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Importin 8 Is a Gene Silencing Factor that Targets Argonaute Proteins to Distinct mRNAs

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SUMMARY

Small regulatory RNAs including small interfering RNAs (siRNAs) and microRNAs (miRNAs) guide Argonaute (Ago) proteins to specific target RNAs leading to mRNA destabilization or translational repression. Here, we report the identification of Importin 8 (Imp8) as a component of miRNA-guided regulatory pathways. We show that Imp8 interacts with Ago proteins and localizes to cytoplasmic processing bodies (P bodies), structures involved in RNA metabolism. Furthermore, we detect Ago2 in the nucleus of HeLa cells, and knockdown of Imp8 reduces the nuclear Ago2 pool. Using immunoprecipitations of Ago2-associated mRNAs followed by microarray analysis, we further demonstrate that Imp8 is required for the recruitment of Ago protein complexes to a large set of Ago2-associated target mRNAs, allowing for efficient and specific gene silencing. Therefore, we provide evidence that Imp8 is required for cytoplasmic miRNA-guided gene silencing and affects nuclear localization of Ago proteins.

INTRODUCTION

Small noncoding RNAs including microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs) are important regulators of gene expression in many different organisms (Filipowicz et al., 2005; Seto et al., 2007; Zamore and Haley, 2005). miRNA genes are transcribed by RNA polymerases II and III generating primary miRNA transcripts, which are further processed to stem-loop-structured miRNA precursors by the nuclear RNase III Drosha and its partner DGCR8/Pasha (Bushati and Cohen, 2007). Pre-miRNAs are exported to the cytoplasm where processing of Dicer, another RNase III enzyme, generates 21–23 nucleotide (nt) long double-stranded (ds) miRNA/miRNA* intermediates with characteristic 2 nt 3′ overhangs and 5′ phosphate groups (Meister and Tuschl, 2004; Zamore and Haley, 2005). After further processing and/or unwinding steps, one strand gives rise to the mature miRNA and is incorporated into miRNA-protein complexes often referred to as miRNPs (Leuschner et al., 2006; Matranga et al., 2005; Mourelatos et al., 2002; Rand et al., 2005).

Members of the Argonaute protein family constitute the cellular binding partners of miRNAs as well as other small RNAs and are therefore key components of miRNPs. The human genome encodes for eight different Argonaute genes, which can be phylogenetically divided into four Ago and four Piwi subfamily members (Peters and Meister, 2007; Tolia and Joshua-Tor, 2007). Expression of the Piwi subfamily members HIWI1-3 and HILI seems to be restricted to testes, and different Piwi subfamily members bind to different classes of testes-specific piRNAs (Aravin et al., 2006; Girard et al., 2006). The mouse Piwi member MILI, for example, binds to a developmentally regulated piRNA cluster and influences transposon expression (Aravin et al., 2007). The individual members of the human Ago subfamily, namely Ago1–4, are ubiquitously expressed and most likely bind to similar populations of miRNAs.

Depending on the degree of complementarity between the miRNA and the target RNA, miRNAs guide sequence-specific cleavage, deadenylation, or translational repression of specific target mRNAs (Pillai et al., 2007). It has been shown by in vitro translation assays that miRNAs inhibit translation of reporter constructs at early stages of translational initiation (Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006). In contrast, on the basis of the finding that miRNAs cosediment with polysomes it has been proposed that miRNAs function on the level of translational elongation (Maroney et al., 2006; Olsen and Ambros, 1999; Seggerson et al., 2002). Other models of miRNA functions suggest rapid ribosome drop-off from mRNAs upon miRNA inhibition or miRNA-guided degradation of the nascent polypeptide chain by the proteasome (Nottrott et al., 2006; Petersen et al., 2006). mRNA profiling studies have recently shown that miRNAs affect

the stability of many mRNAs that contain imperfect miRNA binding sites. Moreover, it appears that miRNA effects on mRNA levels are as common as translational repression (Bagga et al., 2005). Consistently, miRNAs guide mRNA deadenylation processes followed by decapping and degradation (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Humphreys et al., 2005; Wu et al., 2006).

While miRNA processing is well understood, target mRNA recognition and binding is only poorly understood. It has been demonstrated that HuR (ELAV1) can release miRNA repression of the CAT-1 mRNA upon cellular stress (Bhattacharyya et al., 2006). Moreover, a protein termed dead end 1 (Dnd1) can occupy miRNA binding sites on the 3′ untranslated regions (UTRs) of specific target mRNAs and thus inhibit miRNA-guided gene silencing (Kedde et al., 2007). Although only a few mRNA binding proteins with effects on miRNA function have been identified thus far, it is very likely that many more protein factors exist that influence recruitment or stable binding of miRNPs to specific mRNAs.

Here, we report the identification of Imp8 as component of human Ago protein complexes. We show that Imp8 is required for binding of Ago proteins to a variety of mRNA targets and that depletion of Imp8 interferes with miRNA-guided gene silencing. Furthermore, we demonstrate that Imp8 modulates nuclear localization of Ago2. We have therefore identified Imp8 as specificity factor in the miRNA pathway, which may fulfill additional functions in nuclear Ago import.

RESULTS

Imp8 Interacts with Human Ago Proteins

Human Ago1 and Ago2 complexes have been analyzed biochemically in the past (Gregory et al., 2005; Hock et al., 2007; Liu et al., 2004; Meister et al., 2005). However, the function of Ago3 and Ago4 in human cells has not been addressed yet. Therefore, we performed biochemical purification studies using FLAG/HA (FH)-tagged Ago3 and Ago4. HEK293 cells were transfected with FH-Ago3 or FH-Ago4, and immunoprecipitates were analyzed by mass spectrometry (Figure 1A, Tables S1 and S2 available online). We found known Ago-associated proteins such as TNRC6B, but also a variety of factors that have not been linked to Ago function thus far. Among them, we found the Importin β-like import receptor Imp8 (Gorlich et al., 1997). Cosedimentation studies revealed that FH-Imp8 comigrates with Ago proteins in sucrose gradients (Figure S1). For in vitro interaction studies, recombinant GST-Imp8 was immobilized on glutathione-coated beads and incubated with [^{35}S]-labeled Ago1–4 (Figure 1B). All Ago proteins interacted with GST-Imp8 (lanes 1–4), whereas no signal was observed in control reactions where GST alone was immobilized (lanes 6–9). Next, we investigated the interaction of Ago proteins with Imp8 in vivo. HEK293 cells were cotransfected with myc-tagged Ago proteins and FH-Imp8. Consistent with the in vitro data, all myc-Ago proteins were readily detectable in the anti-FLAG immunoprecipitates (Figure 1C, lanes 2, 5, 8, and 11). No myc-Ago was detected in FH-GFP control experiments (lanes 3, 6, 9, and 12). Furthermore, treatment of the immunoprecipitates with RNase A demonstrated that interaction of Ago proteins with Imp8 is independent of RNA (lanes 1, 4, 7, and 10). In contrast, interaction of Ago2 with the poly-A binding protein C1 (PABPC1) was impaired upon RNase A treatment (lane 13), demonstrating efficient RNase A digestion. In order to validate endogenous Imp8-Ago2 interactions, we generated a polyclonal antibody against Imp8. The purified anti-Imp8 serum immunoprecipitates transfected FH-Imp8 (Figure 1D, lane 2) and endogenous Imp8 (lane 4, lower panel). Moreover, with an antibody against Ago2 (Rüdel et al., 2008), endogenous Ago2 was detectable in the Imp8 immunoprecipitate (lane 4, upper panel). Import receptors require interaction with the GTPase Ran for cytoplasmic substrate binding and nuclear transport. Therefore, we added recombinant Ran or RanQ69L, a mutant that promotes the dissociation of the cargo-Importin complexes, to the FH-Imp8 immunoprecipitate (Figure 1E, lanes 2 and 3). RanQ69L strongly reduced Ago2 binding to Imp8 (lane 3) compared to Ran (lane 2) or a sample where no protein was added (lane 1), indicating that Ago proteins bind to Imp8 in a Ran-dependent manner. By using a proteomics approach, we have identified and validated Imp8 as Ago-interacting protein in human cells.

Imp8 and Ago Proteins Colocalize in P Bodies

Human Ago proteins localize to P bodies and stress granules (Leung et al., 2006; Liu et al., 2005; Sen and Blau, 2005). We therefore analyzed the subcellular localization of Imp8. FH-Imp8 was transfected into HEK293 cells, and fixed cells were stained with anti-HA antibodies (Figure 2A). FH-Imp8 was detectable in the nucleus as well as cytoplasmic structures that were positive for the P body marker LSm4, indicating that Imp8 localizes to P bodies. Moreover, Imp8 localizes to arsenite-induced stress granules, as indicated by the colocalization with the known stress granule marker FMRp. As control, FH-Importin 4 (Imp4) was analyzed, and it localizes neither to P bodies nor to stress granules (Figure S2). We next analyzed whether Ago proteins colocalize with Imp8 in P bodies (Figure 2B). Myc-Ago2 and FH-Imp8 were cotransfected and fixed cells were analyzed with anti-myc or anti-FLAG antibodies. Indeed, Ago2 colocalizes with Imp8 in P bodies, suggesting a function for Imp8 in RNA metabolism. Similar results were obtained when other human Ago proteins were analyzed (data not shown and Figure S3B). Consistently, the anti-Imp8 serum stained P bodies in cells expressing FH-Ago2 (Figure 2B, panels 5–8) or the known P body component FH-TNRC6B (panels 9–12) (Meister et al., 2005), indicating that endogenous Imp8 is present in P bodies as well.

The Ran-dependent interaction of Imp8 and Ago proteins prompted us to analyze whether localization of Imp8 to P bodies depends on Ran as well (Figure 2C). FH-Imp8 was cotransfected with myc-Ran (panels 1–4), myc-RanQ69L (panels 5–8), or myc-RanT24N (panels 9–12), a mutant that stabilizes importin-cargo interaction, into HEK293 cells and localization was analyzed with anti-HA antibodies. Coexpression of RanWT or RanT24N had no effect on Imp8 P body localization. However, cotransfection of RanQ69L, which promotes the dissociation of import receptors from their cargo proteins, resulted in disruption of Imp8 P body localization, whereas P body formation itself was not impaired, as indicated by LSm4 analysis. Interestingly, Ago protein localization remained unaffected when RanQ69L was expressed (Figure S3). Of note, in the RanQ69L expression experiments, FH-Imp8 could be detected in cytoplasmic granules that

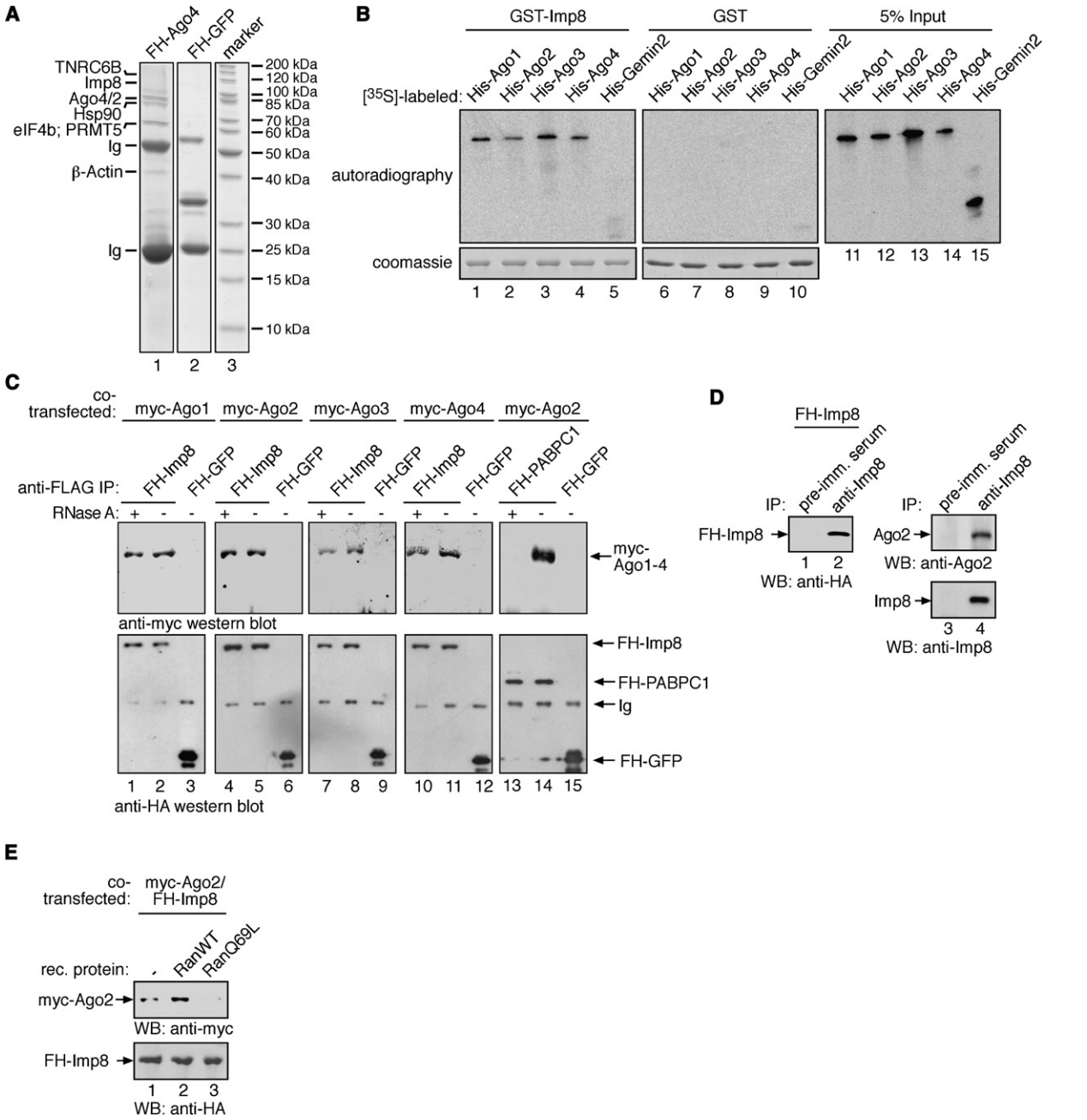


Figure 1. Imp8 Interacts with Human Ago1–4

(A) SDS-PAGE analysis of proteins interacting with human Ago4. FH-Ago4 or FH-GFP plasmids were transfected into HEK293 cells. FH-Ago4 (lane 1) and FH-GFP (lane 2) were immunoprecipitated from cell lysates, and immunoprecipitates were separated by SDS-PAGE. Lane 3 shows a molecular weight standard. Proteins interacting with FH-Ago4 but not with FH-GFP are shown to the left. A complete list of FH-Ago4- and FH-Ago3-interacting proteins is shown in Tables S1 and S2.

(B) GST-Imp8 (lanes 1–5) or GST alone (lanes 6–10) was immobilized on glutathione sepharose beads and incubated with [^{35}S]-methionine-labeled in vitro-translated His-Ago1–4 or His-Gemin2 control protein. Autoradiograms of bound [^{35}S]-labeled proteins (lanes 1–10) and radioactive input signals (lanes 11–15) are shown in the upper panels. Lower panels show coomassie stainings of coupled GST-Imp8 (lanes 1–5) or GST (lanes 6–10).

(C) FH-Imp8, FH-GFP or FH-PABPC1 plasmids were cotransfected with myc-Ago1 (lanes 1–3), myc-Ago2 (lanes 4–6, 13–15), myc-Ago3 (lanes 7–9), or myc-Ago4 plasmids (lanes 10–12) into HEK293 cells. FH-tagged proteins were immunoprecipitated from cell lysates with anti-FLAG sepharose beads in the presence or absence of 20 μg/ml RNase A. The immunoprecipitate was analyzed by anti-myc western blotting (upper panel) and anti-HA western blotting (lower panel). Ig, Immunoglobulin heavy chain.

(D) Left panel: HEK293 cells were transfected with FH-Imp8. Immunoprecipitation was performed from cell lysates using preimmune serum (lane 1) or anti-Imp8 (lane 2). FH-Imp8 was detected with anti-HA antibodies. Right panel: Lysate from untransfected HEK293 cells was immunoprecipitated with preimmune serum (lane 3) or anti-Imp8 (lane 4). The immunoprecipitate was analyzed by anti-Ago2 western blotting (upper panel) and anti-Imp8 western blotting (lower panel).

are clearly distinct from P bodies (panel 7) and might correspond to stress granules. Thus, Imp8 colocalizes with Ago proteins to P bodies in a Ran-dependent manner.

Imp8 Affects Nuclear Localization of Ago Proteins

Import receptors target cargo proteins to the nucleus. Since human Ago proteins have been shown to function in the nucleus (Janowski et al., 2006; Kim et al., 2006), we investigated nuclear Ago protein import (Figure 3). The monoclonal anti-Ago2 (11A9) antibody stained P bodies, as well as the diffuse cytoplasm and the nucleus of fixed HeLa cells (panels 1–4) (see Rüdel et al., 2008 for a detailed characterization of anti-Ago2 [11A9]). Knockdown of Ago2 led to a loss of the cytoplasmic and the nuclear signal, indicating that Ago2 is indeed in the nucleus (Rüdel et al., 2008). We quantified nuclear and cytoplasmic signals from average pixel intensities and calculated ratios of cytoplasmic versus nuclear Ago2 for each set of samples (Figure 3B). We found that upon Imp8 knockdown, the localization of Ago2 is shifted from the nucleus to the cytoplasm, whereas the total amount of soluble Ago2 remained unaffected (Figure 3B). Similar results were obtained when a HEK293 cell line stably expressing EGFP-Ago2 was analyzed by fluorescence correlation spectroscopy (Figure 3C) (Ohrt et al., 2008). As control, Imp4 knockdown was performed, and it had no effect on nuclear EGFP-Ago2 localization. The used Imp4 siRNAs were as efficient as the Imp8 siRNAs (data not shown). In summary, we provide evidence that Imp8 can influence nuclear import of Ago proteins in human cells.

Imp8 Is Required for miRNA-Guided Gene Silencing

The interaction and colocalization of Ago proteins with Imp8 prompted us to analyze whether Imp8 is functionally involved in miRNA-guided gene silencing. We first analyzed the consequence of Imp8 depletion on sequence-specific cleavage of a luciferase reporter perfectly complementary to miR-21 (Figure 4A). As expected, knockdown of the slicer endonuclease Ago2 (Liu et al., 2004; Meister et al., 2004) led to a strong increase of luciferase expression. However, luciferase activity was not altered after Imp8 depletion, indicating that Imp8 is not required for Ago2-mediated cleavage of target RNA (for siRNA validation, see Figure S4).

Next, we investigated whether Imp8 is necessary for miRNA-guided silencing of known target mRNAs (Figure 4B). Luciferase reporters fused to full-length 3′ UTRs of previously validated miRNA targets (Beitzinger et al., 2007) were transfected into HeLa cells where either Imp8 or as controls Ago2 or TNRC6B had been depleted by RNAi. Depletion of Ago2 or TNRC6B, which are required for miRNA function, resulted in elevated luciferase expression from all constructs. Strikingly, knockdown of Imp8 led to an increase of luciferase activity as well, indicating that Imp8 is required for repression of the tested 3′ UTRs. We further tested whether the observed Imp8 effects on gene expression are linked to the miRNA pathway or whether Imp8 functions independently of Ago proteins on 3′ UTRs. For further studies, we used the 3′ UTR of Hmga2, which is strongly derepressed when endogenous let-7a is inhibited with 2′-O-methylated antisense oligonucleotides (Figure 4C). Knockdown of TNRC6B or Imp8 led to increased luciferase activity similar to other miRNA targets. As specificity control, we mutated all let-7a binding sites in the Hmga2 reporter construct (Figures 4C and 4D and Figure S5). Indeed, the mutated 3′ UTR did not respond to Imp8 knockdown anymore, indicating that Imp8 functions together with Ago proteins in the miRNA pathway. Since miRNAs can trigger target mRNA degradation, we investigated whether knockdown of Imp8 affects the level of endogenous miRNA target mRNAs (Figure 4E). We found that Imp8 depletion led to a moderate increase of some miRNA targets relative to GAPDH or β-Actin mRNAs (Figure S6). Thus, Imp8 is required for silencing of endogenous miRNA targets.

Imp8 Is Involved in Loading of Ago Complexes onto mRNA Targets

We next investigated individual steps of the miRNA pathway for Imp8 requirement. It has been suggested that Importin β-like proteins function as chaperones and increase the solubility of RNA binding proteins by preventing unspecific aggregation with nucleic acids (Jakel et al., 2002). Therefore, we analyzed soluble Ago protein levels after Imp8 knockdown in HeLa cells (Figure 5A). Cells transfected with siRNAs against Imp8 or control siRNAs were analyzed by western blotting with specific antibodies against endogenous Ago1–4 or β-actin as loading control. Because of the low Ago3 and Ago4 expression levels, Ago3 and Ago4 were immunoprecipitated prior to western blotting. Knockdown of Imp8 had no effect on the steady-state levels of soluble Ago proteins. Next, we analyzed Ago2 protein turnover using pulse-chase experiments (Figure 5B). HEK293 cells were cotransfected with a construct expressing FH-Ago2 and a plasmid expressing short hairpin RNAs (shRNAs) against Imp8 or control shRNAs. After 3 days, cells were pulsed with [^{35}S]-methionine-containing medium followed by chase in normal medium, and [^{35}S]-labeled Ago2 complexes were immunoprecipitated with anti-FLAG antibodies. Both in control and Imp8 knockdown cells, [^{35}S]-labeled Ago2 was strongly reduced after 13 hr chase. Therefore, our data suggest that Imp8 has no effect on the steady-state levels or turnover of Ago proteins in human cells.

Next, we asked whether Imp8 is involved in miRNA biogenesis, which would explain the effects of Imp8 on miRNA target expression (Figure 5C). HeLa cells were transfected with siRNAs directed against Dicer or Imp8, and total RNA was analyzed by northern blotting with probes specific for let-7a, miR-21, or miR-16. As expected, Dicer knockdown resulted in lower miRNA levels (lane 1). However, Imp8 knockdown had no effect on mature miRNA levels, suggesting that Imp8 is not involved in miRNA biogenesis (lanes 3 and 4). Using anti-Ago2 immunoprecipitations, we further investigated whether Imp8 affects the loading of Ago2 with miRNAs (Figure 5D). Endogenous Ago2 was immunoprecipitated from cell lysates transfected with

(E) myc-Ago2 and FH-Imp8 plasmids were cotransfected into HEK293 cells. FH-Imp8 was immunoprecipitated from cell lysates with anti-FLAG antibodies. The immunoprecipitate was incubated with PBS/5mM GTP containing either 100 μM RanWT (lane 2), RanQ69L (lane 3) or no protein (lane 1). Myc-Ago2 and FH-Imp8 proteins were detected by western blotting (upper panel and lower panel).

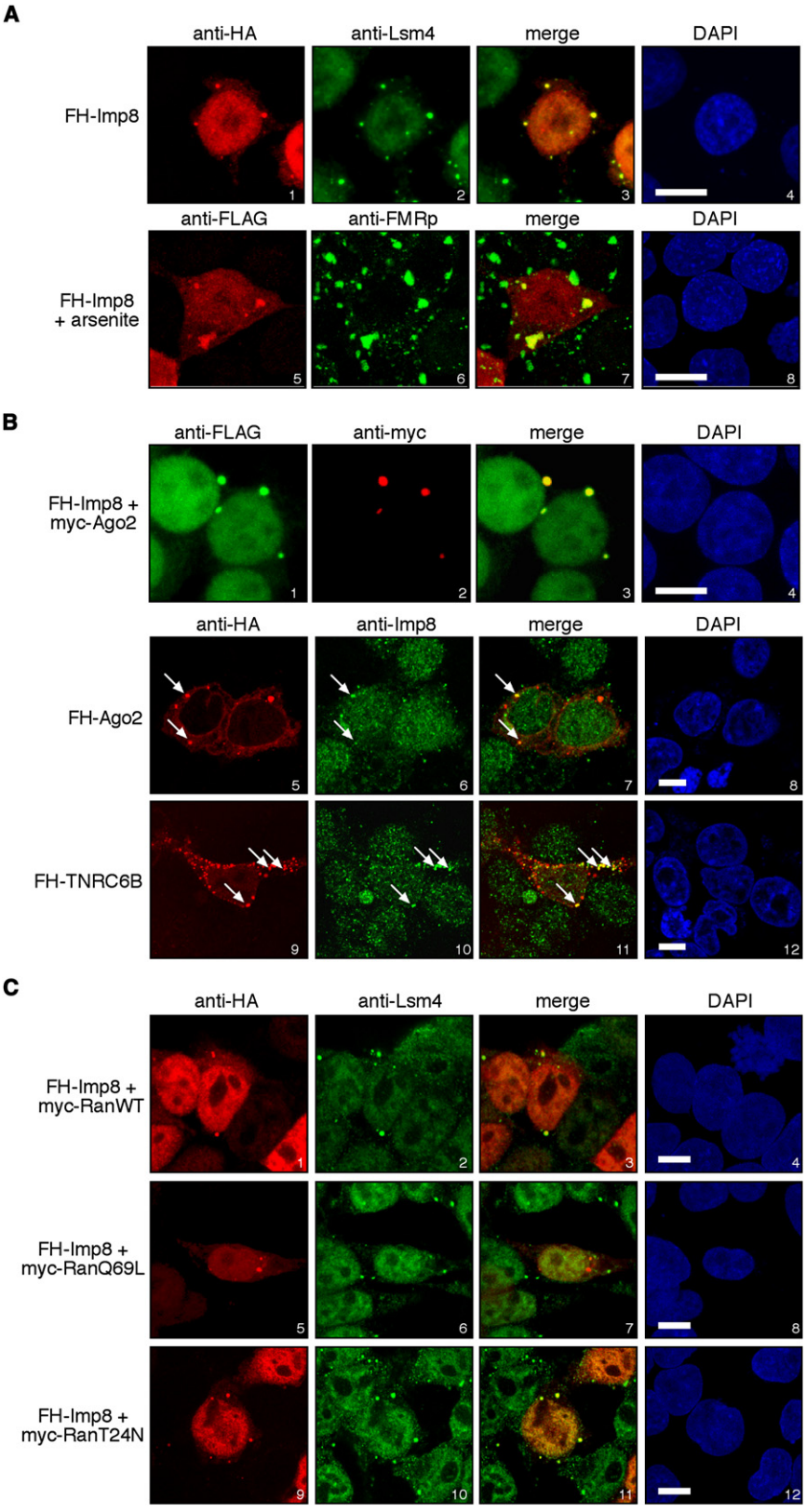


Figure 2. FH-Imp8 Localizes to P Bodies and Stress Granules

(A) HEK293 cells were transfected with FH-Imp8. Cells were fixed and stained for FH-Imp8 with anti-HA antibody (1) and for endogenous LSm4 (2). Lower panel: Cells were treated with 500 μM sodium arsenite for 30 min, fixed, and stained for FH-Imp8 with anti-FLAG antibody (5) and for endogenous FMRp (6). DAPI was used as nuclear counterstain (4 and 8).

(B) Upper panel: HEK293 cells were transfected with FH-Imp8 and myc-Ago2, fixed, and stained with anti-FLAG antibody (1), anti-myc antibody (2), and DAPI (4). Lower panels: HEK293 cells were transfected with FH-Ago2 (5-8) or FH-TNRC6B (9-12), fixed, and stained with anti-HA antibody (5 and 9), anti-Imp8 antibody (6 and 10), and DAPI (8 and 12).

(C) HEK293 cells were cotransfected with FH-Imp8 and myc-RanWT (1–4), myc-RanQ69L (5–8), or myc-RanT24N (9–12), fixed, and stained with anti-HA antibody (1, 5, and 9), anti-LSm4 antibody (2, 6, and 10), and DAPI (4, 8, and 12). Scale bars represent 10 μm.

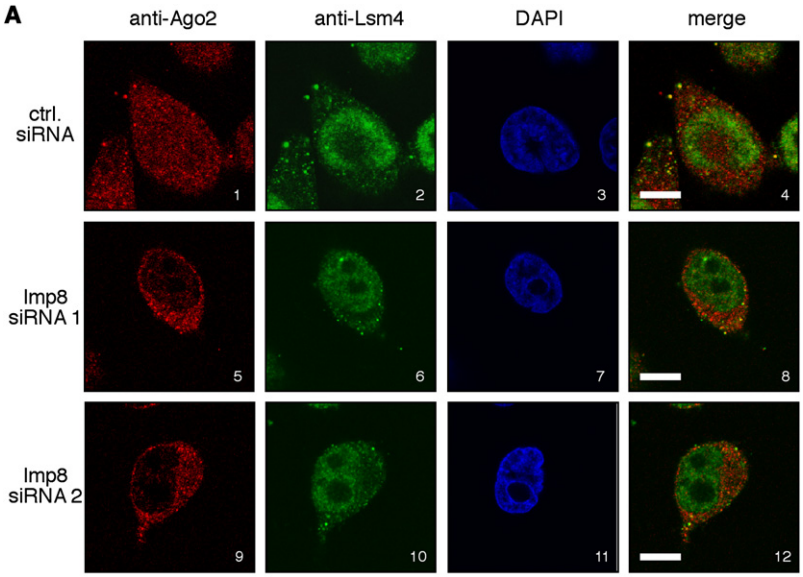
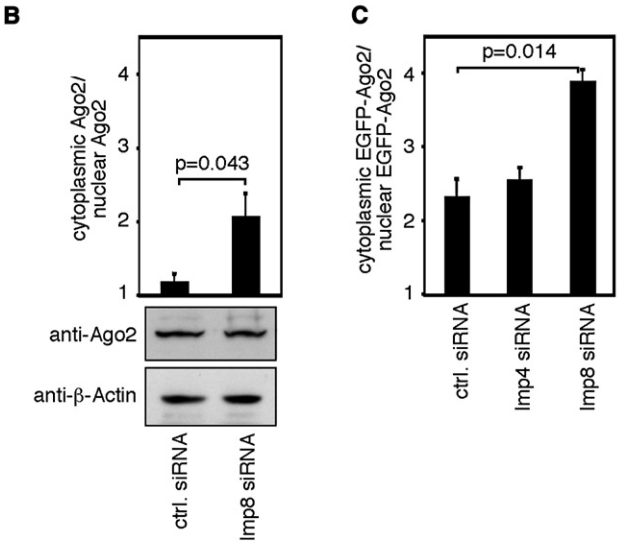


Figure 3. Imp8 Affects nuclear Localization of Ago Proteins

(A) HeLa cells were transfected with a control siRNA (1–4) or Imp8 siRNAs (5–8 and 9–12), fixed, and stained with anti-Ago2 (11A9) antibody (1, 5, and 9), anti-LSm4 antibody (2, 6, and 10), and DAPI (3, 7, and 11). The scale bar represents 10 μm.

(B) Upper panel: Cells were treated as in (A), and nuclear and cytoplasmic Ago2 signal intensities were quantified from at least 15 cells per sample. The figure shows the mean ratio of cytoplasmic versus nuclear signal intensities ± SEM. Lower panel: siRNA-transfected cells were lysed, and total Ago2 levels were assessed by western blotting, with β-Actin used as a loading control.

(C) A HEK293 subline stably transfected with EGFP-Ago2 was transfected with a control siRNA, an Imp4 siRNA, or an Imp8 siRNA. The fluorescence intensities of cytoplasmic and nuclear EGFP-Ago2 were quantified with live microscopy, and mean ratios (± SEM) of cytoplasmic versus nuclear EGFP-Ago2 signals were calculated from 20 cells per sample.



control siRNAs (lanes 1 and 3) or siRNAs directed against Imp8 (lanes 2 and 4), and the presence of let-7a or miR-21 in the immunoprecipitate was analyzed by northern blotting. Knockdown of Imp8 did not change the levels of miRNAs coprecipitated with Ago2 complexes, suggesting that Imp8 is not required for loading of Ago proteins with miRNAs.

Finally, we analyzed Imp8 requirements for binding of Ago2 to miRNA target mRNAs (Figure 5E). We and others have reported earlier that Ago complexes stably associate with miRNA targets (Beitzinger et al., 2007; Easow et al., 2007; Karginov et al., 2007). Endogenous Ago2 complexes were immunoprecipitated with anti-Ago2 antibodies, and the coprecipitated mRNAs were further analyzed by quantitative real-time PCR (qRT-PCR). As expected, endogenous Hmga2 mRNA was strongly enriched in the anti-Ago2 immunoprecipitate. Specific binding was abrogated when either let-7a antisense inhibitor or an Ago2 siRNA was transfected, indicating that the enrichment in the immunoprecipitate is miRNA specific and depends on the presence of Ago2. Strikingly, Hmga2 mRNA enrichment was significantly reduced ($p < 0.01$) when Imp8 was targeted by two different siRNAs, suggesting a role for Imp8 in recruiting Ago proteins to miRNA target mRNAs. Similar results were obtained when myc-Ago2 was analyzed (Figure 5F, left panel).

Our finding that Imp8 is required for Ago2 binding to miRNA targets as well as nuclear Ago import could potentially be explained by recruitment of Ago proteins to target mRNAs in the nucleus. In order to analyze binding of Ago2 to mRNAs in the nucleus, we fused Ago2 to a SV40-NLS, leading to Imp8-independent nuclear localization (Figure S7). Binding of SV40 NLS-Ago2 to the miRNA target HMGA2 is still dependent on Imp8 (Figure 5F, right panel, and Figure S8), suggesting that Imp8 functions in the miRNA pathway independently of nuclear Ago import and that Ago proteins are loaded onto mRNAs in the cytoplasm.

Imp8 Is Required for Binding of Ago2 to a Large Set of Target mRNAs

Next, we analyzed Imp8 effects on global Ago2 transcript binding. Endogenous Ago2 was immunoprecipitated from cell lysates that were transfected with control siRNAs or siRNAs directed against Imp8. Coimmunoprecipitated mRNA was extracted and investigated by affimetrix microarray analysis (Figure 6A and Table S3). The Ago2-associated transcripts that were most enriched compared to total RNA levels were identified in the data set where control siRNAs had been transfected. Indeed, most of the top 30 Ago2-associated mRNAs are reduced in Ago2 immunoprecipitates from Imp8-depleted cells, indicating that Imp8 not only affects Ago2-binding to HMGA2 but also many other mRNA targets (Figure 6B).

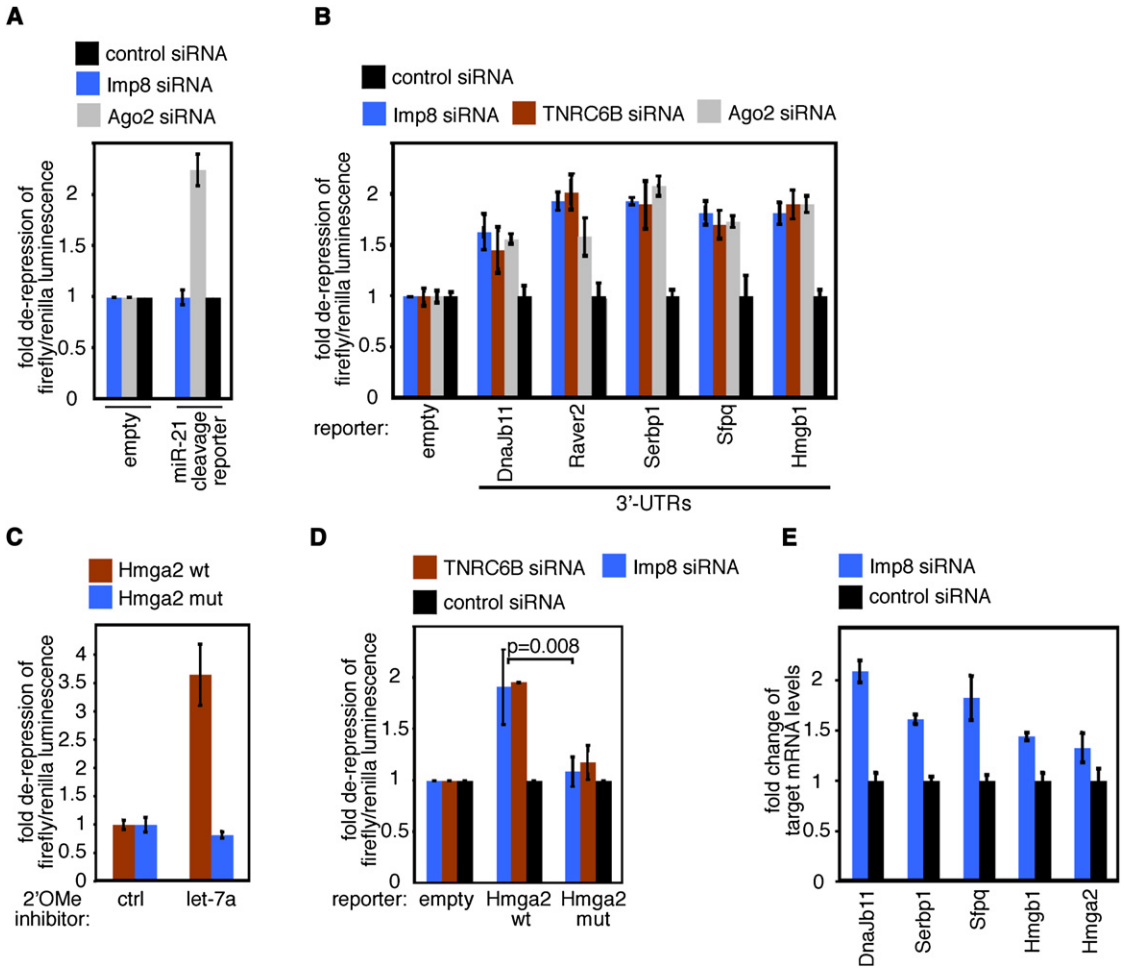


Figure 4. Imp8 Is Required for miRNA Function, but Not for RNAi

(A, B, and D) HeLa cells were sequentially transfected with the indicated siRNAs and pMIR-RL dual luciferase reporter plasmids. Firefly/renilla luminescence ratios are displayed as mean derepression of the reporter constructs (± SEM), which is calculated by normalization of the luminescence ratios of the construct of interest to the corresponding values of the empty plasmid.

(C) A Hmga2 3′ UTR dual luciferase reporter construct or a corresponding reporter construct bearing mutated let-7 binding sites was cotransfected with either hsa-let-7a antisense 2′O-methyl oligonucleotide or a control oligonucleotide into HeLa cells. Data are displayed as mean derepression of luciferase activity (± SEM), normalized to negative control oligonucleotide transfections.

(E) HeLa cells were transfected with Imp8 or control siRNAs. RNA was isolated and reverse transcribed, and miRNA target mRNA levels were quantified by qPCR. Data were normalized to GAPDH mRNA levels and to control siRNA-transfected samples.

The requirement of Imp8 for Ago2 mRNA binding was further validated by qRT-PCR (Figure 6C). Indeed, knockdown of Imp8 reduced the Ago2 association of all mRNAs that have been tested. Moreover, the 30 mRNAs analyzed in Figure 6B are significantly stabilized when TNRC6B, a component of the miRNA pathway guiding mRNA destabilization processes, is depleted (Figure 6D). In contrast, a control set of mRNAs, which is not specifically enriched on Ago2, was not stabilized upon TNRC6B knockdown (Figure 6D). These results strongly suggest that the analyzed Ago2-bound mRNAs are indeed targets of the miRNA pathway. This is further supported by the finding that miRNA seed sequence matches are enriched in Ago2-associated mRNAs (Figure 6E). In order to investigate the step at which Imp8 affects Ago2 mRNA target binding, endogenous Imp8 was immunoprecipitated and the associated mRNAs analyzed by qRT-PCR (Figure 6F). None of the Ago2-associated mRNAs analyzed in Figure 6C, however, were enriched in the Imp8 immunoprecipitate, suggesting that Imp8 does not bind to Ago2 on mRNA targets. Alternatively, Imp8-miRNA target interactions may not be stable enough to resist immunoprecipitation conditions. Our data suggest that Imp8 is required for binding of Ago2 to a broad set of mRNAs. Thus, Imp8 is a general gene-silencing factor that regulates target mRNA repression on the level of miRNP-mRNA interactions.

DISCUSSION

Ago proteins bind to miRNAs and mediate repression of target mRNA expression. However, only little is known about how Ago proteins find their specific binding sites on the 3′ UTRs of

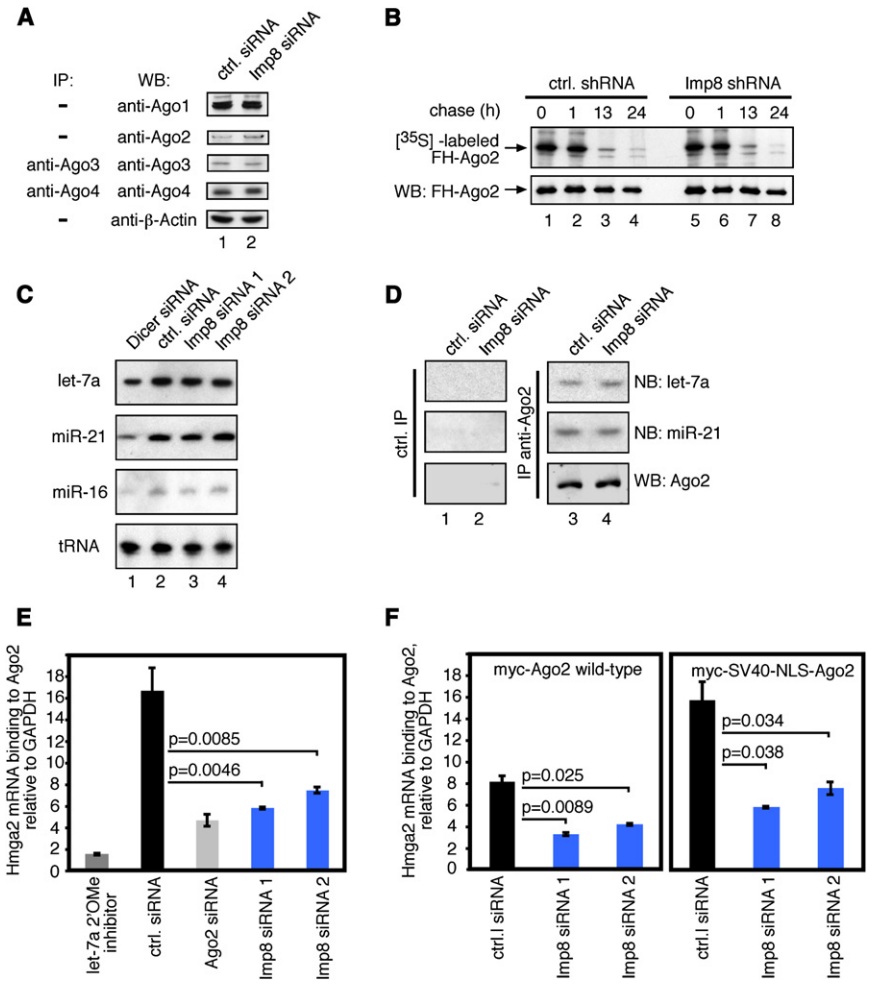


Figure 5. Imp8 Functions in Ago Protein Recruitment to Target mRNAs

(A) RNAi depletion of Imp8 does not affect soluble Ago protein levels in human cells. HeLa cells were transfected with a control siRNA (lane 1) or Imp8 siRNA (lane 2). Cells were lysed, and insoluble cellular fractions were pelleted by centrifugation. Supernatants were analyzed for Ago1/2 levels by SDS-PAGE/western blotting, with β-Actin used as loading control. Note that because of the low abundance of endogenous Ago3 and Ago4 in HeLa cells, Ago3 and Ago4 were immunoprecipitated with specific monoclonal antibodies prior to western blot analysis to obtain detectable signals.

(B) Plasmids for the expression of FH-Ago2 and an Imp8 shRNA (lanes 5–8) or a control shRNA (lanes 1–4) were cotransfected into HEK293 cells. Cells were pulsed with medium containing [^{35}S]-labeled amino acids, incubated with nonradioactive chase medium, and harvested at the indicated time points. FH-Ago2 was immunoprecipitated with anti-FLAG beads. The immunoprecipitate was analyzed for [^{35}S]-labeled FH-Ago2 (upper panel) and for total FH-Ago2 levels by western blotting (lower panel).

(C) HeLa cells were transfected with a Dicer siRNA (lane 1), control siRNA (lane 2), or Imp8 siRNAs (lanes 3–4). Total RNA was analyzed by denaturing RNA-PAGE/northern blotting for let-7a, miR-21, and miR-16, with lys-tRNA used as loading control.

(D) HeLa cells were transfected with a control siRNA (lanes 1 and 3) or Imp8 siRNA (lanes 2 and 4). IP was performed from cell lysates with a control antibody (lanes 1 and 2) or anti-Ago2 (11A9) monoclonal antibody (lanes 3 and 4). The immunoprecipitate was analyzed for Ago2 by western blotting (lower panel) and for let-7a and miR-21 by northern blotting (upper and middle panels, respectively).

(E) Cells were transfected and treated as in (D), except that additional siRNAs and a let-7a antisense 2′O-methyl inhibitor were used. RNA was isolated from immunoprecipitates and reverse transcribed. The levels of Ago2-associated Hmga2 mRNA were quantified by qPCR relative to GAPDH mRNA. For each anti-Ago2 IP sample, relative Hmga2 mRNA levels were normalized to the corresponding input samples.

(F) HeLa cells were sequentially transfected with Imp8 or control siRNAs and myc-Ago2 (left panel) or myc-SV40-NLS-Ago2 (right panel). Myc-tagged proteins were immunoprecipitated from cell lysates with anti-myc beads. Associated mRNAs were analyzed as in (E).

(E and F) Data are displayed as mean ± SEM.

target mRNAs. In this study, we provide evidence that Imp8 is required for efficient binding of Ago proteins to target mRNAs. We have identified Imp8 in proteomic analyses of Ago complex purifications (this study and Hock et al. [2007]) and show that Imp8 is required for binding of Ago2 to the let-7a target Hmga2. Imp8 knockdown had no effect on Ago stability, miRNA biogenesis, or miRNA loading onto Ago proteins. However, Hmga2 mRNA was strongly reduced in anti-Ago2 immunoprecipitations when Imp8 was depleted by RNAi, suggesting that Imp8 is involved in loading of Hmga2 mRNA with Ago2 complexes or in stabilizing the Ago2-target interaction. Similar results were obtained for many other mRNA targets, as shown by affymetrix arrays. However, we show that Imp8 is not stably associated with Ago2-bound mRNAs, suggesting that Imp8 may be required for efficient binding of Ago2 to target mRNAs rather than for stabilization of such interactions. We further show that depletion of Imp8 interferes with efficient miRNA repression of many known miRNA targets. Therefore, we propose that Imp8 might be a general factor in the miRNA pathway. Specific factors negatively regulating miRNA binding to target 3′ UTRs have been identified recently. It has been shown in liver cells that HuR (ELAV1), which binds to AU-rich elements on target mRNAs, antagonizes miR-122 repression on the CAT-1 3′ UTR upon cellular stress (Bhattacharyya et al., 2006). Moreover, Dnd1, a protein expressed in primordial germ cells, prevents miRNA-guided repression by binding to the vicinity of miRNA binding sites (Kedde et al., 2007). In contrast, Imp8 is required for efficient binding of miRNAs to 3′ UTRs and therefore functions in the opposite manner of factors such as HuR or Dnd1. Interestingly, Imp8 is not required for RNAi, suggesting that the assembly of miRNP-mRNA structures might be a highly coordinated process, which may differ from sequence-specific cleavage events. Notably, imb-5, the closest *C. elegans* homolog of human Imp8, has been identified in

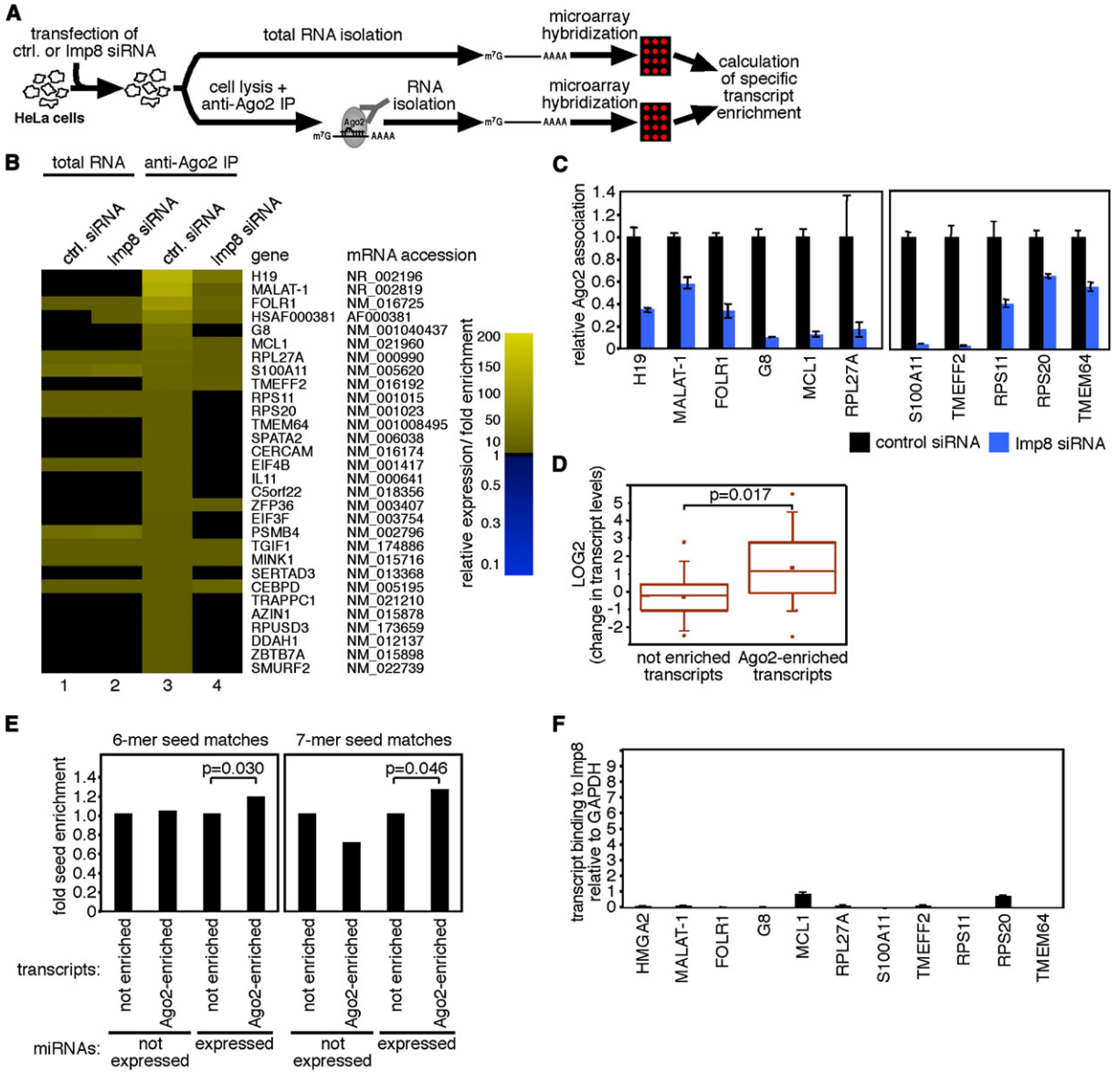


Figure 6. Imp8 Is Required for Binding of Ago2 to a Large Set of Target mRNAs

(A) HeLa cells were transfected with control or Imp8 siRNAs. Each sample was divided into two parts for total RNA extraction (upper arrow), and anti-Ago2 immunoprecipitation followed by RNA extraction (lower arrows). Total RNA samples and immunoprecipitated RNA samples were hybridized to separate Human Genome U133 Plus 2.0 Affymetrix microarrays. The specific enrichment of each individual transcript was calculated from its normalized measurement value in the immunoprecipitation sample, divided by its value in the total RNA sample.

(B) A heat map of 30 transcripts most highly enriched in anti-Ago2 immunoprecipitates from control siRNA-transfected cells. The heat map shows normalized measurements for total RNA from control siRNA-transfected cells (lane 1), Imp8 siRNA-transfected cells (lane 2), and Ago2-associated RNA from control siRNA-transfected cells (lane 3) and Imp8 siRNA-transfected cells (lane 4).

(C) Ago2 association of transcripts was validated by qRT-PCR and quantified relative to GAPDH and to input lysate samples. Ago2 association of transcripts in control siRNA transfected cells was normalized to 1.

(D) Box-whisker plot for log2 changes in transcript abundance after TNRC6B knockdown. The plot shows the top 30 Ago2-associated transcripts (right side) and transcripts which are not specifically associated (enrichment = 1 ± 0.05; left side). Outliers are denoted by asterisks. Mean changes of Ago2-associated transcript levels are significant ($p = 0.017$; Student's t test from four microarray replicates from immunoprecipitated RNA).

(E) The number of 6-mer (left panel) and 7-mer (right panel) seed sequence matches was calculated for 3′ UTRs of all transcripts more than 2-fold Ago2-enriched in at least three out of four microarray replicates compared to seven control groups of not enriched transcripts (enrichment = 1 ± 0.1). Enrichment was calculated for nine miRNAs highly expressed in HeLa cells and nine absent miRNAs, followed by normalization to 3′ UTR length.

(F) HeLa cell lysates were immunoprecipitated with anti-Imp8 antibodies. RNA was isolated from immunoprecipitates, and the specific association of transcripts with Imp8 was calculated relative to the input sample and to GAPDH.

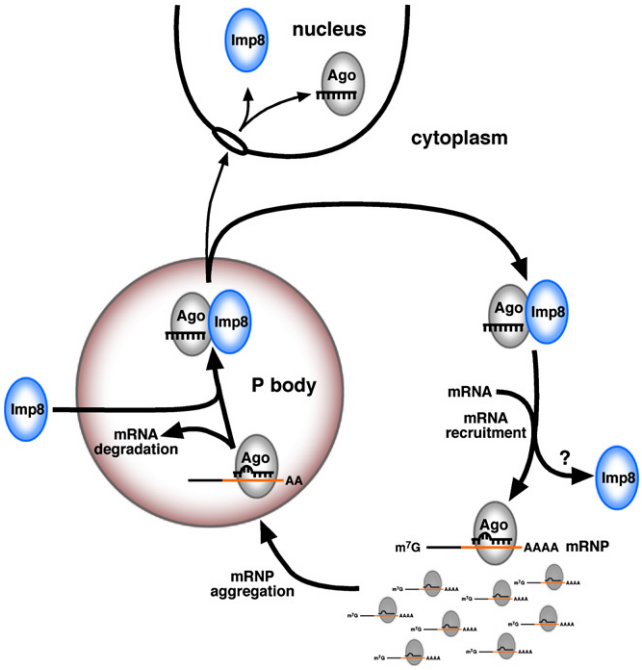


Figure 7. A Model for Imp8 Function

Imp8 may bind to Ago proteins in P bodies, followed by shuttling to the nucleus. Alternatively, Imp8 assists Ago protein recruitment to miRNA target mRNAs in the cytoplasm by delivering Ago proteins to target mRNAs, followed by dissociation of the Imp8-Ago protein complex and aggregation of repressed mRNPs. Please note that the Ran binding cycle was omitted from the figure for simplification.

a genetic screen for factors essential for small RNA-guided gene silencing (Kim et al., 2005).

Notably, among the most highly enriched Ago2-associated transcripts we found the noncoding RNAs H19 and MALAT-1. Most miRNA target predictions published thus far have been restricted to 3′ UTRs of mRNAs. Our data suggest that other noncoding RNAs are targeted by miRNAs as well. On the basis of the analysis of Ago2-associated mRNAs, we suggest that miRNA target searches should be extended to the entire transcriptome and should include larger noncoding transcripts.

Interestingly, Imp8 localizes together with Ago proteins to P bodies in human cells. It is therefore tempting to speculate that Ago proteins bind to Imp8 in P bodies presumably after target mRNA degradation and that Imp8 subsequently transfers free Ago-miRNA complexes to new target mRNAs (Figure 7). Since Imp8 has no obvious RNA binding domain, Imp8 might transiently interact with other protein factors that interact with specific binding sites on target 3′ UTRs. Alternatively, binding of Imp8 to Ago proteins may alter the structure of Ago proteins, allowing for efficient binding to target mRNAs.

Ago proteins have been implicated in transcriptional silencing processes in different organisms (Lippman and Martienssen, 2004). The intriguing finding that the import receptor Imp8 associated with human Ago complexes led us to investigate whether or not Ago proteins are imported into the nucleus. Using a monoclonal anti-Ago2 antibody, we provide evidence that Ago2 is indeed found in the nucleus of human cells. Moreover, depletion of Imp8 reduced the pool of nuclear Ago2, suggesting that Imp8 is involved in targeting Ago proteins to the nucleus. Biochemical analyses have suggested before that miRNAs as well as Ago proteins localize and function in the nucleus (Meister et al., 2004; Robb et al., 2005). siRNAs directed against nuclear RNAs such as 7SK RNA efficiently reduced 7SK RNA levels in nuclear fractions, suggesting that Ago2-mediated cleavage of target RNA occurs in the nucleus (Robb et al., 2005). Notably, a significant portion of Ago2 remained in the nucleus after Imp8 depletion, suggesting that alternative nuclear import pathways for Ago proteins may exist. Human Ago proteins have been implicated in transcriptional silencing processes (Janowski et al., 2006, 2007; Kim et al., 2006; Morris et al., 2004). It is possible that the nuclear Ago pool might also be involved in small RNA-guided transcriptional silencing processes in human cells. It is also conceivable that novel nuclear Ago functions exist that have yet to be defined.

Our data strongly suggest that Imp8 plays a major role in Ago-mediated gene silencing (Figure 7). In the cytoplasm, Imp8 localizes together with Ago proteins to P bodies and is involved in efficient loading of Ago proteins onto a variety of different target mRNAs. Moreover, Imp8 may also direct Ago proteins to the nucleus of human cells. Currently, it is unclear which factors direct Imp8 for nuclear import and which factors for Ago protein loading onto mRNA targets. It is likely that other factors that are present in our proteomic data set might be involved in regulating Imp8 function in small RNA pathways.

EXPERIMENTAL PROCEDURES

Antibodies and Recombinant Proteins

The following antibodies were used: mouse-anti-HA (Covance, Princeton, NJ), rabbit-anti-FLAG (Sigma, St. Louis, MO), rabbit-anti-myc (Abcam, UK), mouse anti-β-Actin AC15 (Abcam, UK), chicken-anti-Lsm4 (Genway, San Diego, CA), anti-rabbit-HRP (Sigma), anti-mouse-HRP (Sigma), anti-rat-HRP (Jackson, West Grove, PA), goat-anti-rat-Texas Red, goat-anti-rabbit-Texas Red, horse-anti-mouse-fluorescein, horse-anti-mouse-Texas Red (all Vector Laboratories, Burlingame, CA), and anti-chicken-fluorescein (Sigma).

Anti-Ago2 clone 11A9 has been described elsewhere (Rüdel et al., 2008). Rat-anti-Ago1 clone 1C9, rat-anti-Ago3 clone 5A3, and rat-anti-Ago4 clone 6C10 were generated as described (Beitzinger et al., 2007). Anti-Imp8 polyclonal antiserum was generated by immunization of KLH-CMQSNNGRGEDEEEEDDDWD into rabbits. Purification of anti-Imp8 polyclonal antibody was performed with CNBr-activated Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's instructions. Recombinant GST-Importin 8 dialyzed against coupling buffer (0.1 M $NaHCO_3$ [pH 8.3], 0.5 M NaCl) served as ligand.

Coupled sepharose beads were washed with 10 ml PBS and subsequently incubated with 10 ml serum overnight at 4°C. Serum was removed from the column by gravity flow, and beads were washed twice with 10 ml PBS. For elution of the purified antibody, 1 ml fractions of elution buffer (0.1 M Glycine [pH 2.3]) were added to the beads, and the column was emptied by gravity flow into reaction tubes containing 0.1 ml 1M Tris-HCl (pH 8.8) to neutralize the eluate.

Recombinant GST-Imp8 was expressed and purified as follows: pGEX6P1-Imp8 was transformed into *E. coli* BL21 Rosetta bacteria. Overnight cultures were diluted into fresh medium, grown to 0.8 OD_{600}, and induced at 18°C with 1 mM IPTG for 18 hr. Bacteria were disrupted by sonication in purification buffer [500 mM NaCl/50 mM Tris/HCl (pH 7.5)/5 mM $MgCl_2$/1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)], and debris was removed by centrifugation. Supernatants were incubated with Glutathione sepharose beads (GE healthcare) for 2 hr. Beads were washed twice with purification buffer (pH 8.0). GST-Imp8 was eluted in purification buffer (pH 8.0) containing 3 mg/ml glutathione and dialyzed against PBS.

Coimmunoprecipitation and Mass Spectrometry

For coimmunoprecipitation (Co-IP) experiments, 3 × 15 cm plates of HEK293 cells per sample were transfected with appropriate plasmids for 2 days. Cells were washed twice with PBS and lysed in 500 μl lysis buffer (150 mM KCl/25 mM Tris-HCl [pH 7.5]/2 mM ethylenediaminetetraacetic acid [EDTA]/1 mM NaF/0.5% NP-40/0.5 mM dithiothreitol [DTT]/0.5 mM AEBSF) per plate. Ribolock (Fermentas, 1 μl per ml of lysis buffer) was added for RNA IPs. Lysates were cleared by centrifugation at 16,000 g for 10 min. For IP of FLAG-tagged proteins, lysates were incubated with 60 μl anti-FLAG M2 agarose beads for 3 hr at 4°C with or without RNaseA (QIAGEN, 20 μg/ml). For IP of endogenous proteins, 6 ml monoclonal antibody-containing hybridoma supernatant was coupled to 60 μl protein G-Sepharose (GE Healthcare) for 2 hr at 4°C. Coupled beads were washed twice with PBS and subsequently incubated with cell lysate for 3 hr at 4°C. All IP samples were washed three times with IP wash buffer (300 mM NaCl/50 mM Tris [pH 7.5]/1 mM NaF, 0.01% NP-40/5 mM $MgCl_2$) and once with PBS. For the detection of proteins, beads were boiled in protein sample buffer. For the detection of associated RNAs, proteins were digested with 1 mg/ml proteinase K for 1 hr at 42°C, followed by phenol/chloroform/isopropyl alcohol extraction and precipitation of RNA in 80% ethanol at −20°C. Mass spectrometry of Ago complexes was performed as previously described (Hock et al., 2007).

Ago2-Associated mRNA Analysis

HeLa cells were reverse transfected in 10 cm plates with Imp8 or control siRNAs. For the analysis of mRNA binding to myc-tagged Ago2, cells were transfected 2 days later with 15 μg/plate pCS2-Ago2 or pCS2-SV40-NLS-Ago2 with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three days after plasmid transfection, cells were lysed and treated analogous to IP samples. IP for myc-tagged proteins was performed for 2.5 hr at 4°C with 40 μl anti-myc beads (Sigma) per sample. For analysis of mRNA binding to endogenous Ago2, siRNA-transfected cells were harvested 5 days after transfection and treated as described for IP samples. Immunoprecipitates were washed three times with IP wash buffer and once with PBS. IP samples and corresponding samples containing 10% of input lysate were proteinase K digested, followed by phenol/chloroform/isopropyl alcohol extraction and precipitation of RNA in 80% ethanol at −20°C. RNA was pelleted, dried, and treated with DNaseI (Fermentas) for 30 min at 37°C, followed by thermal inactivation of DNaseI. RNAs were detected via cDNA synthesis and qPCR. Hmga2 mRNA levels were normalized to GAPDH mRNA or Ile tRNA levels for input and IP samples. Specific binding of Hmga2 mRNA to Ago proteins was calculated from the relative Hmga2 mRNA abundance in IP samples, divided by the relative abundance in the corresponding input samples.

Microarray Hybridization and Data Analysis

RNA for microarray analysis was isolated with PrepEase Kit for total RNA (USB, Cleveland, OH) and phenol/chloroform/isopropyl alcohol extraction for IP samples. RNA was processed and hybridized with the Gene Chip (Affymetrix) kit and the hybridization procedure for eukaryotic samples, used according to the manufacturer's instructions. Samples were hybridized to Human Genome U133 Plus 2.0 arrays. All arrays were performed in at least two biological replicates.

Microarray data were analyzed with Agilent Genespring software. Expression values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. All IP samples were normalized to the corresponding total RNA samples: the IP sample from control siRNA-transfected cells was normalized against the median of the corresponding total RNA sample, and the IP sample from Imp8 siRNA-transfected cells was normalized against the median of the corresponding total RNA sample. Each measurement for each gene in the IP samples was divided by the median of that gene's measurements in the corresponding total RNA samples.

Using this normalization procedure, the normalized expression value of each transcript in IP samples directly reflects its over- or underrepresentation in the immunoprecipitated transcript pool relative to the total RNA pool. So that transcripts bound by Ago2 could be filtered, all transcripts with raw measurements over 50 that were more than 4-fold enriched in immunoprecipitates from control siRNA-transfected cells were displayed (Table S3). p values for enriched transcripts were calculated on the basis of expression levels with the Genespring software.

ACCESSION NUMBERS

The microarray data sets reported in this paper have been deposited in Gene Expression Omnibus with the serial accession number GSE14054.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eight figures, and four tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01628-0.

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