Argonaute (Ago) proteins interact with small regulatory RNAs such as microRNAs (miRNAs) and facilitate gene-silencing processes. MiRNAs guide Ago proteins to specific miRNAs leading to translational silencing or mRNA decay. In order to understand the mechanistic details of miRNA function, it is important to characterize Ago protein interactors. Although several proteomic studies have been performed, it is not clear how the Ago interactome changes on miRNA or mRNA binding. Here, we report the analysis of Ago protein interactions in miRNA-containing and miRNA-depleted cells. Using stable isotope labeling in cell culture in conjunction with Dicer knock out mouse embryonic fibroblasts, we identify proteins that interact with Ago2 in the presence or the absence of Dicer. In contrast to our current view, we find that Ago-mRNA interactions can also take place in the absence of miRNAs. Our proteomics approach provides a rich resource for further functional studies on the cellular roles of Ago proteins.

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Argonaute proteins are a highly conserved protein family found in all kingdoms of life (1, 2). They directly interact with small RNAs and can be classified according to the small RNA class they bind. The Argonaute (Ago) family is ubiquitously expressed and interacts with short interfering RNAs or miRNAs to mediate post-transcriptional gene silencing processes (3, 4). Expression of the Piwi subfamily of Argonaute proteins seems to be restricted to the germline, where they bind to Piwi interacting RNAs and inhibit the expression of mobile genetic elements (5).

Argonaute proteins contain Piwi-Argonaute-Zwille, MID, and PIWI domains. Structural analysis revealed that the Piwi-Ago Argonaute-Zwille domain binds the 3’ end, whereas the MID domain specifically anchors the 5’ end of the bound small RNA (6). The PIWI domain is structurally similar to RNase H and indeed some Argonaute proteins possess endoribonuclease activity. Such proteins are referred to as “slicers” (7, 8).

MiRNAs, the main binding partners of Ago subfamily members in mammals, are transcribed by RNA polymerase II as capped and polyadenylated primary miRNA transcripts (4, 5, 9). In the nucleus, the microprocessor containing the RNase III enzyme Drosophila processes primary miRNA transcripts to stem-loop structured precursors, which are exported to the cytoplasm. Here, the RNase III Dicer cleaves an approx. twenty to twenty-three nucleotide (nt) long double stranded intermediate out of the stem of the pre-miRNA. The mature miRNA duplex is subsequently unwound and one strand gives rise to the mature miRNA whereas the other strand, referred to as miRNA*, is removed by cellular degradation systems. In rare cases, both strands can be selected and function as mature miRNAs (e.g. mir-9 and mir-9*). The mature miRNA binds to Ago proteins and together with other proteins, a miRNA-protein complex, referred to as miRNP, is formed (4, 9).

MiRNAs guide miRNPs to partially complementary sequences often located on the 3’ untranslated region (UTR) of target mRNAs (10, 11). On the target mRNA, a member of the GW protein family (termed TNRC6A-C in mammals) interacts with the Ago protein and induces deadenylation of the target mRNA by recruiting cellular deadenylases. mRNAs with shortened poly(A) tails are either translationally silent or decapped and finally degraded by cellular mRNA decay systems (12).

MiRNA-guided gene silencing is important for almost all cellular processes. Therefore, miRNA function is heavily regulated at many different steps. First, regulation occurs on the various steps of miRNA biogenesis including transcription and processing by Drosha and Dicer (13). Second, Ago protein
levels as well as their activities can be regulated by phosphorylation, hydroxylation, or ubiquitinylination (14–16). Third, Ago proteins are embedded into large protein-RNA structures containing miRNAs as well as translationally repressed mRNAs (17–19). It is becoming more and more apparent that proteins within such RNA-protein complexes (RNPs) can influence miRNA-guided gene silencing activity. For example, RNA binding proteins such as Dead end 1 (Dnd 1) can regulate accessibility of miRNA target sites (20). Another example is the ARE binding protein HuR, which antagonizes miRNA function by interfering with miRNA-target site interactions (21). Furthermore, the RNA binding protein hnRNP-E2 can function as decoy for a specific mature miRNA (22). These examples highlight the importance of the protein composition of an mRNA-protein complex (mRNP) for miRNA-guided gene silencing.

A common approach to analyze protein complex composition is the combination of affinity purification with mass spectrometric analysis (AP-MS) (23). However, despite great advances in this technology, it can have potential limitations especially when used in a nonquantitative format. Indeed, due to improvements in preparation methods and instrument sensitivity the potential to identify false positive as interaction partners has increased. Consequently, samples need to be purified to a high degree, for example by double affinity purification. Such stringent purification approaches require a high amount of sample and risk losing relevant but substoichiometric and weak interactions. Quantitative proteomic approaches present an elegant solution to these problems (24, 25). They directly distinguish between background binders and true interaction partners by quantification between sample and control. Thus, they facilitate high confidence identification of interaction partners from low stringency and single step purifications (26). SILAC is a widespread metabolic labeling technique used in quantitative proteomics (27). SILAC-based proteomics can be applied to compare different states of protein complexes, for example upon stimulation (24, 28) or protein complexes in mammalian cells. Here, we present a SILAC-based interaction partners that specifically associate with Ago2 in the absence of Dicer and miRNAs. Unexpectedly, we find that Ago2 still associates with miRNAs in the absence of miRNAs. Based on our proteomics data, we present an in depth and specific RNA-dependent and -independent Ago2 interaction network.

**EXPERIMENTAL PROCEDURES**

**Lysate Preparation and Immunoprecipitation**—SILAC labeled cells were lysed separately in Lysis Buffer (150 mM KCl, 25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM NaF, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 1× Complete Protease Inhibitor mixture (Roche)). For immunoprecipitation 3–4 mg total lysate protein was incubated with 50 μl M2 FLAG agarose beads (Sigma) for 4 h at 4 °C with rotation. For RNA dependence samples 100 μg/ml RNase A was added to the lysate for the last 20 min of the incubation. Beads were washed three times with IP Wash Buffer (300 mM KCl, 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 0.1% Nonidet P-40, 5% glycerol) and twice with Elution Buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 5% glycerol). Corresponding beads for the SILAC experiments were combined directly after washing and bound proteins were eluted with 500 μg/ml 3x FLAG peptide (N-MDYKDHDGDYKDHDIDYKDDDDK-C) in Elution Buffer for 90 min at 4 °C with 800 rpm.

**Protein Digestion**—Eluates were separated by one dimensional gel electrophoresis on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA) and visualized by staining with the NOVEX Colloidal Blue Stain Kit (Invitrogen). Lanes were cut into 8 slices and proteins were in-gel digested with trypsin (Promega, Madison, WI) (40) using iodoacetamide as alkylation reagent. Peptides were concentrated and desalted using C₁₈ StageTips (41, 42).

**LC-MS/MS Analysis**—Peptides were separated on line to the mass spectrometer by using an easy nano-LC system (Proxeon Biosystems, Dreieich, Germany). Four microliter samples were loaded with a constant flow of 700 nl/min onto a 15-cm fused silica emitter with an inner diameter of 75 μm (Proxeon Biosystems) packed in house with RP ReproSil-Pur C₁₈-AQ 3 μm resin (Dr. Maisch). Peptides were eluted with a segmented gradient of 5–60% solvent B over 105 min with a constant flow of 250 nl/min. The nano-LC system was coupled to a mass spectrometer (LTQ-Orbitrap; Thermo Fisher Scientific) via a nanoscale LC interface (Proxeon Biosystems). The spray voltage was set between 2.0 and 2.2 kV, and the temperature of the heated capillary was set to 200 °C.

Survey full-scan MS spectra (m/z = 300–2000) were acquired in the Orbitrap with a resolution of 60,000 at the theoretical m/z = 400 after accumulation of 1,000,000 ions in the Orbitrap. The five most intense ions from the preview survey scan delivered by the Orbitrap were sequenced by collision induced dissociation (collision energy 35%) in the LTQ after accumulation of 5000 ions concurrently to full scan acquisition in the Orbitrap. Maximal filling times were 1000 ms for the full scans and 150 ms for the MS/MS. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged peptides were rejected. The dynamic exclusion list was restricted to a maximum of 500 entries with a maximum retention period of 90 s and a relative mass window of 10 ppm. Orbitrap measurements were performed with the lock mass option enabled for
SILAC-based Identification of Dicer-dependent AGO2 Interactions

The raw files were processed using the MaxQuant computational proteomics platform (44) version 1.1.1.27. The fragmentation spectra were searched against the IPI mouse database (version 3.68, 56,729 entries) supplemented with frequently observed contaminants, using the Andromeda search engine (45) with the initial precursor and fragment mass tolerances set to 7 ppm and 0.5Da, respectively, and with up to two missed cleavages allowed. Trypsin allowing for cleavage N-terminal to proline was chosen as enzyme specificity. Carbamidomethylated cysteins were set as fixed, oxidation of methionine, and N-terminal acetylation as variable modifications. Maximum false discovery rates (FDRs)—calculated by employing a reverse database strategy—were set to 0.01 both on peptide and protein levels and thus not dependent on the peptide score. Minimum required peptide length was six amino acids. Corresponding forward and reverse experiments were analyzed together and specified as “forward” and “reverse” in the experimental design.

Raw MS data, unfiltered protein groups tables and peptides tables can be downloaded from https://proteomecommons.org/tranche using the following HASH key: a6LrT+dbAF4mNtZcpYJvut2NTFl0Vnhf1qeh4SwQ8l68MSFv5dnfBw35b8RN099yQZUAhTvWHP+1x07dW2SeigAAAAAABX0A==.

All further analysis was done in a script-based manner employing R (http://www.r-project.org). Protein groups were further filtered requiring at least two unique peptides per protein identification, and 2 ratio counts (quantification events) in the forward as well as in the reverse experiment. For all analysis log2 transformed normalized ratios (as computed by MaxQuant) were used.

Median plus 1.2 standard deviations and median minus 1.2 standard deviations was used as significance threshold in the forward and reverse experiment, respectively. Proteins were defined as interactors if they passed the threshold either in the wt versus control, or the knockout versus control experiment in the forward and reverse experiment. For all further analysis, only proteins passing these criteria were considered. For hierarchical clustering only proteins that showed a ratio in four out of six experiments (wt versus control, ko versus control, wt versus ko; each forward and reverse) were considered.

The separate experiments were combined using the Uniprot identifier, and proteins were clustered employing an Euclidian distance measure.

RESULTS

Generation of MEFs Stably Expressing Tagged Ago2—To identify Dicer-dependent and -independent Ago2 interactors, we established Dicer-deficient or Dicer wt MEF cell lines that stably express FH-tagged Ago2 (Fig. 1). We confirmed the stability of the Dicer knock out during culturing by PCR (Fig. 1A) and tested FH-Ago2 expression levels by Western blotting using antibodies against the HA-tag (Fig. 1B). As expected and also observed for endogenous Ago proteins (data not shown), Ago2 levels were slightly lower in the Dicer-deficient cells compared with Dicer wt cells. This is probably due to active destabilization of untagged Ago proteins. The GFP-only expressing cell lines serve as controls for all experiments. To minimize background binding during our biochemical Ago2 complex purification, we asked whether FH-Ago2 can be eluted with the FLAG-peptide (Fig. 1C). Indeed, an excess of FLAG-peptide efficiently removed bound FH-Ago2 from the anti-FLAG antibody matrix. As a further quality control, we analyzed miRNA expression in Dicer-deficient MEFs that express FH-Ago2 (Fig. 1D). Northern blotting against miR-19b showed that miRNAs were neither present in the lysates of Dicer-deficient MEFs nor in anti-FLAG immunoprecipitates from these cells. Therefore, the cell lines are suitable for the analysis of miRNA-dependent and -independent Ago2 interaction partners.

To further increase the robustness and information content of our quantitative mass spectrometry data, we performed forward and reverse (label swapping) experiments. For the forward experiment, we labeled the FH-Ago2-expressing cell line with heavy amino acids and the corresponding GFP-expressing cell line with light amino acids (Fig. 1E, left panel). For the reverse experiment, the labels of the cell lines were swapped (right panel). To prevent potential heavy to light exchange of specific transiently interacting partners during the purification procedure (46, 47), the FH-Ago2 complexes were immunoprecipitated with the FLAG antibody from total lysates for each SILAC state separately and combined during FLAG peptide elution. Eluates were separated by SDS-PAGE and cut into eight slices, proteins were in-gel digested and peptides were analyzed by high resolution LC-MS/MS on an LTQ Orbitrap instrument.

Specific interactors by definition show a high ratio in forward and a low ratio in reverse experiments, whereas unspecific background binders show a ratio close to 1 in both experiments. For data visualization, we plot the logarithmized normalized ratios of the forward and reverse experiments against each other. Every dot represents an identified and quantified protein. Each of the datasets contained on average 1214 identified proteins and 672 of these fulfilled our strict criteria for quantification (see Experimental procedures). Background binders constitute the vast majority of quantified proteins show no ratio change between SILAC pairs and are therefore clustered around zero (Fig. 1F, gray circle). Specific Ago2 interactors show high ratios in the forward and low ratios in the reverse experiment and are outliers from the background distribution in the lower right quadrant (green circle).

Identification of Dicer-independent Ago2 Interactors—To compare miRNA-free (unloaded) and miRNA-containing (loaded) Ago2 complex compositions, we isolated FH-Ago2 from Dicer wt and Dicer-depleted cell lines. GFP-transfected cells were used to identify unspecific binders (Fig. 2). Proteins interacting with Ago2 independently of miRNAs show significant ratios above the cut-off both in Dicer +/+ and Dicer −/− cell lines when measured against the GFP control (Fig. 2A, left and middle panel; specific binders appear in the lower right quadrant). To directly quantify the interaction as a function of the presence or absence of Dicer and miRNAs, we additionally precipitated FH-Ago2 from Dicer +/+ MEFs (heavy label) and Dicer −/− MEFs (light label). Labels were swapped for the reverse experiment. In this experiment, proteins binding independently of miRNAs appear together with the background binders clustering around zero (right panel).

1444
Among the Dicer-independent Ago2 binders (Table I; see supplemental Table S1 for the complete mass spec data set), we found a number of proteins that have been implicated in Ago2 function before, indicating high specificity in our analyses. For example, Hsp90 is involved in loading small RNAs into RISC (48, 49) and it regulates Ago2 localization (50).

**Fig. 1.** Characterization of the F/H-Ago2 expressing MEFs. **A**, Wild type (wt) and Dicer-depleted (ko) MEFs were transduced with an adenovirus carrying F/H-Ago2 and GFP. Genomic DNA was isolated from cells 3 weeks after transduction and amplified by PCR with primers flanking the deleted region in the Dicer gene. PCR products were separated on an agarose gel and visualized by Ethidiumbromide staining. **B**, Expression of F/H-Ago2 was analyzed by Western blotting. Total cell lysates were separated by SDS-PAGE, blotted and probed with anti-HA (upper panel), anti-Ago2 (middle panel) or anti-tubulin antibodies (lower panel). C, Proteins were immunoprecipitated from total lysate with anti-FLAG agarose beads and bound proteins were eluted from the antibody with 3xFLAG peptide. Eluted proteins were analyzed by Western blotting with anti-HA antibodies. The asterisk indicates a degradation product. D, RNA was isolated from lysate or immunoprecipitated F/H-Ago2, separated by 12% denaturing PAGE, blotted and probed for miR-19b (upper panel) or U6 (lower panel). E, Schematic representation of SILAC-based interaction proteomics. For the forward experiment (left panel), the F/H-Ago2-expressing cell line (bait expression) was labeled with heavy amino acids (red) and the corresponding GFP-expressing cell line (no bait) was labeled with light amino acids (blue). For the reverse experiment (right panel), the SILAC label was swapped. F, Forward and reverse data sets are combined for analysis. For data visualization, the logarithmized normalized ratios of the forward and reverse experiments are plotted against each other. Background binders are clustered around zero, as highlighted by the gray circle. Specific interactors can be found in the lower right quadrant as indicated by the green circle.

Among the Dicer-independent Ago2 binders (Table I; see supplemental Table S1 for the complete mass spec data set), we found a number of proteins that have been implicated in Ago2 function before, indicating high specificity in our analyses. For example, Hsp90 is involved in loading small RNAs into RISC (48, 49) and it regulates Ago2 localization (50).
co-chaperones FKBP5 and PTGES have not been described in Ago2 complexes before and but our results suggest that they may function in a similar manner. Another example is the putative DExD box helicase MOV10, which has been identified as RISC component before (17, 18, 34). Other proteins in this group are the mRNA binding proteins IGF2BP1–3, PUM2, DHX30, HNRNPL, a highly specific set of ribosomal proteins (RPS 19, 18, 14, 5, and 3a) and the ARE binding protein DHX36/RHAU. We also find proteins involved in mRNA decay such as the decapping enhancer EDC4, Upf1 and DDX6/Rck (51–53). As mentioned above, GW proteins directly interact with Ago proteins via a specific Ago-interaction domain (12) and consistently, we find TNRC6A and TNRC6B as Dicer-independent Ago2 binders. Finally, the novel Ago2 interacting protein STRAP/Unrip (54) found here has been shown to interact with poly-A binding proteins and are involved translational repression in Drosophila (55). We speculate that this protein is involved in miRNA-guided gene silencing as well.

Identification of Dicer-dependent Ago2 Interactors—We next analyzed proteins that bind to Ago2 preferentially in the
### Table I

**Dicer dependent and independent Ago2 interactors**

| Protein names | Gene names | Uniprot | Keywords | wt | knockout | wt vs knockout |
|---------------|------------|---------|----------|-----|----------|---------------|
|               |            |         |          | Forward | Reverse | Forward | Reverse | Forward | Reverse | Forward | Reverse |
| Bait protein: Ago2 |            |         |          | 31.401 | 0.017 | 35.089 | 0.021 | 1.2876 | 0.952 |
| Protein argonaute-2 | Elf2c2;Ago2 | A1A563 | small RNA mediated gene silencing | 55.043 | 0.016 | 63.056 | 0.016 | 1.177 | 1.017 |
| Proteins binding independently of the presence or absence of mRNAs/Dicer |            |         |          | 33.307 | 0.008 | 22.783 | 0.026 | 0.90288 | 2.010 |
| Peptidyl-prolyl cis-trans isomerase FKBP5 | Fkbp5 | Q64378 | heteromultimeric cytoplasmic complex with HSP90 and HSP70 | 33.102 | 0.015 | 16.950 | 0.044 | 1.0143 | 1.066 |
| Heat shock protein HSP 90- alpha | Hsp90 alpha | P07901 | molecular chaperone ATPase activity | 29.605 | 0.027 | 37.910 | 0.012 | 0.87774 | 1.995 |
| Heat shock protein 84b | Hsp90 beta | Q71LX8 | molecular chaperone ATPase activity | 27.236 | 0.015 | 36.365 | 0.017 | 1.3 | 1.301 |
| Trinucleotide repeat-containing gene 6A protein | Trnc6a | Q3UHK8 | small RNA mediated gene silencing | 17.280 | 0.032 | 9.537 | 0.075 | 1.6645 | 1.106 |
| Cytosolic prostaglandin E2 synthase | Ptges3 | Q9RQ7 | molecular chaperone | 13.611 | 0.064 | 2.622 | 0.537 | 0.52678 | 2.068 |
| Trinucleotide repeat-containing gene 6B protein | Trnc6b | Q8BKI2-1 | small RNA mediated gene silencing | 12.604 | 0.053 | 4.575 | 0.229 | 0.6453 | 1.757 |
| Insulin-like growth factor 2 mRNA-binding protein 1 | Igf2bp1 | Q84777 | RNA binding, mRNA translation and stability | 11.289 | 0.064 | 3.601 | 0.372 | 1.0019 | 1.452 |
| Putative helicase MOV-10 | Mov10 | Q3TFC0 | small RNA mediated gene silencing | 8.727 | 0.084 | no value | no value | 1.9104 | 0.635 |
| Insulin-like growth factor 2 mRNA-binding protein 2 | Igf2bp2 | Q5SF07-1 | mRNA translation, mRNA 5’UTR binding | 6.983 | 0.112 | 3.477 | 0.406 | 1.0228 | 1.330 |
| Probable ATP-dependent RNA helicase DHX36 | Ddx36 | Q8HV9K | mRNA translation and stability, mRNA 3’UTR binding | 6.003 | 0.125 | 2.961 | 0.379 | 1.1648 | 0.585 |
| Putative ATP-dependent RNA helicase DHX30 | Ddx30 | Q99PUB3 | RNA binding and stability, mRNA 3’UTR binding | 5.459 | 0.166 | 2.308 | 0.483 | 1.6228 | 0.454 |
| YTH domain family 2 | Ythdf2 | Q3TWU3 | unknown function | 4.820 | 0.175 | 3.732 | 0.350 | no value | no value |
| 40S ribosomal protein S14 | Rps14 | P62264 | ribosomal protein | 4.387 | 0.280 | 4.217 | 0.298 | 1.4352 | 0.685 |
| Heterogeneous nuclear ribonucleoprotein L | Hnmlp | Q8RDP81 | pre-mRNA binding | 4.080 | 0.198 | 2.338 | 0.790 | no value | no value |
| Ribosomal protein S5;Ribosomal protein S6 | Rps5 | Q91V55 | ribosomal protein | 3.951 | 0.299 | 4.169 | 0.273 | 1.3374 | 0.740 |
| 40S ribosomal protein S19 | Rps19 | Q5M9P3 | ribosomal protein | 3.558 | 0.369 | 4.152 | 0.287 | 1.3051 | 0.827 |
| 40S ribosomal protein S18 | Rps18 | P62270 | ribosomal protein | 3.534 | 0.379 | 4.171 | 0.347 | 1.2577 | 0.765 |
| 40S ribosomal protein S3a | Rps3a | P97351 | ribosomal protein | 3.357 | 0.362 | 4.345 | 0.294 | 1.37 | 0.776 |
| Histone H1.4 | H14 | P43747 | chromatin compaction | 3.063 | 0.229 | 3.692 | 0.337 | 1.5909 | 0.665 |
| Heterochromatin protein 1-binding protein 3 | Hp1bp3 | Q3TEA8-1 | unknown function | 2.229 | 0.248 | 5.789 | 0.297 | 0.81516 | 1.439 |
| Probable ATP-dependent RNA helicase DDX6 | Ddx6 | P54823 | mRNA degradation, mRNA decapping | 7.152 | 0.129 | 1.8026 | 1.118 |
| Enhancer of mRNA-decapping protein 4 | Edc4 | Q3UB99-1 | mRNA decapping | 8.772 | 0.108 | 1.0429 | 0.785 |

**SILAC-based Identification of Dicer-dependent AGO2 Interactions**

Molecular & Cellular Proteomics 11.11
**TABLE I—continued**

| Protein names binding preferentially in the presence of miRNAs/Dicer | Gene names | Uniprot | Keywords | wt Ratio normalized | knockout Ratio normalized | wt vs knockout Ratio normalized |
|---|---|---|---|---|---|---|
| Trinucleotide repeat-containing gene 6C protein | Tnrc6c | Q3UHC0 | small RNA-mediated gene silencing | 23.986 | 0.050 | 13.655 |
| Dicer | Q8R418 | small RNA-mediated gene silencing | 16.371 | 0.037 | 11.599 |
| Regulator of nonsense transcripts 1 | Rent1;Upf1 | Q2EP0U-1 | nonsense mediated mRNA decay | 10.406 | 0.085 | 2.3498 |
| Matrin-3 | Mat3 | Q8K310 | RNA binding | 7.601 | 0.081 | 1.8666 |
| RISC-loading complex subunit 1 | Tarbp2 | Q99M41 | small RNA-mediated gene silencing | 7.550 | 0.066 | 5.5222 |
| Nuclease-sensitive element-binding protein 1 | Ybx1 | P62960 | pre-mRNA splicing, mRNA processing | 7.006 | 0.158 | 4.4745 |
| Polyadenylate-binding protein 1 | Pabp1 | P29341 | RNA binding | 6.248 | 0.142 | 2.9082 |
| Constitutive coactivator of PPAR-gamma-like protein 1 | Fam120A | Q6A0A9 | RNA binding | 6.071 | 0.129 | 1.861 |
| ELAV-like protein 1 | Elav1 | P70372 | AU-rich element binding | 5.352 | 0.204 | 4.4003 |
| Palindromic nuclear matrix protein 3 | Matin3 | Q8K310 | RNA binding | 7.601 | 0.081 | 1.8666 |
| Proteins binding preferentially in the absence of miRNAs/Dicer | Herc5 | Q3UEA7 | E3 ubiquitin-protein ligase | 1.690 | 0.040 | 0.10923 |
| Clathrin heavy chain 1 | Ctlc | Q5SXR6 | vesicel coating | 1.537 | 0.507 | 0.36005 |
| Zinc finger protein 521 | Zfh521 | Q6KAS7-1 | transcription factor | 0.923 | 1.034 | 0.48416 |
| Protein argonaute-3 | Ago3 | Q8CJF9 | small RNA-mediated gene silencing | 2.976 | 0.176 | 0.51235 |
| 60 kDa SS-A/Ro ribonucleoprotein | Ssa2; RoRNP | O08848 | RNA binding | no value | no value | 0.36005 |

**Proteins binding preferentially in the absence of miRNAs/Dicer**

- Putative uncharacterized protein
- Clathrin heavy chain 1
- Zinc finger protein 521
- Protein argonaute-3
- 60 kDa SS-A/Ro ribonucleoprotein
presence of Dicer and miRNAs (Fig. 2B). Together with Dicer itself, this group of proteins is located in the lower right quadrant in the experiments using Dicer +/+ cells versus control (Fig. 2B, left panel) and in the Dicer +/+ versus Dicer −/− experiment (right panel). In the Dicer −/− versus control experiment, these proteins do not appear as specific binders (middle panel).

As expected, we find the Dicer cofactor TARBP2 (TRBP) (56, 57) in this group (Table I). Among the identified factors is the RNA helicase A/DHX9, which has been implicated in siRNA-loading (58). Proteins such as YBX1, Gemin4, Gemin5, HNRNPC, HNRNPUL1, the ARE binding protein ELAVL1/HuR, the poly-A binding proteins PABPC1 and 4, the mitochondrial protein Matri3 and the Fragile X mental retardation protein paralog FXR2 have been found in Ago complex purifications before. In addition, we identified the PABPC1-binding protein LARP1 (59), the mRNA binding proteins FAM120A/Ossa and CSDA, which have not been implicated in Ago2 function before. In contrast to the GW protein family members TNRC6A and B, TNRC6C interacts with Ago2 only in the presence of Dicer, suggesting that it requires Ago2 to be loaded onto miRNA target mRNAs. Of note, it is also conceivable that proteins found in this group associate with Ago2 indirectly via interaction with Dicer.

Proteins that Interact with Ago2 Preferentially in the Absence of Dicer—Proteins that preferentially interact with unloaded Ago2 are found in the lower right quadrant of the plot generated from Dicer-deficient cells (Fig. 2C, middle panel).

In this group, we find the RNA binding protein RoRNP (60), the zinc finger protein ZNF521 (61) and the vesicle coat protein clathrin (CLTC) (Table I). Of note, Ago3 shows an increased association with Ago2 in the absence of Dicer and miRNAs. HERC5, a HECT-type E3 protein ligase that mediates conjugation of ISG15 to target proteins in human (62) is also in this group, raising the possibility that it post-translationally modifies unloaded Ago2.

Ago2 Associates with mRNPs in the Absence of Dicer and Mature miRNAs—Because miRNAs guide Ago proteins to specific mRNAs for gene silencing, RNA-binding proteins are associated with Ago proteins and many of these interactions are bridged by mRNAs (17–19, 34). However, a requirement for miRNA for these interactions have generally not been investigated. To our surprise, we noticed that several mRNA binding proteins bind to Ago2 independently of Dicer and mature miRNAs. Some of these proteins associate with Ago2 in an RNA-dependent manner as has been reported for IGF2BP1 and three for example (17) suggesting that Ago2 may associate with mRNAs even in the absence of mature miRNAs. To test this hypothesis, we immunoprecipitated Ago2 complexes from the F/H-Ago2 expressing Dicer wt and Dicer-depleted MEFs and isolated the bound RNAs (Fig. 3A). GFP expressing cell lines or RNase treatment served as control. The isolated RNAs were analyzed on an agarose gel and Ago2 levels were controlled by Western blotting. A significant amount of longer RNA is bound to Ago2 in the Dicer-depleted cells supporting our hypothesis that Ago2 stably associates with mRNAs in the absence of miRNAs (Fig. 3A).

Because Ago2 may interact with mRNAs in the absence of miRNAs, it was not clear from our SILAC data which interactions were mediated by mRNAs. To analyze mRNA-bridged Ago2 interactions, we established RNase treatment conditions under which the mRNA is completely degraded but the miRNAs are not affected (supplemental Fig. S1). We performed a SILAC experiment in which Ago2-containing mRNPs were isolated and the immunoprecipitate from the light labeled cells was treated with RNase A whereas the immunoprecipitate from the heavy labeled cells was untreated. For reverse experiments, labels were swapped. We obtained 681 quantified proteins in the Dicer wt and 520 in the Dicer-depleted cell lines (supplemental Table S2). We exclusively considered the proteins that were identified as specific interactors in our previous experiment (Table I) and visualized their ratios in plots (Fig. 3B), a heat map (Fig. 3C) as well as a table (Table II). In the ratio plots, RNA-dependent interactors appear in the lower right quadrant and interactors that are not affected by RNase treatment appear together with the background binders in the center of the ratio plots. In the heat map, the red color indicates values above, blue below and gray around zero. Red-blue pairs for forward and reverse experiments are characteristic for an mRNA-dependent interactor.

As hypothesized, a high number of proteins associate with Ago2 in a RNA-dependent manner even in the absence of miRNAs (Table II). In this group, we find many RNA binding proteins including IGF2BP1–3, DHX36, DHX30, HNRNPL, and the ribosomal protein RPS14. Strikingly, genetic data in C. elegans demonstrated that PRS14 modulates mature let-7 function (63). The SILAC data therefore confirms that Ago proteins associate with mRNAs in the absence of mature miRNAs.

Additionally, we find RNA-dependent interactors that are only present when miRNAs are present (Table II). Among them are UPF1, FAM120A, YBX1, CSDA, ELAVL1/HuR, Matri3, HNRNPC and LARP1. Interestingly, the poly-A binding proteins 1 and 4 also appear to require miRNAs for RNA-dependent Ago2 association.

A set of proteins associates with Ago2 in an mRNA-independent manner. This includes the TNRC6 proteins and Dicer. Other mRNA-independent protein-protein interactors of Ago2 are the HSP90 alpha and beta proteins with their cochaperones PTGES and FKBP5. Among the interactors preferentially binding in the absence of Dicer and miRNAs only Ago3, CLTC and ZNF521 were identified in the datasets and they show a direct binding behavior.

Taken together, our mass spectrometry approach revealed that Ago2 associates with larger RNA species even in the absence of small RNAs. Furthermore, several mRNA-binding proteins are specific to miRNA-free and miRNA-containing Ago2-mRNA complexes.
Fig. 3. Dicer- and miRNA-independent mRNA binding of Ago2. A, Ago2-containing RNPs were precipitated from whole cell lysate of Dicer wt (lanes 1–3) and Dicer-depleted (lanes 8–10) MEFs and treated with RNase A as indicated. GFP-only expressing cell lines were used as negative controls (lanes 5–7 and 11–13). RNA was isolated from immunoprecipitates or lysates were separated on an agarose gel and visualized by ethidiumbromide staining (upper panel). F/H-Ago2 was analyzed by Western blotting with anti-HA antibodies (middle panel). Tubulin was used as a loading control (lower panel).

B, To analyze the mRNA requirements for Ago2 interactions, F/H-Ago2 was immunoprecipitated from total cell lysates from SILAC labeled, F/H-Ago2-expressing Dicer wild type (wt) MEFs. One sample was treated with RNase A and beads were combined for elution after washing. Eluates were analyzed by LC-MS/MS and the data is visualized in ratio plots as described in Fig. 1F (upper panel). The experiments were also carried out using the F/H-Ago2-expressing Dicer-depleted MEFs (lower panel). The mRNA-dependent Ago2 interactors are expected to show high H/L ratios in the forward and low ratios in the reverse experiment and appear in the lower right quadrant. Selected Ago2 interactors are indicated in color.

C, The H/L ratios of Ago2 interactors (see Table I) in the RNase treatment experiment are displayed in form of a heat map. Red indicates values above, blue below and white around zero. Gray squares indicate that the protein was not identified in the experiment. Red-blue pairs for forward and reverse ratios are characteristic for an mRNA-dependent interactor. The color intensity indicates the strength of the mRNA dependence.
# Table II
**RNA dependent and independent Ago2 interactors**

| Protein names | Gene names | Uniprot | wt Forward | wt Reverse | knockout Forward | knockout Reverse |
|---------------|------------|---------|------------|------------|-----------------|-----------------|
|              |            |         | normalized | normalized | normalized      | normalized      |
| Bait protein: Ago2 | Eil2;Ago2 | A1AS63  | 0.964      | 1.077      | 0.988           | 1.030           |

### Protein argonaute-2

**mRNA dependent interactions**

| Protein names | Gene names | Uniprot | Ratio normalized | Ratio normalized |
|---------------|------------|---------|-----------------|-----------------|
| Heterogeneous nuclear ribonucleoprotein L | Hnrnpl | Q8R0B1  | 18.787           | 0.039           |
| Putative helicase MOV-10 | Mov10 | Q3TFC0  | 17.880           | 0.026           |
| Insulin-like growth factor 2 mRNA-binding protein 1 | Igf2bp1 | Q86477  | 16.224           | 0.028           |
| Heterochromatin protein 1-binding protein 3 | Hrpbp3 | Q3TEE8-1 | 15.491           | 0.091           |
| Insulin-like growth factor 2 mRNA-binding protein 3 | Igf2bp2 | Q8CPL9N8 | 15.403           | 0.080           |
| 40S ribosomal protein S3a | Rps3a | P97351  | 13.257           | 0.067           |
| Insulin-like growth factor 2 mRNA-binding protein 2 | Igf2bp2 | Q5SF07-1 | 13.066           | 0.046           |
| Probable ATP-dependent RNA helicase DHX36 | Dhx36 | Q8VHK9  | 12.600           | 0.042           |
| 40S ribosomal protein S14 | Rps14 | P62264  | 6.142            | 0.181           |

### Proteins binding preferentially in the presence of miRNAs/Dicer

| Protein names | Gene names | Uniprot | Ratio normalized | Ratio normalized |
|---------------|------------|---------|-----------------|-----------------|
| ELAV-like protein 1 | Elav1 | P70372  | 23.522           | 0.075           |
| Nuclease-sensitive element-binding protein 1 | Ybx1 | P62960  | 16.441           | 0.042           |
| Cold shock domain-containing protein A | CsdA | Q8UKB3-1 | 14.131           | 0.035           |
| Poly(A) binding protein, cytoplasmic 4 | Pabpc4 | Q8BLF8  | 11.727           | 0.071           |
| Polyadenylate-binding protein 1 | Pabpc7 | P29341  | 10.879           | 0.071           |
| Regulator of nonsense transcripts 1 | Rent1;Utp1 | Q8EPU0-1 | 9.236            | 0.081           |
| Heterogeneous nuclear ribonucleoprotein U-like protein 1 | Hnrnpu1 | Q8VDL6-1 | 9.222            | 0.088           |
| Heterogeneous nuclear ribonucleoproteins C1/C2 | Hnrnpc;Hnrnpc | Q8TUL5 | 7.047            | 0.112           |
| La-related protein 1 | Lar1 | Q82Q68-1 | 6.910            | 0.107           |
| Protein FAM120A | FAM120A | Q6A0A9  | 4.762            | 0.186           |
| ATP-dependent RNA helicase A | Dhx9 | O70133-2 | 4.561            | 0.233           |
| Matrin-3 | Matr3 | Q8K12O  | 3.789            | 0.201           |

### Proteins showing a weak preference for binding in the presence of miRNAs

| Protein names | Gene names | Uniprot | Ratio normalized | Ratio normalized |
|---------------|------------|---------|-----------------|-----------------|
| Fragile X mental retardation syndrome-related protein 1 | Fxr1 | Q6584-1 | 3.208           | 0.276           |
| 40S ribosomal protein S19 | Rps19 | Q8M9P3  | 3.604           | 0.319           |
| 40S ribosomal protein S55 | Rps55 | Q9T955  | 3.115           | 0.356           |
| YTH domain family 2 | Ythdf2 | Q8T3WU3 | 1.915           | 0.329           |
| 40S ribosomal protein S18 | Rps18 | P62270  | 1.755           | 0.646           |

### mRNA independent, direct protein-protein interactions

| Protein names | Gene names | Uniprot | Ratio normalized | Ratio normalized |
|---------------|------------|---------|-----------------|-----------------|
| Endoribonuclease Dicer | Dicer | Q8R418  | 0.993           | 0.843           |
| Trinucleotide repeat-containing gene 6C protein | Trnc6c | Q9UHC0  | 0.916           | 1.101           |
| Trinucleotide repeat-containing gene 6B protein | Trnc6b | Q8KBK2-1 | 0.894           | 1.080           |
| Trinucleotide repeat-containing gene 6A protein | Trnc6a | Q9UHK8  | 0.865           | 0.975           |
| Clathrin heavy chain 1 | Cltc | Q5SXR6  | 1.152           | 0.991           |
| Zinc finger protein 521 | Zfp521 | Q9KAS7-1 | 1.027           | 0.864           |
| Protein argonaute-3 | Ago3;Eil2c3 | Q8CJF9 | 0.990           | 1.059           |
Validation of Ago2 Interactions—To further verify our mass spectrometry results, we performed Western blot analyses on a set of identified Ago2 interactors (Fig. 4A). Ago2 complexes were isolated from FH-Ago2-expressing cells (Fig. 4A, lanes 1–4). To test RNA requirements of the interactors, a set of samples was treated with RNase A (Fig. 4A, lanes 3 and 4). Arrows indicate signals specific for the target protein and asterisks indicate background signals. B, Knock down validation by quantitative PCR (qPCR). Total RNA was reverse transcribed and cDNA was amplified using primers specific to ZNF521 (upper panel), CSDA (middle panel) and EDC4 (lower panel). mRNA levels relative to GAPDH mRNA were normalized to control transfections. Values are representative for three different experiments. C, Short interfering RNAs against the indicated proteins were pre-transfected into HeLa cells. After 2 days, a luciferase reporter containing the 3’ UTR of Hmga2 or a mutated Hmga2 3’ UTR lacking the let-7 binding sites were transfected. Mean Firefly/Renilla ratios from seven independent experiments are displayed. Hmga2 values were normalized to those of the mutated vector.

To functionally validate our interaction data, we used luciferase-based miRNA reporters (Figs. 4B and 4C). The 3’ UTR of Hmga2, a well-characterized let-7a target (64), was fused to firefly luciferase and transfected into HeLa cells in which ZNF521, CSDA and EDC4 were depleted by RNAi (Figs. 4B and 4C). In addition, we employed a reporter containing the Hmga2 3’ UTR with mutated let-7a target sites and normalized the data against each other (19) (Fig. 4C). As expected, Ago2 knock down led to increased luciferase activity. Knock down of EDC4, which has been implicated in miRNA function in Drosophila (52), resulted in specific luciferase up-regulation as well, suggesting that EDC4 is indeed involved in silencing of the Hmga2 reporter construct. Similar results were obtained for the mRNP component CSDA. ZNF521, however, is not involved in miRNA-guided gene silencing. Since ZNF521 is a putative transcription factor, it is possible that it cooperates with Ago2 in nuclear Ago functions.
In summary, our validation experiments show that novel Ago2 interactors discovered by proteomics can play roles in miRNA-guided gene silencing, highlighting the specificity of our Ago2 interaction network.

**DISCUSSION**

Although several proteomic and genetic studies aiming at the identification of human Ago interaction partners have been performed they have not discriminated between protein-protein or protein-RNA interactions within Ago protein complexes. Using a powerful quantitative proteomic approach based on SILAC and combined with a Dicer-free cell system, we discovered and classified sets of proteins that bind either by protein-protein interaction or indirectly via RNAs to mouse Ago2.

To analyze Ago2 interactions, we used MEFs in which Dicer has been genetically inactivated. As a consequence, miRNA precursors are not processed and therefore mature miRNAs are not produced. It has been shown that miR-451 is processed by Ago2 independently of Dicer (65, 66). miR-451 is not expressed in the MEFs used here (data not shown) and since no other Dicer-independent miRNA has been reported, Ago2 protein complexes are thought to be miRNA free. However, it has been shown recently that many larger non-coding RNA species such as tRNAs or snoRNAs can give rise to small RNAs and the processing of some of these RNAs might even be Dicer-independent (67–70). To rule out that other so far not characterized Dicer-independent small RNAs guide Ago2 to larger RNAs in the absence of Dicer, we analyzed an Ago2 mutant that is not capable of small RNA binding (71). Preliminary experiments revealed that this mutant might still associate with larger RNAs suggesting that Ago2 is recruited to such RNAs without guidance of small RNAs (data not shown).
MiRNAs are viewed as guides that sequence-specifically target Ago protein complexes to distinct sites on mRNAs (3, 10, 72). Very recently, Ago2-mRNA interactions were analyzed in mouse embryonic stem (ES) cells lacking Dicer using RNA-protein cross linking followed by RNA-seq (73). Interestingly, the authors reported that Ago2 indeed interacts with mRNAs in the absence of Dicer. These results together with our SILAC data suggest that Ago2-mRNA interactions can be independent of small RNAs. Two scenarios of how Ago2 contacts larger RNAs independently of small RNAs might be envisioned. First, Ago2 itself might possess RNA-binding activity toward larger RNAs. This is unlikely, because Ago2 binding affinity toward single stranded RNAs peaks at 21 nucleotides and rapidly decreases with the length of the RNA.

Second and more likely, a set of RNA binding proteins may be involved in recruiting Ago2 proteins to mRNAs. Consistently, it has been reported recently that a complex composed of Pumilio, Ago proteins and eEF1A regulate translation and this regulation might be independent of miRNA binding (74). Many Ago binding proteins co-purify with Ago proteins and these proteins may help stabilizing Ago-mRNA interactions (17, 18).

Hypothesizing that unloaded (miRNA-free) Ago complexes interact with different proteins than Ago proteins that are bound to miRNAs, we compared Ago2 complexes from wt and Dicer-deficient MEFs. Indeed, we found a number of proteins that interact with Ago2 specifically in the absence or the presence of Dicer whereas several proteins interact with Ago2 under both conditions. Our data allows for a detailed mapping of the Ago2 interaction network in MEFs (Fig. 5). Proteins that interact independently of Dicer are shown in green, proteins that require Dicer for the interaction with Ago2 in yellow and proteins that only interact in the absence of Dicer are shown in blue. Strikingly, our proteomics experiments recapitulate all factors that have been implicated in RISC loading or miRNA function so far. In addition we find a number of RNA binding proteins, translational regulators, five ribosomal proteins as well as decapping activators. There are only six other proteins (gray). We note that our approach could be used to analyze whether or not the Ago1, Ago3 and Ago4 interaction networks are identical with the Ago2 network or whether there are differences.

The current model of the mechanism of miRNA-guided gene silencing is that Ago proteins interact with a member of the GW128 protein family, which in turn interacts with a protein binding to the poly(A) tail of the mRNA. This leads to an inhibition of the interaction of PABP with the cap binding complex resulting in reduced translational initiation. GW182 recruits the CCR4/NOT complex to the poly(A) tail, which removes the poly(A) tail leading to decapping and mRNA degradation from the 5' end. Our proteomics data reveals several interesting interactions with regards to the mechanism of miRNA function.

First, although the interactions of the miRNA machinery with deadenylase complexes has been characterized in molecular detail (75–77), not much is known about possible functional interactions of the Ago-miRNA complex with the decapping machinery. Based on our observation that EDC4 interacts with Ago2, it is tempting to speculate that Ago proteins not only stimulate deadenylation via the GW182 proteins but also subsequent decapping via EDC4 leading to efficient mRNA decay and gene silencing. In support of such a model, it has been found that fly EDC4 protein (also referred to as Ge-1) is required for miRNA-guided gene silencing in Drosophila cells (52).

Second, the three mammalian GW-protein TNRC6A, B and C have been implicated in miRNA-guided gene silencing. However, individual functional differences have not been reported. Here, we find that TNRC6A and B interact with Ago2 both in the absence and presence of miRNAs. However, TNRC6C is only found together with Ago2 when miRNAs are processed. We speculate that TNRC6C may only interact with Ago2 on specific mRNA targets while TNRC6A and B interact with Ago2 also in the absence of target RNAs. Alternatively, TNRC6C could be directly recruited to Ago2 by Dicer. Our proteomic data serve to elucidate this and related questions in targeted functional experiments.

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