How Complementary Targets Expose the microRNA 3′ End for Tailing and Trimming during Target-Directed microRNA Degradation

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MicroRNAs (miRNAs) are crucial for posttranscriptional regulation of messenger RNAs. “Classical” miRNA targets predominantly interact with the miRNA seed sequence located near the miRNA 5′ end. Interestingly, certain transcripts that exhibit extensive complementarity to the miRNAs 3′ region, instead of being subjected to regulation, induce miRNA decay in a process termed target-directed miRNA degradation (TDMD). Here, we review recent advances in understanding the molecular mechanisms of TDMD. Specifically, we discuss how extensive miRNA complementarity to TDMD-inducing targets results in displacement of the miRNA 3′ end from its protective pocket in the Argonaute protein. unprotected miRNA 3′ ends are then available for enzymatic attack by still-identified cellular enzymes. Identification of these cellular enzymes and discovery of additional TDMD-inducing transcripts are subjects for future research.

MicroRNAs AND TARGET-DIRECTED microRNA DEGRADATION

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs) that are essential for posttranscriptional regulation of more than one-half of messenger RNAs (mRNAs) in human cells (Bartel 2018). Abrupt levels of cellular miRNAs are linked to disease. miRNAs associate with and act through Argonaute (Ago) proteins, which are the primary components of the RNA-induced silencing complex. In addition, Ago protects miRNAs from degradation (Winter and Diederichs 2011) by selective association with the 5′ and 3′ ends with binding pockets in the Ago MID and PAZ domains, respectively (Wang et al. 2008). Despite this protection, miRNA stability varies greatly (Duffy et al. 2015; Marzi et al. 2016), and miRNA levels are tightly regulated (e.g., during development or the cell cycle [Monticelli et al. 2005; Rissland et al. 2011]). But the mechanisms of miRNA regulation are not well-understood and only two miRNA decay pathways have been described: Tudor SN-mediated miRNA decay (TumiD) (Elbarbary et al. 2017) and target-directed miRNA degradation (TDMD) (Ameres et al. 2010; Baccarini et al. 2011; Libri et al. 2012; Marcinowski et al. 2012; Lee et al. 2013; Bitetti et al. 2018; Cazalla et al. 2018; Ghini et al. 2018; Kleaveland et al. 2018). TDMD is the subject of this review.

“Classical” miRNA targets usually interact with miRNA via base-pairing with the miRNA seed sequence (nucleotides [nt] 2–8 from the miRNA 5′ end); this interaction is sometimes accompanied by supplementary base-pairing to miRNA nt 13–16 (Fig. 1; Bartel 2018). Such interactions result in either miRNA degradation via deamination and decapping and/or translational repression. Interestingly, when a miRNA and its target are perfectly complementary, Ago2—one of four Ago proteins found in humans—is able to cleave the target (Liu et al. 2004), which is then quickly degraded by cellular exonucleases. However, when a target exhibits extensive base-pairing to the 3′ region of the miRNA combined with central mismatches that likely prevent Ago2-mediated cleavage, the miRNA, instead of the target, is subjected to degradation in a process known as TDMD (reviewed recently in de la Mata and Grosshans 2018; Fuchs Wightman et al. 2018).

Targets that are able to selectively induce miRNA decay, known as TDMD targets, represent various RNA classes, including ncRNAs—HSUR1 (small nuclear RNA from herpesvirus saimiri), UL144–145 (intergenic region from human cytomegalovirus), and Cyrano (cellular long ncRNA)—as well as miRNAs: m169 (from murine cytomegalovirus), NREP, and Serpin1 (Table 1; Libri et al. 2012; Marcinowski et al. 2012; Lee et al. 2013; Bitetti et al. 2018; Cazalla 2018; Ghini et al. 2018; Kleaveland et al. 2018). These RNAs vary not only in class and origin, but also seem to have flexible requirements for base-pairing with miRNAs to induce TDMD. In addition, HSUR1 mutagenesis further expanded the range of possible miRNA–TDMD target interactions (Table 2; Sheu-Gruttadauria and Pawlica et al. 2019).

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Thus, bioinformatic attempts to identify additional TDMD targets have proved challenging (Fuchs Wightman et al. 2018; Ghini et al. 2018), and only a handful of TDMD targets have been discovered. Moreover, the molecular mechanism of TDMD remained elusive for nearly 10 years. Our recent paper provides critical structural insights into how TDMD targets trap Ago2 in an extended conformation with the miRNA 3′ end displayed for cellular tailing (nontemplated nucleotide additions) and trimming while remaining bound to Ago proteins (Sheu-Gruttadauria and Pawlica et al. 2019).

**RECENT INSIGHTS INTO TDMD**

Prior to target association, Ago displays only the miRNA seed sequence for base-pairing. Upon binding to “classical” targets, Ago and the miRNA undergo a conformational shift to reveal the supplementary nucleotides in preordered A-form, which then can bind supplementary target sequences (Schirle et al. 2014; Sheu-Gruttadauria et al. 2019). These interactions do not destabilize the miRNA 3′ region, which remains sequestered within a narrow RNA binding cleft with the 3′ end securely bound in the Ago PAZ domain. Thus, when binding canonical miRNA target RNAs, Ago protects the miRNA from degradation. However, when a TDMD target binds, the central RNA binding cleft of Ago opens and the PAZ domain shifts away from the protein body. Importantly, the miRNA 3′ end is dislodged from its binding pocket within the PAZ domain (Fig. 2; Sheu-Gruttadauria and Pawlica et al. 2019).

Confirmation that the function of a TDMD target is to expose the miRNA 3′ end comes from the analysis of miRNAs associated with Ago1 and 2 proteins each containing in the PAZ domain a Phe → Ala substitution predicted to impair miRNA 3′ end binding. Even in the absence of a TDMD target, mutation of the Ago PAZ domain pocket causes the fraction of mature miRNAs bound to Ago to decrease, whereas the fraction of tailed and trimmed miRNA isoforms (isomiRs) increases (Sheu-Gruttadauria and Pawlica et al. 2019). In the presence of a TDMD target these isomiRs disappear, suggesting that the TDMD target further dislodges the miRNA 3′ end or that the Ago2–miRNA complex transitions into a conformation that enables recognition by TDMD-mediating enzymes. Indeed, several cellular enzymes implicated in TDMD can be modeled onto the Ago TDMD confor-

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**Table 1. Known miRNA–TDMD target interactions**

| miRNA   | Target NC RNA | Sequence 5′ miRNA | Sequence 3′ miRNA |
|---------|---------------|--------------------|-------------------|
| miR-27a | HSUR1 ncRNA, HSV | 5′ UUCACAGUGCUAACAGUCCG | 3′ UAGUGUCUAA–AUUCAAGGGUA |
|         | m169 3′ UTR, MCMV | 5′ UUCACAGUGCUAACAGUCCG | 3′ AAGUGUCGAUAA–UAAGGGCUG |
| miR-17  | UL144-145 ncRNA, HCMV | 5′ CAAGUGCUU—AC—AGUGCAAGUAG | 3′ AUUCACGAGAAAAAACUCAGGU |
| miR-20a | UL144-145 ncRNA, HCMV | 5′ CAAGUGCUU—A—AGUGCAAGUAG | 3′ AUUCACGAGAAAAAACUCAGGU |
| miR-29b | NREP 3′ UTR | 5′ UACCCACAUUUGAAUUCAGU | 3′ AUUCGUUAAAGU—AGUCAGCAGA |
| miR-7   | Cyano ncRNA | 5′ UGGAAAGAC—UA—GUAUAGUUGGUUGU | 3′ ACCUUCUGUAACCAUAAACACAA |
| miR-30b | Serpine1 3′ UTR | 5′ UGUAACAC—U—CCUCACUACACUCG | 3′ ACAUUCUGUGCAUGGUGGACAGC |
| miR-30c | Serpine1 3′ UTR | 5′ UGUAACAC—U—CCUCACUACACUCG | 3′ ACAUUCUGUGCAUGGUGGACAGC |

Adapted from Sheu-Gruttadauria and Pawlica et al. 2019, with permission from Elsevier.

Complementary regions are colored green, nucleotides in the central bulge are colored red, and noncanonical base pairs are colored blue.

(TDMD) Targeted directed microRNA degradation, (ncRNA) noncoding RNA, (HSV) herpesvirus saimiri, (UTR) untranslated region, (MCMV) murine cytomegalovirus, (HCMV) human cytomegalovirus, (NREP) neuronal regeneration-related protein.
tion poised to access the exposed miRNA 3′ end. These results, together with the evidence that tailing and trimming of miRNAs take place on Ago proteins (Marcinowski et al. 2012; de la Mata et al. 2015; Haas et al. 2016; Sheu-Gruttadauria and Pawlica et al. 2019), strongly suggest that the enzymes involved in TDMD either are already associated with Ago or are promptly recruited to Ago when the miRNA 3′ end becomes available.

OUTSTANDING QUESTIONS

An important question is which cellular enzymes are responsible for miRNA decay during TDMD. TDMD is associated with miRNA tailing and trimming (Ameres et al. 2010; Marcinowski et al. 2012; de la Mata et al. 2015; Ghini et al. 2018; Kleaveland et al. 2018; Sheu-Gruttadauria and Pawlica et al. 2019). Yet evidence for tailing as an essential step in miRNA decay is lacking. In fact, evidence that tailing is uncoupled from TDMD is accumulating. In mammalian cells there are 11 terminal nucleotidyl transferases (TENTs) with differing activities (mainly either adenylation or uridylation) (Warkocki et al. 2018). Many of these have been implicated in altering miRNA stability via tailing (Jones et al. 2009; Katoh et al. 2009; Boele et al. 2014; Gutiérrez-Vázquez et al. 2017). In addition, deep sequencing of miRNAs in the presence of TDMD targets revealed adenylation (Ghini et al. 2018; Kleaveland et al. 2018), uridylation (Baccarini et al. 2011; Haas et al. 2016; Sheu-Gruttadauria and Pawlica et al. 2019), and mixed A/U tails (Baccarini et al. 2011; de la Mata et al. 2015; Ghini et al. 2018; Sheu-Gruttadauria and Pawlica et al. 2019). However, TUT2 (also known as TENT2, GLD-2) knockout has no effect on Cyrano-mediated TDMD (Kleaveland et al. 2018), and TUT1 (also known as TENT1) knockout likewise does not alter m169-mediated TDMD (Haas et al. 2016). Similarly, single and simultaneous knockouts of two of the main suspects that could catalyze miR-27a U-tailing in the presence of HSUR1 (Sheu-Gruttadauria and Pawlica et al. 2019)—TUT4 (also known as TENT3A or ZCCHC11)

Table 2. Observed base-pairing interactions of miRNAs and their respective TDMD targets

| Seed interaction | Central mismatches | Pairing to miRNA 3′ region |
|------------------|--------------------|---------------------------|
| In miRNA         |                    |                           |
| Start            | Length             | ΔG                        |
| 1–2              | 6–10               | −9.6–21.9                  |
| 8–12             | 1–6                | 0–6.7                     |
| 10–15            | 6–14               | −11.2–20.9                 |
| In target        |                    |                           |
| Start            | Length             | ΔG                        |
| 6–10             | −9.6–21.9          |                           |
| 0–8              | 0–6.7              |                           |
| 6–14             | −11.2–20.9         |                           |

Calculations of the start, length, and hybridization energy (kcal/mol) of the two paired and one unpaired regions in miRNA—TDMD target interactions. Start denotes nucleotides from the miRNA 5′ end (Ameres et al. 2010; Baccarini et al. 2011; Libri et al. 2012; Marcinowski et al. 2012; Lee et al. 2013; Bitetti et al. 2018; Cazalla 2018; Ghini et al. 2018; Kleaveland et al. 2018; Sheu-Gruttadauria and Pawlica et al. 2019). (miRNA) microRNA, (TDMD) targeted-directed microRNA degradation.

Figure 2. Binding of a TDMD target exposes the miRNA 3′ end. Crystal structures of the Ago2–miRNA complex bound to a “classical” miRNA target with seed + supplementary pairing (left, PDB 6N4O) or a TDMD-inducing target RNA (right, PDB 6MDZ). Insets show close-up views of the miRNA 3′ end bound to the PAZ domain in the seed + supplementary structure or exposed to enzymatic attack in the TDMD conformation. (Below) Observed miRNA–target base pairs are shown schematically.
Figure 3. TUT4 and TUT7 are not required for HSUR1-mediated TDMD. (Left) Western blot showing the levels of TUT4, TUT7, and GAPDH in single clones of BJAB cells generated after CRISPR-Cas9-mediated knockout of TUT4, TUT7, or both. (Right) Northern blot showing the impact of single or simultaneous knockouts of TUT4 and TUT7 on HSUR1’s ability to induce TDMD of miR-27. (KO) Knockout, (*) nonspecific band.

and TUT7 (also known as TENT3B or ZCCHC6) do not appear to reverse HSUR1-mediated TDMD (Fig. 3). It therefore seems likely that the exposed miRNA 3’ end can be subjected either to tailing or to trimming, but tailing is not essential for ensuing decay. In addition, the trimming 3’-to-5’ exonucleases responsible for miRNA degradation remain to be identified. Those that have been examined and do not appear to function in TDMD include PARN (Kleaveland et al. 2018) and Dis3L2 (Haas et al. 2016; Kleaveland et al. 2018). Our HSUR1 and Ago mutagenesis studies suggest that TDMD enzymes do not require a lengthy tail of adenylates/uridylates as is needed for Dis3L2 (Faehnle et al. 2014) and PARN (Astrom et al. 1992; Wu et al. 2005), but do require association with Ago protein (Sheu-Gruttadauria and Pawlica et al. 2019). It is also possible that more than one exonuclease acts on an exposed miRNA 3’ end, just as multiple tailing activities clearly participate in both general miRNA decay and TDMD.

An interesting observation from our HSUR1 mutagenesis analyses underscores the role of the target complementarity in the generation of isomiRs that cannot result from earlier miRNA processing steps. Specifically, the miRNA-target architecture on Ago appears to modulate the activity of enzymes that are recruited to the miRNA 3’ end. These results complement those from a study of TUT7, whose activity can change depending on the pairing status of its substrate (Kim et al. 2015). miR-27 isomiR generation in the presence of HSUR1 mutants seems to be enhanced by base-pairing between the target and the extreme 3’ nt of the miRNA, as well as by small and symmetrical central mismatches (Sheu-Gruttadauria and Pawlica et al. 2019). Also, interestingly, certain elongated miR-27 isomiRs may acquire altered ability to repress target miRNAs depending on the mode of miRNA–target interaction (miRNA-like vs. siRNA-like). Moreover, it is becoming clear that miRNA tailing can alter the specificity of miRNA repression, as in the case of miR-27 uridylation, which can compensate for imperfect seed base-pairing and repress noncanonical targets (Yang et al. 2019).

Another outstanding question is what defines a TDMD target. We have learned that base-pairing to the miRNA seed sequence and the miRNA 3’ region, as well as the central mismatches, are necessary, but not sufficient for miRNA decay (Lee et al. 2013; P Pawlica, unpubl. results). It is critical that a miRNA binding site be exposed in an unstructured region of the TDMD target RNA (Pawlica et al. 2016). However, the great majority of transcripts fulfilling these criteria does not induce TDMD. It is possible that additional protein-binding motifs are located near miRNA binding sites (Lee et al. 2013; Kleaveland et al. 2018), whose identification would greatly enhance the pace of discovery of novel TDMD targets. Finally, it remains unclear why TDMD targets appear to have different cell type–specific potency (de la Mata et al. 2015; Kleaveland et al. 2018; P Pawlicka, unpubl. results).

In summary, our study brings us closer to understanding the molecular mechanism of TDMD, but many challenging questions still remain.

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