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A role for the P-body component, GW182, in microRNA function

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Abstract

In animals, the majority of microRNAs regulate gene expression through the RNAi machinery without inducing small RNA-directed mRNA cleavage [1]. Thus, the mechanisms by which microRNAs repress their targets have remained elusive. Recently, Argonaute proteins, which are key RNAi effector components, and their target mRNAs were shown to localize to cytoplasmic foci known as P-bodies or GW-bodies [2,3]. Here, we show that the Argonaute proteins physically interact with a key P-/GW-body subunit, GW182. Silencing of GW182 delocalizes resident P-/GW-body proteins and impairs the silencing of microRNA reporters. Moreover, mutations preventing Argonaute proteins from localizing in P-/GW-bodies prevent translational repression of mRNAs even when Argonaute is tethered to its target in a small RNA-independent fashion. Thus, our results support a functional link between cytoplasmic P-bodies and the ability of a microRNA to repress expression of a target mRNA.

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) enter the RNA-induced silencing complex, RISC, and suppress the expression of target genes, which they recognize by complementary base pairing [1,4]. The precise mechanism of suppression depends upon two factors. First is the degree of complimentarity between the small RNA and its target. In cases of perfect or near-perfect complimentarity, the mRNA can be cleaved by an Argonaute protein. When complimentarity is imperfect, as normally occurs for animal microRNAs, suppression occurs without RISC-mediated cleavage [5]. A second factor is the nature of the Argonaute protein that forms the core of RISC. Not all Argonaute proteins are catalytically active. In mammals, Argonaute-2 is competent for substrate cleavage while Argonautes 1,3 and 4 are inert [6,7]. Thus, at least in mammals, RISC can recognize a substrate and form a complex that is incapable of cleavage even with a perfect small RNA-target interaction. The outcome of such events is presently unknown, but such interactions could potentially lead to cleavage-independent repression.

The mechanisms by which RISC can repress targets in the absence of substrate cleavage are yet to be resolved. Early studies indicated that repression by animal microRNAs occurred without changes in the overall level of the mRNA target [4,8,9]. However, recent studies in mammalian cells and *C. elegans* have indicated that changes in mRNA abundance are observed for the proposed targets of several microRNAs [10,11]. Additionally, several studies detect both bulk microRNAs and some mRNA targets on polysomes, suggesting that suppression might occur during the act of protein synthesis either by changes in initiation or elongation rates or by destabilizing nascent proteins [12–15].

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Recent observations have also led to an alternative model for silencing by miRNAs wherein mRNA interactions with RISC might sequester targeted mRNAs in P-/GW-bodies [2,3,15–17]. These are cytoplasmic foci that contain non-translated mRNAs and exclude the translation machinery [18]. Not only are Argonaute proteins found in mammalian P-/GW-bodies [2,3], mRNA targets of microRNAs become similarly localized in a manner that depends both on the presence of the miRNA and upon miRNA binding sites in the target [2,15]. Such localization could potentially embody part, or all, of the underlying cause of repression or could occur as a downstream consequence of translational repression by RISC. Thus, it is critical to examine the functional significance of the connections between P-/GW-bodies and the RNAi pathway.

To investigate mechanisms of miRNA-mediated repression, we have searched for Argonaute-interacting proteins by MudPIT (Multidimensional Protein Identification Technology) analysis of immunoaffinity purified Ago1 and Ago2 complexes. We recovered a number of previously identified Argonaute binding proteins including HSP90, Dicer, TRBP and DCP1 [2,6,19,20]. Additionally, we repeatedly identified a known component of P-/GW-bodies, GW182, in both Ago1 and Ago2 complexes. To verify these observations, we examined GW182 immunoprecipitates by immunoblotting for epitope tagged Argonaute (Fig. 1a). An interaction between these proteins was easily detectable and was not disrupted by treatment of extracts or immunocomplexes with RNaseA (not shown).

GW182 is present in discrete cytoplasmic foci wherein it co-localizes with the de-capping complex [21,22]. To determine if these foci also contain Argonaute proteins, we used two different GW182-specific autoantisera (IC-6 and 18033) to highlight GW182-containing bodies and an anti-myc antiserum to recognize ectopically expressed Ago2. The two staining patterns show substantial overlap (Fig. 1b), suggesting that at least a portion of the total populations of each of these proteins co-localize.

Considered together, our data identify GW182 as a novel Argonaute-interacting protein. We therefore examined the effects of depleting GW182 on the integrity of P-/GW-bodies and on the ability of small RNAs to silence their targets. By co-transfection with a GFP-GW182 fusion protein, we identified an siRNA that could effectively suppress GW182 expression (Fig. 2a). Transfection of HeLa cells with this siRNA caused a substantial loss of GW182, Dcp1a, and Dcp2 in P-/GW bodies [23] (Fig. 2b, 2c, 2d). In contrast, siRNAs against Dcp2p effectively reduced Dcp2p protein levels, but did not impact the number of GW182 foci observed (data not shown).

Given that GW182 suppression affected the overall integrity of mammalian P-/GW-bodies, we sought to determine whether disruption of these foci impacted small RNA-directed gene silencing. We first examined a cleavage-independent repression event in which a CXCR4 siRNA can bind to 6 imperfect sites in a Renilla luciferase mRNA [24]. Co-transfection of the reporter with the CXCR4 siRNA resulted in an approximately 20-fold repression of luciferase activity under the conditions used for this assay (Fig. 3a). Repression of GW182 but not another P-body protein, XRN1, impaired the ability of the miRNA mimetic to silence its target (Fig. 3a). Suppression of DCP2 also showed a less pronounced, albeit reproducible, effect (Fig 3a). Curiously, suppression of GW182 also had an effect on the ability of the siRNA to suppress a perfectly complementary target via mRNA cleavage (Fig. 3b). Qualitatively similar results were seen with a second reporter that is targeted for repression by an endogenous microRNA, let-7 (Fig. S1). All of these outcomes correlated with inhibition of GW182 expression by the siRNA and with a reduction in the appearance of P-/GW-bodies (Fig. 2 and not shown). Notably, the pattern of Ago1 and Ago2 localization was also disrupted upon repression of GW182 (not shown). These data demonstrate that GW182 has a functional role in RISC-mediated silencing, which is correlated with maintenance of P-bodies.

Cleavage-independent suppression of an mRNA target has previously been accomplished by tethering an Argonaute protein to an mRNA 3' UTR in a manner independent of the small-RNA-target interaction [25]. This was achieved by fusing Ago1 or Ago2 to a phage RNA binding motif (λN) and placing its recognition sequence (boxB) within the reporter. It is difficult to be certain that tethered Argonaute proteins work through precisely the same mechanism as microRNA-directed RISC. However, several lines of evidence are consistent with tethered Ago proteins being able to function in the RNAi pathway similarly to their small RNA-directed counterparts. First, λN-fused Argonaute proteins can complement the silencing defect observed in Ago2 knockout MEF (Fig S2). Second, Argonaute proteins that can suppress their targets through direct protein-mRNA interactions localize to P-/GW-bodies in a manner similar to the native proteins (Fig. 4a). Our previous work indicated that a series of point mutations in the PAZ domain could prevent small RNA binding with an accompanying loss of localization to P-/GW-bodies. The same outcome was observed when this series of PAZ mutations was introduced into λN-fused Ago2 protein (Fig. 4a). Since these recognize their targets in a small-RNA independent fashion, we were afforded the opportunity to examine whether Argonaute binding, *per se*, or localization to P-/GW-bodies correlated with repression.

Expression of Ago2 fused at the amino terminus to λN protein reduced Renilla expression by ~2-fold, provided that the mRNA contained the λN binding site (Fig 4). Also, consistent with previous studies, a λN fusion with HIWI, an enigmatic member of a second Argonaute subfamily had no repressive effect on the reporter. λN-Ago2 (Fig. 4b) proteins containing point mutations in the PAZ domain that prevent small RNA binding neither localized to P-/GW-bodies nor repressed a boxB-containing target mRNA, despite maintaining interaction with the target (Fig. 4, not shown). Notably, Ago2-PAZ9 and -PAZ10 proteins were still present in GW182 immunoprecipitates, indicating that their potential to interact with GW182 was retained, despite the inability of these mutant proteins to localize to P-/GW-bodies or to repress their targets (Fig. 4c).

Previous studies have suggested connections between suppression by RNAi and cytoplasmic foci known as P-bodies or GW-bodies. All four of the mammalian Argonaute proteins that are known to bind to small RNAs are localized to these structures [2,3]. Target mRNAs also entered P-/GW-bodies in a manner that was dependent upon their recognition by small RNAs [2]. Additionally, Argonaute proteins were shown to bind to components of the de-capping complex that reside, at least in part, in P-/GW-bodies [2]. Finally, exogenously added miRNAs can be seen to accumulate within P-/GW-bodies [15].

Results presented here strengthen the correlation and begin to build a functional link between P-/GW-bodies and small RNA-dependent silencing. An analysis of Argonaute complexes revealed a physical interaction with GW182, a core component of P-/GW-bodies. Importantly, silencing of GW182 both disrupts these foci and attenuates suppression of microRNA reporters. Although de-repression was not complete, neither was silencing of GW182 or loss of foci (see Fig. 2b). Thus, it remains possible that a complete ablation of GW182 and P-/GW-bodies might abrogate miRNA function. A similar correlation between P-/GW-body localization and suppression emerged from an analysis of Argonaute proteins that recognize their target by a direct RNA-protein interaction and without the need for a small RNA. Introduction of mutations into the PAZ domains of these λN-fusion proteins both alters their subcellular localization and prevents them from repressing their targets. These results are consistent with observations, reported while this manuscript was under consideration, from *C. elegans* wherein mutations in a homolog of GW182 gave phenotypes very similar to those resulting from defects in core miRNA pathway components and in *Drosophila*, where silencing of a GW182 homolog had effects on both miRNA and siRNA function [26,27].

Recent studies suggest P-bodies represent a pool of translationally repressed mRNPs, which is in equilbrium with the translating pool [18,28]. Thus, the translation status of an mRNA could reflect the competition between interactions favoring assembly of a translation complex and interactions favoring assembly of a translationally repressed mRNP that can aggregate into P-bodies. Given this, an integrated hypothesis it that miRNAs and associated proteins, minimally Argonaute and GW182, alter this equilibrium, either by directly promoting assembly of the repressed mRNP, and/or by directly inhibiting the function of specific translation initiation factors. This model envisions situations in which provision of strong translation promoting signals could override the function of microRNAs, perhaps in a regulated fashion, to retain mRNAs in an actively translating pool.

Interestingly, other mRNA specific translation repression mechanisms that have been correlated with P-bodies require a combined series of events to achieve repression [28]. For example, in *Drosophila* the *Oskar* mRNA assembles a tripartite complex wherein eIF-4E is bound to the cap, but prevented from interaction with eIF-4G by the eIF-4E binding protein *Cup*. Cup is delivered to the mRNA by *Bruno* binding the 3' UTR [29]. Despite the presence of this complex, efficient repression of the *Oskar* mRNA during early development requires the *Drosophila* Me31b protein, whose homolog in yeast and mammals contributes to targeting bulk mRNAs to translational repression and P-bodies [28,30].

Any of the aforementioned models is hard pressed to explain the effects that are seen on silencing by siRNAs that direct mRNA cleavage. One possibility is that product release and turnover of RISC occurs only once the complexes have translocated to P-bodies. Irrespective of what model is considered, emerging links between the RNAi machinery and specific cellular locales suggest that the process can no longer be viewed solely from a biochemical perspective without consideration of the impact that subcellular compartmentalization may have on the assembly and activity of RISC.

Methods

DNA constructs

HA- and Myc-tagged Ago expression plasmids were as described in [2,6]. Ago2 PAZ mutants were subcloned into the LamdaN-HA-fusion vector as described in [25]. GFP-tagged GW182 plasmid was as described in [23]. The miRNA, siRNA and tethering mRNA reporters were as described in [24] and [25].

Cell culture and transfection

Human U2-OS, HeLa and 293 cells were cultured in DMEM (10% FBS) at 37°C with 5% CO_2. Cell transfections were carried out using Mirus TransIT reagent for DNA plasmids and Invitrogen Oligofectamine reagent for siRNAs. Control siRNA, CXCR4 siRNA and siRNAs targeting Dcp2, Xrn1 and GW182 were purchased from Dharmacon. Procedures for immunoprecipitation, immunoblotting and immunoflurescence were described previously [2, 6]. Dual luciferase assays were performed as directed by the manufacturer (Promega).

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Literature Cited

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97. [PubMed: 14744438]
2. Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol 2005;7:719–23. [PubMed: 15937477]
3. Sen GL, Blau HM. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nat Cell Biol 2005;7:633–6. [PubMed: 15908945]
4. Ambros V. The functions of animal microRNAs. Nature 2004;431:350–5. [PubMed: 15372042]
5. Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004;431:343–9. [PubMed: 15372041]
6. Liu J, et al. Argonaute2 is the catalytic engine of mammalian RNAi. Science 2004;305:1437–41. [PubMed: 15284456]
7. Meister G, et al. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell 2004;15:185–97. [PubMed: 15260970]
8. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 1993;75:855–62. [PubMed: 8252622]
9. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843–54. [PubMed: 8252621]
10. Lim LP, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005;433:769–73. [PubMed: 15685193]
11. Bagga S, et al. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 2005;122:553–63. [PubMed: 16122423]
12. Kim J, et al. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. Proc Natl Acad Sci U S A 2004;101:360–5. [PubMed: 14691248]
13. Nelson PT, Hatzigeorgiou AG, Mourelatos Z. miRNP:mRNA association in polyribosomes in a human neuronal cell line. Rna 2004;10:387–94. [PubMed: 14970384]
14. Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 1999;216:671–80. [PubMed: 10642801]
15. Pillai RS, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 2005;309:1573–6. [PubMed: 16081698]
16. Sheth U, Parker R. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science 2003;300:805–8. [PubMed: 12730603]
17. Andrei MA, et al. A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. Rna 2005;11:717–27. [PubMed: 15840819]
18. Teixeira D, Sheth U, Valencia-Sanchez MA, Brengues M, Parker R. Processing bodies require RNA for assembly and contain nontranslating mRNAs. Rna 2005;11:371–82. [PubMed: 15703442]
19. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 2001;293:1146–50. [PubMed: 11498593]
20. Chendrimada TP, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature. 2005
21. Eystathioy T, et al. A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. Mol Biol Cell 2002;13:1338–51. [PubMed: 11950943]
22. Eystathioy T, et al. The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. Rna 2003;9:1171–3. [PubMed: 13130130]
23. Yang Z, et al. GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation. J Cell Sci 2004;117:5567–78. [PubMed: 15494374]
24. Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. Genes Dev 2003;17:438–42. [PubMed: 12600936]
25. Pillai RS, Artus CG, Filipowicz W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. Rna 2004;10:1518–25. [PubMed: 15337849]

26. Ding L, Spencer A, Morita K, Han M. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell 2005;19:437–47. [PubMed: 16109369]

27. Rehwinkel J, Behm-Ansmant I, Gatfield D, Izaurralde E. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. Rna. 2005

28. Coller J, Parker R. General Translational Repression by Activators of mRNA Decapping. Cell 2005;122:875–886. [PubMed: 16179257]

29. Nakamura A, Sato K, Hanyu-Nakamura K. Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Dev Cell 2004;6:69–78. [PubMed: 14723848]

30. Nakamura A, Amikura R, Hanyu K, Kobayashi S. Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. Development 2001;128:3233–42. [PubMed: 11546740]

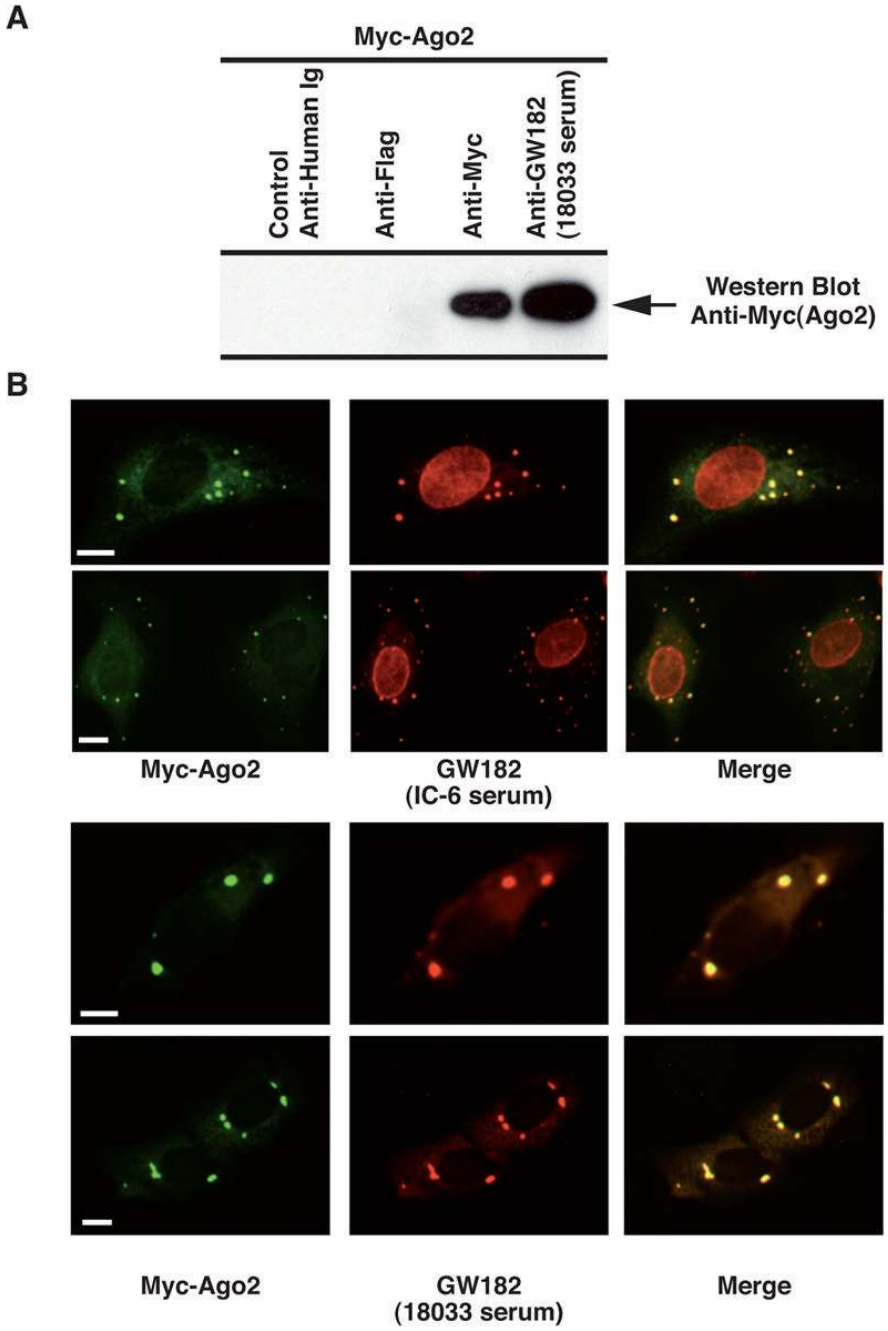

Figure 1. Argonaute proteins interact with GW182, a component of mammalian P-/GW- bodies

(a) Myc-tagged Ago2 protein was expressed in 293 cells. Immunoprecipitates, prepared using a human autoantiserum (18033 serum) that recognizes GW182 or control antibodies, were examined by Western blotting with an anti-myc antibody. **(b)** Argonaute proteins were visualized using and anti-myc epitope antibody and an Alexa Fluor 488 (green) secondary antibody. GW182 was visualized using a human autoantiserum (IC-6 serum or 18033 serum) and an Alexa Fluor 594 (red) secondary antibody. The scale bar indicates 10 μm.

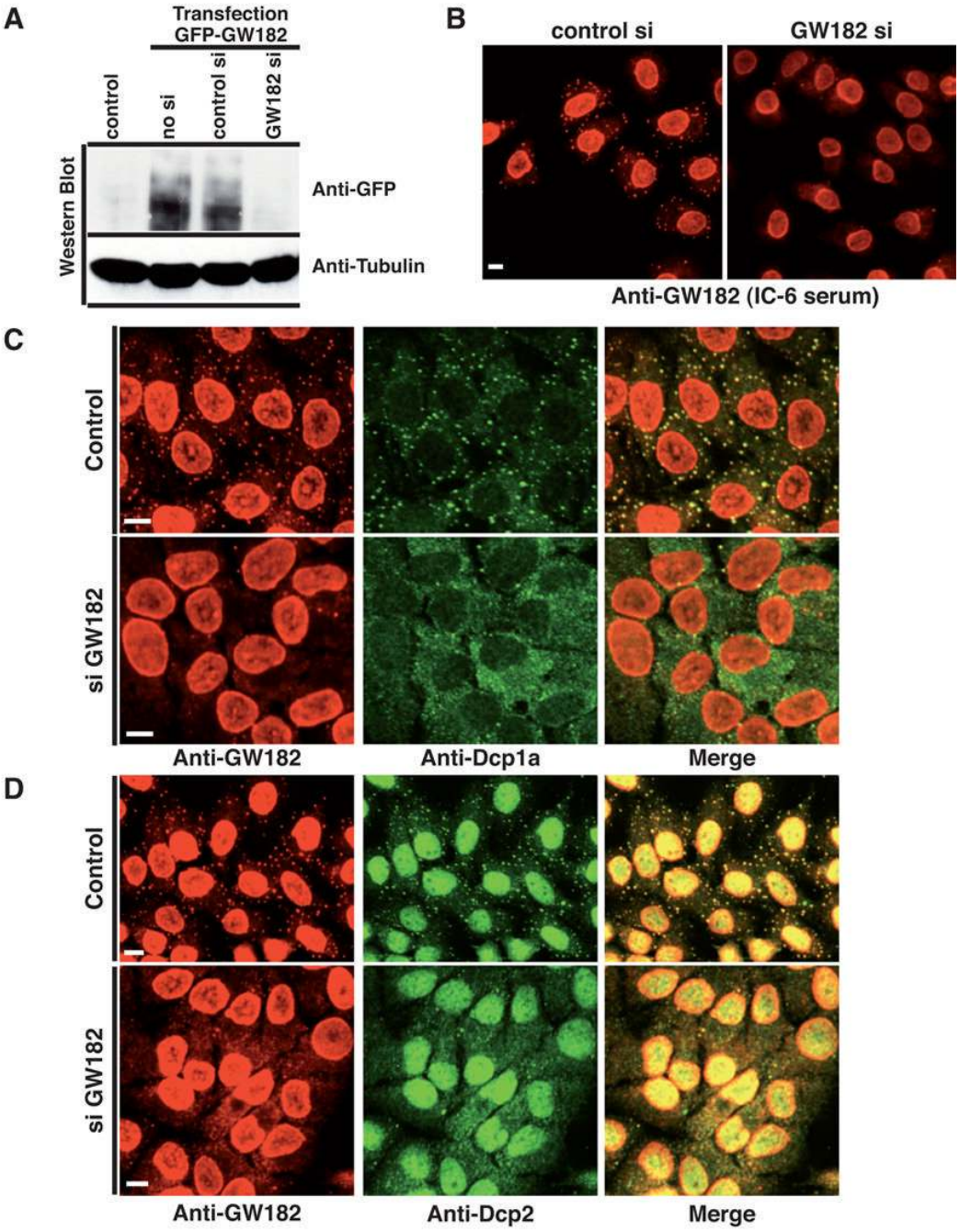

Figure 2. Suppression of GW182 disrupts P-/GW bodies

(a) GFP-GW182 protein was expressed in HeLa cells. The effect of a co-transfected siRNA against GW182 was examined by western blotting with an anti-GFP antibody. **(b)** Transfection of siRNA against GW182 in HeLa cells reduces the number and size of the P-/GW- bodies examined by indirect immunofluorescence microscopy using the IC-6 serum which recognizes GW182 protein. **(c)** Suppression of GW182 expression in HeLa cells reduces the number of Dcp1 foci formation revealed by staining with an anti-Dcp1a antibody. Staining with a human autoantisera that recognizes GW182 (IC-6) is shown for comparison. **(d)** Suppression of GW182 expression in HeLa cells reduces the Dcp2 foci formation revealed by staining with

an anti-Dcp2 antibody. In this case, only the IC-6 antiserum was used to highlight GW182 localization. The scale bar indicates 10 μm.

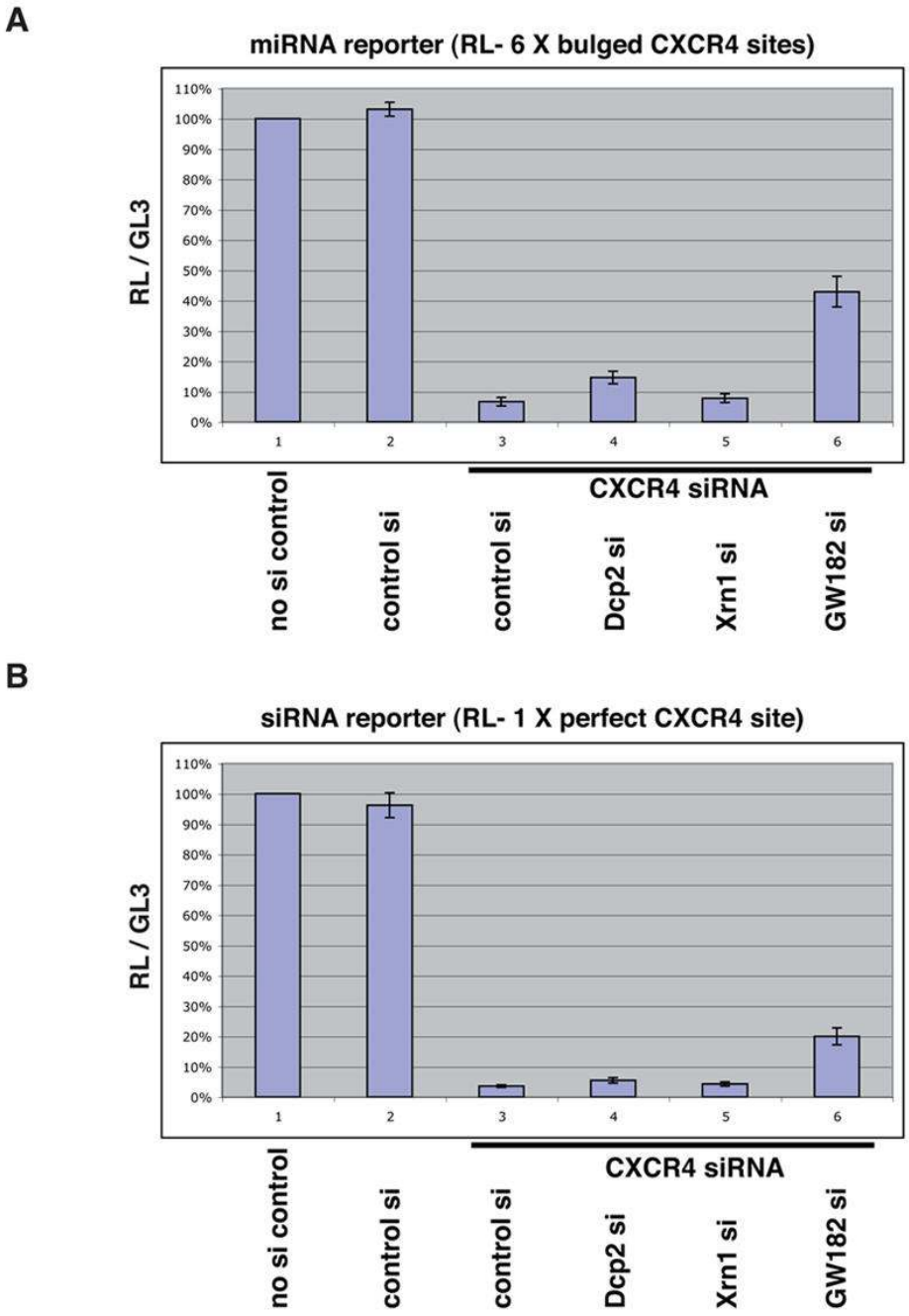

Figure 3. Suppression of GW182 expression impairs gene silencing

(a) Cells were transfected with a miRNA reporter in the presence or absence of the miRNA mimetic CXCR4 siRNA as indicated. Also included were either control siRNAs or siRNAs that suppress Dcp2, Xrn1 or GW182, as indicated. In all cases, transfection rates were normalized using a co-delivered firefly luciferase plasmid. **(b)** The experiment was carried out similarly to (**a**) except that the reporter contained a single perfect binding site for the CXCR4 siRNA.

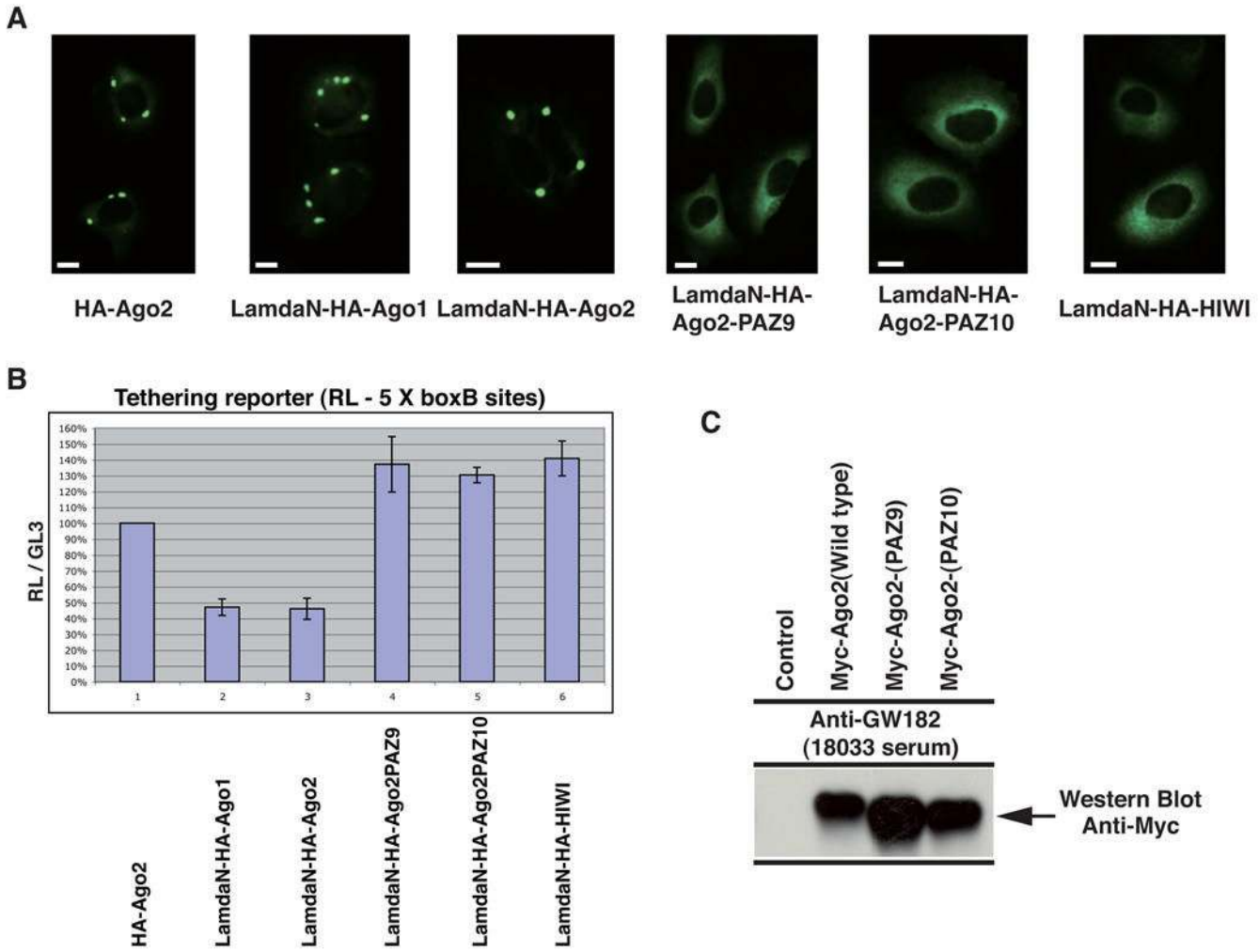

Figure 4. Localization of Argonaute proteins in the P-/GW- bodies is required for suppression of a tethering reporter in small-RNA independent manner

(a) Localization of λN-Ago fusion proteins in HeLa cells. HA epitope tagged fusions between Lamda N protein and Ago1 or Ago2 were expressed in HeLa cells. These localize to discrete cytoplasmic foci as shown by staining with FITC-conjugated anti-HA. A HIWI fusion protein fails to accumulate in these foci. Mutations within the PAZ domain (PAZ9 or PAZ10) disrupt small RNA binding as previously shown [2]. These same mutations disrupt the discrete localization of the λN-Ago fusion proteins. (b) A Renilla luciferase reporter containing 5 BoxB sites, that bind lamdaN, was transfected into HeLa cells. Various Ago fusion proteins were co-delivered as indicated. Also, in each case Renilla activity was normalized to a co-transfected firefly luciferase plasmid. As a control, An HA-epitope-Ago2 fusion that does not bind the boxB sites within the reporter was used to set the 100% level in the assay. λN-fusions to either Ago1 or Ago2 or to mutant Ago2 proteins carrying 9 or 10 mutations in the PAZ domain were tested for their ability to repress as indicated. As was previously shown lamdaN-HIWI was inert in this assay. (c) PAZ mutant Ago2 proteins retain the ability to associate with GW182 protein. The interaction between the mutants and GW182 was examined by immunoprecipitation with 18033 serum followed by western blotting with an anti-myc antibody. The scale bar indicates 10 μm.