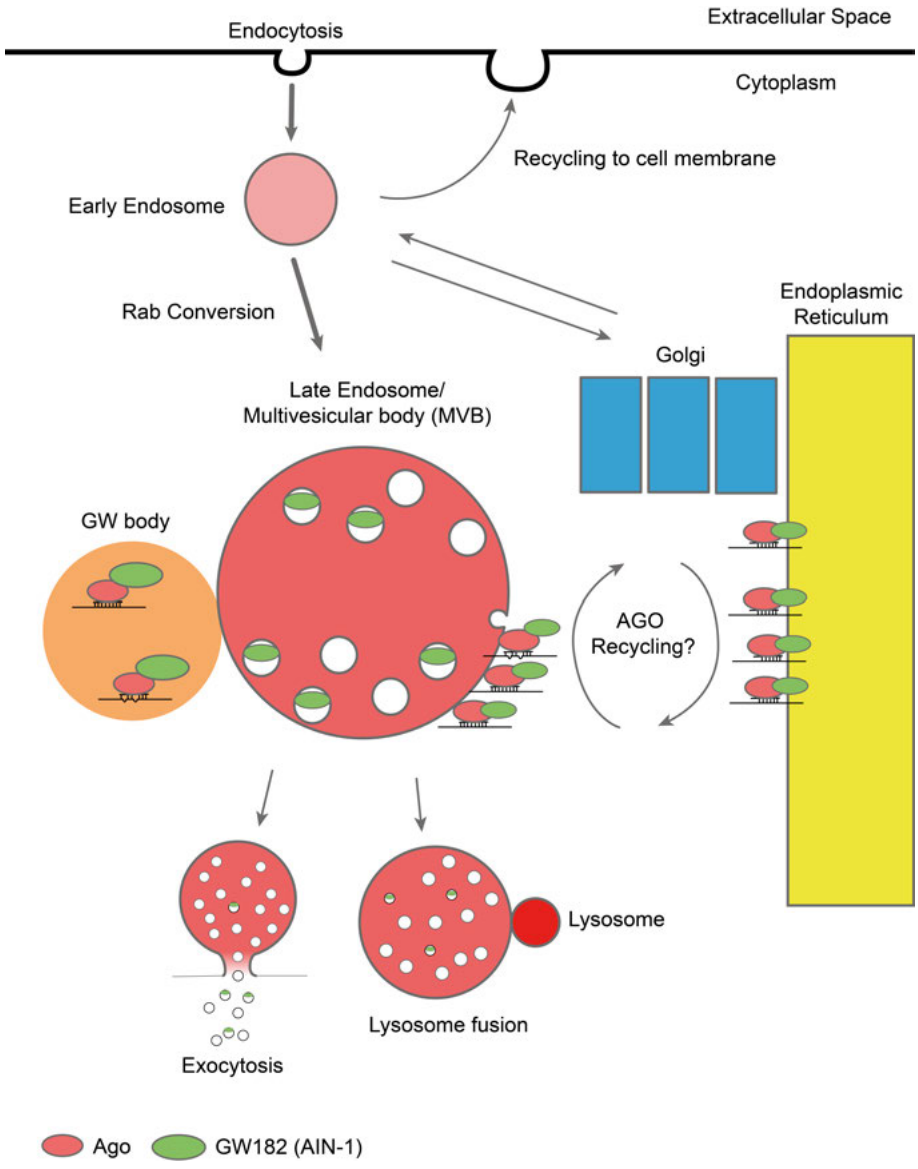

Figure 3. Endosomal transport in a polarized cell. Extracellular cargo is taken up through endocytosis and sorted into early endosomes. These then mature to late endosomes through Rab conversion. Alternatively, they can be recycled back to the cell membrane, or be transported to the Golgi/ER. Late endosomes containing intralumenal vesicles (ILVs) are referred to as multivesicular bodies (MVB). Late endosomes/MVBs can fuse with the cell membrane to release their ILVs, or they can fuse with lysosomes. There is also traffic between late endosomes/MVBs and the Golgi. RISC proteins AGOs and GW182 associate with the endoplasmic reticulum, late endosomes/MVBs, and GW bodies. GW bodies in turn localize close to late endosomes/MVBs.

Quantitative Microscopy

Microscopy is a very powerful class of methods for cell biologists, under the assumption that (ab-)normal function of the labeled product can often be derived from its spatial (mis-)localization. Many conclusions presented in this thesis are based on microscopy images where one or two fluorophore-labeled proteins or cargoes visualize compartments of the endomembrane system. We have used fluorescence microscopy both to determine which endomembrane compartment certain proteins localize to, and also to determine the effect of certain mutant alleles on the size and localization of endosomes.

Quantitative microscopy is the application of image analysis on microscope images for the purpose of extracting information from image data (Dunn et al. 2011). Done properly, quantitative microscopy removes observer bias from the analysis, and simplifies replicating experiments. Quantitative microscopy is often a validating experiment performed after exploratory microscopy, to test whether subjective observations can be confirmed by statistical analysis.

Colocalization is likely the most common property to be quantified from images. Colocalization is the degree by which the intensity in two channels is above zero in the same pixel (co-occurrence) or the degree by which two channels correlate in intensity in the same pixel. Colocalization studies can determine whether two fluorophores occupy the same compartment, and if they have a linear intensity correlation to each other. However, the resolution limit of light microscopy normally precludes determining whether colocalizing proteins are close enough to allow direct interactions between them, in which case the Förster resonance energy transfer (FRET) between two different fluorophores can be measured instead (Sekar and Periasamy 2003). Several different colocalization measures are in use today, depending on the nature of colocalization and the exact question being asked.

Signal Intensity Correlation

For colocalization scenarios where a linear relationship is expected between the intensities of two channels, various intensity correlation coefficient-based (ICCB) methods are available (Bolte and Cordelières 2006). The original – and most commonly used – ICCB method is the Pearson correlation coefficient (PCC); the covariance of the signal intensity per pixel (Manders et al. 1992). A PCC value of 1 means a perfect correlation between the intensities of the two channels, and -1 means perfect inverse correlation of intensity. High Pearson values indicates that the deviation from the mean intensity per pixel tend to be similar in both channels, such that a pixel with relatively strong intensity in one channel will have a relatively strong intensity in the other channel as well. PCC is not dependent on proportionality between the channels, as long as there is a linear relationship in intensity.

Therefore, a relatively bright fluorophore can still be tested for colocalization with a dimmer fluorophore. Large areas of "empty pixels" in the image, such as unlabeled extracellular space, will inflate PCC values, so PCC is normally measured within a smaller designated region of interest (ROI), usually one cell. PCC is mainly useful in cases where there is a simple relationship between the labeled products, such as two strongly bound subunits of a larger protein complex or cargo taken up by the same receptors. Whereas a very high or low PCC value is easy to interpret, an intermediate PCC value might indicate a number of things and will be more difficult to interpret. For example, cases where a small subpopulation of one fluorophore is completely uncorrelated with the other fluorophore might yield the same low PCC value as cases where the colocalization relationship varies slightly. In cases where the relationship in intensity is not expected to be linear, such as when there is a population of compartments strongly positive for one fluorophore and less for the other, PCC might not be an appropriate measure. Intermediate PCC values can be useful in comparative studies to measure differences in colocalization, however.

Signal Co-Occurrence

In cases where co-occurrence of fluorophores is expected rather than strict intensity correlation, the Manders coefficients (MCC) offer a measure of fractional overlap independent of signal intensity correlation (Manders et al. 1993). Pixels with non-zero intensity in both channels are counted as colocalization. For each channel, a fraction is calculated from the number of colocalizing pixels over the total number of non-zero pixels in that channel, resulting in two Manders coefficients when comparing two channels. Splitting the coefficient allows the researcher to determine how much one fluorophore colocalizes with the other instead of how both fluorophores colocalize with each other. The subtle difference in question becomes clear in cases where one fluorophore colocalizes exclusively with a smaller subpopulation of compartments positive for the other fluorophore. A major drawback of MCC is its sensitivity to background pixel noise even in regions empty of fluorophores, resulting in highly overestimated colocalization. Preprocessing of images in the form of background subtraction is therefore normally required to extract signal from the noise (Dunn et al. 2011).

Background subtraction can be as simple as setting a threshold value, pixel intensities under which count as background and are set to zero. More complicated situations, e.g. uneven illumination or high fluorophore density, might require more complicated background subtraction methods, such as subtracting the mean or median intensity of an area around each pixel. Importantly, background subtraction will likely remove the diffuse cytosolic fraction of fluorescence and leave sharply demarcated structures intact. Whether this is appropriate or not depends on the biological question being asked.

Object-Based Colocalization

In object-based colocalization analysis, the focus is not so much on the individual pixels, but rather the structures they compose (Bolte and Cordelières 2006). This often makes sense conceptually, and can in some cases be more descriptive of the biological system than pixel-based colocalization studies. A simple example is cases where the number of colocalizing objects is more interesting than the total number of colocalizing pixels (Lachmanovich et al. 2003). Object-based analysis can also go beyond colocalization analysis, for example to answer whether objects labeled with different fluorophores are localized adjacent to each other. For colocalization, object-based approaches include determining whether the object's centroid or center of mass overlap with an object in the other channel, or of it is within a certain distance of the centroid or center of mass of an object in the opposite channel. Colocalization is then reported as the fraction of colocalizing objects over the total number of objects.

Significant Colocalization

The ultimate gain from quantifying image data is the ability to perform statistical analysis on the results. Following colocalization analysis, the next pertinent step is arguably to test for statistical significance. Comparing two image sets for differences in colocalization is trivial using standard statistical tools such as the two-sample or paired t-test (McDonald and Dunn 2013). This kind of test can be safely performed for both PCC and MCC values. More elaborate methods are required to test one sample group for significant colocalization beyond that which can be explained by chance alone.

One method of one-sample significance testing involves comparing the colocalization of images to the colocalization in derivative images wherein the channels have been shifted out of alignment from each other. For example, one channel can be reversed or shifted relative to the other (van Steensel et al. 1996). In cases of significant colocalization in the source image, colocalization between channels should decrease when they are misaligned. More elaborate methods using the same basic principle have also been developed, such as the confined displacement algorithm (Ramírez et al. 2010).

Another method generates simulated images by scrambling the original image channels independently of each other (Costes et al. 2004). Since pixels are only moved around, the generated images will on average have the same intensity levels as the original, but should reflect the level of colocalization that would be seen in case of no connected localization between channels. Through this method, a large number of scrambled images can be generated, increasing the statistical power. Image scrambling is made more complicated due to spatial autocorrelation, i.e. the tendency of similar values to form clusters in fluorescent microscopy images. Two levels of spatial autocorrelation in microscope images are (1) the fluorescent signal of a sin-

gle fluorophore will be distributed over 2-3 pixels in a well-calibrated image, and (2) the structures, such as endosomes, labeled by the fluorophore. Scrambling individual pixels in an image will not retain the level of autocorrelation of the original image, yielding lower colocalization in the scrambled images than what would be expected. The solution to this is to scramble blocks of pixels, as large as the labeled structures (Ramírez et al. 2010). Block scrambling therefore works best for structures of homogenous size and structure.

Current Investigations

Aim

The overall aim of this thesis is to further investigate the connection between RNAi and the endomembrane system. To do this, we used the systemic RNAi protein SID-5 as a starting point.

We examined the role on RNAi of proteins that interact with SID-5 in a yeast two-hybrid (Y2H) screen (Stagljar et al. 1998). Through this method, we first identified the SNARE SEC-22 as a negative regulator of RNAi (**Paper I**). Despite the known role of the endomembrane system in RNAi, SEC-22 is the first SNARE protein with a role in RNAi. We further discovered that the SNARE protein SYX-6 interacts with both SID-5 and SEC-22, and that like SEC-22, SYX-6 also negatively regulates RNAi (**Paper II**). In this paper, we learned new things about SID-5. SID-5 is able to interact with several different SNAREs, indicating that it might have a role in concentrating SNARE proteins in membrane microdomains. SID-5 also appears important for maturation of late endosomes. We further identified the SID-5 interacting RNA-binding protein C12D8.1 as a negative regulator of RNAi inheritance (**Paper III**). *C12D8.1(-)* mutant animals are also less capable of producing an efficient RNAi response to environmental dsRNA if they have inherited environmental RNAi from their parent. Possibly, C12D8.1 acts to retain RNAi trigger signals in somatic cells. Finally, we have developed a new method for estimating significance of colocalization (**Paper IV**). This method has been very useful for us, and we believe it will be useful for others as well.

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

In recent years, several reports have suggested that RNAi is a membrane-associated process. The cytosolic side of the rough endoplasmic reticulum (rER) has been shown to be an important nucleation site for siRNA-mediated silencing (Stalder et al. 2013). Furthermore, MVBs associate with GW bodies (Gibbings et al. 2009; Lee et al. 2009), which are important sites for

RNAi-dependent RNA degradation. MVBs can fuse with lysosomes, causing degradation of intralumenal cargo. Preventing MVB to lysosome fusion enhances RNAi and increases the amount and sizes of GW bodies. Conversely, preventing MVB formation reduces RNAi efficiency (Gibbings et al. 2009). The SNARE SEC-22 was identified as interacting with SID-5 in the previously performed Y2H screen. SNAREs are membrane proteins that primarily mediate membrane fusion by binding to a corresponding SNARE on a target membrane (Brown and Pfeffer 2010). Despite the central role of SNAREs in membrane traffic, no SNARE protein had been shown to have a role in RNAi prior to this study.

C. elegans sec-22(-) Mutant Animals Display Enhanced RNAi

Following the identification of SEC-22 as a putative SID-5 interacting protein, we tested *sec-22(-)* mutant animals for variant feeding RNAi efficiency phenotypes, targeting various endogenous and transgenic genes. For all gene targets tested (*dpy-13*, *unc-22*, and *act-5*), *sec-22(-)* mutant animals showed enhanced RNAi. To confirm that the enhanced RNAi phenotype of the *sec-22(-)* mutant worms was caused by the *sec-22* mutation, we inserted an extrachromosomal array with functional *sec-22*. In *sec-22(-)* mutant worms with the *sec-22* extrachromosomal array, feeding RNAi levels were restored to wild type levels.

SEC-22 Affects RNA Import or Cell Autonomous RNAi

A variant feeding RNAi phenotype can be the result from deficiencies in one or more of several cellular or extracellular functions. Having established that *sec-22(-)* mutant animals display an enhanced feeding RNAi response, we next wanted to determine which step of feeding RNAi that SEC-22 is involved in. To determine if SEC-22 affects environmental dsRNA uptake or RNAi transport from intestinal cells, we rescued SEC-22 expression exclusively in intestinal cells. *sec-22(-)* mutant animals with an intestinal SEC-22 rescue construct showed a modest rescue of the enhanced RNAi phenotype. In addition, however, *sec-22(-)* mutant animals with SEC-22 rescued exclusively in body-wall muscle cells showed strong rescue for RNAi against body-wall muscle-expressed genes. Indeed, we saw that SEC-22 overexpressed in body-wall muscle cells conferred a reduced RNAi phenotype against body-wall muscle-expressed genes even when compared to wild type worms. This would indicate that SEC-22 mainly act in cell autonomous RNAi/import, but that it may also have a small effect on RNAi transport or uptake of environmental dsRNA.

SEC-22 Localizes to Late Endosomes/MVBs Primarily

To investigate the expression and localization of SEC-22, we constructed an mCherry::SEC-22 fusion protein under the endogenous SEC-22 promoter that was able to rescue the *sec-22(-)* enhanced RNAi phenotype. We saw that SEC-22 is expressed broadly in *C. elegans*. In intestinal cells, mCherry:SEC-22 localizes to vesicle-like structures and small punctae. mCherry::SEC-22 structures localize significantly to the intralumenal space of vesicles positive for late endosomal/MVB markers GFP::RAB-7 and LMP-1::GFP, but not significantly with markers for autophagosomes, Golgi, early endosomes, recycling endosomes. While LMP-1::GFP and GFP::RAB-7 colocalize with markers for acidified lysosomes, mCherry::SEC-22 does not colocalize significantly to the acidified lysosome marker LysoTracker Green. The localization of mCherry::SEC-22 to the intralumenal space of late endosomes rather than to the limiting membrane could suggest that SEC-22 in *C. elegans* intestinal cells localizes mainly to ILVs of MVBs, either following vesicle fusion and preceding lysosomal degradation or recycling, or preceding export of intralumenal vesicles to the extracellular space.

C. elegans sec-22(-) Mutant Animals Have Enlarged Late Endosomes/MVBs

Previous studies have shown that late endosomes/MVBs and the relative balance of late endosomes/MVBs to lysosomes can affect the efficiency of RNAi in *C. elegans.* We wanted to know if the enhanced RNAi phenotype of *sec-22(-)* mutant animals could be explained by altered characteristics of late endosomes/MVBs. To this end, we compared the size of LMP-1::GFP vesicles in *sec-22(-)* mutant worm intestinal cells to the size in wild type worms. We could see that *sec-22(-)* mutant animals have significantly larger late endosomes/MVBs compared to wild type.

sid-5 is Epistatic to *sec-22*

The transmembrane protein SID-5 is required for efficient export of the RNAi signal. Given the interaction of SEC-22 with SID-5 in the Y2H screen, and given that SID-5 can often be seen in foci adjacent to late endosomes/MVBs, we wanted to investigate the relationship between SID-5 and SEC-22. In order to determine if the localization of SID-5 near late endosomes/MVBs was dependent on SEC-22, we compared the localization of immunostained SID-5 in *sec-22(-)* mutant animals compared to that of wild-type worms. We could see no striking difference in the distribution of SID-5. However, when we examined their genetic relationship using a *sid-5;sec-22* mutant strain, we could determine that this strain demonstrated the reduced

RNAi phenotype of *sid-5(-)* rather than the enhanced RNAi phenotype of *sec-22(-)* alone.

In conclusion, SEC-22 negatively regulates RNAi in *C. elegans*. This effect is mainly cell autonomous, but SEC-22 also has a small effect on systemic RNAi. SEC-22 localizes to the intralumenal space of late endosomes/MVBs, and late endosomes/MVBs are enlarged in *sec-22(-)* mutant animals. A simple model would be that lack of SEC-22 affects degradation of RNAi silencing signals, perhaps by negatively affecting late endosome fusion to lysosomes. SID-5 would then act upstream in RNAi, although the exact function of SID-5 is still not known, as well as the nature of interaction between SEC-22 and SID-5. It is also still unclear if *sec-22(-)* mutant animals have altered processing of endogenous RNAi in addition to exogenous RNAi. *sec-22(-)* mutant animals do not show the classical temperature sensitivity or high incidence of male phenotypes that would indicate reduced efficiency of endogenous RNAi (Pavelec et al. 2009), but it is still possible that miRNA or piRNA processing is affected in *sec-22(-)* mutant animals. Additionally, it remains to be seen if SEC-22 is involved in RNAi in other organisms.

RNA Transport Protein SID-5 Interacts with Multiple SNAREs and Affects Membrane Trafficking in *C. elegans* Intestinal Cells (Paper II)

Endosomes mature through Rab conversion, where Rab GTPases, acting as master regulators for endosome maturation, are exchanged for another Rab protein characteristic for the next vesicle type (Barr and Lambright 2010). Following Rab conversion, several other proteins and lipids are recruited to the vesicle. Endosome trafficking is also reliant on proper transport and fusion (homotypic and heterotypic) to perform sorting of endosomal cargo.

Building on Paper I, we further investigated the relationship between SEC-22 and SID-5. We found that SID-5 interacts with several SNARE proteins in a Y2H screen, whereas *C. elegans* SEC-22 appears to interact only with the SNARE SYX-6. In *sid-5(-)* mutant animals, maturation of late endosomes appears to be impaired, with RAB-7 positive vesicles taking on appearance of early endosomes instead. We could also determine that SYX-6, like SEC-22, negatively regulates RNAi.

SID-5 and SEC-22 Affect Formation or Maturation of Late Endosomes/MVBs

In Paper I we demonstrated that the SNARE SEC-22 interacts with transmembrane protein SID-5, and that *sec-22(-)* mutant animals have both enlarged late endosomes/MVBs and enhanced RNAi. Building on this, we

wanted to determine if *sid-5(-)* mutant animals also have late endosome phenotypes. Two labels for late endosomes/MVBs and lysosomes were used: GFP::RAB-7 and LMP-1::GFP.

sid-5(-) mutant animals had a population of enlarged basolaterally localized LMP-1::GFP vesicles, compared to the vesicle sizes normally seen in wild type worms. In contrast, most GFP::RAB-7 vesicles in *sid-5(-)* mutant animals were small punctae positioned close to the apical membrane. In wild type worms, only a small fraction GFP::RAB-7 vesicles look like this, while the majority are larger and more medial. There is normally an overlap in LMP-1::GFP and GFP::RAB-7 size and localization in wild type worms. The size and location of GFP::RAB-7 positive late endosomes in *sid-5(-)* animals is similar to the behavior of early endosomes, indicating that late endosome maturation in the absence of SID-5 is impaired at a step downstream of Rab conversion from RAB-5 to RAB-7.

In *sec-22(-)* mutant animals, we had previously seen enlarged LMP-1::GFP vesicles. Keeping in mind the drastic difference in location and size of LMP-1::GFP and GFP::RAB-7 in *sid-5(-)* mutant animals, we also wanted to see whether a similar difference could be seen for GFP::RAB-7 in *sec-22(-)* mutant animals. We found that vesicles with GFP::RAB-7 in their limiting membrane were enlarged in *sec-22(-)* mutant animals. A large fraction of GFP::RAB-7 also formed solid structures, presumably internalized by late endosomes/MVBs not acidic enough to degrade GFP.

SEC-22 Interacts With SNARE SYX-6, SID-5 with Multiple SNARES in Yeast Two-Hybrid Assays

We performed yeast two-hybrid (Y2H) screens to find syntaxin SNARE proteins interacting with either SEC-22 or SID-5. Of nine syntaxin SNAREs tested in this screen, we found that SEC-22 only interacts with the protein SYX-6. In contrast, SID-5 interacted with six out of nine SNAREs, including SYX-6, in this screen.

mCherry::SYX-6 Localizes to Late Endosomes/MVBs but not Acidified Lysosomes and to Unidentified Mesh-Like Structures

From the Y2H screen results, we were interested to see how the subcellular localization of SYX-6 compared to that of SEC-22 and SID-5. We know from previous studies that SID-5 is located in small foci close to late endosomes/MVBs and that SEC-22 colocalizes significantly to late endosomes/MVBs. We produced an mCherry::SYX-6 under the intestine-specific *vha-6* promoter and crossed that construct into strains carrying markers for different membrane compartments. We saw that mCherry:SYX-6 colocalized strongly with late endosome/lysosome marker LMP-1, and less so with

GFP-RAB-7. Besides late endosome vesicles, mCherry::SYX-6 and LMP-1::GFP both also form a reticulate pattern, which we believe correspond to transport vesicles, or perhaps microtubule-associated ER tubules. mCherry::SYX-6 did not colocalize with acidified lysosome marker LysoTracker Green to any larger extent.

Additionally, small mCherry::SYX-6 foci were also often found in close proximity with medial Golgi marker α-mannosidase::GFP. We speculate that the small mCherry::SYX-6 foci could be either *cis* or *trans*-Golgi compartments.

syx-6(-) Mutant Animals Display Enhanced Feeding RNAi Efficiency

In Paper I, the SNARE protein SEC-22 was shown to negatively regulate RNAi. In order to see if this was true also for SYX-6, we performed feeding RNAi on *syx-6(-)* mutant worms and compared the strength of their RNAi response to that of wild type worms. We saw that two independent *syx-6(-)* mutant strains, both likely null alleles, have an enhanced feeding RNAi phenotype when targeting the intestine-expressed *act-5* gene. We could also see an enhanced RNAi phenotype against the epidermis-expressed gene *dpy-13*.

To conclude, by investigating the potential interacting SNARE proteins to SEC-22 and SID-5, we revealed that the SNARE SYX-6, like SEC-22, is a negative regulator of RNAi. We also found that SID-5, previously only described as necessary for efficient systemic RNAi, likely plays a role in late endosome maturation in *C. elegans* intestinal cells. Lack of functional SID-5 caused late endosome (positive for late endosome marker GFP::RAB-7) to look and localize more like early endosomes. Interestingly, LMP-1::GFP vesicles in *sid-5(-)* mutant animals are enlarged, similar to the effect seen in *sec-22(-)* mutant animals. Late endosomes/MVBs have previously been associated with the efficiency of RNAi. Our results indicate that there is a correlation between the size of GFP::RAB-7 positive late endosomes, and efficient feeding RNAi. The same correlation can not be seen between LMP-1::GFP vesicles and RNAi however, indicating that GFP::RAB-7 positive late endosomes or late endosomes positive for both LMP1- and RAB-7 play a role in RNAi. The exact function of SID-5 is still unknown, but it is possible that SID-5 is important for maturation steps downstream of the RAB-5 to RAB-7 exchange, perhaps in transport of late endosomes from the apical membrane, and/or homotypic fusion with other late endosomes/MVBs.

SID-5 interacts with several SNAREs in our Y2H screen. While we cannot say whether SID-5 truly interacts with all Y2H hits *in vivo*, it does show that SID-5 interacts with SNAREs in a non-specific way. Possibly, SID-5 could localize to membrane microdomains with a high concentration of

SNAREs. The effect of *sid-5(-)* mutations on late endosomes/MVBs could also help explain the role of SID-5 in systemic RNAi. Systemic RNAi could be dependent on MVBs fusing with cell membranes to secrete exosomes or non-exosomal cargo.

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

In *C. elegans*, exogenous RNAi can be passed along from parent to offspring. This inheritance is limited in duration, however. In this study, we used an RNAi-based assay to look for SID-5-interacting proteins with an RNAi phenotype when silenced. Of the three genes we found to have RNAi deficiency phenotypes, we selected C12D8.1 for further characterization. *C12D8.1* mutant animals are not deficient in RNAi however; rather they have an enhanced response. Also, *C12D8.1* mutant animals have an enhanced inheritance of RNAi, at least over one generation. This likely interferes with the ability of the offspring to take up new environmental RNAi.

GFP RNAi is Reduced in Worms with C12D8.1 Expression Silenced

We conducted a screen for variant RNAi phenotype against a body-wall expressed GFP construct, following silencing of hits from our Y2H screen by RNAi. In brief, worms were treated with feeding RNAi against genes of interest, from the L4 stage to the L4 stage of the next generation. These worms were then treated with feeding RNAi against GFP from L4 to L4 in the next generation. The number of GFP-positive body-wall muscle cells was then scored. Using this screen, we have as of this writing found RNAi deficiency phenotypes in three putative SID-5 interacting proteins out of seven tested. Of these, we selected C12D8.1 for further characterization since it is an RNA-binding protein that had also previously been identified as having a reduced RNAi phenotype in a similar screen (Kim et al. 2005).

C12D8.1 is Broadly Expressed and Localizes to the Nucleus and Perinuclear Foci

Using a C12D8.1::mCherry construct under the endogenous C12D8.1 promoter, we could see that in the adult worm, C12D8.1 is expressed in several different tissue types, including pharynx, body-wall muscle cells, intestinal cells, seam cells, and hypodermal cells. In these cells, C12D8.1::mCherry is localized to the nucleus, but also to the cytoplasm and to unidentified peri-

nuclear foci that we believe are either RNA granules or perhaps Golgi. The ratio of localization between these different cellular compartments varies between cell types but also between cells of the same type. We could also detect C12D8.1 expression in embryos, where it appears to localize exclusively to the nuclei. The nuclear localization can be explained by a predicted nuclear localization signal (NLS), which is present in the N-terminus of the C12D8.1a isoform. In addition, a phosphoserine in the NLS could act as a switch for nuclear localization (Chung et al. 2012).

C12D8.1 Mutant Worms Display Enhanced RNAi

To our surprise, when we attempted to recapitulate the reduced RNAi phenotype using mutant *C12D8.1(-)* worms rather than C12D8.1-silenced worms, we observed an enhanced RNAi phenotype instead. This was seen for two different *C12D8.1(-)* mutant strains, against both the original body-wall muscle-expressed GFP as well as the endogenous body-wall muscle target *unc-22*. We were not able to see any RNAi phenotype against the hypodermal cell target *dpy-7*.

In our standard feeding RNAi protocol, we cannot differentiate between a variant RNAi phenotype and variant RNAi inheritance phenotype. We reasoned that the discrepancy in observed RNAi phenotype between C12D8.1-silenced worms and in mutant worms could be an effect of altered RNAi inheritance. To determine if the enhanced RNAi phenotype was an inheritance phenotype, we eliminated the effect of inheritance by performing feeding RNAi in *C12D8.1* mutant animals from the L1 stage to young adults of the same generation. Without the contribution of inherited RNAi, the enhanced RNAi phenotype of *C12D8.1(-)* mutant animals disappears. In addition, the reduced RNAi phenotype of C12D8.1-silenced worms is abrogated.

C12D8.1(-) Mutant Animals Have Enhanced RNAi Inheritance, Which Inhibits RNAi against Novel Targets in Offspring

To further characterize the effect of C12D8.1 on RNAi inheritance, we directly tested whether *C12D8.1(-)* mutant worms have a variant RNAi inheritance phenotype. Indeed, when comparing the number of offspring inheriting the dumpy phenotype following parental *dpy-7* feeding RNAi, we could observe a significant enhanced RNAi inheritance in *C12D8.1(-)* mutant animals. We reasoned that the enhanced inheritance of RNAi signals could interfere with silencing of novel targets in the next generation, and that this could explain the ostensible reduced RNAi phenotype in worms where *C12D8.1* had first been silenced. We found that RNAi in *C12D8.1(-)* mutant animals against *dpy-7* is significantly reduced if the previous generation has been exposed to dsRNA targeting either GFP or the endogenous gene *unc-22*.

In order to see whether this effect was exclusively an effect of inheritance, we performed sequential same-generation RNAi against *unc-22* (L1 to L3 larval stages), followed by RNAi against GFP (L3 to young adult). The effect of RNAi against GFP is unaffected by prior *unc-22* RNAi in *C12D8.1(-)* mutant animals. Interestingly however, wild type worms do display a reduced effect by GFP RNAi if they have been treated with *unc-22* RNAi previously.

Taken together, our interpretation of our observations is that C12D8.1 is an RNA-binding protein that affects the balance of RNAi signal distribution between somatic cells and the germ line. In the absence of C12D8.1, retention in somatic cells of RNAi signal molecules (long dsRNA and/or primary siRNA) appear reduced, shifting the balance towards export of RNAi signals from somatic cells, resulting in a stronger inheritance. In addition to the enhanced inheritance phenotype, this would explain why *C12D8.1(-)* mutant animals are less affected by prior RNAi treatment in the same generation compared to wild type worms, but do not display any enhanced RNAi phenotype in same-generation feeding RNAi: less RNAi signal against the first target is retained in somatic cells, reducing the effect of RNAi saturation.

DESCROD Estimates Significant Colocalization by Randomized Object Displacement (Paper IV)

When characterizing colocalization in preparation for Paper I, we were unable to find a method suitable for our particular needs. This led us to develop a novel, object based method that could estimate significant colocalization between objects. In object-based colocalization, in contrast to pixel-based colocalization, focus is on higher-level structures such as vesicles. This is helpful in certain cases, such as if one fluorescent label is located in the limiting membrane of vesicles, and the other fluorophore is located in the vesicle lumen. Additionally, spatial relationships other than colocalization can be determined, such as proximity. We use the object-based approach to determine significant colocalization above the level of colocalization that would occur by pure chance, in the absence of coupled colocalization between two fluorophores. We do this by generating a large number of images where objects from the two channels have been rearranged independently (object displacement) of each other. The level of colocalization that then occurs between the objects of the two channels in the object displaced images then represents the portion of colocalization that can be expected from uncoupled localization. We then rank the original image against the object displaced images to get a measure of how much the colocalization of the source image deviates from the expected value. We describe a novel method for estimating significant colocalization above what would be expected between two com-

pletely uncorrelated channels. In addition to significance estimation, an estimate of the effect size (M_{AE}) is provided, defined as the fraction of colocalization remaining when the portion of colocalization explainable from uncoupled colocalization has been subtracted. This value can be used in addition to the statistical significance rank to determine if the level of colocalization is large enough to also be biologically relevant. We believe that the method described in this study is a step forward in the field of colocalization studies, and should be a useful tool to answer questions in many areas of cell biology.

DESCROD Can Detect Small Differences in Colocalization Yet is Conservative in Crowded Images

We wanted to confirm that our approach is sensitive even to small increases in colocalization above expected. To test this, we generated simulated images with a portion of "coupled colocalization" between two channels and a portion of randomized "uncoupled colocalization". The composition of the simulated images reflected our own dataset in object size, concentration, and shape. From these simulations, we could detect significant colocalization between channels with as low as 5% coupled colocalization.

We additionally wanted to be certain that our method did not overestimate the significance of colocalization, i.e. in cases where images are highly crowded with objects, or objects are distributed heterogeneously. We generated simulated images with different amounts of object crowdedness and distribution, and tested how this affected the M_{AE} coefficients. To our satisfaction, crowdedness had a negative effect on M_{AE}, demonstrating that this method will give a conservative estimate of significance in cases where uncoupled colocalization is expected to have a larger effect.

LysoTracker Red/LMP-1::GFP Colocalization is Unaffected in *sec-22(-)* Mutant Animals

In Paper I we showed that in *C. elegans* intestinal cells, late endosomes/MVBs are enlarged in the absence of SEC-22. We wanted to see if SEC-22 affected the acidification of late endosomes/MVBs. We therefore used DESCROD to determine if SEC-22 affects the colocalization between late endosome/MVB label LMP-1::GFP and acidified lysosome marker LysoTracker Red. We could see that the majority of LysoTracker Red colocalizes with LMP-1::GFP, but that a large portion of LMP-1::GFP does not colocalize with LysoTracker Red. Both LysoTracker Red colocalization to LMP-1::GFP and the opposite relationship are significant compared to uncoupled colocalization, however. When comparing colocalization between

the two channels in wild type and *sec-22(-)* mutant animals, we could see no significant difference in colocalization.

Adjacency Quantification Shows Significant Increase of Late Endosome/MVB-Adjacent Lysosomes in *sec-22(-)* Animals

From looking at the acquired images, it appeared as though a large number of small LysoTracker Red structures localize proximally to larger LMP-1::GFP vesicles. This likely corresponds to lysosomes or pre-lysosomes poised to fuse with late endosomes/MVBs. This effect appeared to be more pronounced in *sec-22(-)* animals, which might indicate an impaired ability for late endosome/MVBs to lysosome fusion. Using DESCROD, we were able to confirm that *sec-22(-)* animals have a significant increase in LysoTracker Red objects adjacent to LMP-1::GFP objects. The object-based approach allows describing other relationships between visualized structures apart from colocalization. We measured the LysoTracker Red adjacency to LMP-1::GFP objects; e.g. colocalization of LysoTracker Red objects to the immediate vicinity to LMP-1::GFP objects. We then compared this to LysoTracker Red adjacency to LMP-1::GFP in case of completely uncoupled colocalization. We found that a subpopulation LysoTracker Red objects are indeed enriched adjacent to LMP-1::GFP objects in wild type animals. Furthermore, this effect was significantly stronger in *sec-22(-)* animals, confirming our hypothesis.

Objects from Automated Vesicle Segmentation Method is Sensitive Enough to Detect Enlarged Late Endosome/MVB Phenotype in *sec-22(-)* Animals

Prior to using DESCROD, objects (corresponding to subcellular structures, such as vesicles) need to be identified. Images are treated to remove background and segment objects from each other. This process often needs to be adjusted according to the source image and the size and shape of the structures. We developed a set of automated object detection and segmentation ImageJ macros for both solid and hollow (i.e. membrane-labeled vesicles) objects. In Paper I, we manually annotated LMP-1::GFP vesicles in images from wild type or *sec-22(-)* mutant animals, to show that late endosomes/MVBs are enlarged in the absence of SEC-22. With the same dataset, we were able to detect a significant difference in vesicle size using the automatic vesicle detection method, showing that it is sensitive enough to be useful.

Conclusions and future perspectives

RNAi and the Endomembrane System

We are fast approaching an understanding of how RNAi is connected to the cell endomembrane system. In Papers I and II, we have identified two SNARE proteins, important factors for membrane fusion, to play a role in RNAi. In accordance with current ideas (Gibbings and Voinnet 2010), these SNARE proteins are located at late endosomes/MVBs, which have previously been identified as important sites for RNAi. In *sec-22(-)* mutant animals, late endosomes/MVBs were enlarged. Using the method described in Paper IV to measure significant adjacency, we could see that late endosome to lysosome fusion seems to be impaired. As in *Drosophila* when late endosome to lysosome fusion is impaired (Lee et al. 2009), *sec-22(-)* mutant *C. elegans* are also more sensitive to RNAi. One explanation is that late endosome to lysosome fusion is an important step for RISC turnover. In SID-5-deficient animals, GFP::RAB-7 positive endosomes are small and located near the apical membrane, similar to early endosomes. On the other hand, LMP-1::GFP positive late endosomes/MVBs are located medially or basolaterally, and several grossly enlarged vesicles could be seen. The method described in Paper IV will be suitable to quantify the size and localization changes of both LMP-1::GFP and GFP::RAB-7.

Our results implicates that late endosomes potentially have a more active part in RNAi, not just as a step in RISC turnover. One possible explanation is that loaded RISC complexes in late endosomes can escape turnover and return to active gene silencing through retrograde transport, and that this route is favored more strongly in cases of reduced late endosome to lysosome fusion. This would be compatible with our observation that SYX-6, in addition to late endosomes, also localizes to small foci near a marker for the medial Golgi compartment.

Originally identified as a systemic RNAi protein likely involved in RNAi export, SID-5 now appears to have a more central role in late endosome maturation. Our data suggest that SID-5 interacts with a broad range of SNARE proteins. We also know that *sid-5(-)* is epistatic to *sec-22(-)*, such that *sec-22; sid-5* double mutant animals display the reduced systemic RNAi phenotype of *sid-5(-)*. SID-5 could perhaps act in close proximity to sites of high SNARE concentrations, such as membrane microdomains. We still do not know what subcellular structure SID-5 is located in, other than that SID-

5 foci are often located close to late endosomes/MVBs. Possible sites include small transport vesicles or ER contact sites. Both of these locations could help explain the *sid-5(-)* impaired late endosome maturation phenotype. It is interesting to note that despite the radical effect of SID-5 deficiency on the appearance of late endosomes/MVBs, *sid-5(-)* mutant animals are relatively healthy. Possibly, SID-5 acts downstream of a sorting step, thus only affecting endosomes with RNAi cargo.

Another interesting question is the mechanism through which RISC is transported to late endosomes/MVBs, following loading at the rER. Little is known about this transport step, such as how it is regulated and what the determinants for rER to late endosome transport is. Possibly, the endosomal route is required to release and completely degrade mRNA transcripts that are not immediately cleaved. It is also possible that RISC complexes that do not bind a matching transcript in the rER are eventually transported to late endosomes/MVBs for release from the guide strand. Since the amount of secondary AGO proteins is a limiting factor for RNAi in *C. elegans*, stringent resource management and recycling of them is probably of value.

Systemic RNAi

As mentioned above, SID-5 was originally characterized as a systemic RNAi protein. SID-5-deficient animals are still able to silence genes cell-autonomously, but it appears as though cells need SID-5 in order to export RNAi trigger signals. In Paper II, we found that SID-5 affects the maturation of late endosomes, thus connecting systemic RNAi to membrane trafficking and cell-autonomous RNAi. However, it is still not clear if it is the late endosome phenotype in *sid-5(-)* mutant animals that negatively affects RNAi export, or if SID-5 has a more general role for endosome sorting (or specifically for sorting endosomes with RNAi cargo).

Extracellular RNAi triggers include both long dsRNA and a shorter double-stranded siRNA intermediate (Jose et al. 2011). Import does not require the RNAi triggers to be confined in exosomes in *C. elegans.* In other organisms, miRNAs and siRNAs have been detected in exosomes, however. It is as of yet unclear if systemic RNAi in *C. elegans* is mediated through exosomes, or if intercellular transport of RNAi triggers is achieved by some other means. It is also still unclear if the main systemic RNAi trigger is long dsRNA, or a shorter siRNA intermediate.

In *C12D8.1(-)* mutant animals (Paper III), germ line cells seem to act as a stronger sink for systemic RNAi trigger molecules relative to somatic cells. Importantly, C12D8.1 does not seem to have a significant effect on RNAi against somatic genes if the contribution from inherited RNAi is removed. We believe that this is an effect in the somatic cells, where retention of a pool of RNAi signals, perhaps not actively silencing, could be impaired in

favor for export. C12D8.1 is a putative RNA-binding protein that can interact with factors required for nuclear RNAi and mRNA degradation, but also with SID-5. One hypothesis is that C12D8.1 normally keeps RNAi trigger molecules unavailable for export by facilitating transport to the nucleus and/or RNA granules. The genetic relationship between *C12D8.1* and *sid-5* remains to be investigated however. In addition, we still have a list of proteins that interacts with SID-5 in a Y2H screen. So far, the search for RNAi proteins from this list has been extremely fruitful.

RNAi inheritance

In recent years, much has been learned about inheritance of RNAi. More and more, it appears as if the generational limit for inheriting exogenous RNAi, previously believed to be a passive effect of RNAi signal dilution, can be regulated (Anava et al. 2015).

In C12D8.1 we found a negative regulator of RNAi inheritance (Paper III). As mentioned above, secondary AGOs are a limiting factor in *C. elegans* RNAi. An animal inheriting exogenous RNAi must still be able to mount an efficient RNAi response against a novel target. In *C12D8.1(-)* mutant animals, an impaired ability to do so follows from the enhanced inheritance of RNAi. Interestingly, depending on the target gene, the enhanced RNAi inheritance does not always lead to enhanced silencing if feeding RNAi is performed over two generations. Further investigation is required to establish the determinants for this effect. Possibly, this could depend on the mechanism downstream of mRNA recognition that predominantly silences that transcript. Whereas some transcripts are mainly cleaved by the RISC complex, others are silenced mainly through nuclear RNAi. Likewise, inherited siRNA silence gene expression through the nuclear RNAi pathway. Hypothetically, RNAi is then be enhanced if the transcript is predominantly silenced post-transcriptionally, where it would act in parallel with the inherited, transcriptional silencing.

While the enhanced inheritance phenotype in *C12D8.1(-)* mutant animals could potentially be explained as a purely parental effect, we cannot yet be certain if C12D8.1 also has an effect on inheritance in the progeny. In support for this, C12D8.1 is expressed during embryonal development, where it has an exclusive nuclear localization. C12D8.1 in the offspring could act to shift the balance between inherited exo-RNAi and novel exo-RNAi, or between somatic and nuclear RNAi.

In conclusion, the results presented in this thesis have helped us understand the place for the RNAi machinery in the nematode, both spatially and conceptually. However, as always in science, this deeper understanding has helped us formulate more detailed questions that we still need to seek an-

swers to. The gaps in our understanding are now at the seams between cell-autonomous RNAi, systemic RNAi, and inheritance of RNAi. More and more, it seems as though SID-5 and its interacting proteins are vital factors in connecting these fields.

Svensk Sammanfattning

Möjligheten att reglera genuttryck är avgörande för allt liv, och nödvändigt bland annat för miljöanpassningar, försvara sig mot stressfaktorer och styra den embryonala utvecklingen. För att upprätthålla kontroll över genuttryck sker reglering på flera nivåer: transkriptionellt för att förhindra att mRNA-transkript syntetiseras, co-transkriptionellt för att avbryta mRNA-transkription och post-transkriptionellt för att förhindra translation från ett mRNA-transkript. Dessa olika nivåer av reglering möjliggör extremt finjusterad kontroll av uttryck, vilket är nödvändigt t.ex. i komplexa multicellulära organismer.

Under åren har man mer och mer insett betydelsen av små RNA i genreglering. I många eukaryoter är till exempel en stor del av alla gener reglerade av mikroRNA (miRNA) som verkar för att finjustera uttrycket. Funktionerna för små RNA sträcker sig emellertid bortom reglering av de egna generna. Till exempel kan RNA-interferens (RNAi) genom små interfererande RNA (siRNA) fungera som ett försvar mot transposoner och i vissa fall som ett antiviralt försvar.

Celler svarar på miljöanpassningar genom att anpassa uttrycket av sina gener. Reglering av små RNA kan spridas från en ursprungscell för att åstadkomma reglering i distala vävnader (systemisk RNAi). RNAi kan också nedärvas av avkomman i vissa organismer.

Eukaryota celler har ett sofistikerat endomembransystem som används för sortering och transport. Både proteiner som cellen själv har syntetiserat och ämnen som cellen har tagit in utifrån (t.ex. genom endocytos) transporteras genom endomembransystemet. Det har även visat sig att RNAi är starkt associerat med sena endosomer, som är en del av endomembransystemet.

Det övergripande syftet med denna avhandling var att ytterligare undersöka sambandet mellan RNAi, systemisk RNAi, och endomembransystemet. För att göra detta användes det systemiska RNAi-proteinet SID-5 som utgångspunkt. Vi har använt nematoden *C. elegans* som modellorganism. Vi undersökte om proteiner som interagerar med SID-5 i en jäst-tvåhybrid (Y2H)-screening påverkar RNAi. Med hjälp av denna metod identifierade vi först SNARE-proteinet SEC-22 som en negativ reglerare för RNAi (Artikel I). SNARE-protein behövs vid fusion av membran i cellen. Trots att endomembransystemet har visat sig ha en viktig funktion i RNAi är SEC-22 det första kända SNARE-proteinet med en RNAi-fenotyp.

Vi upptäckte även att SNARE-proteinet SYX-6 interagerar med både SID-5 och SEC-22. I likhet med SEC-22 reglerar även SYX-6 RNAi negativt (Artikel II). Vi undersökte också effekten av SID-5 på endomembransystemets funktion. SID-5 kan interagera med flera olika SNAREs, vilket tyder på att det kan ha en roll exempelvis i att koncentrera SNARE-proteiner i mikrodomäner i membran. SID-5 framstår också viktigt för mognad av sena endosomer.

Vi identifierade vidare det SID-5-interagerande RNA-bindande proteinet C12D8.1 som en negativ reglerare av nedärvning av RNAi (Artikel III). C12D8.1-mutanter är dessutom mindre kapabla att åstadkomma effektivt RNAi om de har ärvt RNAi från sina föräldrar. Möjligtvis verkar C12D8.1 genom att hålla kvar RNAi-trigger-signaler i somatiska celler.

Slutligen har vi utvecklat en ny metod för att bedöma betydelsen av kolokalisering (Artikel IV). Denna metod har varit mycket användbar för oss, och vi tror att den kommer att vara användbar även för andra.

Sammanfattningsvis har de resultat som presenteras i denna avhandling hjälpt oss att förstå RNAi-systemets plats och dess funktioner. Denna djupare förståelse har hjälpt oss att formulera mer detaljerade frågor som vi fortfarande behöver söka svaren på. Nu finns de största bristerna i vår förståelse i gränslandet mellan cell-autonom RNAi, systemisk RNAi och nedärvning av RNAi. Mer och mer verkar det som om SID-5 och dess associerade proteiner är viktiga faktorer för att koppla samman dessa fält.

Acknowledgements

Thanks first of all to **Andrea**, for being a rock with an eye for details. Couldn't have done it without you!
Yani: thanks for the snacks and for being a great lab partner. **Dirui,** I hope you're doing well! I have your picture placed very prominently at home. **Linnéa,** thanks for your hard work, much of which ended up in this thesis! **Erik,** hope you're ready for some more worm work ~~~
Marcus, thanks for being a friend, and especially for being you. Never change (except for the puns, they're terrible), **Bow-tie Magnus,** our bizarre conversations are always a pleasure. **Sanna,** Thanks for having a good name policy for your group members. Please have a serious discussion with Marcus about the puns though. Thanks. HEJ **DISA**! **Katrin & Danna**: never a sad face with you guys around
Jonas, if I ever were to teach again I'd like it to be with you. **Zhen,** I can always rely on you. **Fredrik, Bart & Helena** for appreciating the beauty of slime molds
Gerhart, thanks for being a great teacher. **Mirthe,** for lifting all the parties **Maaike,** great office mate**, Sofia** for the fika**, Ced** for the oligos
Staffan, great prefect! **Asgeir**, for caring for the microscope, **Elin,** I have also enjoyed our talks. Thanks to rest of Staffan's group: **Jingyi, Showgy, Dimitra and all the new people whose names I don't remember (sorry)**
Lina, thanks for being a rock and for defending before me so I could get inspired by your thesis. Thanks non-bow-tie **Magnus** for the nice CRISPR discussions
Thanks Mikael and group, Stefan, Marie, Maria Letizia
IMBIM people – **Daniel, Ravi, Wael, Tanel**
Old students from course labs, I hope you've all improved (even those of you who were already good)
Dennis, Andreas, Andris, Jordi, Aaron, Max for rules and rolls
Dr. Falafel for being a true academic role model
Jättetack till familjerna:
Holmgren/Ruuskanen/Sundberg – **Gerd, Lennart** – starkt jobbat med min uppfostran och så, **Aurora & J.P**., **Mormor, Morfar, Farmor, Farfar**.
Andersson/Dahlqvist – **Carola, Lasse, Laila, Jens, Ellan**
Felicia mest av allt dåråå
xii:32:12 171:20:5 630:1:9 518:19:1 208:6:10 100:7:3 877:16:4 **467:28:3**

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