Quantifying Argonaute proteins in and out of GW/P-bodies: Implications in microRNA Activities

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Abstract

MicroRNAs (miRNAs) are a class of ~22nt non-coding RNAs that regulate the translational potential and stability of mRNAs. Though constituting only 1–4% of human genes, miRNAs are predicted to regulate more than 60% of all mRNAs. The action of miRNAs is mediated through their associations with Argonaute proteins and mRNA targets. Previous studies indicated that though the majority of Argonaute proteins is diffusely distributed in the cytoplasm, a small fraction is consistently observed to be concentrated in a cytoplasmic compartment called GW/P-bodies. In this chapter, we will provide a quantitative and dynamic view of the subcellular localization of miRNA function, followed by a discussion on the possible roles of PBs in miRNA silencing.

10.1 Introduction

10.1.1 microRNA

microRNAs (miRNAs) are a class of ~22nt short non-coding RNAs that regulate translational potential and stability of mRNAs in the cytoplasm (Bartel 2009; Fabian et al. 2010; Jackson and Standart 2007; Leung and Sharp 2006). miRNA action is pervasive: they are predicted to regulate over 60% of all mammalian mRNAs (Friedman et al. 2009) and constitute a sizable class of regulators, e.g. ~ 2237 different miRNAs were identified in humans (miRBase release 19 in August 2012; http://www.mirbase.org/), even outnumbering kinases and phosphatases.

miRNAs are first transcribed as primary transcripts (pri-miRNA), which fold into hairpin structures and are subsequently processed – first by Drosha in the nucleus and then by Dicer in the cytoplasm. These processing steps result in a ~22 nucleotide duplex in which one strand, miR*, is degraded while the mature miRNA is selectively loaded into the RNA-induced silencing complex (RISC) complex where Argonaute is the key protein that binds the mature miRNA (Figure 10.1).

10.1.2 Argonaute

In animals, most of the miRNAs only require a short "seed" region (2nd–7th position of miRNA) of perfect complementarity with the mRNA target to trigger translation inhibition and/or acceleration of mRNA decay (Bartel 2009). On the other hand, if the miRNA is

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perfectly complementary to its mRNA targets, this results in mRNA cleavage between the corresponding 10th and 11th position of miRNAs. This cleavage event rarely occurs for animal miRNAs, yet this cleavage function is still intact in animals. This is illustrated by the use of chemically synthesized small interfering RNAs (siRNAs) to knock down specific genes in mammals through this mechanism. In fact, miRNA and siRNA silencing pathways share some common activities. For example, endogenous miRNAs can direct cleavage of exogenously expressed targets that contain sites of extensive complementarity, and siRNAs can function as miRNAs in mediating translational repression (Doench et al. 2003; Hutvagner and Zamore 2002). In humans, there are four Argonaute proteins, Ago1–4, which can all mediate translation repression/mRNA decay, but only Ago2 can trigger miRNA/ siRNA-directed cleavage (Hock and Meister 2008). These four Argonaute proteins share a common bipartite structure (Parker 2010), where the N-terminal half containing a PAZ domain that binds the 3′ end of the miRNA and a C-terminal half containing a Mid domain that binds the 5′ end of the miRNA as well as a PIWI domain that binds to the "seed" region of the miRNA that targets mRNA (Figure 10.1). Moreover, the C-terminal half also binds GW182, a downstream effector to mediate miRNA silencing (Eulalio et al. 2009).

To shed light on the potential mechanisms of miRNA functions, we and other groups have used immunostaining and live cell imaging coupled with genetics tools to dissect the localization of Argonaute proteins in different cellular conditions. In most cases examined, but with a few notable exceptions (Gibbings et al. 2009; Lee et al. 2009; Vasudevan and Steitz 2007), all Argonaute members are enriched in a cytoplasmic structure called GW- or P-bodies (PBs). As reviewed elsewhere in this book, PBs, are devoid of any translation machineries, but are enriched with silenced mRNAs, translational repressors and RNA decay factors that are involved in nonsense mediated decay, AU-rich element decay or miRNA silencing. In this chapter, we will first review the data on Argonaute localization, specifically focusing on its quantitation and dynamics. This is followed by a discussion on the possible roles of PBs in the context of miRNA silencing.

10.2 Quantitation of Argonaute, miRNA and mRNA target localization

10.2.1 Quantitation of Argonaute

To identify where miRNA-mediated silencing occurs in cells, we chose to track the localization of Ago2, the signature component of RISC that can mediate translation repression, acceleration of mRNA target decay as well as miRNA/siRNA-mediated cleavage (Leung et al. 2006). Several HeLa cell lines that stably expressed Green Fluorescent Protein (GFP)-tagged Ago2 were generated and the one with the lowest expression was chosen for quantitation to avoid overexpression artifacts. Consistent with other observations, GFP-Ago2 was enriched at punctate structures that were stained positive for Dcp1a, which is a marker for PBs, and the GFP-Ago2 intensity at PBs is 10-fold higher than the cytoplasm (Figure 10.2). However, when the volume of PBs and the total cytoplasm were taken into account, only ~1% of cytoplasmic GFP-Ago2 was localized at PBs whereas the majority was diffusely distributed elsewhere in the cytoplasm.

To confirm these results, endogenous localization of Argonaute was examined with 4 different antibodies. Surprisingly, though Dcp1a-positive PBs are present in the parental HeLa cell line, < 10% of PBs are co-stained with Argonaute. Several other groups also reported that only a minority of PBs also stain positive for Argonaute. James and colleagues reported that on average only 1 in 7 PBs were stained positively with Ago2 in U2OS cells (James et al. 2010). Vasudevan and Steitz found that Ago2 though co-localized with Dcp1a in HEK293 cells when grown in serum-containing medium, Ago2 dispersed to smaller bodies that no longer co-localized with PBs upon serum starvation (Vasudevan and Steitz 2007). Moreover, homologues of miRNA-binding Argonautes in *Drosophila* S2 cells

(dAgo1) and *Caenorhabditis elegans* epidermal/neuronal cells (Alg1/2) were also found to be diffusedly distributed in the cytoplasm (Behm-Ansmant et al. 2006; Ding et al. 2005). However, these Argonaute members localized to punctate PB-like structures in the cytoplasm only when exogenous Ago or GW182 was ectopically expressed. Similarly, GW182 is not always concentrated as punctate structures and such localization depends on the cell cycle and growth conditions (Yang et al. 2004). Together, this data suggest that the majority of miRNA-mediated silencing is not likely at PBs, but rather its localization seems to be sensitive to the endogenous level of Argonaute and its associated proteins.

10.2.2 Quantitation of mature miRNAs and siRNAs

Several approaches have been used to identify and quantitate the specific localization of mature miRNAs or siRNAs in the cytoplasm. Pillai and colleagues microinjected an *in vitro* transcribed, fluorescently labeled artificial precursor of *let-7a* into nuclei and found that 26% of the cytoplasmic fluorescent signals localized at (18%) or near (8%) PBs (Pillai et al. 2005). However, both the active miRNA strand and the miR* strand of miRNA were equally labeled and therefore cannot easily be distinguished from each other. The signal observed at PBs could represent the accumulation site for miR* prior to degradation, rather than the active strand. Jakaymiw and colleagues labeled the antisense of a siRNA duplex and found that the labeled strand accumulated in PBs (Jakymiw et al. 2005). In this case, it is similarly difficult to be certain that the labeled antisense strand is the one loaded into the RISC as contrasted to the one targeted for decay. Bhattacharyya and colleagues performed *in situ* hybridization using locked nucleic acid (LNA) probes against a highly abundant miRNA, miR122, followed by signal amplification and the accumulation of signals was observed at PBs in hepatocytes (Bhattacharyya et al. 2006). Since the signal has been amplified in a non-linear fashion, it is also difficult to estimate the actual amount of miRNA localized in PBs. Nevertheless, these studies suggest that a fraction of mature miRNAs are present in PBs.

To avoid the ambiguous detection of inactive miRNA/miR* duplexes, or the miR* strand, and to quantitate the signal in the linear range, we have modified a well-characterized siRNA duplex that has been shown to function as a miRNA (Leung et al. 2006). The antisense strand is labeled with tetramethylrhodamine (TAMRA) at its 3′ end and the 5′ phosphate group of the passenger strand is substituted with a black hole quencher 2 (BHQ2) (Figure 10.3). In this way, the fluorescence is quenched in the siRNA duplex form, thereby enhancing the signal detection from the active single strand. Since the 5′ phosphate group is a prerequisite for RISC loading, the substitution of the phosphate group with the quencher presumably favors the selection of the labeled antisense strand for loading into the RISC complex. The siRNA duplex was electroporated into HeLa cells and their localization was examined after 56 hours, a period that allows the decay of endosomally localized siRNAs. Using this modified siRNA, the labeled antisense strand of siRNA was not enriched nor depleted at PBs compared with the cytoplasmic background; instead the signal was diffusively distributed throughout the cytoplasm. The lack of enrichment of siRNA at PBs in HeLa is not surprising given that <10% of PBs are positively stained with anti-Argonaute antibodies (Section 10.2.1). However, we recently found that this modified siRNA can be enriched in PBs in HeLa provided that the cell line overexpresses Ago2. This new data suggest that the level of miRNA/siRNA present in PBs is sensitive to the expression level of Argonaute.

On the other hand, to examine the localization of siRNA duplex, Jagannath and Wood labeled both strands with distinct fluorophores such that they are ~6 nm apart in the native double-stranded configuration (Jagannath and Wood 2009). Such close-proximity configuration resulted in Fluorescent Resonance Energy Transfer (FRET), which requires

<10 nm to occur, from the donor dye at the passenger strand to an acceptor dye at the antisense strand. Using this FRET approach, they found that the native siRNA duplex was also localized in PBs. Importantly, a control experiment was performed by transfecting an equal amount of two siRNA duplexes: (1) the same targeting siRNA duplex, but only the antisense strand was labeled with the acceptor fluorophore this time, and (2) a non-targeting siRNA that is labeled with the donor fluorophore at the passenger strand only. In this way, they delineated that the majority of the FRET signal actually came from the siRNA duplex, rather than from two individual, separated siRNA strands that were proximal by chance in PBs, given that this is physically feasible within such a small structure (~100–300 nm in diameter). The FRET signal was highest at 4 hour post-transfection and lost at 72 hour, yet, at this time point, significant repression was still observed. Consistent with our quantitation data, the miRNA/siRNA in PBs is not the only active fraction. Moreover, this data also raised the possibility that PBs could be the site where miR* and/or the passenger strand of siRNA accumulated.

10.2.3 Quantitation of mRNA targets

To localize repressed targets, Liu and colleagues used GFP-tagged MS2-binding proteins to track luciferase mRNA that has 24 copies of MS2-binding sites behind two tandemly arranged *let-7* binding sites (Liu et al. 2005b). The target was localized to PBs in a miRNA-dependent manner. Moreover, Pillai and colleagues quantitated the *in situ* hybridization signal of *let-7* targeted luciferase mRNA, and, similar to *let-7*, ~21% localized at (13%) or near (8%) PBs (Pillai et al. 2005). We also found similar localization of the mRNA targets at or near PBs, while the rest of the signal is diffusely distributed in the cytoplasm (Leung et al. 2006).

10.3 Dynamics of Argonaute and PBs

Fusion of GFP to a protein of interest not only visualizes the protein localization but also enables quantitative measurements of protein dynamics using photobleaching and photoactivation techniques as well as single-particle tracking. In this section, we will examine the kinetics of Argonaute localization in different cellular conditions, and then review data regarding the dynamics of PBs to understand its possible role in miRNA silencing.

10.3.1 Argonaute localization is sensitive to translation status

First, we examined the Argonaute localization upon limiting translation initiation by hippuristanol, an inhibitor of initiation factor eIF4A (Leung et al. 2006). Under this condition, while the intensity at PBs remains unchanged, 1.2–10.6% of GFP-Ago2 signal was localized in another cytoplasmic structure called stress granules (SGs; Figure 10.2). SGs are aggregates of stalled initiation complexes that are enriched with poly(A)+ mRNAs and RNA-binding proteins that regulate translation potential and stability of mRNAs (Leung and Sharp 2007; Anderson and Kedersha 2008). SG assembly is commonly induced upon multiple types of stress including oxidative stress, heat shock, viral infection and ischemia. Quantitatively, there was at least threefold more GFP-Ago2 localized to SGs than to PBs. Using our quencher-TAMRA based detection, we detected a 1.5 fold enrichment of miRNAs at SGs compared with the neighboring cytoplasmic signal, in contrast to no enrichment at PBs (Leung et al. 2006). Moreover, *in situ* hybridization showed that miRNA-repressed targets also accumulated in SGs (Leung et al. 2006). Given that the amount of Argonaute localized at PBs did not change over the course of hippuristanol treatment, those Argonaute relocalized to SGs (along with miRNA and their targets) are likely originated elsewhere from the diffuse cytoplasm.

Biochemical data consistently showed that Argonaute proteins are associated with polyribosomes (Kim et al. 2004; Nelson et al. 2004; Olsen and Ambros 1999; Seggerson et al. 2002), so we then tested whether GFP-Ago2 in SGs could dynamically exchange with these submicroscopic pools of ribosome-associated mRNAs (Leung et al. 2006). If Argonaute proteins at SGs are in dynamic equilibrium with those in polyribosomes, we would expect addition of another translation inhibitor emetine, a drug that stabilizes the association of mRNA and polyribosomes, will shift the exchanging pool toward the polyribosomes. Indeed, these pre-formed, Argonaute-positive SGs dissociate in the presence of emetine, but the signal at PBs remains unchanged under the same conditions. Therefore, Argonaute proteins in SGs possibly originate from, and dynamically exchange with, polyribosomes in the cytoplasm.

10.3.2 Kinetic behaviors of Ago2 and other PB components

Given the distinct behaviors of Argonaute at PBs and SGs in association with the polyribosomes in the diffuse cytoplasm, we further examined their kinetics using photobleaching (Leung et al. 2006). Photobleaching is a photo-induced alteration of a fluorophore that extinguishes its fluorescence irreversibly (Lippincott-Schwartz et al. 2003). GFP is an ideal tool to study protein dynamics because the chromophore has a high fluorescence yield and is resistant to photobleaching at low illumination. On the other hand, when excited by high illumination levels, the GFP fluorophore can be irreversibly photobleached. These properties were exploited to study the movement of non-bleached GFP fusion proteins into the bleached areas in a technique known as Fluorescence Recovery After Photobleaching (FRAP; Figure 10.4). Though Argonaute proteins at SGs are constantly exchanging with the diffuse cytoplasm, they exhibited much slower kinetics at PBs. The fluorescence at a single photobleached spot at an SG can be fully recovered to its initial intensity level within 6 minutes, whereas the photobleached spot at a PB never recovered during this period. Indeed, ~50% of Argonaute proteins were deemed "immobile" by FRAP analyses. Consistently, photoactivation experiments suggest that 80% of Ago2 remained at PBs, indicative of a very slow off-rate (Figure 10.4). The immobility observed could be because (1) Argonaute anchors to fixed molecules or forms aggregates that are restricted in movement and/or (2) Argonaute is confined to a subcellular region that cannot contribute to fluorescence recovery in a separate compartment. The second scenario seems to be unlikely as decapping enzyme co-activators Dcp1a, Dcp1b and Lsm6, all of which co-localize with Ago2 at PBs, exhibit fast recoveries (Aizer et al. 2008; Andrei et al. 2005; Kedersha et al. 2005; Leung et al. 2006). On the other hand, similar slow rates of exchange were observed at PBs for several Argonaute-associated proteins, including GW182, the cap-binding protein eIF4E and the decapping enzyme Dcp2 (Aizer et al. 2008; Andrei et al. 2005; Kedersha et al. 2005). Though one GW182 is able to physically associate with multiple Argonautes through protein-protein interactions (Takimoto et al. 2009), it is unlikely that GW182 anchors Argonaute to PBs. This is because two Argonaute mutants that are devoid of their PB localization were previously shown to be associated with GW182 at the same or greater affinity when compared with the wild-type (Liu et al. 2005a). Instead, Argonaute may anchor to other fixed molecules or form aggregates with other proteins that are restricted in movement at PBs.

10.3.3 Dynamics of PBs

The understanding of Argonaute protein kinetics would not be completed without the dynamic picture of the PB structure itself. Aizer and colleagues followed the movement of PBs in multiple U2OS cell lines that stably expressing GFP-Dcp1a, RFP-Dcp1b or GFP-Dcp2 at a low level using single-particle tracking technique and subsequent mean square displacement (MSD) analysis (Aizer et al. 2008). MSD analyses suggest that only few PBs were moving in a directional manner on microtubules while most were moved by diffusion

yet confined to an area. In all three different stable cell lines, ~55% of PBs were confined to an area ranging up to 2μm^2 and ~30% between 2–5μm^2. The diffusion coefficient, which describes the relative speed at which a particle moves within a defined area, was measured to be in the range of other cytoplasmic organelles (10^{-3} to 10^{-2} μm^2/sec). Such slow diffusion was commonly ascribed to the crowding cytoplasmic environment (Luby-Phelps 2000). In contrast, cytoplasmic mRNPs diffuse at a much faster rate (0.1 μm^2/sec) (Fusco et al. 2003). As cytoplasmic mRNPs diffuse at a rate that is 100 times faster than PBs and are at least 1,000 times more abundant (Femino et al. 1998; Aizer et al., 2008), it is likely that the random movement of both mRNPs and PBs is sufficient to explain how RNAs enters in and out of the relatively confined PBs. Moreover, unlike other cytoplasmic organelles, PBs are not enclosed with a membrane (Yang et al. 2004), thereby allowing constant exchange of mRNPs in and out of PBs and being subjected to regulation upon cellular cues.

10.3.4 Argonaute protein kinetics in neuronal models

Two recent studies in neurons and in a neurodegenerative disorder model highlighted the importance of Argonaute protein localization and kinetics in miRNA activities (Cougot et al. 2008; Savas et al. 2008). Savas and colleagues reported that Argonaute proteins co-localized at PBs and co-purified with Huntingtin protein (Htt) (Savas et al. 2008). Htt is commonly mutated in Huntington's disease -- a dominant autosomal neurodegenerative disorder. Due to an expansion of CAG triplet in its gene, the mutant protein usually has at least additional 36 polyglutamine (polyQ) at the N-terminus. Both the normal and polyQ mutant co-purified with Ago1 and Ago2. Upon siRNA knockdown of Htt in HeLa cells or upon expression of polyQ mutants in striatal progenitor cells from mouse brain, a reduction in the number of PBs as well as silencing activities were observed. Interestingly, FRAP analyses revealed that the mobile fraction of Ago2 in PBs was reduced from 15% to 8% upon expression of the poly(Q) mutant, but the percentage of mobile fraction remained unchanged upon expression of normal Htt. The reduced mobility implies that an even smaller pool of Argonaute at PBs is available to exchange with the diffuse cytoplasm upon expression of mutant Htt, which possibly contributes to the reduction in miRNA silencing in cells with mutant Htt that model the disease.

Cougot and colleagues reported that Ago2 and Dcp1a co-localized in PB-like structures in dendrites of mammalian neurons (Cougot et al. 2008). Consistent with our kinetic observations (Section 10.3.2), Ago2 exhibited a similar or slightly slower recovery rate in dendrites. On the contrary, though these authors found that Dcp1a exchanged rapidly with the cytoplasm in HeLa cells similar to our studies, the recovery rate of Dcp1a was very slow in the neurons. However, upon neuronal stimulation, such recovery rate of Dcp1a was dramatically increased to a similar rate as in HeLa cells and half of the Dcp1a-positive foci no longer co-stained with Ago2 within 15 minutes of stimulation (the shortest time point examined in the study). It is therefore tempting to speculate that such reduction in Ago2 enrichment in PB structures is related to the relief of miRNA-mediated repression previously observed upon neuronal stimulation (Schratt et al. 2006).

10.4 What determines Argonaute to localize at PBs?

10.4.1 microRNA-dependency?

To determine whether miRNA is required for Argonaute to localize to PBs, we transfected GFP-Ago2 into three clonal mouse embryonic stem (ES) cell lines that lack mature miRNAs by inactivating *Dicer*, the key cytoplasmic processing enzyme to generate miRNAs (c.f. Figure 1; (Leung et al. 2006). Similar to the wild-type ES cells, GFP-Ago2 were still localized to PBs that were Dcp1a-positive in *Dicer*$^{-/-}$ ES cells (Figure 10.5). In contrast, GFP-Ago2 no longer associated with SGs in these cells (Figure 10.5). However, in the

presence of exogenously added *let-7a* miRNA transfected in the form of siRNAs, GFP-Ago2 association with SGs was restored (Figure 10.5). Thus, while the Argonaute localization to SG is miRNA-dependent, its localization to PBs is independent of miRNAs. This is probably because Argonaute can associate with several PB components, including GW182, cap binding protein eIF4E, translational repressor p54/rck and nonsense mediated decay factor UPF1, through direct protein-protein interactions (Chu and Rana 2006; Hock et al. 2007; Jakymiw et al. 2005; Liu et al. 2005a).

Interestingly, Dcp1a-positive PBs are still present in *Dicer*$^{-/-}$ mouse ES cells, suggesting that neither mature miRNAs nor RNA silencing are required for the formation of PBs. These ES cell data, however, are in apparent contrast with other studies, which showed that the number and size of PBs were reduced in *Drosophila* S2 cells (Eulalio et al. 2007) and HeLa cells upon depletion of key miRNA pathway proteins (Pauley et al. 2006). Eulalio and colleagues reported that the number of PBs cannot be restored by translational inhibitor puromycin in these S2 cells (Eulalio et al. 2007), suggesting that the presence of mRNAs that are not undergoing translation is not sufficient to restore the PB assembly. Instead, these mRNAs must be in a repressed state with perhaps enhanced rates of degradation for the restoration. This is illustrated by the PB assembly upon transient transfection of a functional siRNA/miRNA that repress specific endogenous genes, but not with a non-targeting siRNA/miRNA. However, it should be noted that the initial disappearance of PBs in these cells is not due to a lack of repressed targets because the depletion of specific miRNA pathway genes was mediated through RNA silencing in the first place (Eulalio et al. 2007; Pauley et al. 2006). Therefore, the absence of microscopically visible PBs in these cells is likely a kinetic issue and the restoration of PB assembly occurs upon exceeding a critical threshold of repressed mRNP concentration. On the other hand, in the case of mouse *Dicer*$^{-/-}$ ES cells where microscopically visible PBs were observed, perhaps there are already sufficient amount of mRNA degradation/repression mediated through pathways other than RNA silencing in these cells. Consistent with this idea, depletion of translational repressors or RNA decay factors that are not apparently related to RNA silencing is sufficient to trigger PB disassembly (Chu and Rana 2006; Eulalio et al. 2007).

10.4.2 Post-translational modifications

So far, Argonaute localization to PBs was reported to be controlled by two post-translational modifications: phosphorylation and hydroxylation. Zeng and colleagues reported that Ago2 is phosphorylated at serine-387 in the PAZ domain of Ago2 (Zeng et al. 2008). Mutating the serine to alanine resulted in a reduction of Ago2 localization to PBs. The level of Ago2 phosphorylation at this residue was increased upon arsenite-mediated oxidative stress by a downstream kinase of p38 MAPK pathway, MAPKAPK2. However, the significance of the Ago2 phosphorylation at this residue in miRNA silencing abilities and PB localization remains unclear.

On the other hand, stability of Ago2 is modulated by the prolyl 4-hydroxylation at proline-700 in the PIWI domain (Qi et al. 2008). Mutating the proline to alanine reduced the half-life of Ago2 protein from > 10 hours to ~ 6 hours. Similarly, siRNA-mediated knockdown or genetic ablation of enzymes required for hydroxylation reduced the stability of Ago2 protein and the destabilization is mediated by proteasome. Interestingly, though these cells had the same number of Dcp1a-positive PBs, Ago2 was no longer associated with these cytoplasmic structures. Consistent with this observation, Ago2 was delocalized from PBs for proline-700->alanine mutant. Therefore, hydroxylation at this residue can potentially redirect Argonaute to PBs. Alternatively, the observed PB localization could be sensitive to the steady-state level of endogenous Ago2 (c.f. Section 10.2.1).

10.4.3 Hsp90 sensitivity

Even before the discovery of Argonaute function in miRNA silencing, one of the first known protein interaction partners of Ago2 was Hsp90 (Tahbaz et al. 2001). Hsp90 is a stress-induced chaperone that assists a distinct set of ~200 client proteins to adopt their active conformations through ATP binding and hydrolysis (Taipale et al. 2010). Several recent studies in *Drosophila*, human and plant cells have indicated that the HSP90 chaperone machinery facilitates the loading of siRNA duplexes into the RISC complex (Johnston et al. 2010; Landthaler 2010). Addition of Hsp90 inhibitor, such as geldanamycin, resulted in a decrease in the steady state level of endogenous Ago2 through proteasome-mediated degradation, but such decrease can be partially restored by transfecting the cells with siRNA duplex (Johnston et al. 2010). Taken together, these data suggest that Hsp90 stabilizes unloaded Argonaute and facilitates its binding to siRNA duplex. Interestingly, two groups independently observed that Ago2 localization to PBs was abrogated upon treatment of cells with HSP90 inhibitors (Johnston et al. 2010; Pare et al. 2009). This delocalization again, as noted previously, coincides with the low level of steady-state level of endogenous Argonaute (c.f. Section 10.2.1 and 10.4.2.).

10.5 What are the possible roles of PBs in miRNA silencing?

Several lines of evidence suggest that PBs are not required for miRNA silencing. First, the majority of Argonaute, miRNAs and mRNA targets are diffusely distributed in the cytoplasm (Leung et al. 2006). Second, cells with or without microscopically visible PBs are equally competent for miRNA silencing (Behm-Ansmant et al. 2006; Chu and Rana 2006; Eulalio et al. 2007). Third, different aspects of miRNA silencing can be reconstituted *in vitro* using cell extracts that are likely to be devoid of PBs (reviewed in (Standart and Jackson 2007). So, what is the significance of PB localization of Argonaute, miRNAs and their mRNA targets?

In the context of miRNA silencing, PB most likely reflects the underlying network of interactions between different components in the pathway. Though equivalent interactions can probably occur elsewhere in the cytoplasm, the local concentration of factors that participate in related steps of miRNA silencing within a physical structure can potentially increase the overall efficiency. For example, PBs are enriched with RNA decay factors, including deadenylase Ccr4-Not complex, decapping enzyme complex and 5′->3′ exoribonuclease Xrn1. The local concentration of these factors can facilitate the miRNA-mediated mRNA decay process. Yet, in many cases examined in *Drosophila* and human cells, disassembly of these microscopically visible structures does not result in any reduction in siRNA/miRNA-directed cleavage or miRNA-mediated repression both at protein and RNA level (Behm-Ansmant et al. 2006; Chu and Rana 2006; Eulalio et al. 2007). Therefore, the assembly of microscopically visible PBs does not likely confer additional kinetic advantage. Instead, miRNA silencing resulting in mRNA degradation likely occurs in the cytoplasm by submicroscopic complexes that are constantly exchanging with PBs and a majority of these complexes are distributed in the cytoplasm undergoing rapid diffusion.

The existence of such submicroscopic complexes is supported by biochemical fractionation of cell extracts prepared by lysis buffer containing digitonin (Pillai et al. 2005). The resultant pellet from high-speed (14,000 × g) centrifugation of these extracts contains the majority of Argonaute, *let-7* miRNA and endogenous *let-7* targets *K-ras* and *N-ras* mRNAs, along with PB components Dcp1 and Xrn1. Yet, the properties of these submicroscopic complexes must be somehow altered upon/following their entry into PBs. As demonstrated by us and others, Ago2 exhibited very slow exchange at PBs, which was not observed in diffuse cytoplasm (Section 10.3.2 and 10.3.4). Apart from direct protein-protein interactions with other PB components, the slow-exchanging property of Argonaute proteins at PBs

could be due to specific post-translation modifications (Section 10.4.2), which increase their binding affinities at PBs for mRNA degradation. Potentially, those Argonaute proteins retained at PBs can be released back to the cytoplasm in a regulated manner upon change in cellular conditions (as observed in neurons upon stimulation in Section 10.3.4).

There are precedents for PBs to act as storage repositories for specific transcripts in yeast and possibly in mammalian cells (Bhattacharyya et al. 2006; Brengues et al. 2005). Bhattacharya and colleagues previously reported that the translation of *CAT-1* mRNA is repressed by *miR-122*, but such repression is relieved upon amino acid starvation (Bhattacharyya et al. 2006). Correlated with this, a subpopulation of *CAT-1* mRNAs is localized at PBs when repressed by *miR-122* but de-localized from PBs when de-repressed. It is possible that those *CAT-1* mRNAs formally localized in PBs are relocated to polyribosomes upon amino acid starvation, given that such stress-induced translation can still occur in the presence of transcriptional inhibitors. However, this conclusion is qualified only if the majority of *CAT-1* mRNA are localized in PBs at steady state. Another dilemma of this model is that neither poly(A)+ mRNAs nor the poly(A) binding protein Pabpc1 are detected in PBs by *in situ* hybridization and immunostaining, respectively (Anderson and Kedersha 2006; Cougot et al. 2004). As Pabpc1 is highly abundant (8×10^6 molecules per cell) and it binds to a minimum tract of 5As (Gorlach et al. 1994), it is difficult to envisage that those miRNA-targeted mRNAs are not deadenylated upon entry to PBs (Section 10.2.3). Therefore, these transcripts would have to be protected from degradation following deadenylation in PBs and then undergo poly(A) addition by a cytoplasmic poly(A) polymerase, such as mammalian homologue of Gld-2, prior to translation.

Immunoprecipitation data showed that the associations between Argonaute proteins and other PB components, such as Dcp1a, rck/p54 and GW182, were due to protein-protein interactions, rather than through a common RNA scaffold (Chu and Rana 2006; Jakymiw et al. 2005; Liu et al. 2005a; Liu et al. 2005b). Consistent with this, we observed that the PB association with Argonaute protein does not require miRNAs (Section 10.4.1). Since PBs do not contain either ribosomal subunits or initiation factors, one possible model of RNA silencing is that the stable association of Argonaute protein and PBs keeps the bound mRNAs repressed in such a translation-incompetent environment and destined for degradation. On the other hand, the dynamic exchange and distribution of Argonaute in the total cytoplasm suggests an alternative model that silencing at the stage of translation exists elsewhere in the cytoplasm independent of PBs.

10.6 Conclusions and Perspectives

In summary, though most of the miRNA action is likely to be carried out by submicroscopic complexes in the cytoplasm, the distinct kinetics of Argonaute and its associated proteins at PBs indicate roles in translational repression and degradation of mRNAs targeted by miRNAs. To further understand such roles in PBs, it will be important to quantitate the kinetics of protein components required for miRNA silencing in relation with their post-translational modification(s). In addition, efforts should be focused on biochemically purifying PBs such that a quantitative proteomic approach can be used to globally characterize the flux of the full complement in and out of this cytoplasmic organelle under different cellular conditions. Similar effort has previously been applied to the nucleolus, another membrane-less, RNA-rich organelle (Andersen et al. 2005).

Acknowledgments

References

Aizer A, Brody Y, Ler LW, Sonenberg N, Singer RH, Shav-Tal Y. The dynamics of mammalian P body transport, assembly, and disassembly in vivo. Mol Biol Cell. 2008; 19:4154–4166. [PubMed: 18653466]

Andersen JS, Lam YW, Leung AK, Ong SE, Lyon CE, Lamond AI, Mann M. Nucleolar proteome dynamics. Nature. 2005; 433:77–83. [PubMed: 15635413]

Anderson P, Kedersha N. RNA granules. J Cell Biol. 2006; 172:803–808. [PubMed: 16520386]

Anderson P, Kedersha N. Stress granules: the Tao of RNA triage. Trends Biochem Sci. 2008; 33:141–150. [PubMed: 18291657]

Andrei MA, Ingelfinger D, Heintzmann R, Achsel T, Rivera-Pomar R, Luhrmann R. A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. Rna. 2005; 11:717–727. [PubMed: 15840819]

Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136:215–233. [PubMed: 19167326]

Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. 2006; 20:1885–1898. [PubMed: 16815998]

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell. 2006; 125:1111–1124. [PubMed: 16777601]

Brengues M, Teixeira D, Parker R. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. Science. 2005; 310:486–489. [PubMed: 16141371]

Chu CY, Rana TM. Translation Repression in Human Cells by MicroRNA-Induced Gene Silencing Requires RCK/p54. PLoS Biol. 2006; 4:e210. [PubMed: 16756390]

Cougot N, Babajko S, Seraphin B. Cytoplasmic foci are sites of mRNA decay in human cells. J Cell Biol. 2004; 165:31–40. [PubMed: 15067023]

Cougot N, Bhattacharyya SN, Tapia-Arancibia L, Bordonne R, Filipowicz W, Bertrand E, Rage F. Dendrites of mammalian neurons contain specialized P-body-like structures that respond to neuronal activation. J Neurosci. 2008; 28:13793–13804. [PubMed: 19091970]

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; 19:437–447. [PubMed: 16109369]

Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. Genes Dev. 2003; 17:438–442. [PubMed: 12600936]

Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol Cell Biol. 2007; 27:3970–3981. [PubMed: 17403906]

Eulalio A, Tritschler F, Izaurralde E. The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. RNA. 2009; 15:1433–1442. [PubMed: 19535464]

Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem. 2010; 79:351–379. [PubMed: 20533884]

Femino AM, Fay FS, Fogarty K, Singer RH. Visualization of single RNA transcripts in situ. Science. 1998; 280:585–90. [PubMed: 9554849]

Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009; 19:92–105. [PubMed: 18955434]

Fusco D, Accornero N, Lavoie B, Shenoy SM, Blanchard JM, Singer RH, Bertrand E. Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. Curr Biol. 2003; 13:161–167. [PubMed: 12546792]

Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat Cell Biol. 2009; 11:1143–1149. [PubMed: 19684575]

Gorlach M, Burd CG, Dreyfuss G. The mRNA poly(A)-binding protein: localization, abundance, and RNA-binding specificity. Exp Cell Res. 1994; 211:400–407. [PubMed: 7908267]

Hock J, Meister G. The Argonaute protein family. Genome Biol. 2008; 9:210. [PubMed: 18304383]

Hock J, Weinmann L, Ender C, Rudel S, Kremmer E, Raabe M, Urlaub H, Meister G. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. EMBO Rep. 2007; 8:1052–1060. [PubMed: 17932509]

Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science. 2002; 297:2056–2060. [PubMed: 12154197]

Jackson RJ, Standart N. How do microRNAs regulate gene expression? Sci STKE 2007. 2007:re1.

Jagannath A, Wood MJ. Localization of double-stranded small interfering RNA to cytoplasmic processing bodies is Ago2 dependent and results in up-regulation of GW182 and Argonaute-2. Mol Biol Cell. 2009; 20:521–529. [PubMed: 18946079]

Jakymiw A, Lian S, Eystathioy T, Li S, Satoh M, Hamel JC, Fritzler MJ, Chan EK. Disruption of GW bodies impairs mammalian RNA interference. Nat Cell Biol. 2005; 7:1267–1274. [PubMed: 16284622]

James V, Zhang Y, Foxler DE, de Moor CH, Kong YW, Webb TM, Self TJ, Feng Y, Lagos D, Chu CY, Rana TM, Morley SJ, Longmore GD, Bushell M, Sharp TV. LIM-domain proteins, LIMD1, Ajuba, and WTIP are required for microRNA-mediated gene silencing. Proc Natl Acad Sci U S A. 2010; 107:12499–12504. [PubMed: 20616046]

Johnston M, Geoffroy MC, Sobala A, Hay R, Hutvagner G. HSP90 protein stabilizes unloaded argonaute complexes and microscopic P-bodies in human cells. Mol Biol Cell. 2010; 21:1462–1469. [PubMed: 20237157]

Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fitzler MJ, Scheuner D, Kaufman RJ, Golan DE, Anderson P. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. J Cell Biol. 2005; 169:871–884. [PubMed: 15967811]

Kim J, Krichevsky A, Grad Y, Hayes GD, Kosik KS, Church GM, Ruvkun G. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. Proc Natl Acad Sci U S A. 2004; 101:360–365. [PubMed: 14691248]

Landthaler M. Chaperones get RISC loaded. Mol Cell. 2010; 39:161–162. [PubMed: 20670883]

Lee YS, Pressman S, Andress AP, Kim K, White JL, Cassidy JJ, Li X, Lubell K, Lim do H, Cho IS, Nakahara K, Preall JB, Bellare P, Sontheimer EJ, Carthew RW. Silencing by small RNAs is linked to endosomal trafficking. Nat Cell Biol. 2009; 11:1150–1156. [PubMed: 19684574]

Leung AK, Calabrese JM, Sharp PA. Quantitative analysis of Argonaute protein reveals microRNA-dependent Localisation to Stress Granules. Proc Natl Acad Sci U S A. 2006; 103:18125–18130. [PubMed: 17116888]

Leung AK, Sharp PA. Function and Localization of microRNAs in mammalian cells. Cold Spring Harb Symp Quant Biol. 2006

Leung AK, Sharp PA. microRNAs: a safeguard against turmoil? Cell. 2007; 130:581–585. [PubMed: 17719533]

Lippincott-Schwartz J, Altan-Bonnet N, Patterson GH. Photobleaching and photoactivation: following protein dynamics in living cells. Nat Cell Biol Suppl. 2003:S7–14.

Liu J, Rivas FV, Wohlschlegel J, Yates JR 3rd, Parker R, Hannon GJ. A role for the P-body component GW182 in microRNA function. Nat Cell Biol. 2005a; 7:1261–1266. [PubMed: 16284623]

Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol. 2005b; 7:719–723. [PubMed: 15937477]

Luby-Phelps K. Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int Rev Cytol. 2000; 192:189–221. [PubMed: 10553280]

Nelson PT, Hatzigeorgiou AG, Mourelatos Z. miRNP:mRNA association in polyribosomes in a human neuronal cell line. Rna. 2004; 10:387–394. [PubMed: 14970384]

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; 216:671–680. [PubMed: 10642801]

Pare JM, Tahbaz N, Lopez-Orozco J, LaPointe P, Lasko P, Hobman TC. Hsp90 regulates the function of argonaute 2 and its recruitment to stress granules and P-bodies. Mol Biol Cell. 2009; 20:3273–3284. [PubMed: 19458189]

Parker JS. How to slice: snapshots of Argonaute in action. Silence. 2010; 1:3. [PubMed: 20226069]

Pauley KM, Eystathioy T, Jakymiw A, Hamel JC, Fritzler MJ, Chan EK. Formation of GW bodies is a consequence of microRNA genesis. EMBO Rep. 2006; 7:904–910. [PubMed: 16906129]

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. 2005; 309:1573–1576. [PubMed: 16081698]

Qi HH, Ongusaha PP, Myllyharju J, Cheng D, Pakkanen O, Shi Y, Lee SW, Peng J. Prolyl 4-hydroxylation regulates Argonaute 2 stability. Nature. 2008; 455:421–424. [PubMed: 18690212]

Savas JN, Makusky A, Ottosen S, Baillat D, Then F, Krainc D, Shiekhattar R, Markey SP, Tanese N. Huntington's disease protein contributes to RNA-mediated gene silencing through association with Argonaute and P bodies. Proc Natl Acad Sci U S A. 2008; 105:10820–10825. [PubMed: 18669659]

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. Nature. 2006; 439:283–289. [PubMed: 16421561]

Seggerson K, Tang L, Moss EG. Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev Biol. 2002; 243:215–225. [PubMed: 11884032]

Standart N, Jackson RJ. MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. Genes Dev. 2007; 21:1975–1982. [PubMed: 17699746]

Tahbaz N, Carmichael JB, Hobman TC. GERp95 belongs to a family of signal-transducing proteins and requires Hsp90 activity for stability and Golgi localization. J Biol Chem. 2001; 276:43294–43299. [PubMed: 11553639]

Taipale M, Jarosz DF, Lindquist S. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol. 2010; 11:515–528. [PubMed: 20531426]

Takimoto K, Wakiyama M, Yokoyama S. Mammalian GW182 contains multiple Argonaute-binding sites and functions in microRNA-mediated translational repression. RNA. 2009; 15:1078–1089. [PubMed: 19398495]

Vasudevan S, Steitz JA. AU-Rich-Element-Mediated Upregulation of Translation by FXR1 and Argonaute 2. Cell. 2007; 128:1105–1118. [PubMed: 17382880]

Yang Z, Jakymiw A, Wood MR, Eystathioy T, Rubin RL, Fritzler MJ, Chan EK. GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation. J Cell Sci. 2004; 117:5567–5578. [PubMed: 15494374]

Zeng Y, Sankala H, Zhang X, Graves PR. Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. Biochem J. 2008; 413:429–436. [PubMed: 18476811]
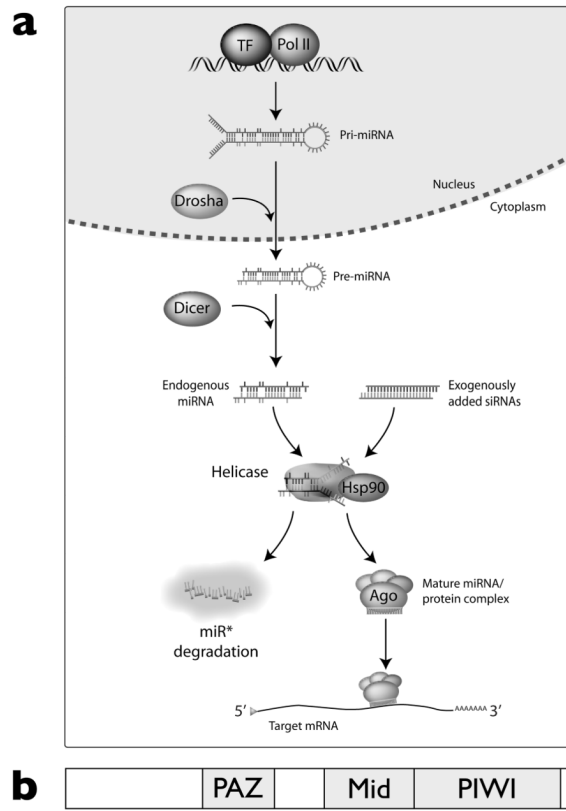
Figure 10.1.
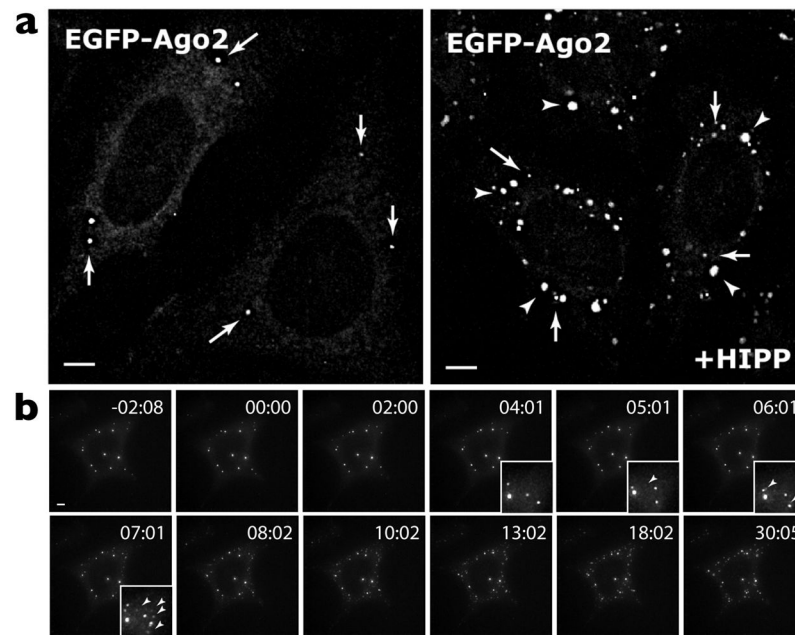Schematics of (a) microRNA biogenesis and (b) Argonaute protein domains

Figure 10.2. Argonaute protein localization
(a) Stably expressed EGFP-Ago2 localized to the cytoplasm and PBs (arrows, left) and, upon addition of 1 μM hippuristanol (HIPP) for 30 min, also localized to SGs (arrowheads, right). (b) Time-lapse micrographs of stably expressed EGFP-Ago2 in a single live cell. The first appearance of EGFP-Ago2 at SGs (arrowheads) occurred between 5 and 6 min after addition of 1 μM hippuristanol (HIPP). Scale bars, 5 μm. (Reprinted, with permission, from Leung et al. (2006) PNAS 103:18125-30; © National Academy of Sciences, U.S.A.)
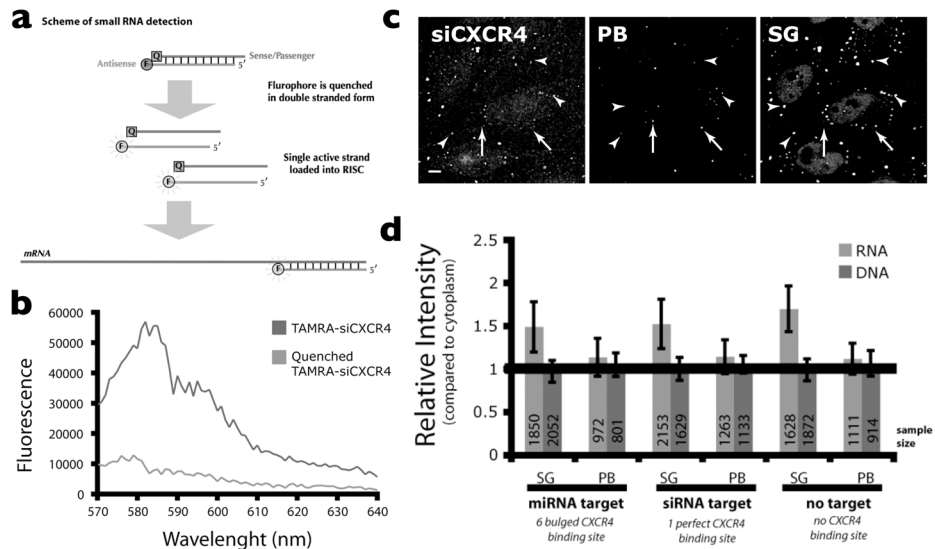
Figure 10.3. Localization of short RNAs

(a) Schematics of active siRNA strand detection. (b) Fluorescence emission profiles of unquenched and quenched TAMRA siRNAs against endogenous gene CXCR4 (siCXCR4). (c-d) The localization of siCXCR4 was compared with PB marker Dcp1a (arrows) and SG marker TIA-1 upon addition of 1 μM hippuristanol (HIPP) for 30 min. In this case, siCXCR4 co-localized with SGs (arrowheads), but not with PBs, when translation initiation was inhibited (Left), as shown by the significant enrichment of the intensities at SGs compared with the cytoplasm (two-tailed t test, $P \ll 0.0001$). Scale bars, 5 μm. (Reprinted, with permission, from Leung et al. (2006) PNAS 103:18125-30; © National Academy of Sciences, U.S.A.)
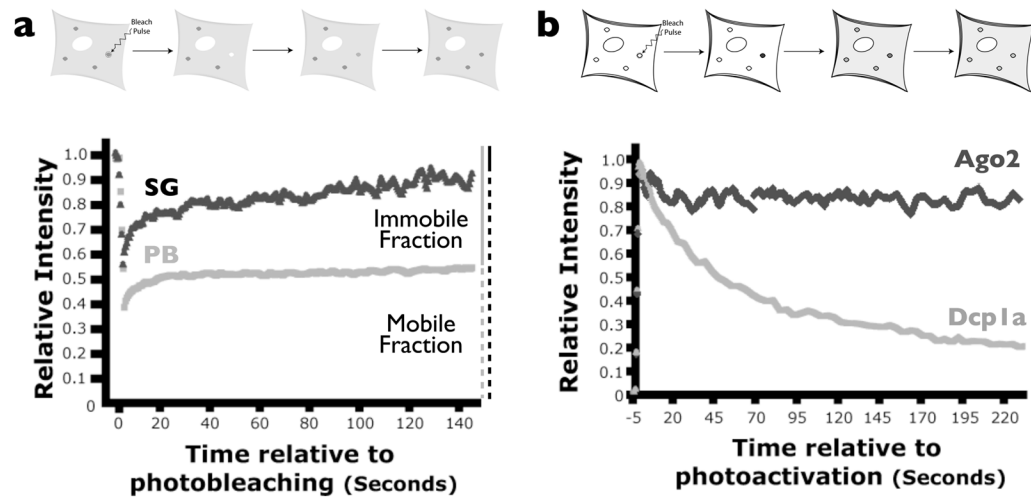
Figure 10.4. Quantitative Dynamics of Argonaute protein
(a) FRAP analyses of EGFP-Ago2 at single PBs (n = 5) and SGs (n = 3) and the intensities at respective structures relative to their initial intensities were compared over time. (b) PAGFP-Dcp1a and PAGFP-Ago2 were photoactivated at single PB labeled by mRFP-Dcp1a for 1 s, and the photoactivated cells were imaged over the next 13 min. (Reprinted, with permission, from Leung et al. (2006) PNAS 103:18125-30; © National Academy of Sciences, U.S.A.)
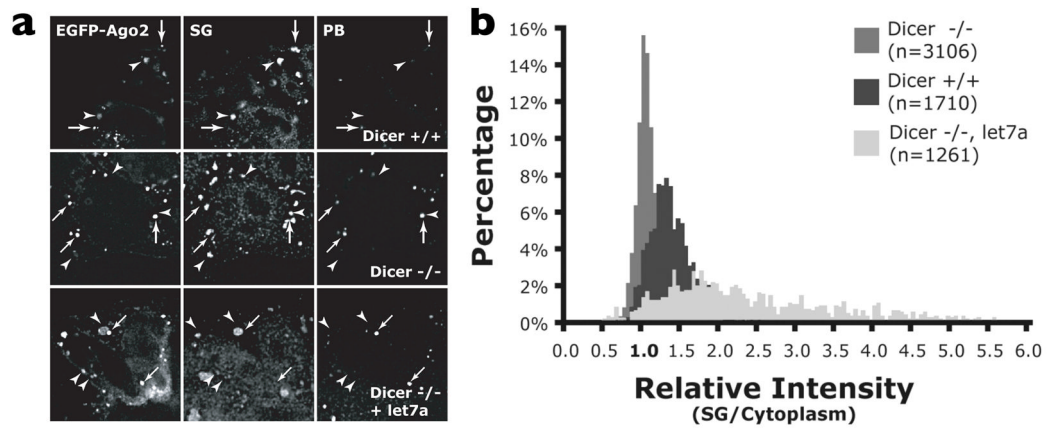
Figure 10.5. The localization of Ago2 at SGs, but not at PBs, depends on the presence of short RNAs

(a) Transiently expressed EGFP-Ago2 still co-localized with PB marker Dcp1a in both *Dicer*$^{+/+}$ (Top, arrows) and *Dicer*$^{-/-}$ (Middle and Bottom) cells. However, EGFP-Ago2 no longer co-localized with SG marker TIA-1 (arrowheads) in *Dicer*$^{-/-}$ cells. Cotransfection of 100 nM of miRNA *let-7a* in the form of siRNA duplex resulted in the localization of EGFP-Ago2 at SGs in *Dicer*$^{-/-}$ cells (Bottom). (b) The intensities of EGFP-Ago2 at SGs were compared with the cytoplasm in each case, and a histogram was plotted with the percentage of SGs as the y axis, for each relative intensity with an interval of 0.05 difference on the x axis. Correlated with the image data in (a), the intensities of EGFP-Ago2 at SGs relative to the cytoplasm were centered at ≈1.0 in *Dicer*$^{-/-}$ cells, suggesting that there is no enrichment of EGFP-Ago2 at SGs in the absence of short RNAs. Scale bars, 5 μm. (Reprinted, with permission, from Leung et al. (2006) PNAS 103:18125-30; © National Academy of Sciences, U.S.A.)