Published in final edited form as:

Methods. 2019 January 01; 152: 18–22. doi:10.1016/j.ymeth.2018.05.010.

Evaluating the susceptibility of AGO2-loaded microRNAs to degradation by nucleases *in vitro*

Reyad Elbarbary^{1,2} and Lynne E. Maquat^{3,4}

¹Department of Orthopaedics and Rehabilitation, Penn State University, College of Medicine, Hershey, PA 17033-0850, USA

²Department of Biochemistry and Molecular Biology, Penn State University, College of Medicine, Hershey, PA 17033-0850, USA

³Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, USA

⁴Center for RNA Biology, University of Rochester, Rochester, New York 14642, USA

Abstract

MicroRNAs (miRNAs) comprise a class of small non-coding RNAs that regulate the stability and/or translatability of most protein-coding transcripts. Steady-state levels of mature miRNAs can be controlled through mechanisms that influence their biogenesis and/or decay rates. Pathways that mediate mature miRNA decay are less well understood than those that mediate miRNA biogenesis. We recently described Tudor-staphylococcal/micrococcal-like nuclease (TSN)-mediated miRNA decay (TumiD) as a cellular pathway that promotes the sequence-specific endonucleolytic decay of miRNAs that harbor a CA and/or UA dinucleotide. Here, we describe an *in vitro* assay for evaluating the susceptibility of AGO2-loaded miRNAs to degradation by different classes of nucleases. This *in vitro* approach can be used to complement *in vivo* studies that aim to identify novel miRNA decay factors.

Keywords

miRNA decay; Tudor-SN; nuclease; *in vitro* assay; immunoprecipitation; AGO2

1. Introduction

miRNAs are short ~22-nucleotide RNAs that regulate many cellular processes [1]. They guide the AGO proteins within the RNA-induced silencing complex (RISC) to degrade and/or repress the translation of their target mRNAs [1, 2]. miRNA biogenesis is a multi-step process that starts with the transcription of each miRNA embedded within a primary miRNA

Correspondence should be addressed to relbarbary@pennstatehealth.psu.edu and lynne_maquat@urmc.rochester.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(pri- miRNA). Each pri-miRNA undergoes Drosha-mediated processing in the nucleus to form a pre- miRNA [3], which is subsequently processed in the cytoplasm by Dicer to form a miRNA : miRNA* duplex. A single strand of the miRNA : miRNA* duplex is then loaded into RISC [3].

As important modulators of gene expression, it is not surprising that the cellular levels of miRNAs are tightly regulated [3, 4]. Regulation can take place at any one of several steps: miRNA gene transcription, pri-miRNA processing, pre-miRNA processing, miRNA loading onto RISC, and mature miRNA turnover [3, 5]. Several regulatory mechanisms have been reported to alter the efficiency of miRNA transcription and processing [3]. In contrast, the regulation of mature miRNA turnover is less well understood. Some exonucleases that degrade mature miRNAs in *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Caenorhabditis elegans*, and human cells have been characterized [5, 6]; however, their specificities and mechanisms of action remain unresolved. It is also known that highly complementary target RNA can trigger miRNA degradation by a mechanism that involves the addition of nontemplated nucleotides to miRNA 3'-ends followed by exonucleolytic degradation [3, 5, 7]. Recently, we identified the evolutionarily conserved endonuclease Staphylococcal nuclease and tudor domain-containing 1 (Tudor-SN, abbreviated TSN) as a mediator of miRNA decay [8, 9]. TSN degrades miRNAs that contain one or more CA or UA dinucleotides, preferring those residing internally to the 5'-most and 3'-most five nucleotides [8]. Our studies thus implicate endonucleases as potential mediators of miRNA decay.

Further studies are required to understand the mechanisms whereby different classes of nucleases gain access to mature miRNAs within RISC. Here we describe an *in vitro* assay for evaluating the susceptibility of RISC-loaded miRNAs to degradation by different classes of nucleases. The prototype exemplified here is the degradation of human miR-31 by human TSN; bacterial RNase I, which is an endonuclease that cleaves between any two nucleotides; or bacterial Exonuclease T (Exo T), which is a 3' -to-5' exonuclease.

2. Methodology overview

The purpose of the protocol described here is to investigate the susceptibility of AGO2-loaded miRNAs to endonuclease- and exonuclease-mediated degradation *in vitro*. A brief overview of the protocol is provided (Figure 1), which can be described as follows:

- 1) Lysates of HEK293T cells that stably express 3xFLAG-AGO2 are prepared. Cells are lysed using detergent-free buffers to be compatible with subsequent steps.
- 2) 5' -[³²P]-labelled miRNA (³²P-miRNA), e.g., ³²P-miR-31, is loaded onto AGO2 by incubating lysates of 3xFLAG-AGO2-expressing cells with *in vitro*-generated ³²P-miR-31 : miR-31 * duplexes.
- 3) 3xFLAG-AGO2 is immunoprecipitated using anti-FLAG antibody to purify ³²P-miR-31—3xFLAG-AGO2 complexes.
- 4) The resulting immunoprecipitations (IPs) are incubated with nucleases of interest, e.g., HIS- tagged human TSN (HIS-TSN), bacterial RNase I, or bacterial Exo T. As

negative controls, IPscan be incubated with buffer alone or with HIS-TSN(SN4), which is a catalytically inactive deletion variant of HIS-TSN.

5) RNA from all IPs is purified, and the level of ^{32}P -miR-31 is analyzed.

The susceptibility of ^{32}P -miR-31 to degradation by each nuclease is assessed by comparing its level in the presence of each nuclease to its level in the negative controls.

In the following sections, the appropriate conditions and required materials for each step are detailed. Reagent volumes can be scaled up according to the number of IPs required. Importantly, the methods described here are general and can be extended to study other miRNAs and/or nucleases.

3. Materials and Methods

3.1. Preparing total-cell lysates

3.1.1. Establishing stably transfected human-cell lines—HEK293T-cell transfectants stably expressing 3xFLAG-AGO2 or, as a negative control, 3xFLAG are generated using the retroviral expression vector pLHCX-3xFLAG-AGO2 or pLHCX-3xFLAG, respectively (see 3.1.3; Tip 1). The protocol for establishing these cell lines was previously described [8, 10] and will not be detailed here.

3.1.2. Cell lysis

1. HEK293T cells described in 3.1.1 are propagated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) that is supplemented with 10% fetal bovine serum (FBS) (Seradigm) and 200 $\mu\text{g}/\text{ml}$ of Hygromycin B (Roche), which is the antibiotic used to select and maintain the stable transfectants.
2. Cells are harvested while in log-growth phase using Corning Cell Lifters (Sigma-Aldrich), and 40×10^6 cells are pooled for each IP experiment (see 3.1.3; Tip 2).
3. Harvested cells are washed 3 times with ice-cold phosphate-buffered saline (PBS), pelleting at 1000 rpm for 5 min after each wash. All centrifugation steps described in this protocol are performed at 4°C .
4. Cell pellets are re-suspended in 1 ml of ice-cold Lysis Buffer (Appendix A.2.1) and placed on ice for 10 min.
5. Cell suspensions are transferred to an ice-cold Dounce homogenizer and lysed using ten strokes of the pestle, avoiding the generation of bubbles.
6. Lysates are centrifuged at 15,000 rpm for 20 min, and the resulting supernatant is transferred to a 1.5-ml microcentrifuge tube.
7. Protein concentration in each sample is quantitated using the Bradford protein assay (Bio- Rad Protein Assay Kit) and a spectrophotometer, and a lysate volume that contains 1 mg of cellular protein is used in the subsequent steps. Notably, the conditions described here generated lysates with protein concentrations of 2 mg/ml, and thus 500 μl were used in the following steps. The remaining 500 μl

were flash-frozen in liquid nitrogen and stored at -80°C for later use (see 3.1.3; Tip 2).

3.1.3. Tips and troubleshooting

1. A high concentration of 3xFLAG-AGO2 is required to obtain reasonable efficiencies of ^{32}P -miR-31 loading. Accordingly, it is advisable to establish a cell line that stably expresses a high level of 3xFLAG-AGO2 to be used in these *in vitro* experiments (see Figure 2A to compare the expression level of 3xFLAG-AGO2 to that of endogenous AGO2 in the stable cell line used in the experiments described here).
2. The number of cells and the amount of total-cell protein required for one IP experiment depends on the particular cell line and the level of 3xFLAG-AGO2 expression. Accordingly, they should be carefully optimized.

3.2. Loading ^{32}P -miR-31 onto 3xFLAG-AGO2, i.e., assembly of ^{32}P -miR-31—3xFLAG-AGO2 complexes

3.2.1. Preparing ^{32}P -miR-31: miR-31* duplexes

1. To prepare ^{32}P -miR-31, synthetic single-stranded miR-31 (5'-aggcaagaugcuggcagcu-3') (Integrated DNA Technology) is 5'- ^{32}P -labelled using γ -[^{32}P]ATP (PerkinElmer) and T4 polynucleotide kinase (New England Biolabs) following the manufacturer's protocol. The reaction is then stopped by incubating with 1 μl of proteinase K (Thermo Fisher Scientific) for 5 min at 37°C , and ^{32}P -miR-31 is purified using the miRNeasy Kit (Qiagen) following the manufacturer's protocol. Radioactivity is measured using a scintillation counter. A volume of ^{32}P -miR-31 solution that contains 100,000 counts per minute (cpm) is used in the subsequent steps.
2. To prepare 5'-phosphorylated miR-31* (P-miR-31*), synthetic single-stranded miR-31* (5'-ugcuaugccaacauauugccau-3') (Integrated DNA Technology) is 5'-phosphorylated and purified using the same procedures described in the previous step, except nonradioactive ATP is used in place of γ -[^{32}P]ATP. The concentration of P-miR-31* is measured using a NanoDrop Spectrophotometer.
3. To generate ^{32}P -miR-31 : miR* duplexes, ^{32}P -miR-31 and P-miR-31* are mixed in a 1:3 molar ratio (see 3.2.2.; Tip 1), 10 μl of 2X Annealing Buffer (Appendix A.2.2) is added, and samples are brought to 20 μl with RNase-free H_2O . For duplex annealing, samples are incubated for 5 min at 95°C using a heat block and then gradually cooled to room temperature (see 3.2.2; Tip 2).

3.2.2. Tips and troubleshooting

1. ^{32}P -miR-31: miR-31* duplex formation is crucial for the successful loading of ^{32}P -miR-31 onto 3xFLAG-AGO2 complexes. Thus, the relative amounts at which ^{32}P -miR-31 and P-miR-31* are mixed should be carefully optimized. We utilized a 1:3 molar ratio based on the results of our optimization experiments (Figure 2B).

2. We phosphorylated the 5'-ends of both miRNA strands before annealing, i.e., utilized ^{32}P -miR-31 and P-miR-31*, to mimic endogenous miR : miR* duplexes.

3.2.3. Loading ^{32}P -miR-31 onto 3xFLAG-AGO2—To assemble ^{32}P -miR-31—3xFLAG-AGO2 complexes, 500 μl of each cell lysate from 3.1.2 are mixed with the 20 μl of ^{32}P -miR-31 : miR-31* duplexes from 3.2.1 and 300 μl of 3.3X Loading

Buffer (Appendix A.2.3). Reactions are then brought to 1 ml with RNase-free H_2O , mixed well, and incubated for 1 hr at 37°C .

3.3. Immunoprecipitating 3xFLAG-AGO2, i.e., purifying ^{32}P -miR-31—3xFLAG-AGO2 complexes

1. Reactions from 3.2.3 are brought to a final concentration of 150 mM NaCl using 5 M NaCl.
2. For each IP, 50 μl of ANTI-FLAG[®] M2 Affinity Gel (Millipore Sigma) are pre-washed in 1 ml of Lysis Buffer (Appendix A.2.1) and mixed end-over-end with reactions prepared in the previous step for 2 hr at 4°C using a tube rotator.
3. The ANTI-FLAG[®] M2 Affinity Gel is collected by centrifugation at 3000 rpm for 1 min and washed 5 times with ice-cold NET-2 Buffer (Appendix A.2.4). For each washing step, 1 ml of the Buffer is added, the tube is shaken by hand until the ANTI-FLAG[®] M2 Affinity Gel is completely re-suspended, the gel is subsequently pelleted by centrifugation at 3000 rpm for 1 min, and the supernatant is discarded.
4. The ANTI-FLAG[®] M2 Affinity Gel is washed 3 times with ice-cold Nuclease Buffer (Appendix A.2.5; see 3.3.1; Tip 1) following the same series of steps described immediately above. Supernatant removed from the third wash is checked for radioactivity using a scintillation counter. If radioactivity is still detected, washing is continued until the supernatant becomes free of radioactivity (see 3.1.1; Tip 2).

3.3.1. Tips and Troubleshooting

1. NET-2 Buffer should be replaced by Nuclease Buffer to prepare the IPs for incubations with nucleases, including removing NP-40, which is a component of NET-2 Buffer that might inhibit the nuclease activity. Thus, at least 3 washing steps with the Nuclease Buffer are required.
2. The centrifugation step at the end of each wash serves to pellet the ANTI-FLAG[®] M2 Affinity Gel, which contains the bound ^{32}P -miR-31—3xFLAG-AGO2 complexes, while unloaded ^{32}P -miR-31 : miR-31* duplexes remain in the supernatant. Accordingly, washing should be performed until a radioactivity-free supernatant is obtained.

3.4. Incubating purified ³²P-miR-31—3xFLAG-AGO2 complexes with nucleases

1. In a PCR tube, the ANTI-FLAG[®] M2 Affinity Gel from 3.3 is mixed with either 5 pmol of recombinant HIS-TSN, 5 U of RNase I (Promega), or 10 U of Exo T (New England Biolabs) (See 3.4.1; Tips 1 and 2). As negative controls, the ANTI-FLAG[®] M2 Affinity Gel is mixed with either Nuclease Buffer that does not contain a nuclease or 5 pmol of recombinant HIS-TSN(SN4), which lacks the SN4 domain and therefore is catalytically inactive. The protocol for producing and purifying HIS-TSN and HIS-TSN(SN4) in *E. coli* has been described previously [8, 11, 12] and will not be detailed here.
2. Nuclease Buffer (Appendix A.2.5) is added to each tube to bring the final volume to 50 µl (see 3.4.1; Tip 3).
3. Tubes are rotated end-over-end for 2 hr at 37°C for all nucleases except for Exo T, which is incubated at 25°C.

3.4.1. Tips and Troubleshooting

1. Since IP efficiency might vary among experiments, it is essential to pool all IPs that contain the same ³²P-miRNA—3xFLAG-AGO2 complexes and aliquot them into equal portions for incubations with different nucleases.
2. It is advisable to use Coomassie blue staining to ensure that comparable amounts of the nucleases are analyzed.
3. We used Nuclease Buffer for incubations with all three nucleases (HIS-TSN, RNase I and Exo T) based on our experiments showing that this Buffer permits the complete degradation of single-stranded, AGO2-free ³²P-miR-31 (prepared as described in 3.2.1, step 1) (Figure 2C) [8] For experiments testing other nucleases, it may be necessary to use a different buffer for optimal nuclease activity.

3.5. Purifying RNA from 3xFLAG-AGO2 IPs

1. One µl of Proteinase K (Thermo Fisher Scientific) is added, and the tube is rotated end-over-end for 5 min at 37°C.
2. RNA is extracted using miRNeasy Kit (Qiagen) and subsequently eluted in 14 µl of RNase- free water.
3. An equal volume (14 µl) of Gel Loading Buffer II (Thermo Fisher Scientific) is added, samples are boiled for 5 min, and subsequently quenched on ice.
4. An aliquot (10 µl) of each sample is electrophoresed in 6M urea-15% polyacrylamide for 40 min at 280 V.
5. Radioactivity in each lane of the gel is visualized using an Amersham Typhoon RGB Biomolecular Imager (GE Biosciences).

4. Results and Discussion

We found AGO2-loaded ^{32}P -miR-31 to be resistant to 3'-to-5' exonucleolytic degradation by Exo T, susceptible to endonucleolytic decay by HIS-TSN or RNase I and, as expected, resistant to decay by the catalytically inactive HIS-TSN(SN4) (Figure 2D) [8]. These results underscore endonucleases as potential mediators of miRNA decay.

The X-ray crystal structure of a human AGO2-miRNA complex [13, 14] revealed higher B-factor values (i.e. more atomic vibrational motion) for the middle part of the miRNA compared to its 3' end. This indicates that AGO2 binds the middle part less tightly than it binds the miRNA 3' end, which is tucked into the PAZ domain of AGO2. These structural features of AGO2-miRNA complexes might explain our finding that AGO2-bound miRNAs are resistant to 3' -to-5' exonucleases while susceptible to the degradative activity of endonucleases.

5. Conclusion

The protocol we describe here provides researchers with a simple *in vitro* approach that uses purified AGO2-miRNA complexes to investigate the ability of AGO2 to protect mature miRNAs from degradation by different types of nucleases. Results obtained from this *in vitro* approach can complement *in vivo* studies that aim to decipher the nuclease susceptibility of RISC-bound miRNAs.

Acknowledgments

Work in the Maquat lab on miRNA decay is funded by NIH R37 GM074593 to L.E.M. Purchase of the Typhoon Phosphorimager FLA 9500 was supported by NIH S10 OD021489-01A1.

Appendix A.

A.1. Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

A.2. Buffers and Formulations

A.2.1. Lysis Buffer

30 mM HEPES-KOH, pH 7.4

100 mM potassium acetate

2 mM magnesium acetate

5 mM DTT

cOmplete Mini, EDTA-free Protease Inhibitor Cocktail Tablets (Roche/Millipore Sigma; Catalog #11836170001); added according to manufacturer's instructions.

A.2.2. 2X Annealing Buffer

20 mM Tris, pH 7.5

100mM NaCl

2 mM EDTA

A.2.3. 3.3X Loading Buffer

100 mM creatine phosphate (Sigma Catalog #27920G)

300 µg/ml creatine kinase (prepared in 1X PBS) (Roche Catalog #10127566001)

4 mM ATP (Roche Catalog #11140965001)

1 mM GTP (Sigma, Catalog #45-G3776–25UMO)

20 mM DTT

1.2 U/µl RNaseOUT Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific; Catalog #10777019)

Loading Buffer should be prepared just prior to use.

A.2.4. NET-2 Buffer

50 mM Tris-HCl, pH 7.4

150 mM NaCl

0.1% Nonidet P-40 (NP-40)

cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche/Millipore Sigma; Catalog #11836170001); added according to manufacturer's instructions.

A.2.5. Nuclease Buffer

50 M HEPES-KOH, pH 7.5

5 mM CaCl₂

100 mM NaCl

1 mM ATP

A.3. Kits, reagents, and equipment

A.3.1. Preparing total-cell lysates

A.3.1.1. Kits and reagents

1. Bio-Rad Protein Assay Kit (Bio-Rad; Catalog #5000001)

A.3.1.2. Equipment

1. Refrigerated microcentrifuge (Biofuge Primo R 75005448, Sorvall)
2. 2 ml Dounce homogenizer (Kontes Dounce Tissue Grinder, Kimble)
3. Colorimetric spectrophotometer (Spectramax M4, Molecular Devices)

A.3.2. Loading ^{32}P -miR-31 onto 3xFLAG-AGO2, i.e., assembly of ^{32}P -miR-31—3xFLAG-AGO2 complexes

A.3.2.1. Kits and reagents

1. ATP, [γ - ^{32}P], 6000 Ci/mmol, 10mCi/ml (PerkinElmer; Product #BLU502Z/NEG502Z)
2. T4 polynucleotide kinase (New England Biolabs; Catalog #M0201S)
3. Proteinase K (Thermo Fisher Scientific, Catalog #MC5005)
4. miRNeasy Mini Kit (Qiagen; Catalog #217004)

A.3.2.2. Equipment

1. Scintillation counter (LS 6000SC, Beckman)
2. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific)
3. Heating block (Heatblock 13259–005, VWR)
4. Amersham Typhoon RGB Biomolecular Imager (GE Biosciences) (used to visualize the gel shown in Figure 2B)

A.3.3. Immunoprecipitating 3xFLAG-AGO2, i.e., purifying ^{32}P -miR-31—3xFLAG-AGO2 complexes

A.3.3.1. Kits and reagents

1. ANTI-FLAG[®] M2 Affinity Gel (Millipore Sigma; Product #A2220)

A.3.3.2. Equipment

1. End-over-end Tube Rotator (Labquake, Barnstead Thermolyne)

A.3.4. Incubating purified ^{32}P -miR-31—3xFLAG-AGO2 complexes with nucleases

A.3.4.1. Kits and reagents

1. RNase One[™] Ribonuclease (Promega; Catalog #M4261)
2. Exonuclease T (New England Biolabs; Catalog #M0265S)

3.5. Purifying RNA from 3xFLAG-AGO2 IPs

3.5.1. Kits and reagents

1. Gel Loading Buffer II (Thermo Fisher Scientific; Catalog #AM8547)

Reference

- [1]. Gurtan AM, Sharp PA, The role of miRNAs in regulating gene expression networks, J Mol Biol 425(19) (2013) 3582–600. [PubMed: 23500488]
- [2]. Iwakawa HO, Tomari Y, The Functions of MicroRNAs: mRNA Decay and Translational Repression, Trends Cell Biol 25(11) (2015) 651–65. [PubMed: 26437588]
- [3]. Rodriguez EK, Boulton C, Weaver MJ, Herder LM, Morgan JH, Chacko AT, Appleton PT, Zurakowski D, Vrahas MS, Predictive factors of distal femoral fracture nonunion after lateral

- locked plating: a retrospective multicenter case-control study of 283 fractures, *Injury* 45(3) (2014) 554–9. [PubMed: 24275357]
- [4]. Di Leva G, Garofalo M, Croce CM, MicroRNAs in cancer, *Annu Rev Pathol* 9 (2014) 287–314. [PubMed: 24079833]
- [5]. Ruegger S, Grosshans H, MicroRNA turnover: when, how, and why, *Trends Biochem Sci* 37(10) (2012) 436–46. [PubMed: 22921610]
- [6]. Kai ZS, Pasquinelli AE, MicroRNA assassins: factors that regulate the disappearance of miRNAs, *Nat Struct Mol Biol* 17(1) (2010) 5–10. [PubMed: 20051982]
- [7]. Ameres SL, Zamore PD, Diversifying microRNA sequence and function, *Nat Rev Mol Cell Biol* 14(8) (2013) 475–88. [PubMed: 23800994]
- [8]. Elbarbary RA, Miyoshi K, Myers JR, Du P, Ashton JM, Tian B, Maquat LE, Tudor-SN- mediated endonucleolytic decay of human cell microRNAs promotes G1/S phase transition, *Science* 356(6340) (2017) 859–862. [PubMed: 28546213]
- [9]. Elbarbary RA, Miyoshi K, Hedaya O, Myers JR, Maquat LE, UPF1 helicase promotes TSN-mediated miRNA decay, *Genes Dev* 31(14) (2017) 1483–1493. [PubMed: 28827400]
- [10]. Popp MW, Maquat LE, Attenuation of nonsense-mediated mRNA decay facilitates the response to chemotherapeutics, *Nat Commun* 6 (2015) 6632. [PubMed: 25808464]
- [11]. Gleghorn ML, Gong C, Kielkopf CL, Maquat LE, Stauf1 dimerizes through a conserved motif and a degenerate dsRNA-binding domain to promote mRNA decay, *Nat Struct Mol Biol* 20(4) (2013) 515–24. [PubMed: 23524536]
- [12]. Park E, Gleghorn ML, Maquat LE, Stauf2 functions in Stauf1-mediated mRNA decay by binding to itself and its paralog and promoting UPF1 helicase but not ATPase activity, *Proc Natl Acad Sci U S A* 110(2) (2013) 405–12. [PubMed: 23263869]
- [13]. Elkayam E, Kuhn CD, Tocilj A, Haase AD, Greene EM, Hannon GJ, Joshua-Tor L, The structure of human argonaute-2 in complex with miR-20a, *Cell* 150(1) (2012) 100–10. [PubMed: 22682761]
- [14]. Schirle NT, Sheu-Gruttadauria J, MacRae IJ, Structural basis for microRNA targeting, *Science* 346(6209) (2014) 608–13. [PubMed: 25359968]

- Established assays for AGO2-loaded miRNA sensitivity to nucleolytic decay.
- AGO2-loaded miRNAs are resistant to 3'-to-5' degradation by Exo T.
- AGO2-loaded miRNAs are degraded by the RNase I endonuclease.
- AGO2-loaded miRNAs with CA and/or UA are degraded by the TSN endonuclease.
- Purified AGO2-miRNA complexes are a simple tool for *in vitro* miRNA decay studies.

Figure 1. Protocol for analyzing the susceptibility of AGO2-loaded ^{32}P -miR-31 to cleavage by various nucleases *in vitro*

Lysates of HEK293T cells stably expressing 3xFLAG-AGO2 are prepared (Step 1), incubated with ^{32}P -miR-31 : miR-31* duplexes (Step 2), and 3xFLAG-AGO2 is immunoprecipitated using anti-FLAG to purify ^{32}P -miR-31—3xFLAG-AGO2 complexes (Step 3). The resulting 3xFLAG-AGO2 immunoprecipitate is incubated with HIS-tagged human TSN (HIS-TSN) produced in and purified from *E.coli*, or commercially available RNase I or Exo T (Step 4). As negative controls, the 3xFLAG-AGO2 immunoprecipitate is incubated without added protein (–) or with HIS-TSN(Δ SN4), which is catalytically inactive (Step 4). RNA is subsequently purified, and the level of ^{32}P -miR-31 is quantitated (Step 5).

Figure 2. AGO2-loaded miRNAs are susceptible to endonucleolytic degradation by HIS-TSN or RNase I, and resistant to 3'-to-5' exonucleolytic degradation by Exo-T.

(A) Western blot of total-cell lysates demonstrating the expression level of 3xFLAG-AGO2 relative to endogenous AGO2 in HEK293T cells that stably express 3xFLAG-AGO2.

(B) Optimizing the relative amounts of ^{32}P -miR-31 and P-miR-31* for ^{32}P -miR-31 : miR-31* duplex formation. ^{32}P -miR-31 and P-miR-31* were mixed at the specified molar ratios, and annealing was performed as described in 3.2.1, Step 3. Samples were electrophoresed in a native 15% polyacrylamide gel at 280 V and 4°C, and radioactivity in the gel was visualized using an Amersham Typhoon RGB Biomolecular Imager (GE Biosciences). Results reveal that increasing the level of P-miR-31* relative to ^{32}P -miR-31 increases ^{32}P -miR-31 : miR-31* duplex formation until all ^{32}P -miR-31 is duplexed at a molar ratio of 1:3.

(C) Phosphorimages of single-stranded, protein-free ^{32}P -miR-31 after incubation with the specified nucleases in Nuclease Buffer. Comparing the level of ^{32}P -miR-31 in each sample with that in the control, which is not incubated with any nucleases (-), reveals comparably efficient nuclease activity of HIS-TSN, RNase I, and Exo T as evidenced by almost

complete degradation of ^{32}P -miR-31. As expected, HIS-TSN(SN4), the catalytically inactive deletion variant of HIS-TSN, did not show any degradative activity.

(D) As in C, except that ^{32}P -miR-31 was purified from 3xFLAG-AGO2 IPs after incubation with the specified nuclease. Results reveal that 3xFLAG-AGO2-loaded ^{32}P -miR-31 is protected from degradation by the 3'-to-5' exonuclease Exo T, but is susceptible to degradation by the endonucleases HIS-TSN and RNase I. As expected, catalytically inactive HIS-TSN(SN4) does not manifest degradative activity. Notably, the absence of ^{32}P -miR-31 in 3xFLAG IPs is consistent with the requirement of 3xFLAG-AGO2 for the formation of RISCs that contain ^{32}P -miR-31.