JPET Fast Forward. Published on June 6, 2022 as DOI: 10.1124/jpet.122.001158
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The Multiplicity of Argonaute Complexes in Mammalian Cells

Maurizio Mauro[1], Massimiliano Berretta[2,3], Giuseppe Palermo[4], Vincenzo Cavalieri[5], and Gaspare La Rocca[6*]

[1]Department of Medicine, Albert Einstein College of Medicine, New York, NY, USA

[2]Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy

[3]Gruppo Oncologico Ricercatori Italiani, GORI ONLUS, Pordenone, Italy

[4]Department of Biomedical and Biotechnological Sciences, University of Catania

[5]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy

[6]Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, United States

Running title: The multiplicity of AGO complexes

*Corresponding Author:

Gaspare La Rocca

Cancer Biology and Genetics Program

Memorial Sloan Kettering Cancer Center

408 East 69th, ZRC-1231

New York, NY, 10065

Phone: 646-888-3069

Fax: 646-422-0871

E-mail: laroccag@mskcc.org

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ORCID numbers:

Maurizio Mauro (0000-0002-5452-0795)

Massimiliano Berretta (0000-0002-9837-9148)

Giuseppe Palermo (0000-0002-3861-3545)

Vincenzo Cavalieri (0000-0003-0906-9963)

Gaspare La Rocca (0000-0003-1277-0566)

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Abbreviations:

CCR4, carbon catabolite repression 4; Fast protein liquid chromatography, FPLC; NOT, negative on TATA-less; PABP, poly(A)-binding proteins; Protein Interacting With PRKCA, PICK1; RISC, RNA-induced silencing complex; Serpine mRNA binding protein 1, SERBP1;
Size exclusion chromatography, SEC; TNRC6, trinucleotide repeat containing 6 protein; TRBP, transactivation response element RNA-binding protein;

Manuscript pages: 27

Number of Figures: 2

Number of Tables: 0

Number of references: 101

Word count of the Abstract: 158

Word count of Introduction: 686

Word count of Discussion: 574

Abstract. Argonautes (AGOs) are a highly conserved family of proteins found in most Eukaryotes, and involved in mechanisms of gene regulation, both at the transcriptional and post-transcriptional level. Among other functions, AGO proteins associate with microRNAs (miRNAs) to mediate the post-

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transcriptional repression of protein-coding genes. In this process, AGOs associate with members of the trinucleotide repeat containing 6 protein (TNRC6) protein family to form the core of the RNA-induced silencing complex (RISC), the effector machinery that mediates miRNA function. However, the description of the exact composition of the RISC has been a challenging task due to the fact the AGO's interactome is dynamically regulated in a cell type- and condition-specific manner. Here, we summarize some of the most significant studies that have identified AGO complexes in mammalian cells, as well as the approaches used to characterize them. Finally, we discuss possible opportunities to exploit what we have learned on the properties of the RISC to develop novel anti-cancer therapies.

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Significance statement. The RNA-induced silencing complex (RISC) is the molecular machinery that mediates miRNA function in mammals. Studies over the past two decades have shed light on important biochemical and functional properties of this complex. However, still many aspects of this complex await further elucidation, mostly due to technical limitations that have hindered full characterization. Here, we summarize some of the most significant studies on the mammalian RISC, and discuss possible sources of biases in the approaches used to characterize it.

Introduction

microRNAs (miRNAs) are short non-coding RNAs that in metazoans act primarily as post-transcriptional repressors of protein-coding genes. miRNAs recognize their target genes through Watson-Crick base pairing at sequences usually located within the 3' UTR of the mRNA (Schirle et al., 2014) (McGeary et al., 2022). Biochemical events initiated by this pairing eventually result in translation inhibition and/or degradation of the mRNA, with consequential decrease of protein output (Hendrickson et al., 2009) (Guo et al., 2010) (Eichhorn et al., 2014).

miRNAs are generated as long Pol II transcripts, named primary miRNAs (pri-miRNAs), which in the nucleus are processed by the RNase III enzyme DROSHA to produce a shorter intermediate named precursor miRNA (pre-miR) (Nguyen et al., 2015). Pre-miRNAs are hairpin-shaped RNAs which are

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exported to the cytoplasm where they undergo a second cleavage step operated by the RNase III enzyme DICER, generating a double stranded miRNA (Hutvagner et al., 2001). From DICER the duplex miRNA is transferred into the grove of one of the four paralogues of the Argonaute protein family (AGO1-4), in a process that also requires the transactivation response element RNA-binding protein (TRBP) (Chendrimada et al., 2005), and that result in the formation of a long-lived complex (Olejniczak et al., 2013). Both strands of the duplex can in principle act as mature miRNA, although only one strand, named *guide*, is maintained as functional mature miRNA, while the other strand, named *passenger*, is usually degraded (Khvorova et al., 2003). Mature miRNAs, as they are passed from protein to protein while traversing the biosynthesis pathway, are therefore never found as naked single stranded RNAs within cells. The association between AGO and miRNA somewhat confers stability to both components of the complex. On one hand, AGO protects the free 5' and 3' ends of the miRNA from exosome and from the 3'-to-5' exonucleases, respectively (Sheu-Gruttadauria et al., 2019) (Beltrami et al., 2015). This is supported by the fact that overexpression of AGOs elevates the levels of miRNAs, while AGO knockout reduces them (Winter and Diederichs, 2011) (Diederichs and Haber, 2007). At the same time, the miRNA increases the half-life of the otherwise "empty" AGO, consistent with the fact that global reduction of cellular miRNAs results in a decreased AGOs' levels (Smibert et al., 2013).

The association between AGO and the miRNA is also beneficial for mRNA targeting, in that AGO induces the optimal spatial orientation of the miRNA and the mRNA bases for the pairing to occur. In this process AGO confers better pairing potential compared to that expected by the encountering of two complementary naked nucleic acids (Schirle et al., 2014) (Jo et al., 2015).

In this minimal complex, AGO and the miRNA are functionally complementary, and their association is required for miRNA-mediated gene repression to occur. Indeed, while the miRNA carries the information necessary to recognize accurately target mRNAs via Watson-Crick base pairing, AGO provides, either directly or indirectly, the enzymatic activities that ultimately trigger mRNA degradation. More specifically, some members of AGO family, such as the vertebrate AGO2, are RNA III-like

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enzymes, which characteristic allows them to *directly* cleave target mRNAs only in the case where the miRNA and the target present complete or extensive complementarity (Hammond et al., 2000) (Liu et al., 2004). This enzymatic property of AGO2 has been extensively harnessed to artificially achieve the down regulation of a gene of interest by exogenous delivery of so called “small interfering RNAs” (siRNA), namely short RNAs with perfect complementary to regions within the sequence of the targeted gene (Martinez et al.). However, in metazoans this mode of gene repression is rarely used within miRNA-mediated gene regulatory networks (Yekta et al., 2004), but it is common in plants, where it is mediated by the several plant AGO-like Argonaute paralogues (Singh et al., 2015). In metazoans, in contrast, miRNAs and targets predominately present only partial complementary, which condition prevents AGO2-mediated mRNA slicing. However, the metazoan miRNA-AGO complex can repress gene expression by acting as platform for the recruitment of a group of scaffolding proteins and enzymatic activities, such as deadenylates and decapping complexes, thereby *indirectly* inducing mRNA degradation (Chen et al., 2009; Duchaine and Fabian, 2019).

The multiplicity of mammalian AGO complexes

The AGO-miRNA unit with the ability to repress gene expression at the post-transcriptional level has been historically referred to as the RNA-induced silencing complex (RISC) (Hammond et al., 2000), although this term has later been used to indicate a myriad of AGO-containing ribonucleoproteins (RNPs) of often undetermined composition. Sizes ranging form of about 100 kDa to several MDa have been attributed to these complexes depending on the cell type studied, and their proliferative and metabolic state (Mourelatos et al., 2002) (Landthaler et al., 2008) (La Rocca et al., 2021) (Martinez et al., 2002) (Hock et al., 2007) (Hammond et al., 2000) (Nykanen et al., 2001) (Gregory et al., 2005) (Chendrimada et al., 2007) (Martinez and Tuschl, 2004) (Detzer et al., 2011).

While the 100 kDa complexes conceivably represents an AGO unit bound to a miRNA (MacRae et al., 2008), larger forms of RISC include several associated proteins, such as heat-shock proteins (Hsp), heterogeneous nuclear ribonucleoproteins, proteins involved in RNA metabolism, mRNA-binding

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proteins, such as poly(A)-binding proteins (PABPs), and helicases (Mourelatos et al., 2002) (Frohn et al., 2012) (Bottini et al., 2017) (Trabucchi and Mategot, 2019) (Robb and Rana, 2007). (Weinmann et al., 2009) (Hock et al., 2007) (Landthaler et al., 2008) (Waninger et al., 2022).

A functional significance has been attributed only to a subgroup of these AGO-associated proteins. For example, Hsp70 and Hsp90 are essential for the maturation of the RISC, as they support the loading of the miRNA duplex originated from DICER cleavage of the pre-miRNA (Iwasaki et al., 2010). DICER also directly binds to AGO, although its role in the loading of the miRNA duplex in vertebrates is still unclear, as artificial small dsRNAs exogenously delivered in DICER knockout cells can still be loaded on AGOs (Murchison et al., 2005). Moreover, some AGO-interacting proteins have been found to work as modulators of RISC function, such as Nova1 (Storchel et al., 2015), PICK1 (Antoniou et al., 2014), SERBP1 (Barbato et al., 2022), and Mov10 (Meister et al., 2005).

Members of the Trinucleotide Repeat Containing 6 protein family (TNRC6A/GW182, TNRC6B, TNRC6C) appear in almost any attempt to isolate AGO complexes (Meister et al., 2005) (Baillat and Shiekhattar, 2009) (Landthaler et al., 2008) (Kalantari et al., 2016), which reflects an underlying central role of the partnership between AGO-TNRC6 in gene regulatory functions.

TNRC6 is a scaffolding protein found only in animals (Zielezinski and Karlowski, 2015). It can bind to up to three AGO proteins simultaneously (Elkayam et al.) and acts as a docking platform for the recruitment of a large complex that includes PABP, and the two major deadenylation machineries CCR4–NOT and PAN2–PAN3 (Braun et al., 2013). AGO and TNRC6 form the core of the mammalian effector complex, often referred to as *miRISC*, that mediate miRNA-mediated post-transcriptional regulatory functions that do not involve AGO2's slicing activity (Behm-Ansmant et al., 2006) (**Figure 1**). In this review, however, we use the generic term RISC regardless of its final mode of post-transcriptional gene regulation.

In TNRC6-pull down assays from cytoplasmic lysates from human cells, while AGO is detected, as expected, DICER is not. Accordingly, DICER pull down assay captured AGO but not TNRC6 (Landthaler et al., 2008) (Baillat and Shiekhattar, 2009). These observations support a current model of

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RISC maturation where TNRC6 binds to AGO only after AGO has been loaded with the duplex miRNA and detaches from DICER.

An element that further complicates the analysis of the different AGO complexes found in a cell is their diversity in subcellular localization. Indeed, AGO complexes have been found from being homogeneously dispersed in the cytoplasm (Rudel et al., 2008) (Tan et al., 2009), to being associated with cytoplasmic structures, and with most organelles, including the mitochondria, where they seem to mediate regulative mechanisms independent on TNRC6 (Zhang et al., 2014).

Processing bodies (PBs) are one of the cytoplasmic structures that contain enzymes responsible for mRNA decapping, deadenylation, and degradation, and where RISC components and miRNAs have been found. It has been proposed that PBs might be the sites of, and required for, miRNA-mediated repression (Leung et al., 2006) (Pillai et al., 2005) (Bhattacharyya et al., 2006) (Liu et al., 2005b) (Sen and Blau, 2005) (Jakymiw et al., 2005) (Liu et al., 2005a) (Eystathioy et al., 2002). However, it is also possible that PBs represents sites where target mRNAs may be stored until re-entering translation (Standart and Weil, 2018).

There is evidence that RISC components localize on the rough endoplasmic reticulum (ER) (Barman and Bhattacharyya, 2015) (Maroney et al., 2006) as well as on the endosomal trafficking pathway (Kim et al., 2014). Interestingly, the association with the endosomal trafficking pathway it has been proposed to allow the turnover of RISC components, as well as the turnover of target mRNAs (Gibbings et al., 2009) (Lee et al., 2009).

RISC components shuttle also between the cytoplasm and the nucleus of mammalian cells (Wei et al., 2014) (Gagnon et al., 2014) (Gonzalez et al., 2008) (Meister et al., 2004) (Hicks et al., 2017) (Kalantari et al., 2016) (Castanotto et al., 2009; Schraivogel et al., 2015) (Nishi et al., 2013), where they are associate both with the nucleoplasm and with chromatin, and where they are involved in a myriad of nuclear-specific functions, such as splicing, and transcriptional and post-transcriptional gene regulation (Matsui et al., 2013) (Sarshad et al., 2018) (Gong et al., 2021) (Jain et al., 2016).

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By using real time fluorescence correlation spectroscopy in living cells Ohrt and collaborators have compared the abundance and size of the cytoplasmic and nuclear RISCs. They have found that, in mammalian cell lines the nuclear RISC pool differs from its cytoplasmic counterpart, as these complexes show a size of about 160 kDa and 3MDa, respectively (Ohrt et al., 2008). These observations are consistent with the fact that functionally distinct roles of AGOs in the two compartments may exist.

Finally, a further layer of complexity in the dynamics of RISC assembly and subcellular localization stems from the ability of AGO and TNRC6 to form higher order structures that condensate into phase-separated droplets within cells. These condensates appear to attract both miRNA targets and deadenylases, creating a microenvironment where target deadenylation and degradation are accelerated (Sheu-Gruttadauria and MacRae, 2018).

The versatility of functions that AGO complexes can adopt, as well as the complexity of the regulatory mechanisms that control their assembly and function, in part explain the diversity in composition and subcellular localization that the various RISCs described in literature have shown. However, each approach that has been used to investigate these complexes comes with its own technical limitations, which opens the possibility that some of the observations gathered over the years may not accurately reflect endogenous mechanisms. Below, we discuss some of the technical limitations that may have contributed to the observational inconsistencies found in the literature.

Approaches used to characterize the composition of the RISC

Immunoprecipitation (IP) followed by mass spectrometry (MS) analyses of the IP eluate has been widely used by several groups to isolate proteins associated with AGO in mammalian cells (Meister et al., 2005) (Baillat and Shiekhattar, 2009) (Frohn et al., 2012) (Bottini et al., 2017) (Landthaler et al., 2008) (Weinmann et al., 2009) (Kalantari et al., 2016). This approach, however, comes with limitations. First, the specificity and efficiency of the IP is strictly dependent on the availability of well-performing antibodies. This limitation has been partially overcome by overexpressing tagged AGOs in cell lines, for

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which potent antibodies, such as anti-HA and anti-Flag tag, have been extensively optimized. However, as discussed below, several studies have shown that the behavior of tagged AGOs somewhat diverges from that of the endogenous counterparts, both in terms of subcellular localization and in terms of interactions with other proteins. Second, IP may enrich for specific RISC subpopulations. For example, antibodies may preferentially pool down AGO monomers as opposed to multiunit complexes, as the epitopes against which antibodies are raised may be more accessible in the former case.

Sucrose density gradient centrifugation has been extensively used to infer the size of AGO complexes and their association to membranes, organelles and polysomes. AGO complexes identified in sucrose gradients have shown the appearance of particles containing RNA, either associated to polysomes or as ribosome-free RNPs (Hock et al., 2007; Mourelatos et al., 2002; Ohrt et al., 2008; Weinmann et al., 2009) (Landthaler et al., 2008). For example, a 15S (about 550 kDa) RNP also containing miRNAs and Gemin3 and Gemin4 was found as a predominant complex in HeLa cells (Mourelatos et al., 2002). In contrast, other studies on whole lysates have described three distinct human AGO complexes distributed through the sucrose gradient, which were named “ complexes I–III” (Hock et al., 2007; Ohrt et al., 2008; Weinmann et al., 2009) (Landthaler et al., 2008). The majority of AGO was found in complex I, which showed a molecular weight of about 250–350 kDa. Then, complex II, of about 600–700 kDa (or 19S) in size, represented a second prominent, although smaller, peak. Finally, the minority of AGOs fractionated as complex III of at least 900 kDa (or 25–30S). Fractions in which complex I sedimented, also contained DICER (Landthaler et al., 2008), suggesting that complex I represents the RISC loading complex (Chendrimada et al., 2005; Wang et al., 2009) (Park et al., 2019) containing AGO, DICER and TRBP, within which the miRNA duplex is transferred from DICER to AGO. However, this approach may not provide an accurate picture of the relative abundance of the different complexes, as sucrose gradients are run over a period of several hours, which increases the probability to incur into disassembly of the complexes (Ohrt et al., 2008).

Size exclusion chromatography (SEC) is a technique usually employed at the polishing steps at the end of protein purification procedures (Freitag, 2014), and has been instrumental to shed light on the size

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and composition of AGO complexes (MacRae et al., 2008) (Baillat and Shiekhattar, 2009). Several classes of resins used to pack the chromatographic columns, each optimized to discriminate a specific range of molecular weight, are available and are particularly useful to study complexes smaller that 1-2 MDa. As sucrose density gradients are usually employed to study large complexes, such as free ribosomes and polysomes, the two techniques can be considered complementary of each other.
Early studies from Baillat et al. have used SEC-based analyses to show that in cell lines AGO is present preferentially as part of a complex of about 700 kDa, which contains also TNRC6 (Baillat and Shiekhattar, 2009). As the expected size of the AGO-TNRC6 dimer would be of only about 300 kDa, this implies that other factors are associated to it. As mentioned earlier, DICER does not associate to TNRC6 which excludes the possibility that DICER may contribute to the 700 kDa complex. However, as TNRC6 can bind up to three AGOs simultaneously (Elkayam et al., 2017), it is possible that the 700 kDa complex observed by Baillat et al. represents one TNRC6 unit bound to three Flag-tagged AGOs– which would reach an approximate molecular weight of about 500-600 kDa–plus some other unidentified factor. This scenario, although plausible, has not been further investigated in this study. Although each of the techniques described above comes with its own limitations, they have been instrumental to reveal the nuts and bolts of the RISC, both at the structural and functional level.

Potential sources of technical biases

Several studies aimed to characterize the properties of the RISC, have often relied on the overexpression of tagged versions of AGO (Weinmann et al., 2009) (Ohrt et al., 2008) (Landthaler et al., 2008) (Hock et al., 2007). While this approach overcomes issues related to low efficiency of pull-down and antibody specificity, it may contribute to the emergence of artifacts. For example, Kalantari et al. have compared interacting partners of Flag-AGO2 and endogenous AGO2 and found several additional candidates in the former case (Kalantari et al., 2016). The binding of these additional proteins to the Flag-AGO may be a consequence of either the transgene overexpression, or of potential independent interactions that the Flag tag may have formed with cellular proteins. Moreover, they have

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shown that, while endogenous AGO2 is equally distributed between the nucleus and the cytoplasm, 5-fold-overexpressed Flag-AGO2 has a distribution of 67% cytoplasmic and 33% nuclear (Kalantari et al., 2016).

Moreover, Kedersha et al. have shown that proteins can non-specifically localize to the PBs when overexpressed (Kedersha and Anderson, 2007). These observations open the possibility that the massive localization of AGO and TNRC6 observed within PBs in previous studies may have been, at least in some experimental settings, the results of their overexpression. Indeed, miRNA-mediated repression is unaffected in cells devoid of microscopically visible PBs (Eulalio et al., 2007) (Chu and Rana, 2006), suggesting that PBs formation itself is not required for the repression and such association may represent secondary events.

Finally, the introduction of tags within the structure of the protein under study can in principle alter its properties, resulting in the emergence of non-endogenous behaviors of the complexes the protein can form (Booth et al., 2018) (Gibson et al., 2013). In support of this scenario, our lab has observed that the presence of tags fused to AGO affects its ability to reconstitute in a fully assembled RISC. Specifically, SEC-based analyses have shown that while endogenous AGO tends to eluted prevalently as part of high molecular weight complexes in conditions that support cell proliferation (La Rocca et al., 2015), tagged AGOs assemble in multiple complexes of variable size during the same conditions (Li et al., 2020), reflecting possible non-physiological behaviors of the RISC. A possible explanation for this abnormal AGO behavior could be that the presence of a tag may reduce the ability of AGO to assemble a functional RISC for steric reasons. Nevertheless, previous biochemical analyses have shown that tags, as long as introduced at the N terminal of AGO, has minimal consequences on the localization and repressive function of the AGO complexes (Leung et al., 2006) (Ohrt et al., 2008) (Sen and Blau, 2005). However, it is still possible that this may hold true only within specific windows of AGO expression, and that different tags may affects the assembly and function of the RISC at a different extent. For example, overexpression of AGO beyond a certain threshold may affect the relative proportion of the various RISC components leading to abnormal RISC assembly, subcellular

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localization, and function. This scenario seems plausible as studies have shown that, when interacting, AGO and TNRC6 affect each other's subcellular distribution (Nishi et al., 2013) (Schraivogel et al., 2015). Moreover, AGO mutants with impaired ability to bind to mRNAs are not able to retain TNRC6 in the cytoplasm (Schraivogel et al., 2015). This is compatible with a model where the stable association of AGO-TNRC6 to the mRNA is required for its cytoplasmic localization. It is therefore possible that, when overexpressed, AGO may outnumber the miRNA binding sites on mRNA, as well as altering the balance of the number of AGO proteins relative to the endogenous number of TNRC6 proteins. This condition may leave a pool of AGO-miRNA complexes "floating" in the cytoplasm. Regardless of the cause, an excess of AGO in the soluble compartment may, not only skew the formation of RISC, but also AGO localization within a cell.

As several biochemical studies of the RISC have been based on overexpression of tagged RISC components, this may have contributed to the variability of observations compared to analysis based on endogenous AGOs. As discussed in the next section, our group has attempted to minimize the above-mentioned biases by studying the behavior of endogenous proteins and using *ad hoc* analytical approaches aimed to preserve the native functions of the protein complexes under study.

SEC fractionation of whole lysates

Aware of the possible technical limitations encountered in previously published analyses, we have previously revisited extant purification and analytical protocols with the goal of maintaining and studying the native characteristic of the RISC (Olejniczak et al., 2013) (La Rocca et al., 2015) (La Rocca et al., 2021). First, to avoid the aforementioned artifacts caused by protein overexpression and by the presence of tags, we have focused on the behavior of endogenous AGO and TNRC6 proteins. Second, as starting material we used whole lysates, as opposed to IP eluates, thereby minimizing the possibility of capturing only the subpopulations of AGO complexes in which epitopes recognized by the antibody are easily accessible, potentially leaving behind AGO complexes in which AGO-associated factors may hinder access to the epitope. Third, to minimize the possibility of disassembly of the AGO complexes,

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we employed automated SEC, which allows the assessment of the size of complexes from whole lysates in less than 2 hours at 4°C, therefore skipping long processing times typical of sucrose gradient fractionation. Finally, lysates were produced in a buffer containing only minimal amounts of detergents, moderate salt concentration (respectively 1/10 and 1/3 of the salt concentration previously used by Baillat et al.) and physically lysed by snap freezing and gently Dounce-homogenized, to further minimize the possibility to induce complex disassembly and preserve protein interactions. Moreover, lysis was conducted in EDTA-free conditions to preserve ribosome and polysome integrity (**Figure 2**).

The size of AGO complexes observed in our studies ranges from particles larger than 2 MDa, to small complexes of just 100 kDa, whose abundance depends on the cell type, organ, and conditions in which lysates are prepared. The sizes of the complexes identified are consistent with those previously observed in the context of sucrose gradient analyses (Mourelatos et al., 2002) (Landthaler et al., 2008) (Martinez et al., 2002) (Hock et al., 2007) (Hammond et al., 2000) (Nykanen et al., 2001) (Gregory et al., 2005) (Chendrimada et al., 2007) (Martinez and Tuschl, 2004) (Detzer et al., 2011). However, our assay has shown a different picture in terms of the relative abundance between the different AGO complexes detected. For example, when we analyzed whole cell lysate of multiple mammalian cells (i.e. MEFs, DLD1, FL5.12, Hut78) maintained in nutrient- and growth factor-rich media that support exponential proliferation, we observe that the majority of AGO complexes elute in the void of the column, indicating that they are larger than 2 MDa (La Rocca et al., 2015). With “high molecular weight RISC” (HMWR) we refer to the AGO complexes eluting in the void, while we refer to as “low molecular weight RISC” (LMWR) a minor pool of AGO that in these whole extracts elutes as a complex of about 100 kDa, conceivably representing AGO alone (MacRae et al., 2008).

Although, we have designated the AGO complex eluting in the void with the generic term HMWR, the resolution limit of the column employed in these analyses–i.e. Superose 6–does not allow to determine whether the HMWR is in fact a heterogeneous group of large AGO complexes. However, various lines of evidence suggest that the HMWR represents the pool of fully assembled RISC associated either with polysomes or with large mRNPs: First, the HMWR disassembles upon RNase-A treatment, upon

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depletion of TNRC6A, B and C, and when mature miRNAs are depleted following deletion of *Dicer* or *Drosha* (La Rocca et al., 2015). Moreover, HMWR disassembles when an excess of TNRC6B's AGO-binding domain (T6B)–which interferes with the AGO-TNRC6 interaction–is expressed (La Rocca et al., 2015). Depletion of mature miRNAs, or RNase-A treatment leads to the accumulation of a complex of about 500 kDa, which conceivably results from the binding of multiple AGOs to a single TNRC6 (La Rocca et al., 2015). All together these observations in whole cell extracts suggest that our modified SEC-based assay can assess the relative abundance of free AGO, AGO associated with TNRC6, and AGO associated to mRNA.

It should be noted that, while sucrose gradients fractionations of lysate of proliferating cells have reported a predominance of complexes below 700 kDa (Hock et al., 2007) (Ohrt et al., 2008), our SEC based assay has instead revealed that cells grown in similar conditions are enriched in HMWR, namely RISC pool associated to mRNA. Below, we discuss possible explanations of these discrepancies.

RISC assembly and function varies among tissues and is dynamically regulated.

Internal and external cues induce a profound remodeling and re-localization of the RISC (La Rocca et al., 2015) **(Castanotto et al., 2018)**. This characteristic of the RISC may have also contributed to the emergence of apparently contrasting results even among studies conducted on the same cell type.

T cell activation represents an explicative illustration where changes in RISC function and assembly take place simultaneously and are possibly mechanistically interconnected. Splenic resting T cells present AGO almost exclusively in low molecular weight complexes of about 100 kDa (La Rocca et al., 2015), most likely representing a single AGO unit (MacRae et al., 2008). Interestingly, when T cells are stimulated *ex vivo* to engage in exponential proliferation, AGO assembles in HMWR larger than 2 MDa (Olejniczak et al., 2013). Conversely, exponentially proliferating MEFs, with a predominance of HMWR, undergo an almost complete disassembly of the RISC when deprived of nutrients or growth factors resulting in proliferative arrest (Olejniczak et al., 2013).

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The molecular mechanisms responsible for this condition-specific shift between HMWR to LMWR are largely unknown, although some clues are beginning to emerge. For example, the assembly of HMWR in T cells upon *ex vivo* activation is paralleled by and accumulation of TNRC6, which may enable the RISC to stably bind to the mRNA, thereby forming large complexes (La Rocca et al., 2015). Accordingly, in resting T cells, which only present LMWR, TNRC6 levels are close to undetectable levels (La Rocca et al.).

Considering that TNRC6 proteins are under the control of multiple intracellular pathway (Olejniczak et al., 2016), it is conceivable that RISC assembly is regulated, at least in part, by the modulation of TNRC6 expression in a condition-specific fashion.

Although RISC disassembly can be actuated by making one of its components (i.e. TNRC6) limiting, other mechanisms can control the ability of AGO to form multiunit complexes. For example, specific AGO post-translational modifications dictate whether a functional RISC can stably associate with target mRNAs (Golden et al., 2017) (Horman et al., 2013) (Zeng et al., 2008) (Bridge et al., 2017) or with other components of the RISC (Rajgor et al., 2018) (Bridge et al., 2017).

To further support the notion that the RISC is not a defined molecular entity is also the fact that its size widely varies among mammalian adult tissues. For example, there are tissues, such as the intestine, liver, and thymocytes, in which AGO is assembled in HMWR. In contrast, tissues like heart, skeletal muscle, and lung shown AGO mostly associated with LMWR (La Rocca et al., 2015).

Although the composition and functional roles of HMWR and LMWR *in vivo* are not fully characterized, we have speculated that their relative abundance in a cell may reflect the extent of engagement of the miRNA pathway in gene repression. In one scenario, HMWR represents the active RISC engaged in target repression, therefore its relative abundance in a cell would be directly proportional to miRNA function. This idea is consistent with studies showing that for the first 2-3 hours after its encountering with the target, the RISC meditates translational inhibition without any appreciable mRNA degradation (Bethune et al., 2012). This phase would last enough time for the RISC to be captured as HMWR by SEC analysis. In contrast, the LMWR represents “free” AGO, either *de novo* produced, or released from

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the HMWR after completion of a cycle of mRNA degradation. It is conceivable that, the free AGO would be immediately re-assembled into a HMWR for a new cycle of mRNA repression. Given the narrow time window within which AGO is free, proliferating cells would only show small amounts of LMWR. There is evidence supporting the latter scenario. For example, HMWR is the only form of RISC present in cell lines, such as MEFs and HEK293, widely used in miRNA reporter assays and shown to carry active miRNA function (La Rocca et al., 2015). Moreover, we have shown that the potency by which miRNAs repress their putative targets increases from resting to stimulated T cell, mirroring the above-described shift from LMWR to HMWR between these two cell states (La Rocca et al., 2015). Alternatively, LMWR could reflect the presence of a short translational repression phase, followed by a fast mRNA degradation phase. The AGO pool released after mRNA degradation would be immediately bound by TNRC6 and recruited into a new cycle of target repression. In this scenario, the enrichment of LMWR would then reflect high RISC turnover.

Our group has recently attempted to discriminate between the two above-mentioned scenarios, and aimed to assess the functional significance of the HMWR and LMWR *in vivo*. To this aim, we have generated a genetically engineered mouse strain in which the shift between the HMWR and LMWR RISC can be triggered by expressing an inducible transgene encoding the small T6B protein, which interferes with the binding between AGO and TNRC6 (La Rocca et al., 2021). We reasoned that a tissue would be affected by the expression of T6B, and therefore by loss of miRNA function, by an extent proportional to the levels of the functional form of the RISC present, which presumably indicates a higher reliance of that tissue on miRNA function. We have found that tissues are affected by the expression of T6B regardless of their enrichment in either HMWR or LMWR. For example, heart, skeletal muscle, and lung are tissues which are equally enriched in the LMWR form (La Rocca et al., 2015). However, while both heart and skeletal muscle are heavily damaged following T6B expression, the lung is not (La Rocca et al., 2021). Therefore, it appears that the relative abundance of LMWR versus HMWR in a tissue does not reflect its reliance on miRNA function. Our groups are currently investigating the mechanisms underlying such a great variability in the relative abundance of LMWR

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and HMWR among tissues, and why some tissues more than others are affected by loss of miRNA function. Given the crucial role of miRNAs function in animal biology, the information obtained during these studies can potentially reveal important aspects on the mechanisms that govern gene expression *in vivo*.

Conclusions and future directions

More than two decades of biochemical and functional studies on the RISC have revealed an intricate *modus operandi* of this ribonucleoprotein complex in mammalian biology. Much has been discovered in terms of regulation of its turnover, post-translational modifications of its components, and regulation of its function. Yet, important questions need to be addressed. For example: why is the RISC functionally regulated, and what are the implications for our understanding of gene expression dynamics? One can speculate that the evolution of a regulated, rather than a constitutively active RISC may provide several advantages in maintaining and regulating cell functions. RISC activity may be enhanced in conditions that demand prompt adjustments to the gene expression program, such as during cell proliferation or stress response. During these conditions, rapid changes of gene expression programs are required, and miRNAs may enable this process by decreasing the half-life and translation rate of targets, avoiding the accumulation of undesired proteins and providing an advantage in terms of transcriptome responsiveness to external stimuli. Inactivation of the RISC, instead, would result in increased mRNA half-life and translation rates, allowing optimal protein synthesis when changes in the gene expression programs are not desired, for example during homeostasis.

Another important question is whether we can exploit the information on the biochemistry of the RISC to develop novel therapeutical interventions. One opportunity may come from the observation that significant differences in terms of RISC composition between cancer cells and normal tissue counterparts exist. Indeed, cancer cell lines consistently express exclusively the HMWR form, in contrast to most post-mitotic cells which present a significant portion of AGO associated with the LMWR (La Rocca et al., 2015) (reviewed in (La Rocca and Mauro)). Although we still do not fully

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understand the functional significance of the HMWR and LMWR in cells, the differences in their relative abundance between cancer cells and normal counterparts may reflect an underlying peculiarity in terms of reliance on miRNA function during tumorigenesis. For example, it is possible that cancer cells rely on the presence of the HMWR, for reasons yet to be elucidated. It follows that pharmacological disruption of the HMWR may result in the dysregulation of processes necessary for tumor progression. In support of the scenario are several studies showing that small molecules that interfere with the assembly of a full RISC can potentially repress tumor development. For example, it has been reported that trypaflavine, which blocks RISC loading, hinders the growth of xenografts (Watashi et al.), while a molecule, named BCI-137, that targets the miRNA binding domain of AGO2 improves the retinoic acid-induced differentiation of acute promyelocytic leukemia cells (Masciarelli et al.). Of note, a screen of small molecules able to specifically inhibit RISC loading has found several potential candidates (Tan et al.) each of which may be eventually tested for tumor suppressive function.

Alternatively, RISC disassembly can be induced by interfering with the interaction between AGO and TNRC6, as demonstrated by the use of T6B peptide described in the previous section (Hauptmann et al.) (La Rocca et al.). Ongoing studies in our lab are aiming to assess whether, indeed, the presence of HMWR in cancer cells is a requirement, rather than the expression, of the tumorigenic process, and whether interferences with the AGO-TNRC6 binding can lead to the inhibition of tumor progression. If confirmed, this information would further stimulate the chase for the development of new classes of molecules able to functionally impair the RISC, opening new avenues of drug discovery for cancer treatment.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Mauro, Berretta, Palermo, Cavalieri, and La Rocca.

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Footnotes.

Funding: This research was funded by the National Cancer Institute, grant number [P30 CA08748] to G.L.

Conflicts of Interest: No author has an actual or perceived conflict of interest with the contents of this article.

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Figure legends:

Figure 1. Mechanisms of post-transcriptional gene repression mediated by the RISC. miRNAs guide the RISC to partially complementary binding sites within the 3' UTR of a target mRNA. Next, decapping and deadenylase complexes are recruited on the target mRNA. Upon deadenylation and decapping, mRNAs become substrate for cellular exonucleases, and are rapidly degraded.

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Figure 2. SEC-based analysis of RISC size in whole cell and tissue lysates. Whole cell lysates are prepared as described in the main text, cleared by centrifugation, and loaded on a fast protein liquid chromatography (FPLC) automated system. 1 mL fractions are collected, processed for TCA precipitation, and analyzed by Western blotting for detection of RISC components.

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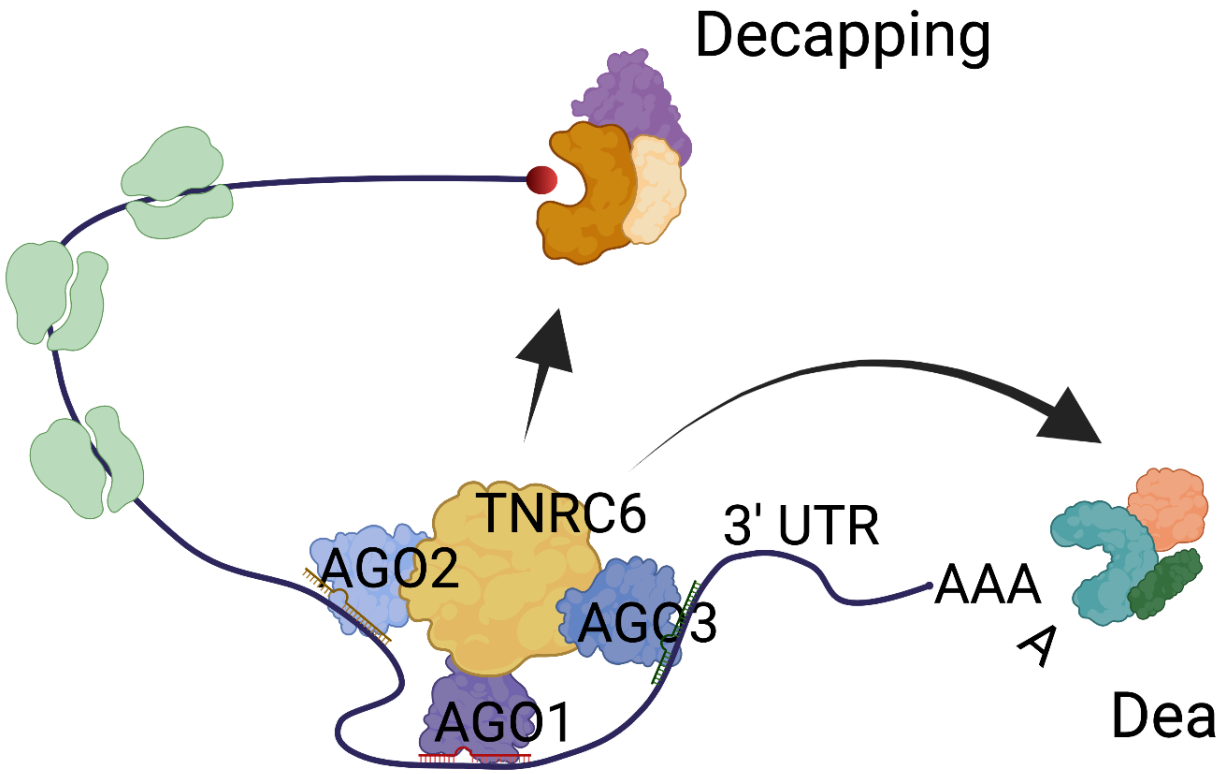

Figure 1

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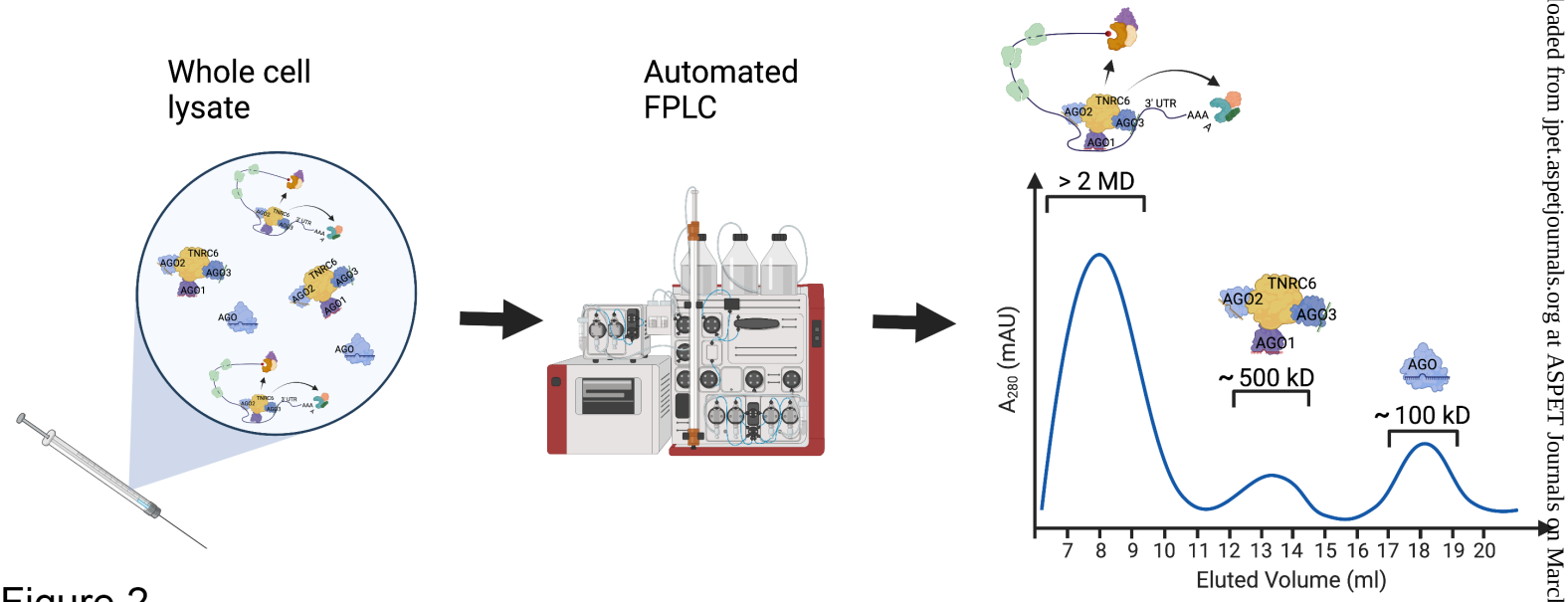

Figure 2

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