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

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In animals, the majority of microRNAs regulate gene expression through the RNA interference (RNAi) machinery without inducing small-interfering RNA (siRNA)-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 that prevent Argonaute proteins from localizing in P-/GW-bodies prevent translational repression of mRNAs even when Argonaute is tethered to its target in a siRNA-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 on two factors. The first factor is the degree of complimentarity between the siRNA 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 in 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, whereas 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 siRNA-target interaction. The outcome of such events is currently 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 *Caenorhabditis elegans* have indicated that changes in mRNA abundance have been observed for the proposed targets of several microRNAs[10,11]. Additionally, several studies have detected both bulk microRNAs and some mRNA targets on polysomes, indicating 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].

Recent observations have also led to an alternative model for silencing by microRNAs 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], but mRNA targets of microRNAs become similarly localized in a manner that depends both on the presence of the microRNAs and on 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 crucial to examine the functional significance of the connections between P-/GW-bodies and the RNAi pathway.

To investigate the mechanisms of microRNA-mediated repression, we have searched for Argonaute-interacting proteins using MudPIT (Multidimensional Protein Identification Technology) analysis of immunoaffinity-purified Argonaute-1 (Ago1) and Argonaute-2 (Ago2) complexes. We recovered a number of previously identified Argonaute binding proteins, including HSP90, Dicer, TRBP and DCP1 (refs 2,6,19,20). Additionally, we have 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 (data not shown).

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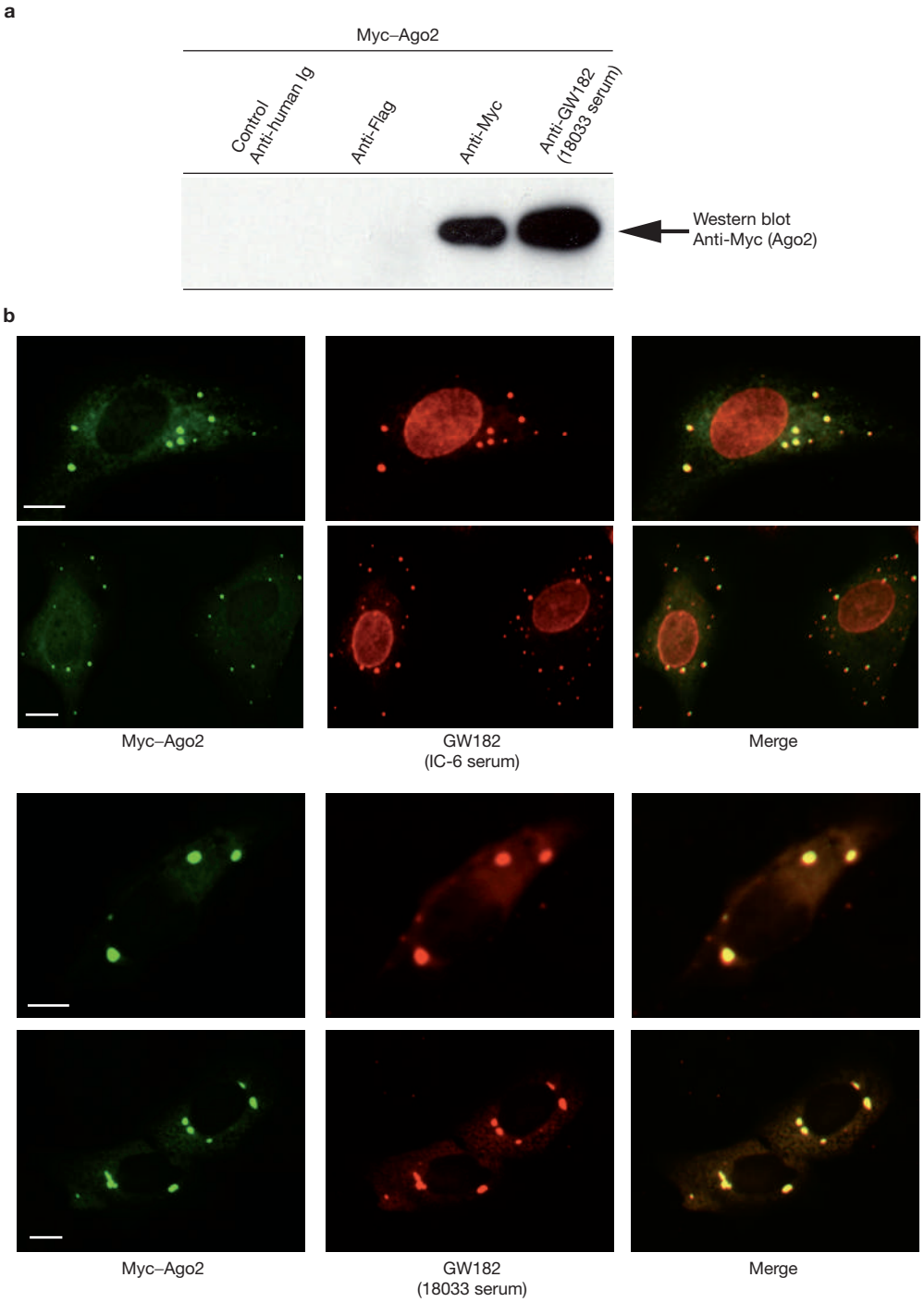

Figure 1 Argonaute proteins interact with GW182, a component of mammalian P-/GW-bodies. (**a**) Myc-tagged Ago2 protein was expressed in 293 cells. Immunoprecipitates, prepared using a human autoantiserum (18033 serum) that recognizes GW182 or control antibodies, were examined by western blotting with an anti-Myc antibody. (**b**) Argonaute proteins were visualized using 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. Scale bar, 10 μm.

GW182 is present in discrete cytoplasmic foci wherein it co-localizes with the de-capping complex[21,22]. To determine whether 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 showed substantial overlap (Fig. 1b), indicating 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 siRNAs 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]

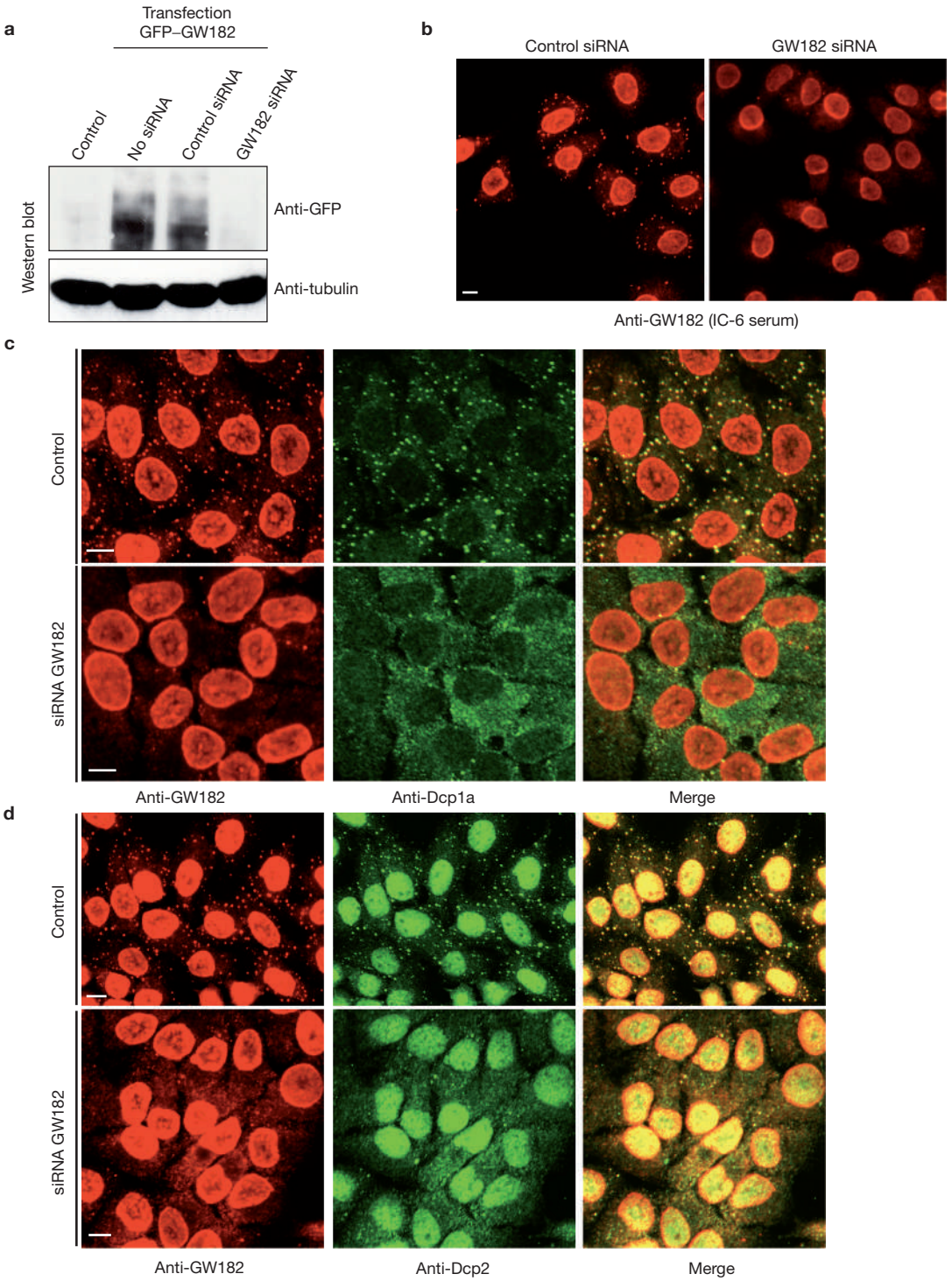

Figure 2 Suppression of GW182 disrupts P-/GW-bodies. (**a**) GFP–GW182 protein was expressed in HeLa cells. The effect of a co-transfected small interfering RNA (siRNA) against GW182 was examined by western blotting with an anti-GFP (green fluorescent protein) antibody. (**b**) Transfection of siRNA against GW182 in HeLa cells reduces the number and size of the P-/GW-bodies, as 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, as 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, as revealed by staining with an anti-Dcp2 antibody. IC-6 antiserum was used to highlight GW182 localization. Scale bar, 10 μm.

(Fig. 2b, c, d). By contrast, siRNAs against Dcp2 effectively reduced Dcp2 protein levels, but did not have an impact on the number of GW182 foci observed. In each case a second siRNA was also used to verify the phenotype (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 had an impact on siRNA-directed gene silencing. We first examined a cleavage-independent repression event in which a *CXCR4*

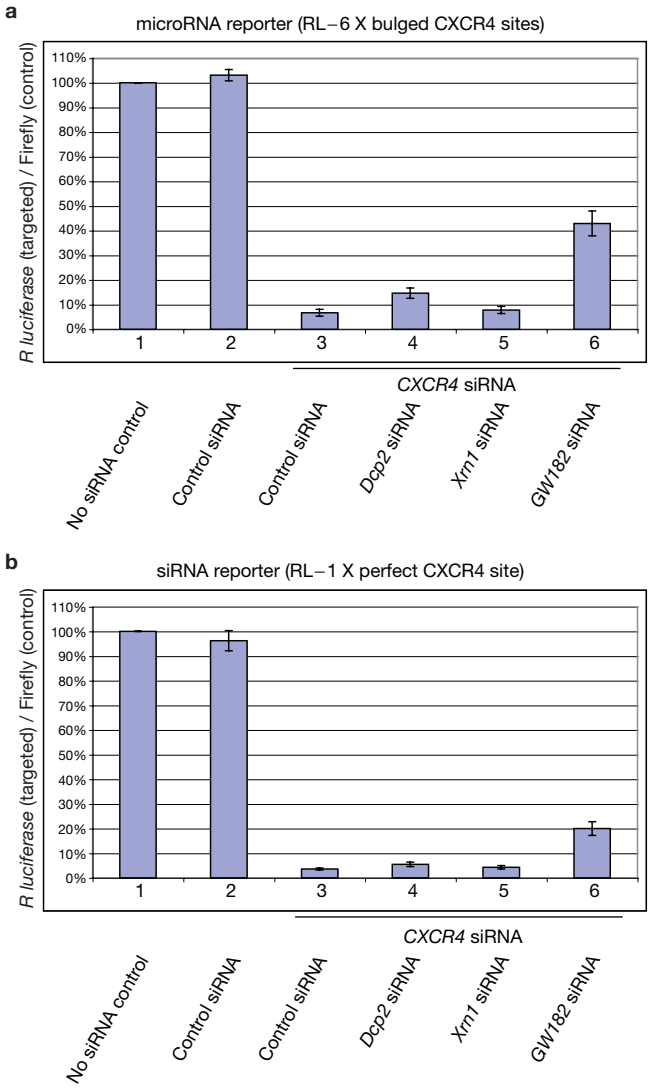

Figure 3 Suppression of GW182 expression impairs gene silencing. (**a**) Cells were transfected with a microRNA reporter *R. luciferase* mRNA (RL) in the presence or absence of the miRNA mimetic CXCR4 siRNA, as indicated. Also included were either control siRNAs or siRNAs that suppress Dcp2, Xrn1 or GW182, as indicated. In all cases, transfection rates were normalized using a co-delivered firefly luciferase (GL3) plasmid. (**b**) The experiment was carried out similarly to (**a**), except that the reporter contained a single, perfect binding site for the *CXCR4* siRNA.

siRNA can bind to six 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 that were used for this assay (Fig. 3a). Repression of GW182 but not another P-body protein, XRN1, impaired the ability of the microRNA 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 (see Supplementary Information, Fig. S1). All of these outcomes correlated with the inhibition of GW182 expression by the siRNA and with a reduction in the appearance of P-/GW-bodies (Fig. 2 and data not shown). Notably, the pattern of Ago1 and Ago2 localization was also disrupted following repression of GW182 (data not shown). These data demonstrate that GW182 has a functional role in RISC-mediated silencing, which is correlated with the maintenance of P-bodies.

Cleavage-independent suppression of an mRNA target has previously been accomplished by tethering an Argonaute protein to an mRNA 3′ untranslated region (UTR) in a manner that is independent of the siRNA-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 Argonaute proteins being able to function in the RNAi pathway in a similar way to their siRNA-directed counterparts. First, λN-fused Argonaute proteins can complement the silencing defect that is observed in *Ago2*-knockout mouse embryonic fibroblasts (see Supplementary Information, Fig. S2). Second, Argonaute proteins that can suppress their targets through direct protein–mRNA interactions localize to P-/GW-bodies in a manner that is similar to the native proteins (Fig. 4a). Our previous work indicated that a series of point mutations in the PAZ domain could prevent siRNA 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 proteins (Fig. 4a). As these Argonaute proteins recognize their targets in a siRNA-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 that was fused at the amino terminus to the λN protein reduced *Renilla luciferase* expression by ~twofold, as long as the mRNA contained the λN binding site (Fig. 4). In addition, as is consistent with previous studies, a λN fusion protein 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 siRNA binding, neither localized to P-/GW-bodies nor repressed a boxB-containing target mRNA, despite maintaining interactions with the target (Fig. 4, and data not shown). Notably, Ago2–PAZ9 and Ago2–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 that a relationship exists 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 siRNAs are localized to these structures[2,3]. Target mRNAs also entered P-/GW-bodies in a manner that was dependent on their recognition by siRNAs[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 siRNA-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 the 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

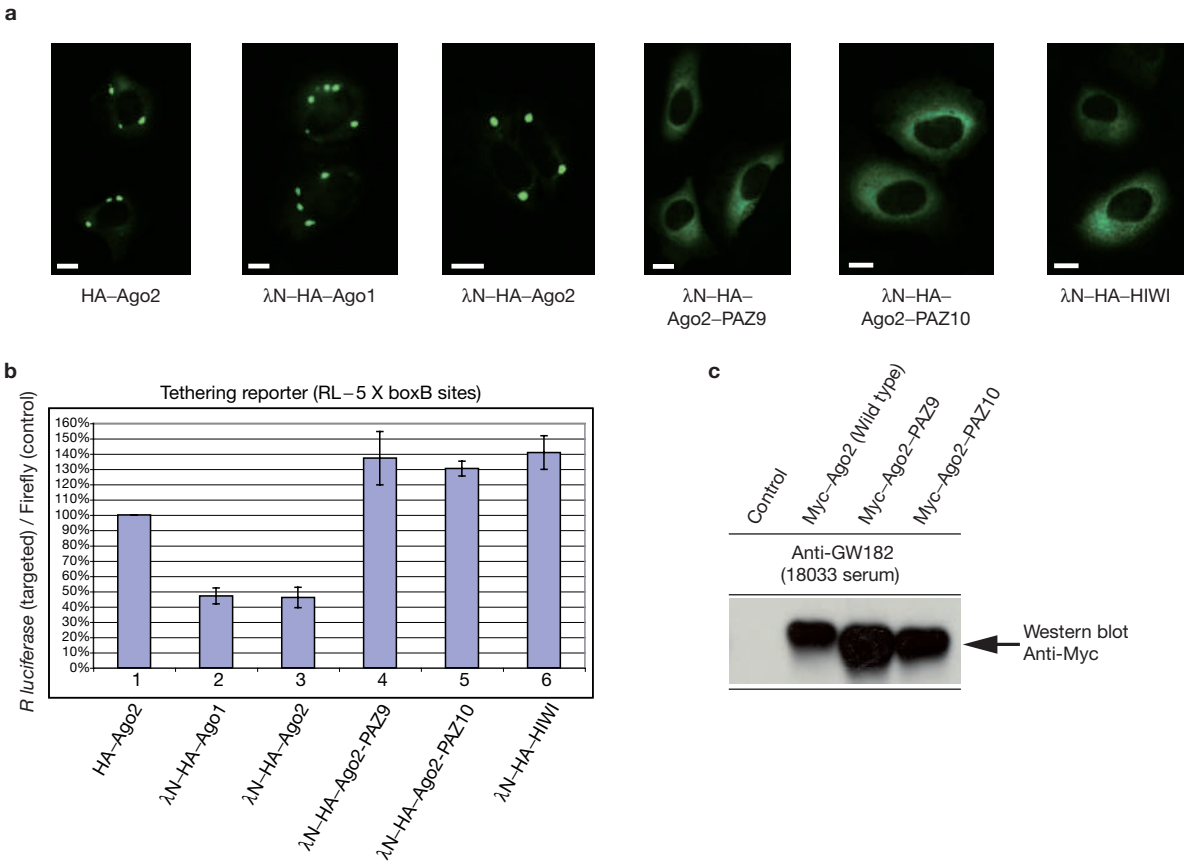

Figure 4 Localization of Argonaute proteins in the P-/GW-bodies is required for suppression of a tethering reporter in siRNA-independent manner. (**a**) Localization of λN–Ago fusion proteins in HeLa cells. HA-epitope-tagged fusions between λN protein and Ago1 or Ago2 were expressed in HeLa cells. These localize to discrete cytoplasmic foci, as shown by staining with FITC-conjugated anti-HA. A HIWI fusion protein failed to accumulate in these foci. Mutations within the PAZ domain (PAZ9 or PAZ10) disrupted small RNA binding, as previously shown [2]. These same mutations disrupted the discrete localization of the λN–Ago fusion proteins. (**b**) A *R. luciferase* reporter (RL) containing five boxB sites that bind λN were transfected into HeLa cells. Various Ago-fusion proteins were co-delivered, as indicated. In addition, *R. luciferase* activity was normalized to a co-transfected firefly luciferase (GL3) plasmid in each case. As a control, a 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-fusion proteins 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, λN–HIWI was inert in this assay. (**c**) PAZ mutant Ago2 proteins retain the ability to associate with GW182 proteins. The interaction between the mutants and GW182 was examined by immunoprecipitation with 18033 serum followed by western blotting with an anti-Myc antibody. Scale bar, 10 μm.

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 siRNAs. 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 that were reported while this manuscript was under consideration, that mutations in *C. elegans* homologue of GW182 produced phenotypes similar to those resulting from defects in core miRNA-pathway components and in *Drosophila melanogaster*, in which silencing of a GW182 homologue had effects on both miRNA and siRNA function[26,27].

Recent studies indicate that P-bodies represent a pool of translationally repressed messenger ribonucleoproteins (mRNPs), which is in equilibrium with the translating pool[18,28]. Therefore, the translation status of an mRNA could reflect the competition between interactions that favour the assembly of a translation complex and interactions that favour the assembly of a translationally repressed mRNP that can aggregate into P-bodies. Given this, an integrated hypothesis is that microRNAs and associated proteins, such as Argonaute proteins 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 is prevented from interactions with eIF-4G by the eIF-4E binding protein Cup. Cup is delivered to the mRNA by *Bruno*, which binds the 3′ UTR[29]. Despite the presence of this complex, efficient repression of the *Oskar* mRNA during early development requires the *Drosophila* Me31b protein, the homologue of which contributes to targeting bulk mRNAs to translational repression and P-bodies in yeast and mammals[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 which model is considered, emerging links between the RNAi machinery and specific cellular locales indicate 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 Argonaute expression plasmids were as described previously[2,6]. Ago2 PAZ mutants were subcloned into the LamdaN–HA fusion vector as described previously[25]. GFP-tagged GW182 plasmid was as described previously[23]. The microRNA, siRNA and tethering mRNA reporters were as described previously[24,25].

Cell culture and transfection. Human U2-OS, HeLa and 293 cells were cultured in DMEM (10%) 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 (Lafayette, CO). Sequences of siRNAs (antisense strand) used in this study were as follows: GW182, UAGCGGACCAGACAUUUCUU; Dcp, UCUAUGACAAGGCAAUUUCUU; Xrn1, GGUAACUCCUUGCCAUGACdTdT. Procedures for immunoprecipitation, immunoblotting and immunoflurescence were described previously[2,6]. Dual luciferase assays were performed as directed by the manufacturer (Promega, Madison, WI).

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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ERRATUM

Owing to a technical error, the pages of this manuscript were originally mis-numbered by a 100 pages. This has now been corrected online. The corrected online manuscript is numbered 100 pages higher than the mis-numbered version.

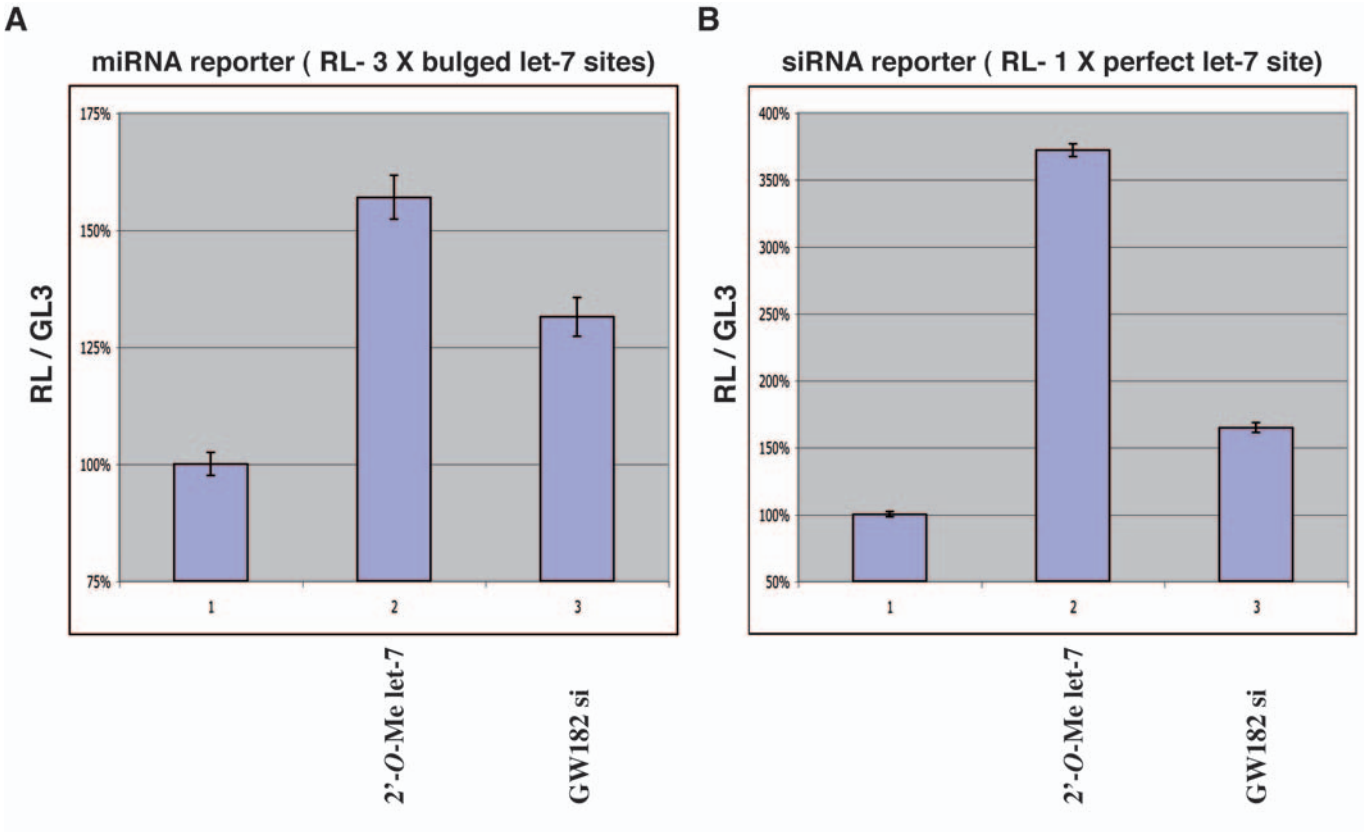

Figure S1 De-repression of a let-7 responsive reporter by suppression of GW182. (A) HeLa cells were co transfected with a Renilla luciferase expression plasmid that contains 3 bulged binding sites for the endogenous let-7 microRNA [15] (bar 1). As a control for de-repression, this reporter was co-transfected with a 2'-O-methyl RNA oligonucleotide complementary to the let-7 miRNA as a decoy (bar 2). To test the effect of GW182 suppression, the reporter was co-transfected with a GW182 siRNA. In all cases, the signal from Renilla luciferase was normalized to a co-transfected firefly luciferase expression plasmid. (B) Experiments in B were exactly as in A except that the reporter contained a single site that is perfectly complementary to the endogenous let-7 miRNA [15].

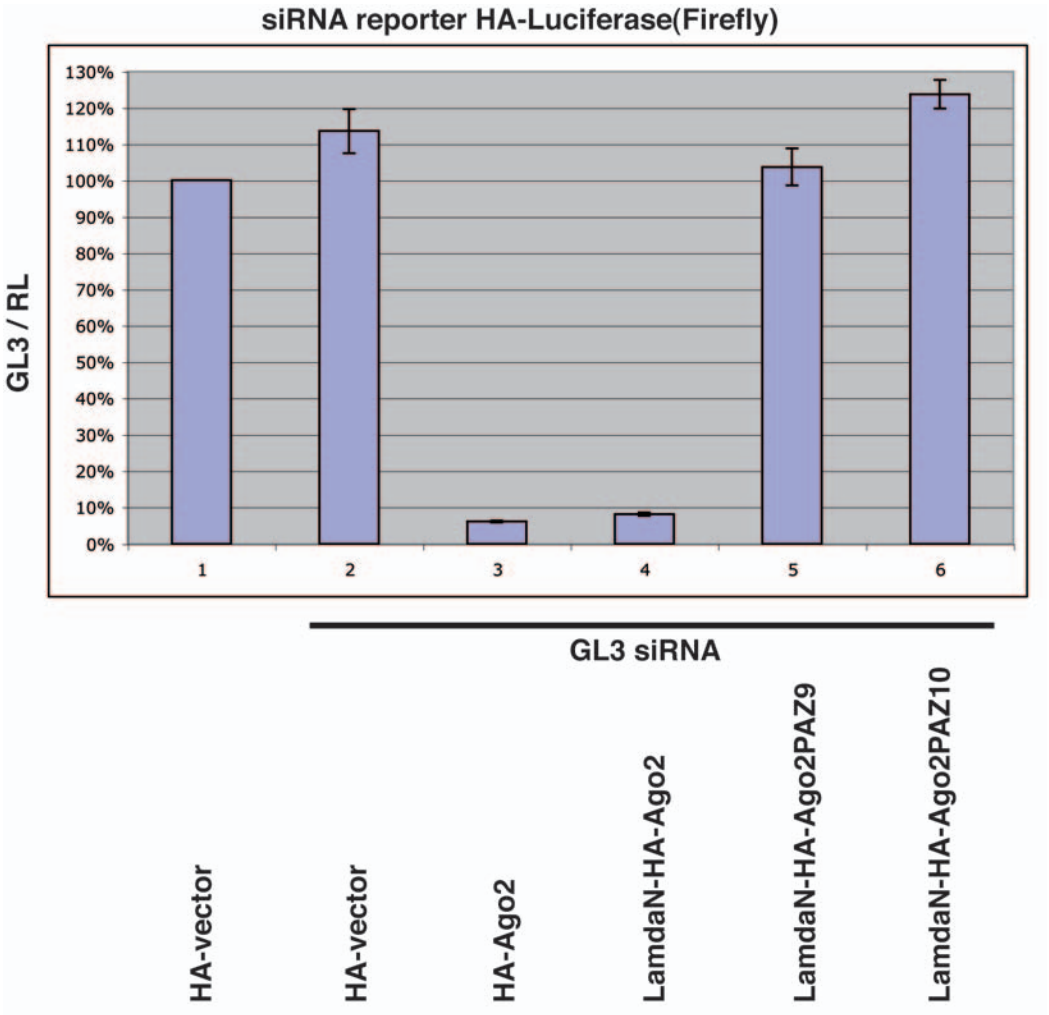

Figure S2 Function of λN-Ago fusions in the RNAi pathway. Ago2 -/- MEF were transfected with a combination of Renilla and firefly luciferase expression plasmids with or without (as indicated) an siRNA that targets firefly luciferase mRNA. Because of the absence of a catalytically competent Ago protein, this siRNA fails to suppress. Suppression can be rescued by transfection with an expression vector that directs production of HA-tagged Ago2 or a λN-Ago fusion protein. However, λN-Ago fusions that contain a series of 9 or 10 mutations in the PAZ domain cannot rescue, presumably due to their inability to bind siRNAs.