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Research paper

# Detection of the argonaute protein Ago2 and microRNAs in the RNA induced silencing complex (RISC) using a monoclonal antibody

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## Abstract

MicroRNAs (miRNAs) are short RNA molecules responsible for post-transcriptional gene silencing by the degradation or translational inhibition of their target messenger RNAs (mRNAs). This process of gene silencing, known as RNA interference (RNAi), is mediated by highly conserved Argonaute (Ago) proteins which are the key components of the RNA induced silencing complex (RISC). In humans, Ago2 is responsible for the endonuclease cleavage of targeted mRNA and it interacts with the mRNA-binding protein GW182, which is a marker for cytoplasmic foci referred to as GW bodies (GWBs). We demonstrated that the anti-Ago2 monoclonal antibody 4F9 recognized GWBs in a cell cycle dependent manner and was capable of capturing miRNAs associated with Ago2. Since Ago2 protein is the effector protein of RNAi, anti-Ago2 monoclonal antibody may be useful in capturing functional miRNAs.

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**Keywords:** Argonaut protein; GW182; GW bodies; Monoclonal antibody; MicroRNA; P body; RNA interference

## 1. Introduction

Post-transcriptional gene silencing or RNA interference (RNAi) is a crucial regulatory pathway of eukaryotic gene expression that occurs in a wide variety of organisms (Meister and Tuschl, 2004). One key player in this pathway is the microRNAs (miRNAs), which are 21 to 24 nucleotide long RNA molecules that bind to partially complementary sequences within the 3'-untranslated region of target mRNAs leading to translation suppression and/or degradation. miRNAs are encoded in the genome and are transcribed into primary miRNA (pri-miRNA) molecules. These pri-miRNAs are

*Abbreviations:* Ago, argonaute; ELISA, enzyme-linked immunosorbent assay; GWBs, GW bodies; IP, immunoprecipitation; IIF, indirect immunofluorescence; IMS, immune mouse sera; kDa, kilodalton; mRNA, messenger RNA; miRNA, microRNA; miR-16, microRNA-16; miR-24, microRNA-24; NMS, normal mouse sera; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase-polymerase chain reaction; RISC, RNA induced silencing complex; RNAi, RNA interference.

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processed in the nucleus by the RNase III enzyme Droscha and its partner protein DGCR8 (Gregory et al., 2004). The resulting miRNA precursors (pre-miRNAs) are then transported to the cytoplasm by Exportin-5 (Lund et al., 2004). Finally, pre-miRNAs are further processed by the cytoplasmic RNase III enzyme Dicer, and the resulting mature miRNAs enter the RISC. The key components of the RISC complex are Argonaute (Ago) proteins with molecular masses of approximately 100 kDa and containing PAZ and PIWI domains (Carmell et al., 2002; Sasaki et al., 2003). Four of these, Ago1 to Ago4, have been demonstrated to associate with miRNAs in humans (Liu et al., 2005b). However, only Ago2 has been demonstrated to possess the activity of miRNA-guided mRNA cleavage or translational inhibition (Meister et al., 2004). Thus, Ago are the key proteins that mediate miRNA function and the significance for “free” or non-Ago bound miRNA subsets is unclear. Furthermore, Ago2 has been shown to associate with GW182, the 182 kDa mRNA-binding protein (Eystathioy et al., 2002) and a critical component of GW bodies (GWBs) (Jakymiw et al., 2005). GWBs are proposed to be cytoplasmic sites for mRNA storage and degradation (Eystathioy et al., 2002), and possible sites for RNAi in mammalian cells (Jakymiw et al., 2005). GW182 is also reported to be required for efficient miRNA mediated repression of mRNA (Liu et al., 2005a). Our recent report showed that the formation of GWBs is a consequence of miRNA genesis based on the observation that the inhibition of miRNA maturation led to disassembly of GWBs (Pauley et al., 2006). In this study, we describe a novel Ago2 monoclonal antibody 4F9 capable of capturing Ago-associated miRNAs in mammalian cells. This monoclonal antibody may be useful in characterizing functional subsets of miRNA in different systems.

## 2. Materials and methods

### 2.1. Purified recombinant Ago2 protein

A human Ago2 cDNA encoding the full length of the Ago2 protein was subcloned into pDEST<sup>TM</sup>17 (Invitrogen<sup>TM</sup>, Carlsbad, CA, USA) expression vector. The recombinant protein was produced in *Escherichia coli* BL21 (DE3) and purified using Ni<sup>2+</sup> affinity chromatography as per the manufacturer’s instructions (Qiagen, Valencia, CA, USA). The soluble recombinant protein was subsequently used in the immunization protocol and enzyme-linked immunosorbent assay (ELISA) described below.

### 2.2. Production of mouse monoclonal antibody to Ago2

We have recently reported that anti-Ago2 autoantibodies are detected in a significant number of patients with autoantibodies to the Su antigen (Jakymiw et al., 2006). Anti-Su antibodies have also been described in mice treated with pristane (2,6,10,14-tetramethylpentadecane, or TMPD). One of the major antigenic targets of anti-Su is Ago2 but there are other unidentified Su antigens including the 200 kDa protein (Jakymiw et al., 2006). To generate anti-Ago2 antibodies, BALB/cJ mice were intraperitoneally injected with 0.5 ml pristane. Sera were collected 12 weeks later and screened for anti-Ago2 antibody using an antigen-capture ELISA as described (Satoh et al., 1995). Anti-Ago2 positive mice were then given an intravenous booster injection of purified soluble recombinant Ago2 protein in phosphate buffered saline (PBS) three days prior to the harvest of spleen cells for hybridoma fusion. The hybridoma cells were cultured and selected using Dulbecco’s modified Eagle’s minimal essential medium that contained 15% horse serum and hypoxanthine-aminopterin-thymidine. The production of monoclonal antibodies to Ago2 was screened by ELISA and indirect immunofluorescence (IIF) as described below. These studies were approved by the institutional animal care and use committee of the University of Florida.

### 2.3. Enzyme-linked immunosorbent assay

Purified soluble recombinant Ago2 protein was diluted in PBS to a final concentration of 2 µg/ml and coated on MaxiSorp<sup>TM</sup> microtiter plates (Nunc<sup>TM</sup>, Naperville, IL, USA). Undiluted hybridoma culture supernatants were initially screened and mouse anti-Ago2 sera were diluted 1:1000 and used as positive control. Horseradish peroxidase-conjugated goat anti-mouse IgG (CALTAG<sup>TM</sup> Laboratories, Burlingame, CA, USA) was used at a dilution of 1:2000 and the optical density (OD) at 405 nm was obtained as described (Rubin, 1997).

### 2.4. Immunoprecipitation (IP)

Human K562 cells, HeLa cells, HEP-2 cells, and murine NIH-3T3 cells were metabolically labeled with [<sup>35</sup>S]-methionine (MP Biomedicals, Irvine, CA, USA), resuspended at 10<sup>7</sup> cells/ml in 500 mM NaCl NET buffer (500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5) containing 0.3% Nonidet-P40 and Complete Protease Cocktail Inhibitors (Roche, Mannheim, Germany), sonicated for 60 s, and cleared by centrifugation at

10,000  $\times g$  for 10 min. The full-length human Ago cDNAs (Ago1: clone 30344513, GenBank BC063275; Ago2: pCMV-SPORT6 kindly provided by Dr. Tom Hobman, University of Alberta, Edmonton, AB, Canada; Ago3 clone CS0DB008YP10, GenBank AL522515; Ago4: clone 4373725, GenBank BF979532) were used as templates for *in vitro* transcription and translation (TnT<sup>®</sup>, Promega, Madison, WI, USA) in the presence of [<sup>35</sup>S]-methionine as described (Jakymiw et al., 2006). In brief, IP of K562 cell extracts and *in vitro* translated products were used in IP reactions by combining 100  $\mu$ l of a 10% Protein A-Sepharose CL-4B beads (Amersham Biosciences, Piscataway, NJ, USA) with 100  $\mu$ l of 4F9 culture supernatant or 5  $\mu$ l of sera, 500 mM NaCl NET buffer containing 0.3% NP-40, and the individual radiolabeled proteins. After 1 h incubation at 4 °C, the suspension was washed four times in 500 mM NaCl NET buffer containing NP-40, and eluted in 25  $\mu$ l sample buffer. For IP experiments involving TnT products, *in vitro* translated radiolabeled Luciferase protein was added to the IP mix to monitor the specificity of IP reactions. Immunoprecipitates were analyzed by 10% gel SDS-PAGE followed by autoradiography (Jakymiw et al., 2006).

### 2.5. Indirect immunofluorescence (IIF)

HeLa or HEp-2 cells were cultured as monolayers, fixed in 4% paraformaldehyde in PBS (pH 7.4) for 10 min, permeabilized in 0.1% Triton X-100 and 0.15% Saponin in PBS for 10 min, incubated with mouse monoclonal or human antibodies, washed and subsequently detected by Alexa Fluor<sup>®</sup> 488 or Cy3-con-

jugated goat anti-IgG antibody appropriate for the species (Invitrogen, Eugene, OR, USA). All of the fixation, permeabilization and incubation with antibodies were performed on ice. Nuclei in the cell substrates were counterstained with 4',6-diamidino-2-phenylindole (DAPI) that was included in the VectaShield Hard Set Mounting Medium (Vector Laboratories, Burlingame, CA, USA). In addition, monoclonal antibodies were also analyzed on commercial HEp-2 cell slides (HEp2000, ImmunoConcepts, Sacramento, CA, USA). Rabbit anti-phosphohistone H3 was obtained from Upstate Biotech (Charlottesville, VA, USA) and used at a dilution of 1:300 to monitor cells in late S and G2 phases. Images of fixed cells were obtained using 63 $\times$ 1.4 NA objectives on a Zeiss Axiovert 200M microscope. For 8-bit color images, pixel intensity levels were adjusted using Adobe Photoshop version 7.0 so that maximal and minimal values were 0 and 255 in each channel.

### 2.6. MicroRNA RT-PCR assay

RT-PCR was performed using 20 ng of RNAs isolated from the Protein A Sepharose and antibody complexes, and HeLa cell extracts after IP as described above except using unlabeled cells. The RNAs were isolated from Protein A beads utilizing the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) which was used according to the manufacturer's protocol. RT-PCR was performed using the mirVana qRT-PCR miRNA Detection Kit (Ambion). Briefly, miRNAs were reverse transcribed by using miRNA RT Primer (Ambion) incubated at 37 °C for 30 min and then 95 °C for 10 min. After the RT step, the miRNA cDNA was amplified using miRNA PCR

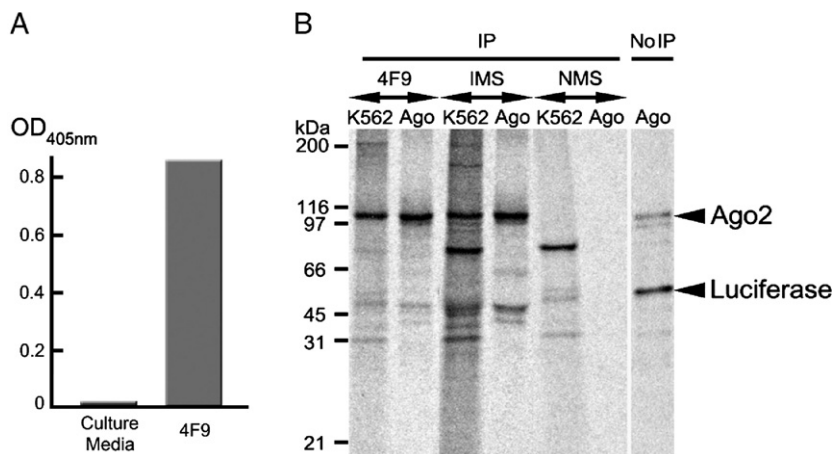


Fig. 1. Development of mouse monoclonal anti-Ago2 antibody 4F9. (A) ELISA reactivity of 4F9 with purified soluble recombinant Ago2 protein. (B) Representative immunoprecipitation analysis of 4F9 reactivity using K562 cell extracts (K562) and *in vitro* translation product of Ago2 recombinant protein (Ago) both labeled with [<sup>35</sup>S]-methionine. Immune mouse anti-Ago2 serum (IMS) is used for positive control and normal mouse serum (NMS) as negative control.

primers with 3 min initial denaturation at 95 °C, and followed by 30 cycles of 95 °C for 15 s and 60 °C for 30 s. The PCR products were confirmed using a 3.5% high resolution agarose gel in Tris-Acetate-EDTA buffer stained with ethidium bromide.

### 3. Results and discussion

#### 3.1. Development of anti-Ago2 hybridoma from pristane treated mice

Intraperitoneal injection of pristane into mice leads to autoantibody responses analogous to those described in

some human systemic lupus erythematosus sera (Sato and Reeves, 1994). Recently, we reported that pristane injected BALB/cJ mice produce anti-Ago2 autoantibodies and these autoantibodies are also detected in rheumatic disease sera (Jakymiw et al., 2006). To further support our initial findings, anti-Ago2 autoantibody positive mice were selected for hybridoma production and ~800 hybridoma clones were screened with recombinant Ago2 coated ELISA plates. A single IgG1 $\kappa$  clone, 4F9, showed strong reactivity with recombinant Ago2 by ELISA (Fig. 1A). Subcloning using standard limited dilution cloning was performed twice over a 3 month period.

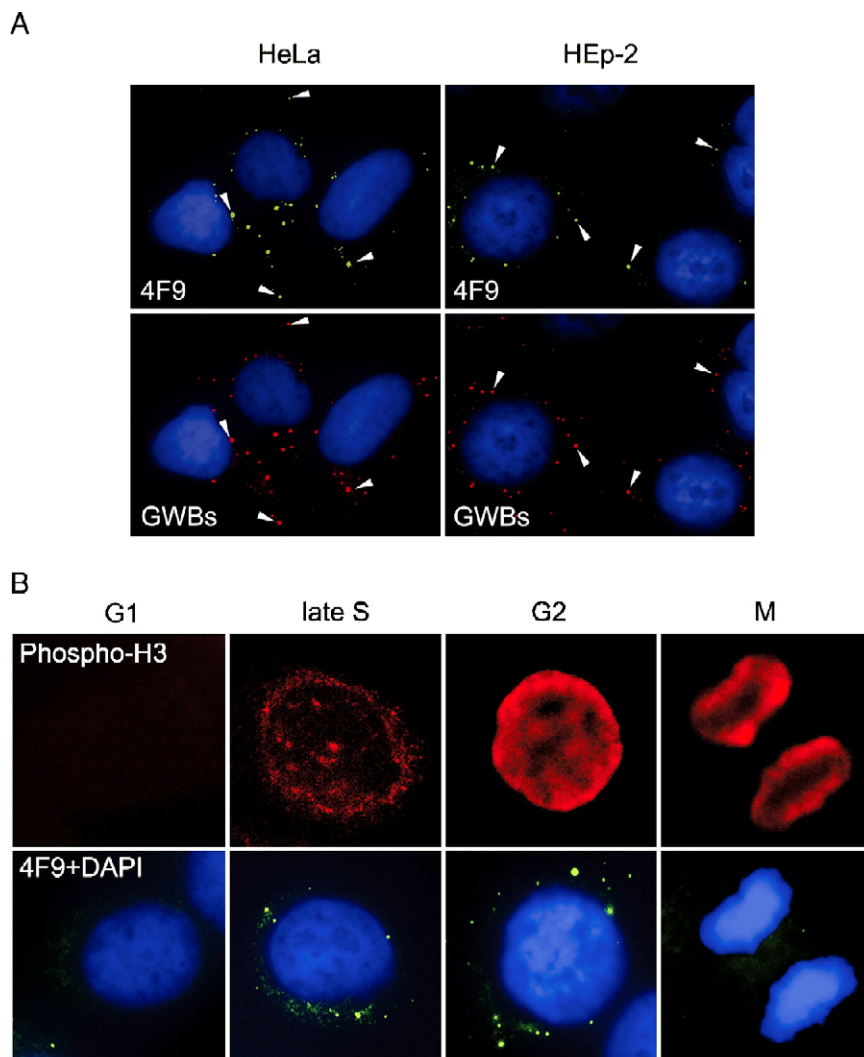


Fig. 2. Immunofluorescence analysis of 4F9. (A) Monoclonal 4F9 (green) recognized cytoplasmic foci identified as GW bodies (GWBs) by costaining with a human index serum known to contain antibodies to anti-Ago2 and GW182 (red). HeLa cells (left panels) were fixed as described in Methods. HEp-2 cells (right panels) were on commercially prepared slides (ImmunoConcepts Inc). Arrowheads show representative GWBs. (B) Monoclonal 4F9 showed staining of GWBs in a cell cycle dependent manner. HEp-2 cells were costained with 4F9 (green foci, bottom panels) and rabbit anti-phosphohistone H3 serum (red, top panels).

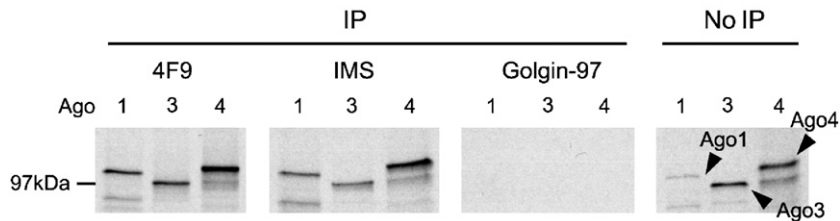


Fig. 3. Monoclonal 4F9 crossreacted with Ago1, Ago3 and Ago4. Immunoprecipitation analysis of 4F9 was performed using *in vitro* translation products of Ago1, 3 and 4 recombinant proteins labeled with [<sup>35</sup>S]-methionine. IMS and mouse anti-golgin-97 antibody were used as positive and negative control, respectively.

### 3.2. 4F9 recognized a conserved conformational epitope

To confirm that 4F9 recognized Ago2, IP was performed using K562 cell extracts and *in vitro* translated Ago2 protein both radiolabeled with [<sup>35</sup>S]-methionine. A 100 kDa protein that comigrated with the *in vitro* translated Ago2 protein as well as the positive control was IP by the immune mouse anti-Ago2 serum (IMS, Fig. 1B). In contrast, normal mouse serum (NMS) did not immunoprecipitate Ago2 from K562 cell extracts or *in vitro* translated Ago2. The inability of 4F9 and IMS to immunoprecipitate the *in vitro* translated Luciferase product also demonstrated the specificity of 4F9 and IMS for Ago2. Additionally, 4F9 immunoprecipitated Ago2 from radiolabeled HeLa, HEp-2 and NIH-3T3 cell extracts (data not shown). 4F9 showed very weak or no reactivity in western blot analysis using HeLa and HEp-2 cell extracts (data not shown). Furthermore, epitope mapping was attempted using sequential overlapping peptides of 15 amino acids spanning the full-length Ago2 protein synthesized on a membrane as previously described (Eystathioy et al., 2003). When 4F9 was applied to the membrane for immunoblotting, specific reactivity was not detected (data not shown). Additionally, no specific reactivity for either mouse or human anti-Ago2 sera was detected with the Ago2 epitope mapping membrane. Interestingly, this lack of reactivity with denatured or linear epitopes exhibited by 4F9 is highly consistent with published data for human and mouse anti-Ago2 autoantibody (Satoh et al., 1994). Based on these observations, we concluded that 4F9 recognized a conformational dependent epitope similar to autoantibody reported for Ago2.

### 3.3. 4F9 recognized GWBs in a cell cycle dependent pattern

4F9 recognized cytoplasmic foci identified as GWBs because there was costaining with a human index serum known to contain antibodies to Ago2 and GW182 (Fig. 2A); the latter is a marker protein for

GWBs. This staining pattern was confirmed using both HeLa cells fixed and permeabilized as described in Methods and the commercially prepared HEp-2 cells. To determine optimal fixation parameters, various fixation methods that included methanol, acetone/methanol, and ethanol were used but none of these retained the 4F9 staining although GWB staining was clearly detected (data not shown).

As shown in Fig. 2B, 4F9 staining of GWBs was cell cycle dependent. By costaining with rabbit anti-phosphohistone H3 antibody, 4F9 staining was consistent with the reported staining for GWBs, i.e. larger size during late S and G2 phases of the cell cycle and diminished staining during mitosis and G1 (Yang et al., 2004). Since Ago2 is a key component of RISC, the observation that Ago2 staining is cell cycle dependent suggests the possibility that Ago2 and GW182/GWBs may be involved in controlling expression and/or silencing of selected mRNAs involved in the cell cycle (Liu et al., 2005a).

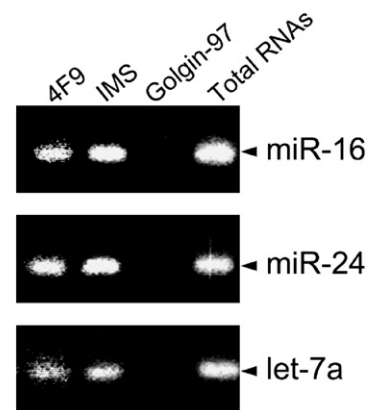


Fig. 4. Argonaute monoclonal antibody captured microRNAs. MicroRNAs were detected in the immunoprecipitates of 4F9 and IMS using RT-PCR. Immunoprecipitates of an unrelated mouse monoclonal anti-golgin-97 were analyzed as a negative control. Total RNAs isolated from human heart were used as a positive control for the RT-PCR.

### 3.4. All Ago proteins could be recognized by 4F9

Mouse and human anti-Ago2 sera reacted not only with Ago2 but also with Ago1, 3, 4 (Jakymiw et al., 2006). Consequently, we performed IP analysis of 4F9 using *in vitro* translated Ago proteins. 4F9 immunoprecipitated all Ago proteins as did a mouse anti-Ago2 serum (IMS, Fig. 3). The human Ago proteins have 77.6 to 83.6% sequence identity with each other (Sasaki et al., 2003) and 4F9 most likely recognizes a shared conformational epitope found in all Ago proteins. In addition, it was previously reported that all 4 Ago proteins localized to GWBs (Liu et al., 2005b). Taken together, we concluded that 4F9 binds all Ago proteins.

### 3.5. 4F9 captures miRNAs

As discussed above, Ago proteins are known to bind miRNAs and we speculated that unless 4F9 binds to the RNA binding domain of Ago proteins and blocks the interaction of miRNA with Ago, the monoclonal antibody may be useful in capturing cellular miRNA bound to Ago proteins. In order to evaluate whether miRNAs are co-precipitated with Ago proteins from HeLa cell extracts, miRNA RT-PCR was performed on the immunoprecipitates of 4F9, mouse anti-Ago2 serum and an unrelated control mouse monoclonal antibody to the Golgi protein golgin-97. Total RNAs from human heart were used as a positive control for the RT-PCR. After miRNA RT-PCR, microRNA-16 (miR-16), microRNA-24 (miR-24) and let-7a were detected in both 4F9 and IMS immunoprecipitates but not in anti-golgin-97 immunoprecipitates (Fig. 4). In addition, a human anti-rRNP/Sm serum and a human anti-SS-A/Ro serum were used as unrelated antibody control for miRNAs capture and no miRNAs were detected in the immunoprecipitate using these human anti-ribonucleoprotein sera (data not shown). These results are consistent with a previous report that Ago proteins, especially Ago2 protein, and miRNAs form ribonucleoprotein complexes (miRNPs) (Meister et al., 2004). The three miRNAs chosen in this assay have a wide variety of biological functions. miR-16 and let-7a regulate tumor necrosis factor- $\alpha$  (Jing et al., 2005) and Ras (Johnson et al., 2005; Akao et al., 2006), respectively, and miR-24 is related to the regulation of cell growth (Cheng et al., 2005).

Among the several hundreds human miRNAs identified to date (Bentwich et al., 2005; Griffiths-Jones et al., 2006), only relatively few have known biological activities. The continuing discovery of novel small RNA such as the class characterized by 26–31 nucleotides and containing “piwi-interacting RNAs”, or piRNA, impli-

cated in spermatogenesis (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006) will increase the need for improvements in differentiating miRNAs from other small RNAs. In summary, we developed a novel monoclonal antibody 4F9 with the capability of capturing Ago-associated miRNAs in mammalian cells and may help to elucidate their target mRNAs.

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