Review

MicroRNA turnover: when, how, and why

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MicroRNAs (miRNAs) are short (~22 nucleotide) RNAs that are important for the regulation of numerous biological processes. Accordingly, the expression of miRNAs is itself tightly controlled by mechanisms acting at the level of transcription as well as processing of miRNA precursors. Recently, active degradation of mature miRNAs has been identified as another mechanism that is important for miRNA homeostasis. Here we review the molecular factors and cellular conditions that promote miRNA turnover. We also discuss what is known about the physiological relevance of miRNA decay.

Degradation facilitates dynamic miRNA expression patterns

MicroRNAs are a large class of small regulatory RNAs, ~22 nucleotides long. They bind to partially complementary sequences in target mRNAs and silence them translationally or by inducing mRNA degradation [1]. miRNAs are important for gene regulation in numerous cellular and developmental processes [2], therefore it is perhaps of little surprise that miRNAs themselves are subject to extensive regulation. Indeed, a large body of literature connects dysregulation of miRNAs with disease [3], highlighting a need for robust regulation of miRNA activity. Several such regulatory mechanisms have been shown to affect miRNA biogenesis, a well-understood process (Box 1), and miRNA activity [4]. By contrast, regulation of miRNA levels through degradation of the mature, functional miRNA has received less attention. This may be owed in part to the perception of miRNAs as inherently stable molecules, consistent with the finding that mature miRNAs persist for many hours or even days after their production is arrested (e.g., by transcriptional shut-down through chemical inhibitors or depletion of miRNA processing enzymes) [5–8].

Nonetheless, many miRNAs show a dynamic expression pattern during development, including rapid downregulation in some instances [9–12]. Moreover, specific mature miRNAs have been found to be expressed in a tissue- or stage-specific manner without variation in the expression pattern of the precursor forms (pri- and pre-miRNAs), supporting the notion of regulatory mechanisms acting on the mature miRNA [13,14]. These findings suggest that steady-state levels of miRNAs can be regulated through both biosynthetic and decay processes. Here, we summarize how turnover of mature miRNAs contributes to their homeostasis and permits their dynamic regulation. We focus in particular on reviewing the cellular states that affect miRNA stability as well as molecular mechanisms of miRNA degradation. However, we do note that currently, studies consolidating physiological triggers of miRNA destabilization with molecular mechanisms remain largely elusive. Furthermore, insights into the physiological relevance of mature miRNA degradation are just beginning to emerge.

Cellular conditions affecting miRNA stability

In contrast to the view of miRNAs as generically stable molecules, recent studies have shown that individual miRNAs, or miRNAs in specific environments, are subject to accelerated decay (Table 1), altering miRNA levels and hence activity. This section focuses on discussing cellular conditions and extracellular cues that influence miRNA stability.

The cell cycle

Several miRNA families function in cell cycle regulation; for example, by targeting components of cyclin/CDK complexes [15]. Intriguingly, the reverse is also true; that is, cell cycle stage affects accumulation of certain miRNAs [16–18]. miR-29b is the first example of these [16]. In HeLa cells, miR-29b is polycistronically transcribed together with its 'sister' miR-29a, from which it differs by a nucleotide at position 10 as well as its six 3′-terminal nucleotides. However, whereas miR-29a levels change little during progression through the cell cycle, miR-29b is enriched in mitotic cells. When mature synthetic miR-29b was transfected into cells as an miR:miR*-like duplex siRNA, it was similarly found to accumulate preferentially in mitotically arrested cells, indicating that regulation of miR-29b takes place after it has been processed into the mature form. 'Pulse-chase'-like experiments using transfection revealed a half-life of miR-29b of 4 h in cycling cells, compared to >12 h in mitotically arrested cells, whereas miR-29a has a half-life of >12 h in either case [16]. Mutational analysis suggested that the uracils at nucleotide positions 9–11 are necessary, although not sufficient, for the fast degradation of miR-29b [16,19]. Factors recognizing this element and mediating degradation remain to be discovered. Additionally, because these experiments [19] relied mostly on transfection of synthetic miRNA duplexes at rather high levels (40 nM), it is unclear what fraction of these RNAs is indeed loaded into Argonaute (AGO; Box 1),

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Box 1. miRNA biogenesis and function

MicroRNAs (miRNAs) are typically transcribed by RNA polymerase II as primary transcripts (pri-miRNAs) that are subsequently matured in a multi-step biogenesis process to generate the mature, functional form [4] (Figure I). Pri-miRNAs are capped and polyadenylated and are usually several kilobases long. They possess hairpin structures that comprise the future mature sequence (red) in their stem. Alternatively, the pre-miRNA may reside in introns of mRNAs or other non-coding RNAs. In either case, the nuclear RNase III-type enzyme Drosha, in a complex with its co-factor DGCR8 (DiGeorge syndrome critical region 8 homolog), cleaves near the base of the stem, releasing an approximately 70 nucleotide-long stem-loop precursor miRNA (pre-miRNA) [4]. The pre-miRNA is exported from the nucleus by Exportin 5. In the cytoplasm, another RNase III-type enzyme, Dicer, with its co-factors TRBP (TAR RNA-binding protein 2, also known as TARBP2), cleaves off the terminal loop, resulting in an RNA duplex of ~22 nucleotides [4]. Following Dicer cleavage, the short RNA duplex is bound by an AGO (Argonaute) protein, a component of a multisubunit complex termed miRISC (miRNA-induced silencing complex) [4]. Subsequently, one of the two strands, the so called passenger strand (also referred to as miR*), is released and degraded whereas the other strand, termed guide strand or miR, is retained within miRISC. This strand selection follows a 'thermodynamic asymmetry rule' in that the strand whose 5′-terminus is less stably base-paired is destined to become the guide strand. The guide strand targets miRISC to mRNAs with partially complementary sequences and silences them [1]. The so-called 'seed' region, nucleotides 2–8 from the 5′-end of an miRNA, is particularly important for target recognition; hence, miRNAs that share the seed region but differ outside are frequently considered to form a 'family' of miRNAs with largely overlapping sets of targets [54]. miRISC-bound mRNAs are subjected to translational repression, mainly inhibition of translation initiation, and/or degradation following deadenylation by the CCR4-NOT (carbon catabolite repressor protein 4–General negative regulator of transcription) complex and decapping (not shown) [68]. Irrespective of the mechanism, members of the GW182 (glycine-tryptophane protein of 182 kDa) protein family are essential components of RISC for mRNA silencing [68].

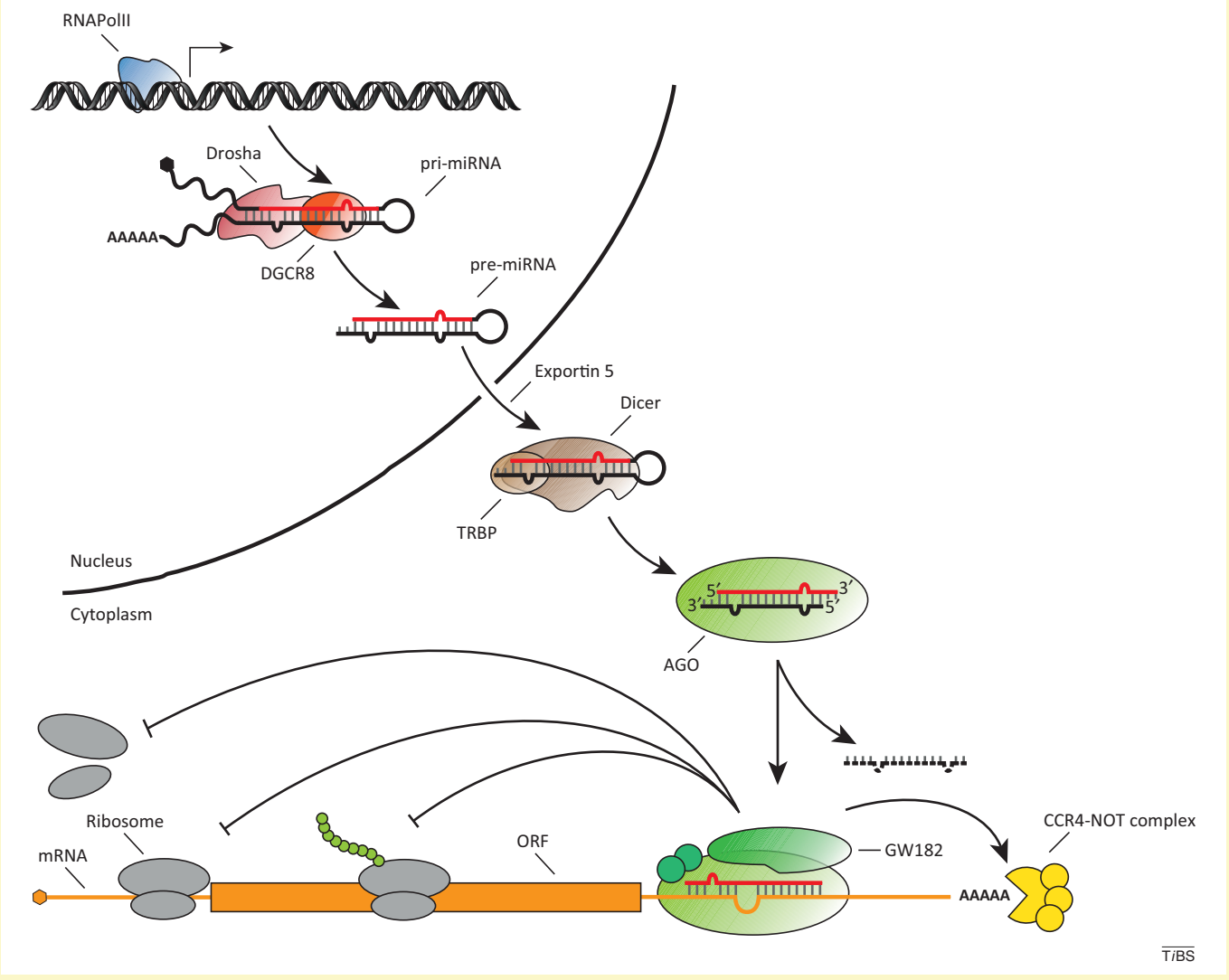

Figure I. Schematic view of miRNA biogenesis and mechanism of action.

and whether loading, which would presumably promote stability [20,21], is equal for all miR-29b mutant variants.

Several other endogenous miRNAs display a cell cycle-dependent expression pattern in HeLa cells [22]. HeLa cells complete a cell cycle in less than 1 day; therefore, this suggests a relatively rapid turnover of these miRNAs, although exact half-lives were not determined. It is also unknown whether stability of these miRNAs, similar to miR-29b, is regulated in a cell cycle-dependent manner. Alternatively, continuously low stability across all cell cycle phases might permit rapid decreases of miRNA levels upon slowed biogenesis. Indeed, Rissland and colleagues

observed that this is true for the rapid downregulation of miR-16 family members in mouse NIH 3T3 cells during transition from G0 to G1 phase [17]. Levels of several members of this family rapidly decreased at the G0–G1-transition. Among these, miR-503, an extended miR-16 family member that differs from other family members at nucleotide eight of the seed region (Box 1), decreased most strongly and with an apparent half-life of 3.6 h. Strikingly, a similarly short half-life was observed for miR-503 that was ectopically expressed from a repressible

Table 1. miRNAs that exhibit accelerated decay

miRNA	Half-life[a] (h)	Destabilizing element	Organism/cells	Nuclease	Condition	Refs
miR-29b	4±1.4 ([16]), 7.3±1.8 ([19])	uagcaccauuu gaaaucaguguu	human HeLa, mouse NIH 3T3 cells	ND	Destabilized in cycling cells, stable in mitotic cells	[16,19]
miR-503, miR-15a/b, miR-322, miR-16	3.6 (miR-503), 5.8 (miR-322)	uagcagcg ggaacag uacugcag (miR-503)	Mouse NIH 3T3 cells	ND	Inherently unstable; Decreased at G0–G1 transition due to transcriptional shut-off	[17]
miR-134, miR-155, miR-188, miR-191, miR-198, miR-202, miR-212, miR-320, miR-370, miR-452, miR-498, miR-564, miR-572, miR-575, miR-601, miR-629, miR-630, miR-638, miR-662, miR-663, miR-765, miR-654, miR-671	~1	ND	Human MCF10A immortalized breast and HeLa cervical cancer cells	ND	Destabilized by EGF exposure	[18]
(miR-183/96/182, miR-204, miR-211)*, let-7b, miR-29c, miR-15a, miR-16, miR-124, miR-128, miR-134, miR-138	~1.5 (for light-regulated ones*)	ND	Retinal mouse neurons (*in vivo*), cultured rodent cortical and hippocampal neurons, ES cell-derived neurons	ND	Unstable in neurons; stabilized when neuronal activity is blocked	[23]
miR-125b, miR-132, miR-146a, mir-183, miR-9	0.7 (miR-9) – 3.5 (miR-125b)	ND	Primary human neuron cultures, postmortem human brain tissues	ND	ND	[24]
miR-124, miR-184	ND	ND	Cultured *Aplysia* neurons	ND	Levels reduced upon serotonin exposure	[25]
miR156, miR159, miR163, miR167, miR172, miR173, siR1003 (endo-siRNA),	ND	ND	*A. thaliana*	Combined depletion of SDN1/2/3 and At3g50090	ND	[31]
miR912, miR909.1, miR1157, siRNAs	ND	ND	*Ch. reinhardtii*	RRP6	Quality control mechanism (loss of 2′-*O*-methylation)	[39]
(let-7, miR-48, miR-84, miR-241, miR-237, miR-34, miR-85, miR-240, miR-234, miR-245, miR-77)$^{\circ}$ (let-7, miR-241, miR-77, lin-4)$^{+}$	ND	ND	*C. elegans*	XRN-1^{+}/XRN-2°	ND	[41,42]
miR-382	1.2	gaaguugu ucguggu ggauucg	HEK 293 cells	RRP41/XRN1?	ND	[47]
miR-221, miR-222, miR-106, miR-103, miR-107, miR-183, miR-193a-3p, miR-210, miR-214, miR-29b, miR-320, miR-518a-5p-527, miR-572, miR-612, miR-617, miR-628-3p, miR-630, +12 not yet annotated miRs	ND	ND	Human melanoma cells	PNPase^{old-35}	Destabilized by interferon-β exposure	[48]

Table 1 (*Continued*)

miRNA	Half-life[a] (h)	Destabilizing element	Organism/cells	Nuclease	Condition	Refs
miR-20a	3–6	ND	Human glioma cells, human and murine astrocytes, HEK 293T cells	ND	Stabilized by binding to QKI	[53]
miR-27	ND	Partial complementarity to HSUR1 from HVS and m169 from MCMV	HVS-infected primate T cells, MCMV infected mouse NIH 3T3 cells	ND	Destabilized by binding to viral non-coding transcript (HSUR1 in HVS, m169 in MCMV)	[60–62]
let-7	3.53	Partial complementarity to miR-107	Several human cancer cell lines and mouse mammary tumor cells/tissues	ND	Destabilized by binding to miR-107	[64]

[a]Only the shortest measured half-life is given for those examples where decay rate was shown to increase by a given stimulus/context.
ND, no data.

transgene within G0. Moreover, whereas its precursor, pre-miR-503, was observed by northern blot in G0-arrested cells, it became undetectable upon cell cycle re-entry, suggesting cell cycle-regulated transcription or processing. Thus, it is not miR-503 stability that is altered during transition into G1. Instead, constitutively low stability of miR-503 permits rapid changes in its levels in response to altered transcription or processing.

Expression of variant pre-miRNA from transgenes revealed that residues in the seed region and at the 3′-end coordinately destabilize miR-503 [17]. The nuclease that rapidly turns over miR-503 awaits identification, and the physiological relevance of fast miR-16 family regulation is currently not clear. However, given that the known and predicted miR-16 target genes include several genes that function in the G1-S transition (e.g., Cyclin D1/2/3, Cyclin E1, and CDK6) and that repression of miR-503 target reporters was reduced in G1- compared to G0-phase [17], a role in modulating cell cycle progression appears likely.

Growth factors

In human MCF10A immortalized breast epithelial cells, the levels of several miRNAs rapidly decreased upon epidermal growth factor (EGF) stimulation [18]. After MCF10A cells had been starved of serum to arrest their proliferation, stimulation by addition of EGF caused a reduction by $\geq$50% of 23 miRNAs within 1 h. Similar observations were made for human HeLa cervical cancer cells, although there was only minor overlap in the set of miRNAs that was affected. Known or predicted targets of the miRNAs downregulated in MCF10A cells include several 'immediate early genes'; that is, genes that are rapidly upregulated in response to EGF [18]. This suggests that rapid miRNA downregulation contributes to the physiological responses (i.e., proliferation or migration) of a cell to EGF. Conversely, prior to their degradation, the miRNAs might prevent inappropriate activation of these targets in the absence of serum or EGF. However, it remains unclear whether EGF acts by inducing miRNA degradation or, analogously to the situation of miR-503, alters transcription or processing of inherently unstable miRNAs.

Neuronal activity

Strikingly, although rapid degradation affects only subsets of miRNAs in the examples discussed so far, it appears to be a prevailing feature of neurons [23]. Krol and colleagues found that the miRNA-cluster miR-183/96/182, as well as miR-204 and miR-211, are light-regulated in the mouse retina: when mice were shifted from light to dark, levels of these miRNAs fell to roughly 50% of the starting level within ~90 min, and then remained constant. However, like miR-503, the miRNA decay rate was not altered but invariably fast, and rapid decreases in miRNA levels upon dark adaptation were induced by transcriptional repression. Moreover, even miRNAs whose accumulation was not light-regulated (let-7b, miR-29c, miR-15, and miR-16) displayed fast turnover in retinas. However, they were not transcriptionally regulated and so their steady-state levels were unaffected by dark adaptation. Thus, fast miRNA turnover might be a general property of neurons. In support of this notion, non-differentiated mouse ES cells did not exhibit rapid turnover, whereas pyramidal neurons differentiated thereof did [23]. That turnover of miRNAs is generally fast in neurons is also in accordance with the earlier observations in primary human neuron cultures and postmortem human brain tissues that miRNA half-lives were not longer than 3.5 h for all of five tested miRNAs [24].

Notably, fast turnover of neuronal miRNAs is dependent on neuronal activity. Blocking action potentials by using tetrodotoxin or by blocking glutamate receptors prevented fast turnover [23]. However, miR-132, another miRNA that is enriched in neurons, showed the opposite behavior: blocking glutamate receptors activated its decay, whereas addition of glutamate slowed its decay [23].

Rapid downregulation of neuronal miRNAs was also observed in the sea slug *Aplysia californica*, for which treatment with the neurotransmitter serotonin resulted in a decrease of miR-124 and miR-184 levels with an apparent half-life of <3 h [25]. At this point, it is not known whether this finding reflects accelerated decay or decreased miRNA biogenesis rates for miRNAs displaying constitutively rapid decay. The serotonin-induced reduction of miR-124 levels was shown to contribute to learning-related synaptic plasticity, enhancing the switch from short- to long-term facilitation through derepression of the miR-124 target cAMP response element-binding protein 1 (CREB1).

Taken together, cell cycle progression, growth factor signaling, and neuronal activity were identified as

physiological triggers affecting miRNA stability. A small set of miRNAs, including miR-141, was additionally reported to be rapidly downregulated upon seeding cells at low density, but recent work has now established that this as an artifact caused by differences in miR-141 extraction efficiency when Trizol reagent was used with different amounts of starting material [26].

In these different instances of rapid miRNA degradation, cis-acting elements have been mapped along the entire length of miRNAs: the seed region, central part, and the 3′-end (Table 1). As none of these motifs sufficed for rapid turnover, there appears to be a complex interplay between miRNA decay factors and different parts of a miRNA. It remains enigmatic how trans-acting turnover factors would access particular sequence motifs. Structural and chemical probing data suggest an unequal accessibility for trans-acting factors to different parts of an AGO-loaded miRNA [27,28]. Because both ends of an miRNA are anchored in specific binding pockets within AGO [29], they are likely to require release from AGO in order to become accessible for exonucleases. Interestingly, structural studies of bacterial AGO show that the 3′-end of a DNA guide strand is dislodged from its binding pocket upon binding to target RNA exhibiting extensive complementarity to the guide 3′-half [30]. The freed 3′-end might then become sensitive to nucleotidyl-transferases and 3′-to-5′ exonucleases, underpinning the emerging role of target RNAs as trans-acting regulators of miRNAs, a fact we discuss later.

We note that in the instances of rapid miRNA downregulation discussed thus far, several examples illustrate the use of constitutive miRNA destabilization to predispose some or all miRNAs of a cell to rapid expression level changes through modulation of their transcription or maturation [17,23]. However, there does not appear to be an inherent constraint against modulating miRNA decay rates [16], and we highlight additional examples in later sections.

Finally, in most of the examples we have discussed, determining the physiological relevance of rapid miRNA decay awaits further study. This is particularly true for the intriguing observation of globally accelerated miRNA decay in neurons. Similarly, identification of trans-acting factors that mediate constitutive or induced miRNA turnover in the studies discussed in this section remains a future challenge. However, different ribonucleases (RNases) have already been implicated in miRNA degradation, and we discuss these next.

miRNA-degrading enzymes

Several miRNA-degrading enzymes have been identified, including both 3′-to-5′ and 5′-to-3′ exoribonucleases, but so far no endoribonucleases (Figure 1). Distinct RNases were found to function in turnover of different sets of miRNAs

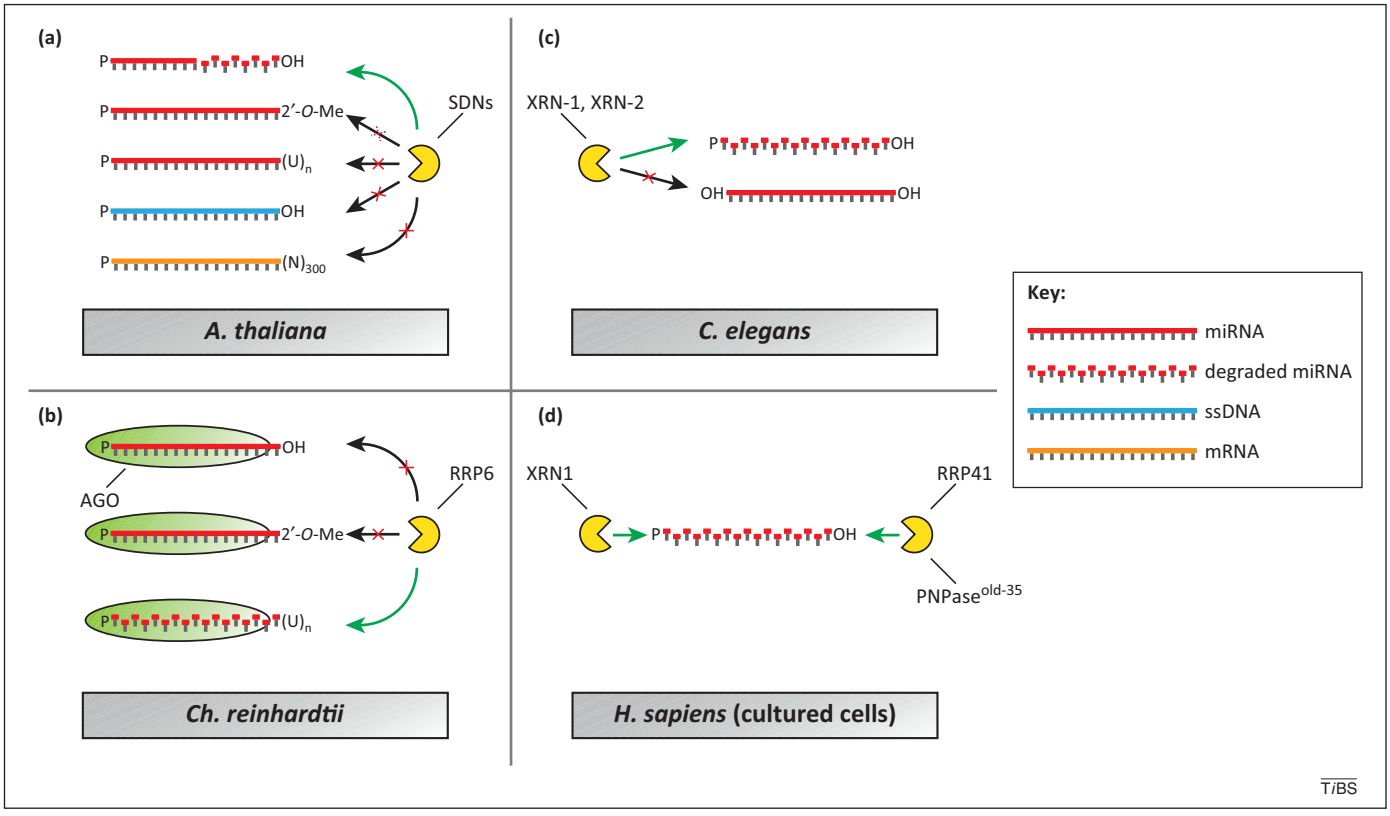

Figure 1. microRNA-degrading enzymes. Distinct enzymes have been implicated in miRNA turnover in various organisms. (**a**) In *Arabidopsis thaliana*, small RNA degrading nucleases (SDNs) degrade miRNAs containing a 3′-OH group yielding a product of 8–9 nt [31]. A 2′-*O*-Me group delays degradation kinetics and oligo-uridine tails render miRNAs degradation-insensitive. Single-stranded DNA (ssDNA; blue) or longer RNAs (orange) are also not targeted by SDNs. (**b**) In the green alga *Chlamydomonas reinhardtii*, ribosomal RNA processing protein 6 (RRP6) degrades Argonaute- (AGO-) loaded miRNAs containing oligo-uridine tails but not miRNAs containing an OH or 2′-*O*-Me group at the 3′-end [39]. RRP6 is proposed to function in a quality control mechanism, eliminating subfunctional small RNAs from AGO. (**c**) In *Caenorhabditis elegans*, exoribonuclease 2 (XRN-2) degrades miRNAs containing a 5′-monophosphate but not an OH group [42] and this is presumably also true for XRN-1 [41]. (**d**) In cultured human cells, ribosomal RNA processing protein 41 (RRP41) [47] and polynucleotide phosphorylase (PNPase^{old-35}) [48] degrade specific miRNAs in the 3′-to-5′ direction, whereas XRN1 [47] acts in the 5′-to-3′ direction. Green arrow: degradation permitted, black arrow with red cross: degradation inhibited.

and/or different organisms, but because these results are only from a small number of studies, substrate specificity and phylogenetic conservation of individual miRNA turnover enzymes remains largely unknown. For convenience, we will refer to miRNA degrading enzymes as 'miRNases', but we emphasize that the substrate spectrum of many or all of these RNases is likely, or in cases even known, to extend beyond miRNAs.

Arabidopsis thaliana: *the small RNA degrading nucleases (SDNs)*

Active degradation of miRNAs was initially reported in *Arabidopsis thaliana*, where it is mediated by the small RNA degrading nucleases (SDNs). The simultaneous depletion of all five SDN family members increased the levels of various miRNAs two- to threefold *in vivo*, and caused pleiotropic developmental phenotypes [31] (Figure 1a). SDNs are homologous to yeast Rex1/2/3/4p, which are involved in the 3′-end processing of rRNAs (yeast does not have miRNA) [32,33]. However, there is no evidence to support a function of plant SDNs in rRNA processing [31], and it is currently not known whether SDN-homologs in other eukaryotes, including animals, function in miRNA degradation.

Experiments with recombinant SDN1 and synthetic miRNAs have revealed that SDN1 uses a 3′-to-5′ exonucleolytic mechanism, yielding a final degradation product of 8–9 nt. SDN1 can degrade single-stranded RNA in the range of 17–27 nt with comparable efficiency, but not pre-miRNAs, longer RNAs, double-stranded RNA or single-stranded DNA. *In vivo*, plant miRNAs are 2′-*O*-methylated at their 3′-ends [34,35]; this feature slowed down but did not prevent miRNA degradation by SDN1 *in vitro* [31]. 2′-*O*-methylation by the methyltransferase HEN1 (HUA ENHANCER1) also stabilizes miRNAs *in vivo* by preventing 3′-end oligouridylation by HESO1 (HEN1 SUPPRESSOR1), a terminal nucleotidyl transferase [36,37]. However, because uridylation, at least *in vitro*, failed to promote and in fact attenuated SDN1-mediated degradation [31], it appears that uridylation influences miRNA degradation through distinct enzymes that remain to be identified. Interestingly, HEN1-mediated 2′-*O*-methylation also prevents uridylation and degradation of other classes of small RNAs, namely piRNAs in various animals and siRNAs in plants and *Drosophila* [38]. Nevertheless, it remains to be shown whether HEN1 is used as a physiological regulator of miRNA degradation.

Chlamydomonas reinhardtii: *MUT68 and RRP6*

Uridylation of miRNAs and siRNAs also contributes to their decay in the green alga *Chlamydomonas reinhardtii* [39]. The terminal nucleotidyl transferase MUT68 was found to uridylate the 3′-ends of these small RNAs *in vivo* and to stimulate their degradation by RRP6 (ribosomal RNA-processing protein 6), a component of the 3′-to-5′ exosome RNase complex, *in vitro* (Figure 1b). Furthermore, depletion of RRP6 elevated miRNA and siRNA levels *in vivo* [39]. The 2′-*O*-methyl group present on endogenous *C. reinhardtii* miRNAs prevented both uridylation and degradation *in vitro*.

Although *MUT68* mutation caused an accumulation of small RNAs, paradoxically, it also resulted in accumulation of an mRNA targeted by siRNA expressed from an inverted repeat transgene [39,40]. The interpretation of this finding is not straightforward, because MUT68 also adenylated the 5′ terminal product that resulted from siRNA-directed cleavage of this mRNA [40]. Hence, it has not been ruled out that stabilization of the full-length mRNA might be an indirect consequence of impaired clearance of its cleavage product. Nonetheless, a reasonable scenario proposed by Cerutti and colleagues [39] is that MUT68 and RRP6 define a quality control pathway that eliminates non-functional miRNAs, which might otherwise compete with functional miRNAs for access to AGO or other components of the miRNA machinery. Instead of contributing to general control of miRNA stability, HEN1 and HESO1 might then be part of an analogous quality control system in *Arabidopsis*.

Caenorhabditis elegans: *XRN-1 and XRN-2*

In the nematode *Caenorhabditis elegans*, the 5′-to-3′ exoribonucleases XRN-1/XRN1 and XRN-2/XRN2/Rat1p were shown to modulate miRNA activity through degradation [41,42] (Figure 1c). XRN1 and XRN2 are conserved across eukaryotes and have been implicated in exonucleolytic degradation and/or processing of various RNA substrates including rRNA, tRNA, small nucleolar (sno)RNA, pre-mRNA, and mRNA [43]. In *C. elegans*, RNAi-mediated depletion of *xrn-1* or *xrn-2* led to an accumulation of several mature miRNAs, whereas levels of pri- and pre-miRNA remained unchanged [41,42]. Depletion of *xrn-1* or *xrn-2* also suppressed mutant phenotypes, such as bursting through the vulva, that are associated with a point mutation in the seed sequence of the *let-7* miRNA. This mutation leads to a reduction of mature *let-7* miRNA levels by affecting its biogenesis [44] and/or stability [41], but *let-7* levels were restored by depletion of *xrn-1* or *xrn-2*. Diminished mRNA levels of the *let-7* targets *daf-12* and *lin-41* provide a molecular basis for the rescue of *let-7* mutant phenotypes by *xrn-2* depletion, and further demonstrate that XRN-2 targets actively repressing rather than scavenging non-functional miRNAs.

RNAi against *xrn-2* led to a $\geq$2-fold increase of nine out of 12 endogenous miRNAs tested *in vivo* [42]. Whether the lack of an effect on the remaining miRNAs reflects a true substrate specificity of XRN-2 or its inefficient depletion at specific times or in specific tissues remains to be determined. *In vitro*, each of four synthetic miRNAs that were tested was degraded irrespective of its sequence.

A few XRN co-factors involved in RNA degradation have been described. In yeast, processive RNA decay by Rat1p/Xrn2p requires binding of its co-factor Rai1p (RAT1 interacting protein). However, the metazoan Rai1p homolog Dom3Z (Downstream of MES-3 homolog Z) does not interact with XRN2 [45], leaving it unclear if metazoan XRN2 requires a processivity-stimulating co-factor. In mouse embryonic fibroblasts, XRN2 degrades 'non-targeting' siRNAs in complex with the endoplasmatic reticulum-resident protein NPGPx (non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase; or glutathione peroxidase GPX7) [46]. NPGPx expression was induced by stress from accumulation of siRNAs lacking cognate targets. Degradation of the siRNA by the

NPGPx/XRN2 complex appeared to release this stress. However, because the authors did not investigate miRNA levels and used high concentrations of non-targeting siRNAs in their experiments (20–160 nM), the relevance of murine NPGPx and XRN2 for physiological miRNA turnover remains unclear.

Humans: XRN1, RRP41, and PNPase^{old-35}

In human embryonic kidney (HEK293T) cells, Bail *et al*. implicated XRN1 and the exosome in miRNA turnover. Using microarrays to determine the levels of miRNAs following transcriptional shutoff by actinomycin D treatment, they found that 95% of miRNAs remained stable for at least 8 h [47]. Among the miRNAs with a half-life <8 h, miR-382 was verified by RT-qPCR (reverse transcription-quantitative polymerase chain reaction) to be unstable. Knock-down of RRP41 (ribosomal RNA-processing protein 41), a core component of the exosome complex, yielded a modest 1.5-fold increase in miR-382 levels (Figure 1d); a 1.3-fold increase was observed upon XRN1 knockdown. XRN2 depletion had no effect. A HEK293T cytoplasmic lysate was found to recapitulate rapid miR-382 turnover relative to a more stable miR-378 control miRNA, but only if the mature miRNA was derived from processing a pre-miRNA with Dicer-overexpressing lysate. By contrast, synthetic mature miR-378 and miR-382 decayed at equal rates. How *in vitro* processing contributes to destabilization of miR-382 remains unclear, but the coupled processing-degradation system permitted demonstration that the 3′-terminus, positions 16–22, was required for rapid decay of miR-382. How this element leads to accelerated decay and the functional relevance of miR-382 destabilization remain to be elucidated.

Finally, the human polynucleotide phosphorylase (PNPase^{old-35}; aka PNPT1 or polyribonucleotide nucleotidyltransferase 1, mitochondrial) degrades certain mature miRNAs in human melanoma cells without affecting pri- or pre-miRNA levels [48] (Figure 1d). PNPase^{old-35} is an interferon (IFN)-inducible 3′-to-5′ exoribonuclease that has been implicated in the degradation of bacterial small non-coding RNAs [49] and IFN-induced growth arrest of human melanoma cells [50]. Microarray profiling of human melanoma cells highlighted the downregulation of several mature miRNAs (including miR-221, miR-222, and miR-106b) upon ectopic expression of PNPase^{old-35}. RT-qPCR and northern blotting further confirmed the downregulation of miR-221, miR-222, and miR-106b by ectopic or interferon-beta- (IFN-β-) induced expression of PNPase^{old-35}. The reduction in miR-221 and miR-222 levels was accompanied by an upregulation of p27^{kip1}, a validated target of these miRNAs [51]. Interestingly, miR-221 overexpression rendered human melanoma cells insensitive to IFN-β, supporting the notion that IFN-β-mediated growth arrest depends, at least partially, on miR-221 degradation. Of note, several miRNAs (including let-7a, miR-184, and miR-25) did not decrease upon ectopic expression of PNPase^{old-35}. When total RNA from HO-1 cells was incubated with *in vitro* translated PNPase^{old-35}, these same miRNAs were also refractory to degradation, whereas miR-221, miR-222, and miR-106b were sensitive. PNPase^{old-35} might thus have inherent substrate specificity. PNPase $^{old-35}$ seems to be preferentially, and possibly exclusively, localized in the mitochondrial inner membrane space [52], therefore, an interesting question is where it degrades miRNAs.

In summary, several 5′-to-3′ and 3′-to-5′ miRNA-degrading enzymes have been identified (Figure 1). Although the miRNases described thus far are widely conserved proteins among eukaryotes, evidence for evolutionary conservation of miRNA turnover pathways has, in fact, not yet been produced. Difficulties in pinpointing orthologous miRNases might arise from (partially) redundant degradation pathways, as illustrated by the need to co-deplete several SDNs in *A. thaliana* to elicit a phenotype *in vivo* [31]. Individual studies also tended to focus on changes in the levels of one or only a few miRNAs, therefore, it is further conceivable that miRNase activity might have been missed due to substrate or tissue specificity. Lastly, several of these studies investigated phenotypes upon depletion of candidate RNases by RNAi rather than gene knockout. Residual RNase activity might thus hamper detection of miRNA turnover defects.

Although miRNases have now been shown to function in maintaining miRNA homeostasis in several organisms, it remains to be identified whether and how these enzymes are regulated. The human RNA-binding protein Quaking (QKI) has recently been shown to bind to and stabilize miR-20a, a function that appears to contribute to the tumor suppressive function of QKI in glioblastoma multiforme [53]. However, it remains unknown which RNase normally degrades miR-20a and how QKI mechanistically prevents degradation. More generally, we have little knowledge of factors that possibly convey processivity, substrate- or tissue-specificity to miRNases. However, recent work has revealed an important role of target RNAs in determining the stability of their cognate miRNAs.

Reversing a relation: regulatory functions of target RNAs on miRNAs

At the heart of miRNA-mediated mRNA regulation lies the sequence-specific interaction of the miRNA and the mRNA [54]. The extent of sequence complementarity between miRNA and mRNA determines the mode of mRNA silencing. Extensive complementarity, reminiscent of the siRNA–mRNA interaction, can result in endonucleolytic cleavage of the target mRNA, and constitutes a major means by which miRNAs regulate mRNAs in plants [55]. In metazoans, miRNAs base-pair with mRNAs mainly through partial complementarity, resulting in translational repression or exonucleolytic degradation [1]. Recent studies now provide evidence for reciprocal regulation, such that target RNAs can modulate miRNA stability (Figure 2). The degree of sequence complementarity appears be fateful for the miRNA as well.

Highly complementary targets can induce miRNA degradation in animals

In flies, mice, and human HeLa and HEK293T cells, miRNAs are destabilized if they are supplied with an artificial target exhibiting extensive complementarity [6,56,57] (Figure 2a). The decline of a miRNA in the presence of a highly complementary target is accompanied by the emergence of longer ('tailed'; typically multiple [56] or individual

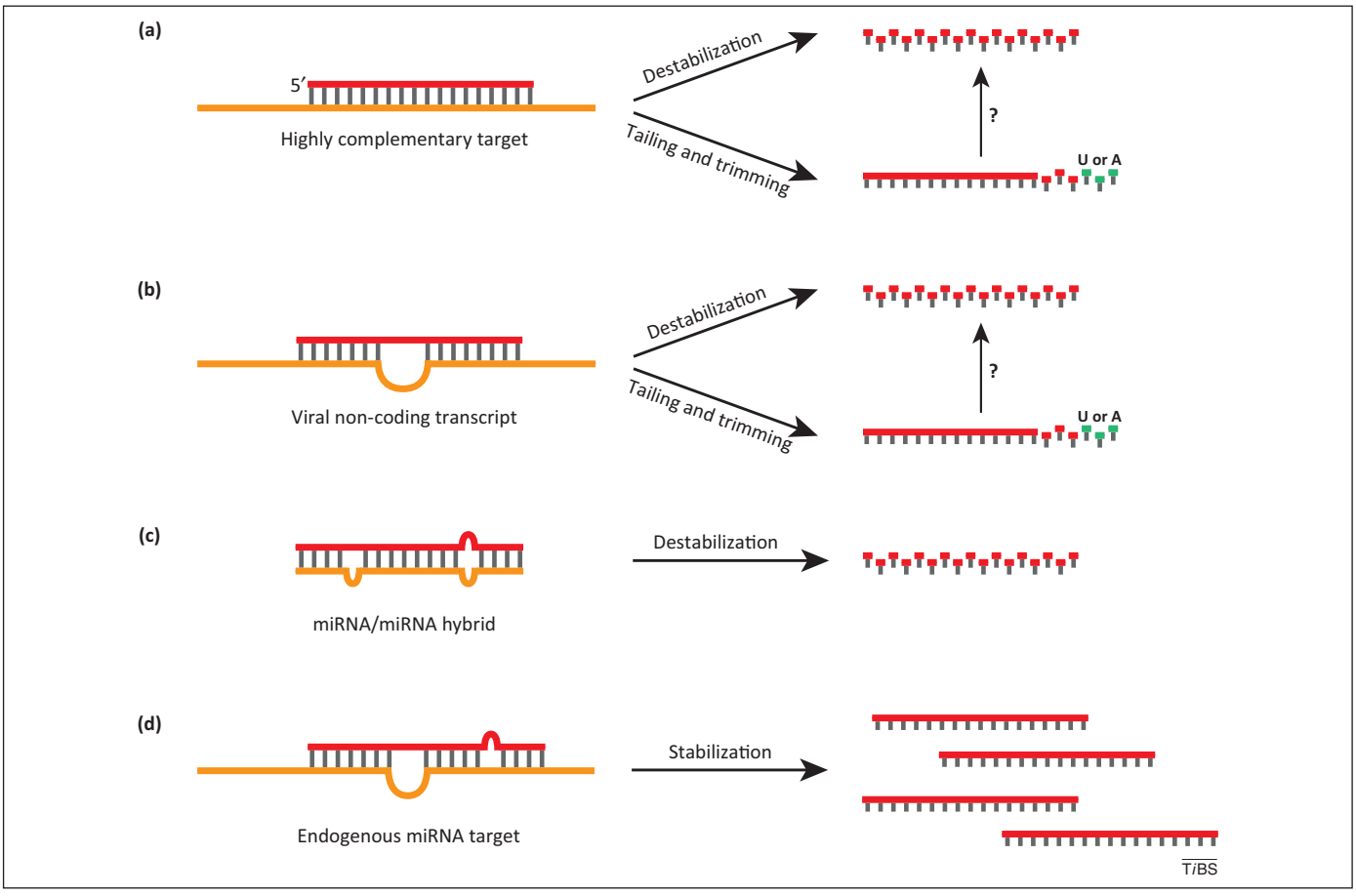

Figure 2. Effects of target RNAs on miRNAs. Target RNAs can stabilize or destabilize cognate miRNAs. (**a,b**) Highly complementary targets and viral non-coding transcripts lead to destabilization as well as tailing and trimming of miRNAs [6,56,57,60–62]. The tails (in green) consist predominantly of uridines and adenines. It is currently unknown if and how target-dependent tailing and trimming stimulates miRNA degradation. (**c**) A miRNA/miRNA hybrid has been shown to lead to destabilization of one of the two interacting strands (miR-107/let-7 in [64]). (**d**) Artificial and endogenous miRNA targets stabilize *Caenorhabditis elegans* miRNAs in a trimeric complex consisting of AGO, target, and miRNA [41].

[6,57] added uridines or adenosines) and shorter ('trimmed'; [56,57]) species of the original miRNA. At this point, it is not known whether tailing precedes trimming, or rather defines a separate miRNA fate upon binding highly complementary targets. For instance, when Baccarini and colleagues turned off miR-223 transcription using a 'tet-off' system, levels of mono-uridylated species of miR-223 increased over time, relative to those of the unmodified miRNA [6]. This could mean that uridylation occurs prior to, and promotes, miR-223 degradation. Alternatively, uridylation might stabilize miR-223 so that it decays less rapidly than the unmodified species. Solving this issue will require the identification of the enzymes mediating tailing and trimming in a target-dependent manner. Although *Drosophila* Nibbler, a member of the DEDD family of exonucleases, trims the 3′-ends of some miRNAs by a few nucleotides [58,59], it does not appear to function in miRNA turnover.

Some degree of mismatching between miRNA and target RNA does not abolish tailing and trimming, and in particular base-pairing of the eight 3′ terminal nucleotides is dispensable [56]. However, miRNA 'seed' binding sites, that is, those with complementarity to nucleotides 2–8 of the miRNA only, do not induce tailing and trimming. Hence, because miRNA complementarity is limited to the seed for most endogenous targets [54], these targets will not usually induce miRNA degradation [56].

Viruses employ targets to destabilize host miRNAs

No endogenous cellular mRNA has been found to induce tailing and trimming in flies or mammals. By contrast, viruses exploit target-dependent miRNA destabilization to affect gene expression of host cells [60–62] (Figure 2b). In *Herpesvirus saimiri* (HVS) infected primate T cells, small non-coding HSUR RNAs (*H. saimiri* U-rich RNAs) bound to the partially complementary miR-142-3p, miR-27 (comprising miR-27a and miR-27b, which differ by one nucleotide), and miR-16 [60]. However, only miR-27, which base-pairs with HSUR 1, was destabilized by this interaction, causing an increase of FOXO1, a validated miR-27 target. By contrast, binding of HSURs to miR-142-3p or miR-16 did not alter their levels, perhaps due to less extensive base-pairing. How the virus benefits from miR-27 degradation remains unclear.

Similar to HVS, infection of mouse cells with murine cytomegalovirus (MCMV) induces the rapid downregulation of cellular miR-27 through an HSUR-unrelated viral mRNA, m169, which contains a miR-27 binding site in its 3′-UTR [61,62]. Despite hundreds of predicted miR-27-binding sites in the MCMV transcriptome, m169 is solely responsible for miR-27 degradation [61]. Moreover, expression of m169 3′-UTR in uninfected NIH 3T3 cells sufficed for miR-27 degradation, suggesting that no other viral factors are required for this process [62]. Marcinowski

and colleagues found that miR-27 degradation was preceded by tailing and trimming, and that degradation and tailing and trimming alike were dependent on an intact miR-27 binding site in m169 [62]. However, 3′-end heterogeneity was observed for several miRNAs even in uninfected mouse cells, possibly suggesting that tailing and trimming also occurred in uninfected cells and that MCMV infection enhances this process specifically for miR-27 [62]. Although the mechanism by which miR-27 degradation facilitates viral infection is unknown, m169-mutant viruses that were unable to degrade miR-27 exhibited attenuated titers in various organs, strongly implying that degradation of miR-27 is important for efficient virus replication *in vivo* [62].

In *Drosophila* embryo lysates, tailing and trimming requires a high degree of complementarity between miRNA and target, such that a central loop of more than 3 nt impaired trimming [56]. By contrast, base-pairing between miR-27 and m169 involves a larger loop, and miR-27 degradation occurred, to a low extent, even with an m169 version carrying a point mutation in the 'seed-match' sequence [62]. It thus appears possible that the requirements for target-induced tailing and trimming might differ for different miRNAs or in different systems. For instance, depending on the thermodynamics of miRNA-target interaction, high expression levels of targets could be permissive for a lower degree of complementarity.

Target-induced miRNA degradation in plants

Unlike in animals, plant target mRNAs are frequently highly complementary to their cognate miRNAs. Moreover, there is precedence for the idea of tailing and trimming, which occurs in *A. thaliana* when 2′-*O*-methylation of small RNA 3′-termini is lost through mutation of *hen1* [34,35]. The tails almost exclusively consist of uridines and also occur on trimmed small RNAs [35]. Although it remains to be shown that endogenous targets can indeed induce plant miRNA degradation, artificial, highly complementary target RNAs containing two target sites were found to cause a severe reduction of cognate miRNA levels [63]. That primary miRNA levels did not decrease confirmed that the effect was post-transcriptional, and partial restoration of mature miRNA levels in *sdn1 sdn2* double mutant plants provided further evidence that targets acted by inducing miRNA degradation. However, as discussed earlier, SDNs are thought to be specific for single-stranded RNA, therefore, it is unclear how target binding could promote SDN-dependent miRNA degradation. It also remains possible that SDNs function in a parallel pathway; that is, their mutation restores miRNA levels by bypassing rather than reversing the miRNA-reducing effect of targets.

Destabilization of one miRNA by another

In human cancer cell lines, miR-107 can reduce the stability of mature let-7 miRNA, but not pri- or pre-let-7, via base-pairing interactions [64] (Figure 2c). Whereas ectopic expression of miR-107 decreased let-7 levels and enhanced the levels of the let-7 targets HMGA2 and RAS, depletion of endogenous miR-107 stabilized let-7 levels and reduced let-7 targets. In a mouse tumor model, let-7-dependent tumor suppression was abolished by transfection of miR-107 but not mutant versions thereof. Examination of mutant variants of let-7 and miR-107 suggested that miR-107-induced let-7 destabilization involved formation of a miR-107/let-7 duplex, but a mechanism remains to be determined. In this regard, it will be of particular interest to determine whether miR-107 is AGO-bound when interacting with let-7. It will also be interesting to determine whether other regulatory miRNA–miRNA interactions exist [65].

Stabilization of a miRNA through its targets

Contrasting with target-induced degradation of miRNAs, target mRNAs in *C. elegans* have been found to stabilize miRNAs *in vivo* by preventing their release from AGO proteins [41] (Figure 2d). Chatterjee *et al*. found that reduced availability of endogenous targets decreased accumulation of the cognate miRNAs, whereas miRNA levels increased in the presence of artificial target RNAs. This process, termed target-mediated miRNA protection (TMMP) counteracts miRNA decay mediated by XRN-1 and XRN-2. Together, miRNA decay and TMMP could thus serve as a proofreading mechanism that ensures preferential occupation of AGO with functional, that is, target-engaged miRNA.

Although miRNA* (also known as passenger; Box 1) strands are not normally loaded onto AGO proteins, miR-241* was stabilized when an artificial target RNA was provided *in vitro* or *in vivo* [41]. This effect may be limited to a subset of miRNA duplexes that, like miR-241:miR-241*, do not conform to the thermodynamic asymmetry rule for selection of the miRNA guide strand (Box 1). Nonetheless, it offers the possibility that TMMP might provide a way to drive evolution of new miRNAs by stabilizing previously unused miRNA* strands once targets, and thus potential biological functions, have been acquired. Such a mechanism might explain how the ratios of miR and miR* levels can vary in different tissues of an organism or during development [9,66,67].

In sum, target RNAs have been found to affect partially complementary miRNAs in various systems, causing either miRNA stabilization or destabilization. At this point, it remains unclear whether targets can mediate miRNA stabilization as well as destabilization in the same system, or whether these are distinct miRNA regulatory systems occurring in different organisms. Interestingly, Kuchen and colleagues observed that in mouse A70 proB and human HEK293T cells, expression of RNAs with eight or 16 highly complementary target sites, containing a four nucleotide central bulge, resulted in reduced accumulation for five out of six targeted miRNA passenger strands, and increased accumulation of one [66]. Although it was not investigated in these experiments whether the effects were related to mature miRNA turnover, the results emphasize a need for a detailed investigation of how target architecture, including the extent of complementarity, and expression levels of the target and/or its cognate miRNA determine the outcome of miRNA binding by target mRNA.

Box 2. Outstanding questions

- What are the (patho-)physiological functions of miRNA turnover processes? For instance, are there developmental processes or responses to environmental cues that rely on rapid miRNA turnover?
- Is there a 'major', widely conserved miRNA turnover pathway or do different miRNAs, cells, organisms, etc. rely on distinct miRNA degrading enzymes?
- Is miRNA turnover regulated and if so, how? How is specificity provided in situations where individual miRNAs are selectively degraded?
- To what extent and to what end do endogenous targets modulate miRNA levels? Are there specific 'regulatory' targets? Which factors decide the outcome of miRNA binding by targets, that is, stablization or destabilization? In particular, what is the contribution of target site architecture, target expression levels, and cell type or experimental system?
- Where does miRNA turnover occur within the cell? Is localization of the turnover machinery important for regulation of miRNA degradation, for example, by restricting access to specific substrates?

Concluding remarks

Although miRNAs were initially considered to be highly stable molecules, rapid and active miRNA degradation has now been demonstrated in many different organisms and experimental systems. Somewhat surprisingly, there appears to be a great diversity of miRNA degrading enzymes and, thus far, little evidence for conserved usage of individual enzymes across phylogeny. However, given that this field is still in its early days, further studies may force us to revise this view by revealing pathways that are used more broadly.

Degradation can be specific or affect large sets of miRNAs; however, it is currently largely unclear how specificity is achieved (Box 2). The use of target RNAs to modulate miRNA stability, positively or negatively, would provide an elegant solution, but it remains to be shown how widely this approach is used. For now, endogenous targets that alter miRNA levels are largely unknown. However, if targets were shown to broadly modulate miRNA levels, this would also challenge our current concept of miRNAs; rather than thinking of miRNA regulation as a one-way street leading to target mRNA silencing, we might need to consider a more complex network of mutual regulation of miRNAs and their targets.

Finally, miRNA turnover occurs widely in various organisms and systems, implying that it represents an important aspect of miRNA regulation. Indeed, the striking observation that miRNA turnover is generally accelerated in neurons strongly suggests a major role for this pathway in neuronal development, homeostasis, and/or function. Nonetheless, identification of the precise physiological function of miRNA turnover remains a major challenge in this as well as most other instances (Box 2).

Note added in proof

Eri1 (3′-to-5′ exoribonuclease 1) has recently been implicated in miRNA turnover in murine immune cells [69]. Previously, *C. elegans* mutant for *eri-1* had been found to accumulate siRNAs derived from exogenously supplied double-stranded RNA [70], although this may be a consequence of enhanced biogenesis rather than, or in addition to, impaired turnover of the siRNAs [71,72]. Thomas *et al.* now found that loss of Eri1 impaired mouse natural killer cell development, maturation and function. It also increased the levels of many miRNAs by approximately twofold in these, and to a lower extent in T-cells [69]. It remains to be determined if Eri1 alters mature miRNA levels directly, by degradation, and whether it is miRNA overexpression or some other consequence of Eri1 deficiency that causes the observed immune cell phenotypes.

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