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Analysis of sDMA modifications of PIWI proteins

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Summary

Arginine methylation is an important post-translational protein modification that modulates protein function for a wide range of biological processes. PIWI proteins, a subclade of the Argonaute family proteins, contain evolutionarily conserved symmetrical dimethylarginines (sDMAs). It has become increasingly apparent that the sDMAs of PIWI proteins serve as binding elements for TUDOR-domain containing proteins and that sDMA-dependent protein interactions play crucial roles in the biogenesis and function of PIWI-interacting RNAs (piRNAs). We describe a method for detecting PIWI sDMAs and purifying PIWI/piRNA complexes using anti-sDMA antibodies.

Keywords

PIWI; piRNA; Arginine methylation; Symmetrical dimethylarginine (sDMA); Y12; SYM10; SYM11

1. Introduction

Arginine methylation is an important post-translational protein modification that plays crucial roles in numerous biological processes, such as structural remodeling of chromatin, signal transduction, mRNA splicing and DNA repair [1–5]. Arginine methylation is mediated by two types of protein methyltransferases (PRMTs): type I enzymes (e.g., PRMT1) catalyze asymmetrical dimethylarginine (aDMA) in which two methyl groups are placed on one of the terminal nitrogen atoms of the guanidino group, whereas type II enzymes (e.g., PRMT5) catalyze symmetrical dimethylarginine (sDMA, Fig 1A) in which one methyl group is placed on each of the terminal nitrogens [1–5]. sDMA modifications occur in “sDMA motifs” comprising arginines flanked by glycines (GRG) or alanines (GRA or ARG), which are often found as repeats. sDMAs are known to specifically bind to TUDOR domains of proteins and regulate protein–protein interactions [1–5]. For example, in mammals, sDMAs of Sm proteins, components of small nuclear ribonucleoproteins

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[4]Y12 purifies snRNPs containing snRNAs [29] as well as piRNPs containing piRNAs. Therefore, the long RNAs observed in Y12 immunoprecipitate (Fig 3) include snRNAs.

(snRNPs), promote their binding to the TUDOR domain of survival of motor neuron (SMN) protein, which facilitates snRNP assembly [6,7].

Arginine methylation and its mediated protein-protein interactions have attracted increased attention as key molecular factors in small regulatory RNA pathway in the germline. PIWI proteins, a subclade of the Argonaute family proteins, are predominantly expressed in the germline and bind to 25–31 nucleotide (nt) PIWI-interacting RNAs (piRNAs) to form PIWI-ribonucleoproteins (piRNPs) [8–10]. Mice express three PIWI proteins, MIWI, MILI and MIWI2 [11–13], and *Bombyx mori* (silkworm) expresses two PIWI proteins, SIWI and BmAGO3 [14]. piRNPs play critical roles in germline development by regulating transposons and other targets to maintain genome integrity. PIWI proteins contain evolutionarily conserved sDMAs that are synthesized by PRMT5 [15]. Putative sDMA motifs are typically clustered at the N-terminus of PIWI proteins (Fig 1B); in mouse and *Drosophila*, sDMA positions were further determined by mass spectrometry [16–19]. Several members of the TUDOR domain-containing protein family, such as Spindle-E, Tudor, Krimper, and Tejas in *Drosophila* [19–23] and TDRD1-9 in mice [16–18,24–26], have recently been studied for their PIWI interactions and functional involvements in piRNA biogenesis and function [27,28].

Here, we describe a method for detecting sDMA modifications of PIWI proteins. There are three highly-specific anti-sDMA antibodies: Y12, SYM10 and SYM11. Y12 is a monoclonal antibody that was generated from a hybridoma of lupus erythmatosus-like syndrome mice developing autoantibodies against Sm proteins [29,30]. The epitope that Y12 recognizes on Sm proteins comprises sDMAs of the proteins [31]. SYM10 and SYM11 are polyclonal antibodies derived from rabbit serum immunized with peptides containing sDMAs, K(sDMA)G(sDMA)G(sDMA)G(sDMA)G and KAAILKAQVAA(sDMA)G(sDMA)G(sDMA)GMG(sDMA)G, respectively [32,33]. We describe an utilization of these antibodies to detect sDMAs of MIWI and MILI, which were purified from mouse testicles, and SIWI and BmAGO3, which were transiently expressed and purified from BmN4, a *Bombyx-mori* ovary derived cultured cell line [34].

In addition, we describe a method to purify piRNP using immunoprecipitation with the Y12 antibody. For piRNA purification and identification, piRNPs are typically purified by anti-PIWI immunoprecipitation. However, we previously reported successful piRNP purification using Y12 immunoprecipitation for mouse testicles and *Xenopus* oocytes [15]; we here demonstrate that it can also be used for BmN4 cells. Our results suggest that the Y12 antibody can be widely used to purify piRNPs for identifying piRNA sequences in various organisms for which antibodies against PIWI proteins have not yet been generated.

2. Materials

1. Recombinant protein G agarose beads (Invitrogen)
2. Anti-Flag® M2-agarose from mouse (Sigma)
3. Y12 antibody (mouse monoclonal, a gift from G. Dreyfuss, University of Pennsylvania; Note 1)

4. SYM10 antibody (rabbit polyclonal, Millipore)
5. SYM11 antibody (rabbit polyclonal, Millipore)
6. Anti-MIWI antibody (rabbit polyclonal [23]; Note 1)
7. Anti-MILI antibody (mouse monoclonal clone 17.8 [15]; Note 1)
8. Non-immune mouse and rabbit serum
9. Mouse testicle (Pel-Freez Biochemicals)
10. Lysis buffer: 20 mM Tris-HCl (pH 7.4); 200 mM NaCl; 2.5 mM $MgCl_2$; 0.5% NP-40; 0.1% Triton X-100; one tablet of Complete protease inhibitor EDTA-free (Roche) per 50 mL of lysis buffer.
11. 7 mL Dounce tissue grinder (Wheaton)
12. Bioruptor sonication system (Diagenode)
13. BmN4 cell line (a gift from S. Katsuma, University of Tokyo)
14. Expression plasmids for Flag-SIWI and Flag-BmAGO3: The N-terminal Flag/His-tagged SIWI or BmAGO3 were cloned into a pIZ/V5-His vector (a gift from S. Katsuma, University of Tokyo) [34].
15. Insect-Xpress medium (LONZA)
16. Sf-900™ III SFM (1×), liquid (Invitrogen)
17. ESCORT transfection reagent (Sigma)
18. NuPAGE LDS sample buffer (Invitrogen)
19. β-Mercaptoethanol
20. NuPAGE 4%–12% Bis-Tris gel (Invitrogen)
21. NuPAGE MOPS SDS running buffer (Invitrogen)
22. SilverQuest staining kit (Invitrogen)
23. Nitrocellulose/filter paper; 0.45 μm pore size (Invitrogen)
24. Transfer buffer: 62.5 mM Tris; 18 mM Glycine; 20% Methanol
25. TE70 ECL semi-dry transfer unit (GE Healthcare)
26. PBS (TEKNOVA)
27. PBST: PBS containing 0.1% Tween 20
28. Blocking solution: 5% non-fat dry milk in PBST
29. ECL anti-rabbit IgG, horseradish peroxidase linked F(ab') 2 fragment from donkey (GE Healthcare)

[1]The Y12, anti-MIWI, and anti-MILI antibodies are commercially available from various distributors.

30. ECL anti-mouse IgG, horseradish peroxidase linked F(ab') 2 fragment from sheep (GE Healthcare)
31. ECL plus western blotting detection system (GE Healthcare)
32. ChemiDoc™ XRS+ system (Bio-Rad)
33. Trizol (Invitrogen)
34. Glycogen (Ambion)
35. 3 M NaOAc, pH5.5 (Ambion)
36. Isopropanol (Sigma)
37. Centrifugal evaporator (myVac)
38. Alkaline phosphatase, calf intestinal; CIP (NEB)
39. T4 Polynucleotide Kinase; T4 PNK (NEB)
40. ATP [γ-^{32}P] (American Radiolabeled Chemicals)
41. 15% PAGE solution with 7 M Urea (1L): 420.42 g Urea (Sigma); 376 mL 40% acrylamide and bis-acrylamide solution (19:1, Bio-Rad); 100 mL Ultrapure™ 10×TBE buffer (Invitrogen) and MilliQ water to prepare 1L. After filtration, store at 4°C (protect from light).
42. Ammonium persulfate (Sigma)
43. Ultrapure™ TEMED (Invitrogen)
44. SE-400 electrophoresis system (Hoefer)
45. 2×Loading buffer for Urea PAGE: 5.4 g Urea (Sigma); 6 mg Bromophenol blue (Sigma); 6 mg Xylene cyanol (Sigma) and MilliQ water for 10 mL.
46. Phosphor autoradiography plate (Kodak)
47. Molecular Imager PharosFX System (Bio-Rad)

3. Methods

3-1 Purification of MIWI and MILI from mouse testicles by immunoprecipitation

3-1-1 Preparation of antibody-bound agarose beads

1. Wash protein G agarose beads (10 μL bed volume) three times with 1 mL of lysis buffer.
2. Add either anti-MIWI (10 μL), anti-MILI (2.5 μL), non-immune mouse serum (NMS, negative control) or non-immune rabbit serum (NRS, negative control) to the beads in 700 μL of lysis buffer.
3. Rotate for 1 h at room temperature (RT).
4. Discard the buffer containing antibody and wash five times with 1 mL of lysis buffer.

3-1-2 Preparation of mouse testicle lysate

1. Use one mouse testicle per immunoprecipitation (500 μL of lysis buffer). Homogenize testicles in lysis buffer with a Dounce tissue grinder in a cold room (4°C).
2. Sonicate the homogenate using the Biorupter sonication system according to manufacturer's instructions (on: 5 sec, off: 7 sec, cycles: 14, strength level: medium).
3. Centrifuge the lysate at 20,000 g for 10 min at 4°C. Collect the supernatant.

3-1-3 Immunoprecipitation

1. Add the testicle lysate to the prepared beads with antibodies and rotate for 1 h at 4°C.
2. Wash the beads five times with 1 mL of lysis buffer.

3-1-4 Visualization of purified proteins on SDS-PAGE by silver-staining

1. Add 30 μL of NuPAGE LDS sample buffer containing 10% β-Mercaptoethanol to the beads and incubate at 70°C for 15 min.
2. Run 10 μL of immunoprecipitate samples on NuPAGE 4%–12% Bis-Tris gel.
3. Stain with SilverQuest staining kit according to the manufacturer's instructions (Fig 2A).

3-2 Purification of SIWI and BmAGO3 from BmN4 cells by immunoprecipitation

3-2-1 Transient expression of Flag-SIWI and Flag-BmAGO3 in BmN4 cells

1. Spread and culture 5×10^6 BmN4 cells on a 10 cm dish in Insect X-press medium at 27°C for 24 h, then change the medium with 5 mL of Sf-900 medium.
2. Gently mix 13 μg of expression plasmid with 40 μL of ESCORT reagent and 650 μL of Sf-900 medium by pipetting, and then incubate at RT for 15 min.
3. Add this mixture to cells, incubate at 27°C for 6 h, and add 5 mL of Sf-900 medium.
4. After three days, collect cells with a scraper and wash the cells twice with 1 mL of PBS.

3-2-2 Preparation of BmN4 cell lysate

1. Resuspend cells in lysis buffer (5×10^6 cells in 200 μL of lysis buffer per immunoprecipitation).
2. Sonicate the suspension and collect the supernatant as described in 3-1-2.

3-2-3 Anti-Flag Immunoprecipitation

1. Wash anti-Flag agarose beads (10 μL bed volume) three times with 1 mL of lysis buffer.

2. Add the 200 μL of lysate to the beads and adjust total volume to 500 μL with lysis buffer so that the beads are not stacked within the tube during rotation.
3. Rotate for 1 h at 4°C, and then prepare samples for SDS-PAGE as described in 3-1-4.

3-3 Detection of PIWI sDMAs by western blots using anti-sDMA antibodies

3-3-1 SDS-PAGE and Transfer

1. Run 4 μL of immunoprecipitate samples on NuPAGE 4%–12% Bis-Tris gel.
2. Immerse the gel, nitrocellulose membrane and filter paper in transfer buffer and perform transfer procedure with TE70 ECL semi-dry transfer unit according to the manufacturer's instructions (run at 90 mA for 45 min).

3-3-2 Immunoblotting and detection

1. Rinse the membrane with MilliQ water, and then incubate the membrane with blocking buffer at 70 rpm for 1 h at RT.
2. Discard the blocking buffer and rinse the membrane twice with PBST.
3. Incubate the membrane with a primary antibody solution (anti-Flag 1:1000; Y12 1:500; SYM10 1:1000; and SYM11 1:1000; each diluted in blocking buffer) at 4°C overnight.
4. Rinse the membrane briefly and wash three times with PBST for 10 min.
5. Incubate the membrane with a secondary antibody solution (1:5000; diluted in PBST) at RT for 1 h.
6. Rinse the membrane with PBST, wash three times with PBST for 10 min, and immerse in PBS.
7. Detect bands using ECL-Plus detection solution and ChemiDoc according to the manufacturer's instructions (see Fig 2B, 2C and Note 2).

3-4 Isolation of piRNPs from BmN4 cells by immunoprecipitation with Y12 antibody

3-4-1 Immunoprecipitation with Y12 antibody

1. Prepare protein G agarose beads (10 μL bed volume) bound to Y12 (5 μL) as described in 3-1-1.
2. Prepare BmN4 cell lysate (1×10^7 cells per immunoprecipitation) as described in 3-2-2.
3. Add the lysate to the beads and rotate at 4°C for 2 h.
4. Wash the beads five times with 1 mL of lysis buffer.

[2]The affinities of the respective anti-sDMAs for PIWI proteins have certain differences. SYM10 and SYM11 recognize MIWI and SIWI well. This is because both of these PIWI proteins contain a long GRG/ARG/GRA repeat (Fig 1B) that is similar to the sequences of the peptides used for producing SYM10 and SYM11 [32,33]. In contrast, the Y12 antibody attaches to MILI much more strongly than to MIWI on western blot (Fig 2B) and in immunoprecipitation [15].

3-4-2 Isolation of piRNAs

1. Add 500 μL of Trizol reagent to the immunoprecipitate beads and vortex for 30 sec.
2. Add 100 μL of chloroform, vortex for 15 sec, and let stand at RT for 2 min.
3. Centrifuge at 20,000 g for 30 min at 4°C. Collect the upper aqueous phase (carefully exclude the interphase).
4. Add 2 μL of glycogen (5 mg/mL), vortex briefly, add 350 μL of isopropanol, and vortex again. Cool the tube at −20°C for 20 min.
5. Centrifuge at 20,000 g for 30 min at 4°C. Carefully remove the supernatant and dry the pellet for 1 min with a centrifugal evaporator.
6. Dissolve the pellet in 14 μL of MilliQ water with thorough pipetting.
7. Store the piRNA solution at −80°C.

3-4-3 5’-end radiolabeling of isolated piRNAs

1. To remove the 5’-end phosphate of piRNAs, incubate 7 μL of the isolated piRNA solution with 0.5 μL of CIP, 2 μL of 10×buffer and 10.5 μL MilliQ water (total 20 μL) at 37°C for 30 min.
2. Adjust the total volume to 100 μL with MilliQ water, add 100 μL of phenol and thoroughly vortex.
3. Centrifuge at 20,000 g for 5 min at RT and then carefully collect the upper phase.
4. Add 2 μL of glycogen (5 mg/mL), 10 μL of 3M NaOAc and briefly vortex. Then, add 275 μL of chilled 100% ethanol, vortex well and cool the tube at −80°C for 30 min.
5. Centrifuge at 20,000 g for 30 min at 4°C. Carefully remove the supernatant and dry the pellet for 1 min with a centrifugal evaporator.
6. Dissolve the pellet with 7.5 μL of MilliQ water with thorough pipetting.
7. For 5’-end labeling of piRNAs, incubate 7.5 μL of dephosphorylated piRNA solution with 1 μL of [γ-^{32}P]ATP, 0.5 μL of T4 PNK and 1 μL of 10×buffer (total 10 μL) for 1 h at 37°C.

3-4-4 Separation and detection of labeled piRNAs

1. Set up the gel apparatus (SE-400 system) using 18×24 cm glass plate and 0.75 mm thick combs.
2. Add 150 μL of 10% ammonium persulfate and 10 μL of TEMED to 30 mL of 15% 7M Urea PAGE solution. Mix gently and immediately pour into the apparatus.
3. Run the gel at 300 V for 30 min and wash the wells with a syringe to remove accumulated urea in the wells.
4. Add an equal volume of 2×loading buffer to the labeled piRNA solution.

5. Run 10 μL of the samples at 700 V until the dye (BPB) front reaches the bottom of the gel.
6. Disassemble glass plates and peel out one side of glass plate with a spatula. Cover the gel with a wrap and expose the gel to a phosphor autoradiography plate in a cassette for 1 h–overnight at −80°C.
7. Scan the plate using a PharosFX phosphor imager (see Fig 3, Note 2 and Note 3).

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[3]It has been reported that immunoprecipitation with SYM10 or SYM11 could be used to pull down various sDMA-containing proteins, including Sm proteins [32,33,35]. Based on these reports, we attempted to perform SYM10 and SYM11 immunoprecipitation using lysates from mouse testicles or *Drosophila* ovaries to purify piRNPs. However, despite using different lysis buffers containing different salt concentrations (100–200 mM), we were unable to detect PIWI proteins and piRNAs in these immunoprecipitates. It was observed that, among the anti-sDMA antibodies, the Y12 antibody was particularly useful for purifying piRNPs.

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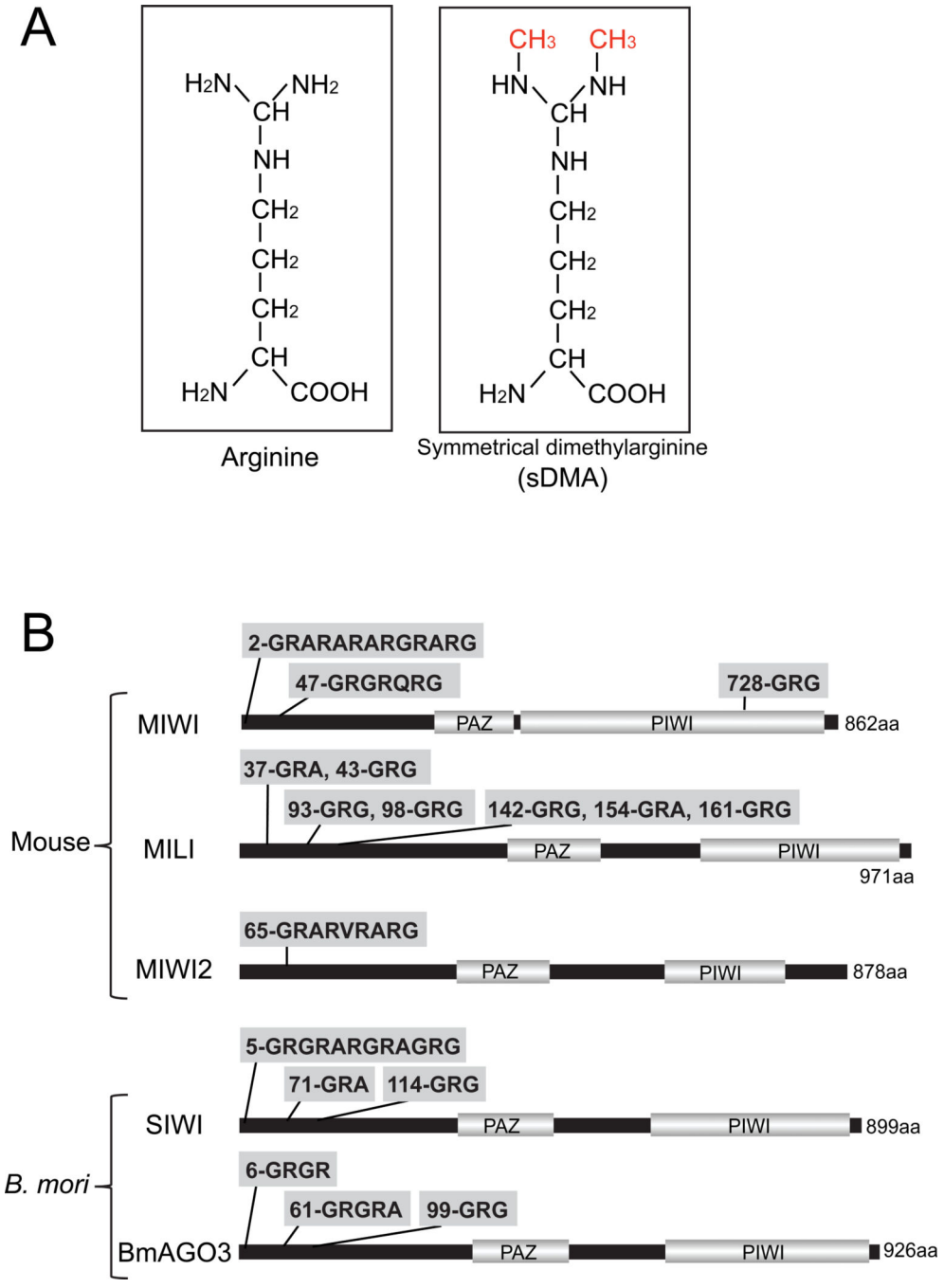

Fig 1. sDMA motifs in PIWI proteins

(A) Chemical structure of arginine and sDMA.

(B) The structures of PIWI proteins (MIWI, MILI and MIWI2 for mouse; SIWI and BmAGO3 for *Bombyx mori*) are shown with sDMA motifs comprising GRG or GRA/ARG sequences. PAZ and PIWI are the two major protein motifs that exist in the Argonaute family proteins.

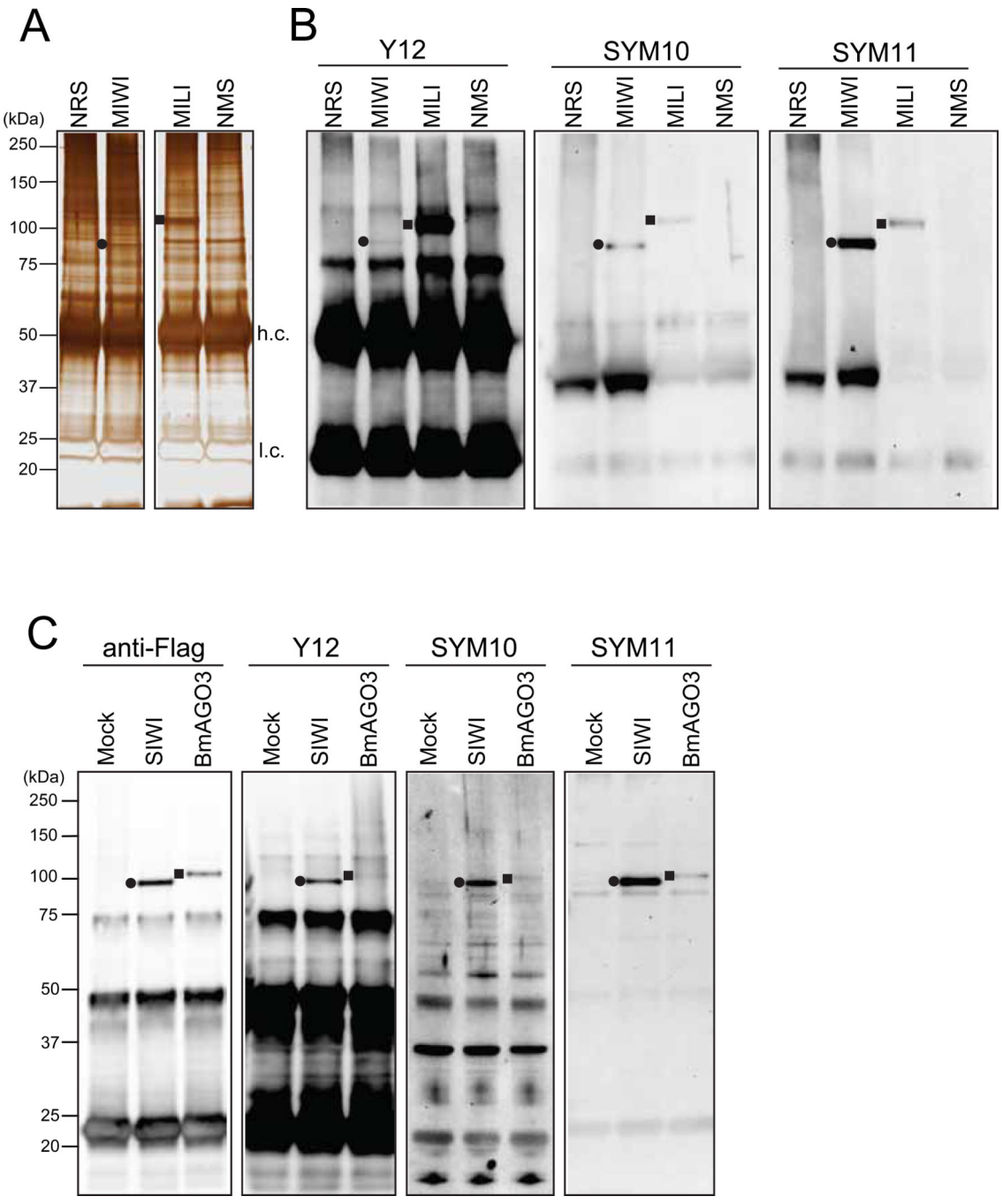

Fig 2. Detection of PIWI sDMAs by western blot with anti-sDMA antibodies

(A) MIWI and MILI immunoprecipitates from mouse testicles were visualized by silver staining (NMS: non-immune mouse serum; NRS: non-immune rabbit serum). The filled circle and square indicate the bands for purified MIWI and MILI, respectively. h.c. and l.c. indicate antibody heavy and light chains, respectively.

(B) Immunoprecipitates from mouse testicles were probed on western blots with the indicated antibodies. The Y12, SYM10 and SYM11 antibodies recognized both MIWI

(filled circle) and MILI (filled square), which indicated that MIWI and MILI contain sDMAs.

(C) Flag-SIWI or Flag-BmAGO3 expressed in BmN4 cells were immunopurified with an anti-Flag antibody (Mock: the sample from BmN4 cells with no transient protein expression) and probed on western blots with the indicated antibodies. All these antibodies recognized SIWI (filled circle) and BmAGO3 (filled square), indicating the presence of sDMAs in SIWI and BmAGO3.

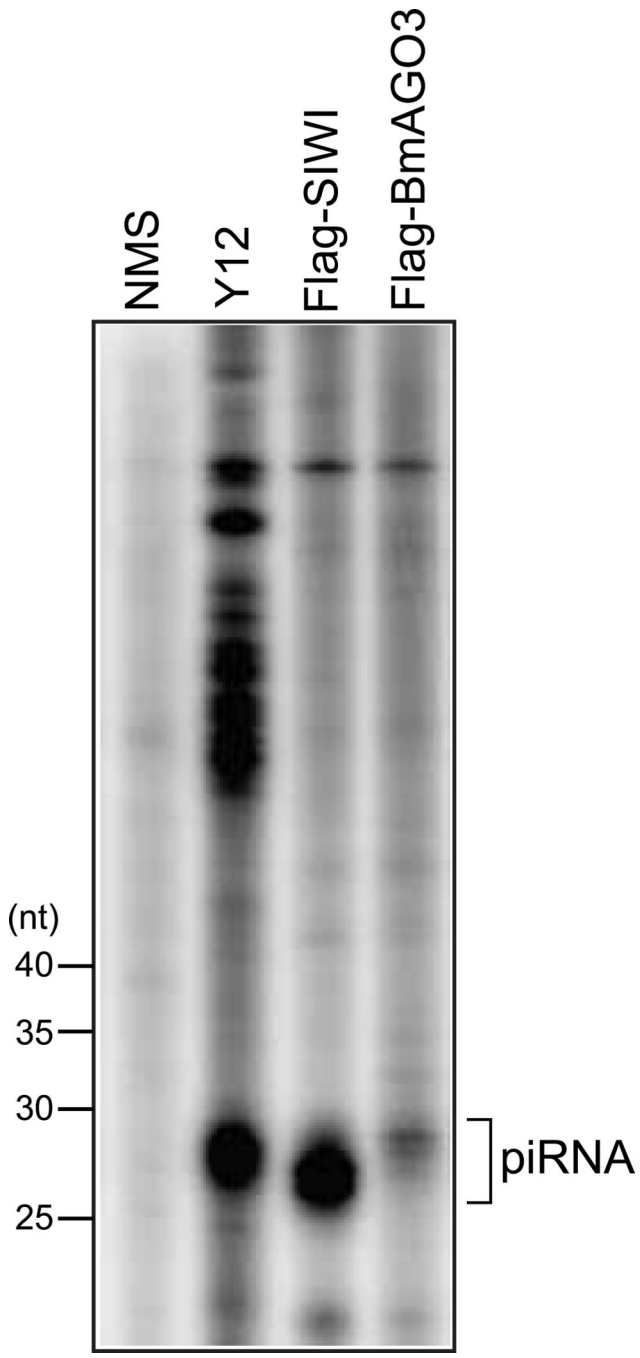

Fig 3. piRNP purification by immunoprecipitation with the Y12 antibody

Y12 immunoprecipitate from BmN4 cells, and anti-Flag immunoprecipitates from BmN4 cells expressing Flag-SIWI or Flag-BmAGO3 were subjected to RNA extraction, 5’-end labeling of the extracted RNA, and denaturing PAGE. piRNAs were clearly observed in Y12 immunoprecipitate as well as PIWI’s, which indicated the successful purification of piRNPs with the Y12 antibody.