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Traffic into silence: endomembranes and post-transcriptional RNA silencing

Yun Ju Kim¹, Alexis Maizel^{2,*} & Xuemei Chen^{1,3,**}

Abstract

microRNAs (miRNAs) and small interfering RNAs (siRNAs) are small RNAs that repress gene expression at the post-transcriptional level in plants and animals. Small RNAs guide Argonaute-containing RNA-induced silencing complexes to target RNAs in a sequence-specific manner, resulting in mRNA deadenylation followed by exonucleolytic decay, mRNA endonucleolytic cleavage, or translational inhibition. Although our knowledge of small RNA biogenesis, turnover, and mechanisms of action has dramatically expanded in the past decade, the subcellular location of small RNA-mediated RNA silencing still needs to be defined. In contrast to the prevalent presumption that RNA silencing occurs in the cytosol, emerging evidence reveals connections between the endomembrane system and small RNA activities in plants and animals. Here, we summarize the work that uncovered this link between small RNAs and endomembrane compartments and present an overview of the involvement of the endomembrane system in various aspects of RNA silencing. We propose that the endomembrane system is an integral component of RNA silencing that has been long overlooked and predict that a marriage between cell biology and RNA biology holds the key to a full understanding of post-transcriptional gene regulation by small RNAs.

Keywords Argonaute; endoplasmic reticulum; microRNA; MVB; siRNA

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An introduction to RNA silencing and its cell biology

As a conserved and widespread regulatory mechanism in eukaryotes, post-transcriptional gene silencing (PTGS) impacts many biological processes such as development, defense, and stress responses. Small RNAs (sRNAs) of 20–24 nucleotides (nt) in size serve as a core component in PTGS by imparting sequence specificity to target gene repression; as such, PTGS is often referred to as post-transcriptional RNA silencing (see Box 1).

The functions of sRNAs are not restricted to PTGS. Based on their sequence origin and biogenesis, sRNAs are categorized into several classes including microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) (Carthew & Sontheimer, 2009). miRNAs are final products of *MIR* genes encoded in the genome in both plants and animals (Carthew & Sontheimer, 2009; Chen, 2009). siRNAs are derived from long double-stranded RNAs that can be of exogenous or endogenous origin. In animals, exogenous siRNAs are widely used as a tool for gene silencing and are introduced into cells or animals as siRNA/siRNA* duplexes or their precursors. piRNAs derive from both transposons and genes and are only found in animals (Siomi *et al*, 2011). While miRNAs and exogenously applied siRNAs act in PTGS, piRNAs and certain endogenous siRNAs (such as heterochromatic siRNAs in *Schizosaccharomyces pombe* and plants) guide the deposition of repressive chromatin marks to result in transcriptional gene silencing (TGS) (Carthew & Sontheimer, 2009; Law & Jacobsen, 2010; Siomi *et al*, 2011). Here, we will not discuss the sRNAs involved in TGS, but we note that several factors required for piRNA biogenesis are associated with the outer surface of mitochondria (Pane *et al*, 2007; Malone *et al*, 2009; Saito *et al*, 2009, 2010; Haase *et al*, 2010; Olivieri *et al*, 2010; Watanabe *et al*, 2011; Shiromoto *et al*, 2013; Vagin *et al*, 2013), raising the possibility that aspects of piRNA biogenesis are connected to the endomembrane system. In this review, however, we focus on the connection between endomembranes (see Box 2) and the PTGS activities of miRNAs and siRNAs.

MIR genes are transcribed by RNA polymerase II (Pol II) into pri-miRNAs, which are stabilized by a 5'-cap and a 3' poly A tail. A pri-miRNA is cropped into a stem loop pre-miRNA by the RNase III enzyme Drosha, and the pre-miRNA is further processed into a mature miRNA/miRNA* duplex by the RNase III enzyme Dicer (Carthew & Sontheimer, 2009). In plants, DICER-LIKE1 (DCL1) is responsible for both processing steps and the 3' end nucleotides of a mature miRNA/miRNA* duplex are 2'-O-methylated by the methyltransferase HEN1 to promote the stability of miRNAs (Chen, 2009). In siRNA biogenesis, long double-stranded precursor RNAs formed through intramolecular base pairing or by the activities of RNA-dependent RNA polymerases are processed into mature siRNA/siRNA* duplexes by Dicer in animals or DICER-LIKE proteins in

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Box 1: RNA silencing

RNA silencing is a set of mechanistically related and conserved pathways by which the expression of one or more genes is attenuated or entirely suppressed by small non-coding RNAs. RNA silencing can occur at both post-transcriptional (PTGS) and transcriptional levels (TGS). Three primary classes of small non-coding RNAs have been identified: microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA). These small non-coding RNAs are distinguished by their tissue of origin (soma and germline for miRNAs/siRNAs and germline for piRNAs) and their biogenesis, rather than their mechanism of action. All sRNAs program a multiprotein complex, termed RISC (for RNA-induced silencing complex), invariably containing a member of the ARGONAUTE (AGO, siRNAs/miRNAs) or PIWI (piRNAs) families. RISC specifically interacts with any RNA molecule presenting sequence homology to the loaded small RNA. The outcome of this interaction is variable: mRNA deadenylation followed by exonucleolytic decay, mRNA endonucleolytic cleavage, translational inhibition, or TGS through DNA methylation or histone methylation.

miRNAs derive from long, single-stranded RNA (ssRNA) precursors that adopt a stem loop structure containing an imperfect double-stranded RNA (dsRNAs) stem. These precursors are sequentially processed by RNase III proteins of the Drosha/Dicer families, releasing 21–24 nt dsRNA. The miRNA strand of the dsRNA is loaded into an AGO protein and acts in trans by regulating mRNAs that exhibit strong complementarity to the 5' end of the miRNA sequence.

siRNAs derive from the Dicer-mediated processing of long, perfect dsRNAs produced by transcription of inverted repeat sequences, convergent transcription of sense–antisense gene pairs, or synthesis by RNA-dependent RNA polymerases (RDRs). As multiple siRNAs from both strands of the precursor are programmed into RISC, siRNAs can potentially act both in cis and in trans by targeting the elements from which they derive as well as unrelated elements with substantial complementarity to their sequence.

The biogenesis of piRNAs is not yet fully understood, but piRNAs seem to result from single-stranded transcripts originating from specific clusters in the genome. The primary transcripts are processed through at least two nucleolytic steps into primary piRNAs, which could initiate secondary piRNA biogenesis through the ping–pong cycle (Siomi *et al*, 2011).

plants (Meister & Tuschl, 2004; Xie *et al*, 2004). The miRNA or siRNA guide strand is incorporated into an Argonaute protein (note that Argonaute will be abbreviated as AGO for general reference or for *Arabidopsis* proteins and as Ago for animal proteins in this article) to form the RNA-induced silencing complex (RISC), the functional entity in RNA silencing (Carthew & Sontheimer, 2009).

AGO proteins are the main effectors in RNA silencing. An AGO protein contains four functional domains, N, PAZ, MID, and PIWI. The PAZ and MID domains serve as anchors of the 3' and 5' ends of the bound sRNA, respectively (Jinek & Doudna, 2009). The PIWI domain is structurally similar to RNase H, and through mutational studies on the catalytic core motif DEDH or DEDD followed by biochemical analyses, it was shown that the PIWI domain of an AGO protein possesses endonuclease activity to cleave target RNAs (Liu *et al*, 2004; Baumberger & Baulcombe, 2005; Rivas *et al*, 2005; Nakanishi *et al*, 2012; Schurmann *et al*, 2013). In many organisms, multiple AGO proteins are encoded and can bind to sRNAs with differential preference for sequence or structural features. In *Drosophila*, siRNAs processed by Dicer2 have nearly perfect complementarity in the small RNA duplexes and are specifically loaded into dmAgo2, whereas miRNAs processed by Dicer1 contain mismatches

in the small RNA duplexes and are loaded into dmAgo1 (Tomari *et al*, 2007). In mammals, miRNAs are associated with all four Ago proteins (Ago1–4) *in vivo*, but only Ago2 displays endonuclease activity and acts as the major effector of exogenous siRNAs in RNA silencing (Meister *et al*, 2004). In *Arabidopsis thaliana*, 5' nucleotide identity and small RNA length are critical for the partitioning to different AGO proteins. atAGO1-associated small RNAs tend to have a 5' U and are 21–22 nt in length, thus include almost all miRNAs and species of endogenous siRNAs that act at the level of PTGS and making atAGO1 the major effector of sRNAs in PTGS (Baumberger & Baulcombe, 2005; Mi *et al*, 2008).

RISC is recruited to targets through base pairing between the miRNA or siRNA guide and the target transcript, resulting in target gene repression. For siRISC in both plants and animals, the siRNA is fully complementary to the target transcript and siRISC cleaves the target transcript through the endonuclease activity of the AGO protein, such as atAGO1 in *Arabidopsis* and Ago2 in mammals (Baumberger & Baulcombe, 2005; Matranga *et al*, 2005; Rand *et al*, 2005; Leuschner *et al*, 2006). For miRISC, the mode of target recognition is different between plants and animals. Animal miRNAs show restricted complementarity to targets and the most common mode of target recognition involves the pairing of the seed sequence (nucleotides 2 to 7 or 8 from the 5' end) with targets (Lewis *et al*, 2003, 2005). The limited pairing prevents most animal miRNAs from guiding the cleavage of their target mRNAs, and instead, they cause translational repression as well as destabilization of their target transcripts (Lim *et al*, 2005; Wu *et al*, 2006). Conversely, in plants, miRNAs exhibit a high degree of sequence complementarity to their target mRNAs and are able to guide AGO1 to cleave their target transcripts (Mallory *et al*, 2004; Fahlgren & Carrington, 2010). However, plant miRNAs are also able to inhibit the translation of their target mRNAs (Aukerman & Sakai, 2003; Chen, 2004; Gandikota *et al*, 2007; Brodersen *et al*, 2008; Dugas & Bartel, 2008; Beauclair *et al*, 2010; Yang *et al*, 2012; Li *et al*, 2013).

In the past decade, sRNA-mediated PTGS has been extensively studied and the basic molecular framework of sRNA biogenesis, RISC formation, and target repression is thus well established; however, in comparison, little is known about the subcellular localization of the PTGS machinery or its activities. Processing bodies (P-bodies), cytoplasmic foci containing enzymes acting in mRNA turnover, were first implicated as sites of RNA silencing (Jakymiw *et al*, 2005; Liu *et al*, 2005; Pillai *et al*, 2005). In animals, Ago, miRNAs, and target mRNAs were detected in P-bodies and P-bodies were proposed to be sites of sRNA-mediated translational inhibition (Jakymiw *et al*, 2005; Liu *et al*, 2005; Pillai *et al*, 2005). However, visible P-body formation was found to be dispensable for miRNA-mediated gene silencing, raising the possibility that the localization of the RNA silencing machinery and target mRNAs to P-bodies is the consequence rather than the cause of RNA silencing activity (Chu & Rana, 2006; Eulalio *et al*, 2007). In plants, P-bodies are also considered the site of mRNA turnover since these aggregates contain homologs of mammalian or yeast proteins involved in mRNA decay (Pomeranz *et al*, 2010; Xu & Chua, 2011); however, only circumstantial evidence links P-bodies to RISC action in plants (Brodersen *et al*, 2008; Pomeranz *et al*, 2010).

While it remains to be seen whether RNA silencing occurs in P-bodies, an unexpected theme that has emerged from recent studies is the intimate association between RNA silencing and the

Box 2: The endomembrane system and its dynamics in animals and plants

The endomembrane system of eukaryotic cells allows the spatial and temporal compartmentalization of macromolecule synthesis, sorting, delivery, and degradation. It consists of a variety of organelles that are connected either directly or through transport vesicles, the formation of which necessitates the selective recruitment of coat proteins (clathrin, COPI, COPII, and retromer), various GTPases (Sar1, Arf1, and Rabs), and the endosomal sorting complex required for transport (ESCRT) complexes (Kirchhausen, 2000; Nickel *et al*, 2002; Gabe Lee *et al*, 2009; Hurley & Hanson, 2010). The correct targeting or fusion of the transport vesicles relies on organelle-specific tethering factors and SNARE complexes (Faini *et al*, 2013). The main organelles of the endomembrane system are the endoplasmic reticulum, the Golgi complex, trans-Golgi network (TGN), endosomes, and lysosomes or vacuoles. Flows of membranes and proteins to (outward) and from (inward) the plasma membrane go through these organelles. Endosomes sit at an essential position within the endomembrane system and have a core sorting function: They are the first point of fusion for endocytic vesicles, which mediate inward flow of extracellular materials as well as the outward transport of cargoes either from the Golgi to the lysosome/vacuole or their return from lysosomes to the Golgi. Animal endosomes are classified into early, recycling, intermediate, and late endosomes/multivesicular bodies (MVBs) (Spang, 2009). Early and recycling endosomes receive and recycle both endocytosed membrane proteins back to the plasma membrane and vesicles from the vacuole back to the TGN. Intermediate and late endosomes/MVBs sort membrane proteins into endosomal intraluminal vesicles to be degraded by vacuolar/lysosomal hydrolases upon fusion with vacuoles/lysosomes. In addition, they also mediate the transport of new vacuolar proteins from the Golgi to lysosomes/vacuoles (Gruenberg & Stenmark, 2004). Endosomes coordinate the recognition, concentration, and packaging of cargo proteins by different complexes such as ESCRT (for sorting to the vacuoles/lysosomes) and the retromer (for recycling to the plasma membrane) (Seaman, 2005). Two mechanisms underpin cargo sorting in the endosomes, either direct interactions between the cargo proteins and the sorting complexes or by post-translational modifications of cargo proteins. Endocytosed cargo destined for degradation becomes ubiquitinated at the plasma membrane, and this signal causes them to be recognized by the ESCRT complex and sorted into the intraluminal vesicles of MVBs. This step is critical for the termination of signaling cascades that continue even after internalization of the receptor–ligand complex (Raiborg & Stenmark, 2009). The organelles of the plant endocytic pathway are overall similar to those present in mammalian cells. However, unlike the situation in animal cells, higher plants do not have separate TGN and early endosome compartments (Scheuring *et al*, 2011). In addition to lysosome/vacuole-mediated destruction of extracellular material and plasma membrane proteins delivered via the endocytic pathway, cytosolic material and organelles can also be delivered to the lysosome by autophagy. Autophagy is an umbrella term for different pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy. In macroautophagy, a phagophore sequesters a portion of the cytoplasm, including soluble materials and organelles, to form the autophagosome, which fuses with endosomes before fusion with lysosomes. In microautophagy, domains of the cytoplasm are engulfed by inward invagination of the lysosomal membrane. Like the formation of MVBs in endosomes, this process requires the ESCRT complex. In chaperone-mediated autophagy, target proteins harboring a distinctive peptide are recognized by Hsc70 and directly translocated to the lumen of lysosomes. Autophagosomes are generated on or in close proximity to the ER and their number can increase upon starvation and other stresses (Mizushima & Komatsu, 2011).

endomembrane system (Box 2). Here, we provide an overview of the data linking RNA silencing to endomembranes in plants and animals and attempt to integrate the myriad of findings into a tentative framework that summarizes and rationalizes the impacts of membranes on intra- and intercellular RNA silencing.

Rough endoplasmic reticulum: site of translation for most cellular mRNAs?

Cellular mRNAs are partitioned into two subcellular compartments for translation: the cytosol and the endoplasmic reticulum (ER). ER with ribosomes dotting its cytosolic surface is referred to as rough ER (rER, Fig 1). The classic view holds that mRNAs encoding cytosolic or nucleoplasmic proteins are translated on cytosolic ribosomes, whereas mRNAs encoding secretory or membrane proteins are translated on the rER. The latter class of mRNAs is docked onto the rER co-translationally through the recognition of N-terminal signal peptides emerging from translating ribosomes by the ER-localized signal recognition particle (SRP) (Schwartz, 2007). However, this classic view has been challenged by observations suggesting that rER may serve as the site of translation for a broad range of mRNAs rather than a restricted set of secreted factors. In an early study, the partitioning of mRNAs between free and membrane-bound polysomes was investigated in mammalian cells (Lerner *et al*, 2003). Surprisingly, a set of mRNAs encoding soluble proteins was found associated with membrane-bound polysomes. Treatment with protein synthesis inhibitors shows that these mRNAs are bound to functionally active ribosomes and that the ER association of the mRNAs does not depend on translation. Furthermore, cDNA microarray analysis using total and ER-associated RNAs reveals that, in mammalian cells, the majority of mRNAs encoding cytosolic proteins are also present on ER-bound polysomes (Lerner *et al*, 2003).

A number of studies in cell culture and animal systems have since reported findings consistent with the notion that rER translates a broad range of cellular mRNAs (Pyhtila *et al*, 2008; Chen *et al*, 2011; Reid & Nicchitta, 2012). These studies show that a set of mRNAs encoding cytosolic or nucleoplasmic proteins is dually localized in the cytosol and at membranes, suggesting the existence of an alternative mechanism for the translocation of mRNAs to the ER besides the SRP pathway. Indeed, in stable *SRP54*-depleted cell lines in which SRP activity was compromised, the cytosol-membrane partitioning of mRNAs was not affected (Pyhtila *et al*, 2008). In fact, in *Saccharomyces cerevisiae*, several mRNAs that encode membrane/secreted proteins were shown to be targeted to the ER in a translation- and SRP-independent manner (Kraut-Cohen *et al*, 2013). Another study examined the cytosolic and rER translome through subcellular fractionation followed by ribosomal profiling (Reid & Nicchitta, 2012) and revealed that a large portion of the transcriptome is translated on the ER. Interestingly, when two groups of mRNAs, encoding either cytosolic/nucleoplasmic proteins or ER-targeted proteins, were compared, the transcript levels as well as their ribosome loading density were similar in the ER fraction. In addition, ER-bound mRNAs showed higher levels of ribosome loading density compared to cytosolic mRNAs, which may reflect higher translational efficiency on the ER (Reid & Nicchitta, 2012).

Rough endoplasmic reticulum: site of miRNA-mediated translational repression

Translational repression is a widespread and fundamental activity of miRNAs in both animals and plants (Fabian *et al*, 2010;

Meister, 2013). For animal miRNAs, which are also known to induce target mRNA destabilization through 3' deadenylation, 5' decapping, and subsequent exonucleolytic decay (Wu *et al*, 2006; Fabian *et al*, 2009), it has been debated whether translational repression contributes much to target gene regulation (Guo *et al*, 2010).

Three studies examined the temporal kinetics of the two activities of miRNAs, mRNA decay and translational repression, and support that translational repression is an integral activity of miRNAs. It was found that, upon the induction or transfection of a miRNA, translational repression occurred at a time when there was no sign of target mRNA decay (Bazzini *et al*, 2012; Bethune *et al*, 2012; Djuranovic *et al*, 2012). This indicated that translational repression is a fundamental activity of animal miRNAs. Plant miRNAs pair with target mRNAs with nearly complete sequence complementarity and guide the cleavage of target mRNAs through the slicing activity of AGO1 (Mallory *et al*, 2004; Fahlgren & Carrington, 2010). Although mRNA cleavage was once thought to be the major activity of plant miRNAs, translational repression of plant miRNA targets has gradually garnered support over the years, as more and more studies examined expression of miRNA target genes at both RNA and protein levels and interrogated mutants compromised in miRNA activities (Aukerman & Sakai, 2003; Chen, 2004; Gandikota *et al*, 2007; Brodersen *et al*, 2008; Dugas & Bartel, 2008; Beauclair *et al*, 2010; Yang *et al*, 2012; Li *et al*, 2013). For example, several mutants were isolated from forward genetic screens aimed to identify players in miRNA action in *Arabidopsis* and, in some of the mutants, the expression of many miRNA target genes was elevated at the protein but not the RNA level (Brodersen *et al*, 2008; Yang *et al*, 2012; Li *et al*, 2013). This suggested that miRNAs inhibit the translation of their target mRNAs. In fact, in one such mutant, *altered meristem program 1* (*amp1*), protein synthesis from miRNA target genes was shown to be elevated, indicating that plant miRNAs indeed repress translation (Li *et al*, 2013). In addition, and confirming the observation made earlier in animal systems, atAGO1 and miRNAs were found to be associated with polysomes (Lanet *et al*, 2009; Reynoso *et al*, 2013). These results showed that translational repression is another major mode of action of miRNAs in plants but left open the question of where in the cell it takes place.

A recent study provided a location to the translational repression activity of miRNAs in plants by linking it to the rER. In *Arabidopsis*, the *AMP1* gene, despite its unknown molecular function, has continuously graced the scientific literature in the past 15 years as a gene that impacts almost all aspects of plant development (Conway & Poethig, 1997; Helliwell *et al*, 2001; Vidaurre *et al*, 2007; Griffiths *et al*, 2011; Shi *et al*, 2013). Recently, it was found that the molecular function of *AMP1* lies in mediating the translational repression activity of plant miRNAs (Li *et al*, 2013). The wide-ranging impacts of *AMP1* on plant development could thus reflect the importance of miRNA-induced translational repression activity. An *amp1* mutant was found to be deficient in stimulating miRNA activity, and intriguingly, miRNA target protein levels were elevated in this mutant with no corresponding changes in target mRNA levels. Pulse-labeling experiments showed that protein synthesis from miRNA target genes was reduced in a miRNA- and *AMP1*-dependent manner, demonstrating that translational repression is an activity of plant miRNAs and that this

activity requires *AMP1* (Li *et al*, 2013). Although the mechanism by which *AMP1* mediates the translational repression activity of plant miRNAs remains unknown, it was found that the association of miRNA-targeted mRNAs with membrane-bound polysomes is repressed by miRNAs in an *AMP1*-dependent manner. This finding, together with the rER localization of the *AMP1* protein, suggests that translational repression by plant miRNAs occurs on the rER (Fig 1).

Consistent with the ER being a site of miRNA-mediated translational repression in *Arabidopsis*, the major miRNA effector atAGO1 was found to be partially membrane-associated (Brodersen *et al*, 2012; Li *et al*, 2013). AGO1 partitions into soluble and membrane fractions, and its membrane association is disrupted by high salt or high pH conditions, suggesting that atAGO1 is a peripheral membrane protein, consistent with its lack of a transmembrane domain. Co-localization with an ER marker showed that atAGO1 partially resides on the ER (Li *et al*, 2013) (Fig 1). How atAGO1 becomes membrane-associated is unknown, but isoprenoid biosynthesis may play a role. A mutant in *HMG1*, which encodes 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase in the mevalonate pathway for isoprenoid biosynthesis, was isolated in a genetic screen as a miRNA action-deficient mutant (Brodersen *et al*, 2008, 2012) and the AGO1 membrane association was reduced in hypomorphic *ago1* and *hmg1* mutants (Brodersen *et al*, 2012). Intriguingly, in *Caenorhabditis elegans*, enzymes in the mevalonate pathway were found to be required for RNA silencing, pointing to an ancient and conserved involvement of isoprenoids and tentative membrane association of AGO in RNA silencing (Shi & Ruvkun, 2012).

Another study supports the functional significance of the rER in miRNA-mediated translational repression in *Drosophila* (Wu *et al*, 2013). The authors found that serum deprivation in S2 cells led to enhanced miRNA-mediated translational repression and the concomitant appearance of two non-canonical miRISCs. These are the polyribosomal miRISC (P-miRISC) and a dmAGO1-containing complex associated with membranous organelles (Fig 1). P-miRISC is composed of dmAGO1, miRNA, and Loqs-PB (a double-stranded RNA-binding protein) and devoid of GW182, an essential component in canonical miRISC (referred to as G-miRISC) (Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2008). The membrane-associated dmAGO1 complex lacks both GW182 and Loqs-PB and is considered to be an intermediate in the turnover of miRISC or P-miRISC. The P-miRISC, rather than G-miRISC, was thought to mediate translational repression observed under the serum deprivation condition, as GW182 was found to be dispensable for this activity. P-miRISC is associated with polysomes and co-sediments with ER. In addition, P-miRISC is still polysome-associated upon cycloheximide treatment, suggesting that it inhibits translational elongation rather than initiation. Interestingly, it was reported that the level of miRNA-mediated translational repression is 5- to 10-fold higher under serum starvation, implying that translational repression through P-miRISC is more efficient than that of G-miRISC (Wu *et al*, 2013). Given that a wide range of the transcriptome associates with ER and that translation at rER is more efficient than in the cytosol (Reid & Nicchitta, 2012), it was proposed that miRNA-mediated translational repression on rER could allow a fast response to various environmental stimuli in both plants and animals (Ando & Leung, 2013; Li *et al*, 2013).

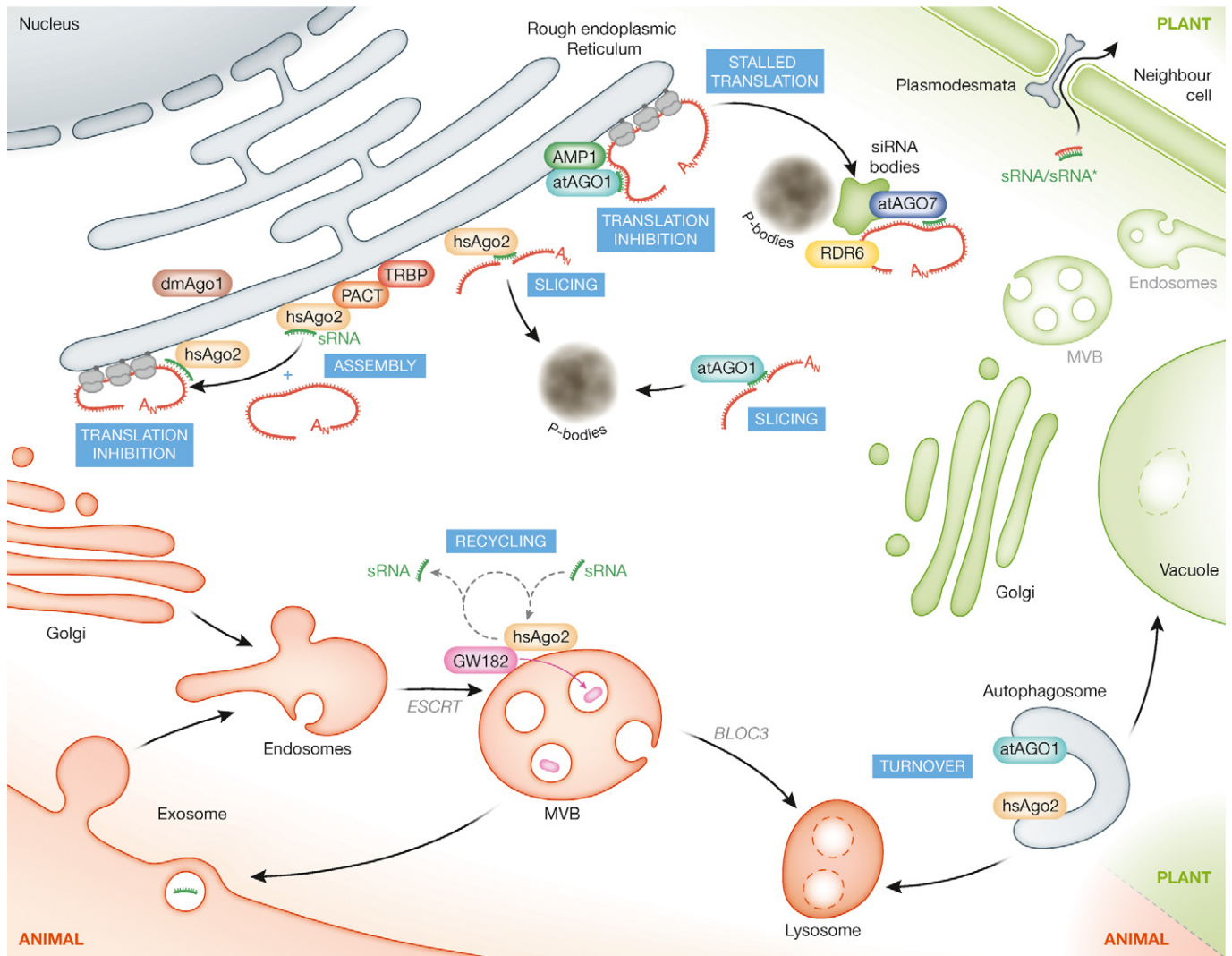


Figure 1. Connections between endomembranes and post-transcriptional gene silencing in animals and plants.

In both plant and animal cells (shaded in green and pink, respectively, in the diagram), the rough endoplasmic reticulum (rER) is probably the site of miRNA-mediated target repression. In *Arabidopsis*, atAGO1 is partially membrane-associated and congregates with AMP1 at the rER to inhibit the translation of target mRNAs. In *Drosophila*, dmAGO1 is associated with the rER. In human cells, the formation of hsAgo2 RISC, a process requiring PACT and TRBP, and target RNA slicing both occur on the rER. In animal cells, endosomes and multivesicular bodies (MVBs) are likely sites of RISC recycling. In mammalian cells, GW182 removal at MVBs by the ESCRT machinery and concomitant AGO reloading are thought to contribute to RISC recycling. In both plant and animals, autophagy regulates the turnover of AGO proteins by selectively addressing them to lysosomes (animal) or the vacuole (plants) for degradation. In plants, mRNAs stalled in translation accumulate in siRNA bodies, an intermediary compartment between the rER and Golgi where atAGO7 and RNA-dependent RNA polymerase proteins congregate. No evidence has been provided for a membrane-P-body connection, but in plants, siRNA bodies and P-bodies are in close proximity. Intercellular transfer of silencing RNAs involves, in plants, the plasmodesmata, an ER-containing organelle, and perhaps endosome/MVB-derived exosomes in animals.

Rough endoplasmic reticulum: site of RISC loading and siRNA-mediated slicing

Mammalian Ago2, an effector in RISC and the only mammalian Argonaute protein with slicer activity, was originally identified as GERp95, an ER- and Golgi-associated protein (Cikaluk *et al*, 1999). Later, it was shown that Dicer is also associated with ER and Golgi in neurons and various other cell types (Tahbaz *et al*, 2004; Barbato *et al*, 2007).

In a recent study, extensive subcellular fractionation efforts were made to document the membrane association of various RNA silencing factors (Stalder *et al*, 2013). miRNA- or siRNA-loaded human (hs)

Ago2 populations co-sediment on membranes together with the RISC-loading complex (RLC) factors Dicer, protein activator of the interferon-induced protein kinase (PACT), and TAR RNA-binding protein (TRBP), but only TRBP fractionated sharply with Golgi and rER marker proteins. Fractionation and membrane co-immunoprecipitation further confirmed that siRNA-loaded hsAgo2 physically associates with the cytosolic side of the rER membranes and demonstrated that TRBP and PACT are required for anchoring RISC to ER in a target RNA-independent manner (Stalder *et al*, 2013). Moreover, loaded and active hsAgo2 was found to be mostly membrane-associated, while the slicing product was solely associated with rER fractions. Taken together, these findings led to the

conclusion that both hsAgo2 RISC formation and target RNA slicing occur on the rER and that the outer rER membrane thus acts as a central nucleation site for siRNA-mediated RNA silencing (Fig 1).

In summary, the classical view of the ER as the site of protein synthesis or folding for secreted and membrane-embedded proteins is challenged. Mounting evidence documents the association of a wide range of the transcriptome, including miRNAs and their target mRNAs, as well as RNA silencing factors with the ER. The rER is likely the site of translation for a large portion of cellular proteins and a platform for siRNA-mediated slicing and miRNA-mediated translational repression.

Endosomes: RISC assembly and turnover

Endosomes are membrane-bound compartments and an integral part of the transport pathway from the plasma membrane to the lysosome. They are a major hub for secretory and endocytic trafficking and receive cargoes from endocytosed material from the plasma membrane as well as from the trans-Golgi network (TGN). Cargoes can leave endosomes to return to the plasma membrane in recycling endosomes or travel to the TGN. Endosomes can also bud vesicles into their interior using the endosomal sorting complex required for transport (ESCRT) machinery to form multivesicular bodies (MVBs). MVBs can fuse with lysosomes or the Golgi, in a process requiring the BLOC-3 machinery, to permit the degradation of their content, or with the plasma membrane to release exosomes into the extracellular space (the reader is referred to Gruenberg & Stenmark, 2004; Li et al, 2004; Raiborg & Stenmark, 2009 for in-depth reviews).

Work in *Drosophila* and mammalian cells has revealed an intriguing association of the RNA silencing machinery with the endosomal trafficking pathway. In human cells, hsAgo2 and GW182 (also called TNRC6), two essential components of RISC, have been shown to co-purify and co-localize with endosome and MVB fractions and define subcellular structures that are distinct from P-bodies (Gibbings et al, 2009). It was also observed in *Drosophila* that GW182 bodies are closely juxtaposed to the cytosolic face of MVBs and/or lysosomes (Lee et al, 2009). In agreement with this association between endomembranes and the RNA silencing machinery are the early observations mentioned above reporting the co-localization and fractionation of AGO and Dicer with membranes related to the ER and Golgi apparatus (Tahbaz et al, 2004). In human cells, miRNAs and target mRNAs were also shown to accumulate in endosomes/MVBs (Gibbings et al, 2009), suggesting that these compartments are sites of miRNA-loaded RISC accumulation and, possibly, action (Fig 1). The significance of this association between RISC components and endosomes/MVBs and its implications for RISC function were assayed by interfering with ESCRT-dependent sorting of cargoes into MVBs. Depletion of ESCRT components leads to a decrease in miRNA activity, a concomitant reduction in MVBs and an increase in GW182 abundance (Gibbings et al, 2009; Lee et al, 2009). Conversely, a *Drosophila* mutant in *HPS4*, a component of the BLOC-3 machinery that catalyzes the fusion of MVBs with lysosomes (Suzuki et al, 2002; Li et al, 2004), leads to an increase in miRNA- or siRNA-mediated RNA silencing (Lee et al, 2009). In fact, *hps4* mutant extracts exhibit faster RISC loading *in vitro* (Lee et al, 2009).

Interference with ESCRT and BLOC-3 machineries thus exerts opposite effects on RISC loading and miRNA activity—ESCRT promotes RISC assembly, while BLOC-3 impinges on RISC assembly. A model coupling RISC disassembly and reloading at MVBs has been proposed (Gibbings & Voinnet, 2010): GW182 removal at MVBs by the ESCRT machinery contributes to AGO release from miRNA and target mRNA. This process is coupled to AGO reloading (by interaction with Dicer and acquisition of the miRNA-miRNA* complex), a process that also occurs on MVBs but is slowed down by BLOC-3-dependent trafficking of RISC toward lysosomes (Fig 1). The proposal that RISC loading occurs on MVBs conflicts with the model presented above placing RISC formation at the rER (Stalder et al, 2013). A way to reconcile observations from the different studies is to assume that upon inhibition of the BLOC-3 machinery Ago2 could be sent back to the ER from MVBs through retrograde trafficking where it would be reloaded. The endo-/lysosomal compartments would therefore act downstream of RISC loading (Fig 1).

Autophagosomes: RISC turnover

Autophagy (macroautophagy) is an intracellular degradation process contributing to cellular homeostasis in which cytoplasmic material is engulfed and then delivered to and degraded in the lysosome by autophagosomes. Autophagy-related (ATG) proteins act in a hierarchical manner to control the formation of autophagosomes. Targeting of specific components to the autophagosomes requires autophagy receptors, among them NDP52 (Thurston et al, 2012). More details for these processes are described extensively elsewhere (Mizushima & Komatsu, 2011). Connections between autophagy and the endosomal pathways have become apparent recently: Autophagosomes can fuse with endosomes and the ESCRT machinery regulates autophagy (Lee et al, 2007). In human cells, the levels of hsAgo2, hsAgo1, and DICER proteins are increased by depletion of the core autophagy components ATG5, ATG6, ATG7 or the autophagy receptor NDP52. Under the same conditions, the levels of GW182 were not affected. In accordance, hsAgo2 and DICER, but not GW182, co-purify with autophagosomes (Fig 1) and co-localize with NDP52-labeled autophagosomes, and NDP52 co-immunoprecipitates with DICER (Gibbings et al, 2012). Together, these data suggest that DICER and hsAgo2 are targeted for autophagic degradation through their association with NDP52. Recently, autophagy was also implicated in the modulation of miRNA-mediated silencing in *Caenorhabditis elegans* (Zhang & Zhang, 2013). Mutations in several autophagy components rescue the developmental phenotypes of animals with hypomorphic mutations in either the Dicer homolog *dcr-1*, the AGO homolog *alg-1*, or the GW182 homolog *ain-1* (ALG-1-interacting protein 1). Interestingly, in *C. elegans*, autophagy seems to regulate the effector step of miRNA-mediated silencing by selectively degrading AIN-1, unlike the situation in human cells where GW182 levels are not controlled by autophagy (Gibbings et al, 2012). In both mouse and *Drosophila*, existing evidence indicates that the stability of AGO protein is post-translationally controlled by miRNA availability. In mouse, mutations in the miRNA biogenesis proteins Dicer or DGCR8 led to decreased Ago2 levels by a process involving the lysosome, but not the proteasome (Martinez & Gregory, 2013). Analogously, in *Drosophila*, levels of

dmAgo1 are reduced in cells depleted of *drosha*, *pasha*, and *Dcr-1* (Smibert et al, 2013). However, unlike the situation in mouse, the reduced stability of dmAgo1 in these cells could be reversed by blocking the action of the proteasome, suggesting that the turnover of unloaded dmAgo1 involves the proteasome (Smibert et al, 2013).

Further links between autophagy and RNA silencing have been independently obtained in plants. Virus-derived small RNAs are used to program RISC to degrade the corresponding viral RNA. To counteract this line of defense, viruses have evolved viral suppressors of RNA silencing that suppress, by different strategies, the silencing response of the host. One such gene, the polerovirus P0, encodes an F-box protein that hijacks the host ubiquitin-protein ligase E3, SKP1-cullin 1-F-box protein (SCF), to promote the degradation of *Arabidopsis thaliana* AGO1 (atAGO1), the key component of RISC (Pazhouhandeh et al, 2006; Baumberger et al, 2007; Bortolamiol et al, 2007). However, the P0-mediated atAGO1 degradation occurs independently of the 26S proteasome pathway (Baumberger et al, 2007; Csorba et al, 2010). Derrien et al observed that the cysteine protease inhibitor E64d, an inhibitor of the degradation of autophagic cargo inside autolysosomes, and 3-methyladenine (3-MA) that blocks autophagosome formation via the inhibition of type III phosphatidylinositol 3-kinases (PI-3K), led to atAGO1 stabilization in the presence of P0 (Derrien et al, 2012). This indicates that autophagy could mediate atAGO1 turnover. A GFP-tagged version of atAGO1 co-localizes with ATG8a-positive bodies when atAGO1 degradation is impaired. Further support for the involvement of autophagy in atAGO1 turnover was provided by the over-accumulation of atAGO1 in the *amsh3* mutant in which vacuole formation is compromised and autophagosomes accumulate (Isono et al, 2010) and in the TOR-overexpressing mutant line G548 (Deprost et al, 2007) in which autophagy is reduced (Derrien et al, 2012). Electron microscopy imaging revealed enrichment of atAGO1 in proximity to the Golgi apparatus (Derrien et al, 2012), reminiscent of the situation in animal cells where both AGO and Dicer localize and fractionate with membranes of the Golgi apparatus (Cikaluk et al, 1999; Tahbaz et al, 2004). However, unlike the situation in animals, Derrien et al did not observe localization of atAGO1 to MVBs, arguing against their involvement in routing AGO1 to the vacuole. The relevance of autophagy-mediated turnover of atAGO1 beyond antiviral responses was established by showing that atAGO1 is also degraded by the autophagy pathway when miRNA production or stability is compromised. Two conclusions arise from this observation. First, in plants, endogenous SCF(s) trigger(s) atAGO1 degradation through the autophagy pathway. Second, and in agreement with the observations made in mouse and fly (Martinez & Gregory, 2013; Smibert et al, 2013), defective loading of AGO by a miRNA appears to signal AGO degradation in plants.

Based on these findings, autophagy-mediated modulation of miRNA action emerges a conserved theme across phylae and kingdoms (Fig 1). Autophagy may ensure the homeostatic regulation of miRNA-mediated RNA silencing by preventing blatant perturbations that could be induced by various stresses of abiotic or biotic origin. Ramifications of this are numerous and can potentially link processes seemingly unrelated. Autophagy is modulated in response to nutrient availability, differentiation, and pathogen infection (Mizushima & Komatsu, 2011) and has also been linked to pathology. A hallmark of cancer is inhibition of autophagy (Levine, 2007),

which could lead to a decrease in miRNA levels and a global impingement of the RNA silencing machinery.

Intermediary compartments and “cytoplasmic bodies”

In *Arabidopsis*, several pieces of evidence indicate that endomembranes may play a role in siRNA biogenesis. Trans-acting siRNAs (ta-siRNAs) are plant-specific endogenous siRNAs that guide the cleavage of specific mRNA targets *in trans*. ta-siRNAs result from DICER-LIKE 4 (DCL4)-mediated cleavage of a double-stranded RNA (dsRNA) produced by the cellular RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) with the aid of the SGS3 RNA-binding protein (Peragine et al, 2004; Vazquez et al, 2004; Allen et al, 2005; Dunoyer et al, 2005; Gascioli et al, 2005). The RDR6/SGS3 substrate results from miRNA-mediated cleavage of a *TAS* precursor transcript. Ta-siRNA produced from the evolutionarily conserved *TAS3* precursors controls the developmental timing of the transition between juvenile and adult leaves, contributes to the specification of leaf adaxial/abaxial polarity and regulates the growth of lateral roots (Adenot et al, 2006; Axtell et al, 2006; Fahlgren et al, 2006; Garcia et al, 2006; Hunter et al, 2006; Nogueira et al, 2009; Douglas et al, 2010; Marin et al, 2010; Yoon et al, 2010). The processing of *TAS3* by a miR390-loaded-AGO7 complex is essential in directing the *TAS3* precursor to the siRNA pathway (Montgomery et al, 2008). Subcellular fractionations have shown that AGO7, miR390, and SGS3 co-purify with membranes (Jouannet et al, 2012). A GFP-tagged version of AGO7 congregates with RDR6 and SGS3 in cytoplasmic siRNA bodies (Jouannet et al, 2012) that are linked to the ER/Golgi endomembrane system since they also accumulate the viral protein 6 (VP6) of the tobacco etch virus (Skog et al, 2008), a membrane-associated protein marking an intermediary compartment between the ER and the cis-Golgi (Schaad et al, 1997; Lerich et al, 2011). It therefore appears that in *Arabidopsis*, the production of some siRNAs involves an endomembrane-associated compartment (Fig 1). It would be interesting to examine the link between these siRNA bodies and other foci implicated in RNA processing.

In plants and animals, mRNAs stalled at the translation initiation stage accumulate in so-called cytoplasmic stress granules. Although a distinctive feature of RDR6/SGS3 substrates in siRNA bodies is their poor ability to be translated (a result of their very poor coding potential, as for *TAS3* (Ben Amor et al, 2009), and/or the absence of a 5'-cap or 3' polyA tail required for efficient translation), siRNA bodies and stress granules are distinct (Jouannet et al, 2012). Indeed, siRNA bodies, unlike stress granules, are readily detected under normal growth conditions (Weber et al, 2008; Jouannet et al, 2012) and animal stress granules are so far not known to be linked to endomembranes.

The enzymes responsible for the elimination of aberrant RNAs such as ones lacking a 5'-cap or 3' polyA tail accumulate in discrete cytoplasmic P-bodies. It is interesting to note that although siRNA bodies never co-localize with P-bodies (Jouannet et al, 2012; Moreno et al, 2013), these two sets of bodies are usually found juxtaposed, are highly dynamic, and move together in the cytoplasm (A. Maizel, unpublished observation). The dynamic nature of membrane-containing siRNA bodies and their association with P-bodies suggests that they are sites of mRNA triage, wherein mRNAs could be sorted for degradation by P-bodies or enter the siRNA pathway (Fig 1).

Endomembranes and small RNA's intercellular transfer

One of the most fascinating aspects of RNA silencing is its ability to spread from cell to cell. The spread of RNA silencing was first reported in plants and in *C. elegans* (Palauqui *et al*, 1997; Voinnet & Baulcombe, 1997; Fire *et al*, 1998; Timmons & Fire, 1998). Intriguingly, non-cell autonomy of silencing bears point of convergence with the endomembrane system.

The observation made in animals and in plants that RISC associates with endosomes, a compartment acting as a hub for cargo traffic between cells and their exterior environment, raises the possibility that RISC may be secreted from cells (Fig 1). In animals, different populations of miRNAs have been consistently retrieved in biological fluids derived from healthy or cancerous individuals, making these miRNAs tentative diagnostic and predictive biomarkers of cancers (for review, see Turchinovich *et al*, 2012). Reports indicate that small RNAs are present in exosomes exported from cultured cells (Valadi *et al*, 2007; Skog *et al*, 2008) and that purified exosome-like vesicles contain single-stranded, mature miRNAs in addition to high levels of GW182 and low levels of hsAgo2 (Gibbings *et al*, 2009). One may speculate that a controlled targeting of miRNAs and/or RISC components by factors modulating endosomal trafficking could serve as a mechanism for the selective secretion in exosomes. In agreement with this idea, a ceramide-dependent secretory mechanism that induces endosome sorting into MVBs (Trajkovic *et al*, 2008) was found to actively regulate the release of exosomal miRNAs (Kosaka *et al*, 2010). The exosome-associated RISCs may have the potential to be internalized by recipient cells, where they modulate gene expression and trigger functional effects (Valadi *et al*, 2007; Skog *et al*, 2008; Pegtel *et al*, 2010; Zhang *et al*, 2010; Mittelbrunn *et al*, 2011; Hergenreider *et al*, 2012; Montecalvo *et al*, 2012). However, the view that exosome-associated RISC and/or miRNAs is the sole form of extracellular miRNAs is challenged by observations that extracellular miRNAs are not only membrane-vesicle-free but are also associated with AGO (Wang *et al*, 2010; Arroyo *et al*, 2011; Turchinovich *et al*, 2011). In addition to secretion/uptake-mediated intercellular small RNA transfer, siRNAs and miRNAs have also been implicated to travel through immune synapses in mammalian cells (Rechavi *et al*, 2009). However, few studies have investigated the intercellular transfer of small RNAs and its functional consequence under physiologically relevant conditions, and the extent and impact of small RNA intercellular transfer in mammals are still debatable. In *C. elegans*, the systemic nature of RNA silencing has long been established (Winston *et al*, 2002), and four SID proteins have been implicated in the uptake and spreading of dsRNAs (Hinas *et al*, 2012). Whereas SID-1, SID-2, and SID-3 are associated with the plasma membrane, SID-5 is endosome-associated (Hinas *et al*, 2012), implicating the endomembrane system in the systemic spreading of RNA silencing in *C. elegans*.

On the contrary, the functional implications of non-cell autonomous RNA silencing in plants are substantial (a topic reviewed elsewhere Melnyk *et al*, 2011). The long-distance movement of RNA silencing through the vasculature not only forms the cornerstone of systemic antiviral defense (Schwach *et al*, 2005), but also has been shown to direct epigenetic changes systemically (Molnar *et al*, 2010) and be implicated in miRNA-regulated stress responses (Buhtz *et al*, 2008; Pant *et al*, 2008). Many species of small RNAs have been found in the phloem sap, suggesting that the phloem is

one of the routes for the long-distance transport of small RNAs (Buhtz *et al*, 2008; Varkonyi-Gasic *et al*, 2010). In addition to the systemic movement of RNA silencing, short-range movement of RNA silencing (by a few to tens of cell layers) patterns leaves and roots (Chitwood *et al*, 2009; Schwab *et al*, 2009; Carlsbecker *et al*, 2010) (for review, see Furuta *et al*, 2012). Plant cells are connected by plasmodesmata (PD), channels traversing the cell walls of plant cells, ensuring cytoplasmic continuity between cells and enabling transport and communication between them. The center of the PD contains a tube of appressed endoplasmic reticulum that runs between two adjacent cells (Maule, 2008) and accumulation of the glucose polymer callose at the PD controls permeability to cargoes (Simpson *et al*, 2009). Genetic screens have not yet revealed plasmodesmata-associated proteins that would influence the symplastic movement of silencing RNA, presumably since mutants affecting symplastic communications between cells would suffer from limited or no viability. However, evidence points toward PD being the channel by which small RNAs transit between cells (Fig 1) (Vatén *et al*, 2011): A dominant mutation in the *CALS3* gene that directs callose synthesis causes the accumulation of callose in PD and inhibits the intercellular transit of proteins and small RNAs through PD (Vatén *et al*, 2011). As trafficking to and through the PD requires the endomembrane system (Wright *et al*, 2006; Tagami & Watanabe, 2007), it is tempting to speculate that links may exist between PD-mediated intercellular traffic of silencing and the congregation of RNA silencing factors on endomembranes.

Concluding remarks

We have come a long way from the simple view of RNA silencing as a solely cytosolic process. The studies discussed above show that many aspects of RNA silencing, such as RISC formation, action, and turnover, involve endomembranes (Fig 1). Although overwhelming and convincing, the results obtained highlight that our understanding of the role of endomembranes in RNA silencing is still very lacunar. We can, however, speculate about the potential implications of this RNA silencing-membrane connection. Endomembranes could serve as a platform for partitioning silencing processes within the cell. The association of the silencing complexes with a specific type of endomembranes would ensure their sorting and allow the encounter with the proper set of targets or partners. It could also determine the ability or magnitude of intercellular transfer for a particular sRNA. The differential mobility of plant siRNAs and miRNAs (Felippes *et al*, 2010) could find its origin in the type of endomembranes associated with the two populations of sRNAs. It is essential for future research to elucidate the intracellular dynamics of various RNA silencing complexes. The rER emerges as a nexus for genome expression where a large portion of the transcriptome is translated and where miRNA-mediated translational repression occurs. Several competing models for miRNA-mediated translational repression have been proposed: inhibition of initiation, disruption of the mRNA loop, competition with eIF4E, or inhibition of elongation (Filipowicz *et al*, 2008). The important role of the rER should be taken into consideration when these models are evaluated. Beyond the obvious parallels between plants and animals described above, it is tempting to surmise that the central role of endomembranes in partitioning RNA processes in distinct subcellular

compartments is evolutionarily ancient, since work in bacteria has shown the RNA degradation machinery to congregate at the bacterial membrane, perhaps ensuring its separation from transcription and translation (Liou *et al*, 2001). The intricate association of RNA silencing—a fundamental coordinator of gene expression in eukaryotes—with the endomembrane system could ensure its coupling to signaling pathways and allow prompt responses to changes in the environment (Gibbings & Voinnet, 2010). In a broader perspective, this draws an interesting parallel to the emerging paradigm in cellular signaling that the biological context can influence the outcome of a ligand–receptor interaction (Barton & Kagan, 2009).

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Author contributions

YK, AM, and XC wrote and revised the manuscript; AM created the figure.

Conflict of interest

The authors declare that they have no conflict of interest.

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