RNAi AND miRNA PATHWAYS IN MAMMALS I – MOLECULAR MECHANISMS

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ABSTRACT

RNA silencing denotes sequence-specific repression mediated by small RNAs. In mammals, there are two closely related pathways, which share several protein factors: RNA interference (RNAi) and microRNA (miRNA) pathway. The miRNA pathway regulates endogenous protein-coding gene expression. It has been implicated in many biological processes and majority of mammalian genes appear to be directly or indirectly exposed to miRNA-mediated regulations. RNAi generally serves as a form of innate immunity targeting viruses and mobile elements, although it occasionally also acquired function in protein-coding gene regulation. The function of RNAi in mammals is still poorly understood but it is clear that proteins supporting RNAi are also involved in miRNA biogenesis and function. Because of the volume of information, the review of mammalian miRNA and RNAi pathways was divided into two parts, where first one presented here reviews components of the pathways and the second one reviews function and significance of the pathways.

Introduction

Mammals belong (with birds and fishes) to the group *Craniata* of the phylum *Chordata*. Mammals are homeothermic animals distinguished by mammary glands, hair, middle ear bones, and neocortex. There are ~5000 extant mammalian species, most of which are placental mammals {Margulis, 1998 #2572}. The synapsid animal lineage leading to mammals branched of the sauropsid lineage leading to dinosaurs and birds. The mammalian miRNA, RNAi and other dsRNA-responding pathways are among the most studied dsRNA-responding pathways. Because of the large volume of the literature, I divided the mammalian material into two articles. In this first one, I will define molecular components and will review key functional implications in the second article. The first reviewed pathway will be the miRNA pathway (Fig. 1).

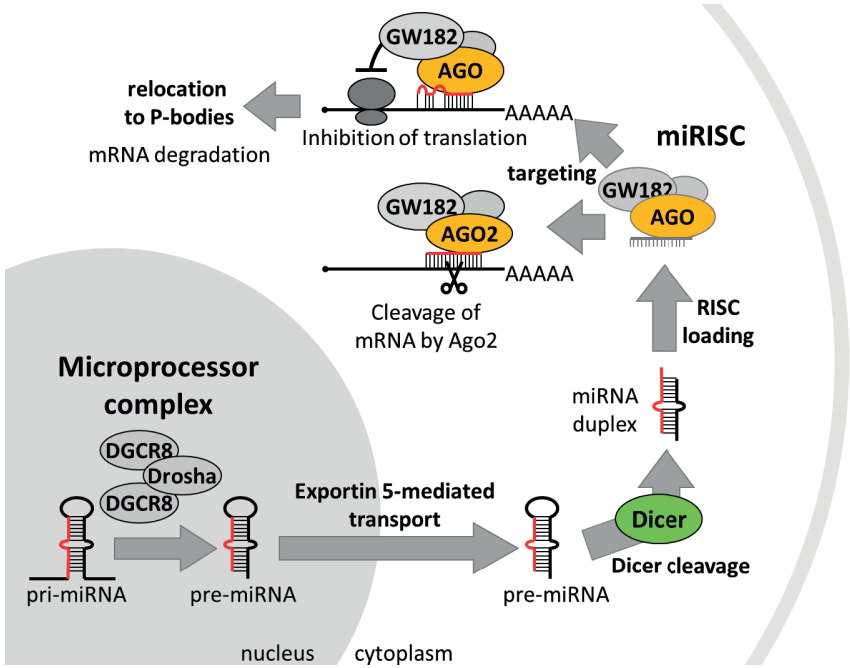

Figure 1 Overview of the molecular mechanism of the mammalian miRNA pathway. See text for details.

The Microprocessor complex – nuclear initiation of miRNA processing

The canonical primary miRNAs (non-canonical precursors are described separately further below) are transcribed by polymerase II (polymerase III-transcribed miRNA precursors are uncommon (Borchert et al., 2006; Canella et al., 2010)) and carry local hairpins, which are released in the nucleus as stem-loop precursors of approximately 70 nucleotides (pre-miRNAs) by the activity of the so-called Microprocessor complex, which is composed of RNase III Drosha and DGCR8 proteins (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004).

Drosha

Drosha, the active component of the Microprocessor complex, was discovered in 2000 as a new member of RNase III in *Drosophila* with a conserved human homolog (Filippov et al., 2000). Structurally, Drosha protein carries a single C-terminal dsRNA-binding motif (dsRBM), tandem catalytic domains, a proline-rich region (PRR) and an RS domain (Fortin et al., 2002). Drosha was recognized as the nuclease that executes the initiation step of miRNA processing in the nucleus (Lee et al., 2003) and the Microprocessor complex was reported in a series of papers in 2004 (Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). The current understanding of the Microprocessor complex includes the

resolved X-ray structure of Drosha with the C-terminal helix of DGCR8 (Kwon et al., 2016). Drosha contains two DGCR8-binding sites, one on each RNase III domain, which mediate the assembly of the Microprocessor complex. The overall structure of Drosha is similar to that of Dicer (see further below) despite no sequence homology except of the C-terminal part. This suggests that Drosha could have evolved from a Dicer homolog and would be consistent with the proposed classification as a non-canonical Dicer in a single RNase III family (Jaskiewicz and Filipowicz, 2008). In addition, Drosha exhibits unique features, including non-canonical zinc-finger motifs, a long insertion in the first RIIID, and a kinked link between a Connector helix and RIIID explaining the 11-bp-measuring "ruler" activity of Drosha. The structural analysis is consistent with biochemical characterization of the complex, which suggested that the Microprocessor complex exists as a heterotrimeric complex (Herbert et al., 2016; Nguyen et al., 2015). In addition, Drosha and DGCR8, respectively, recognize the basal UG and apical UGU motifs in pri-miRNAs, which determines orientation of the complex (Nguyen et al., 2015). These results clarified inconsistencies and unknowns existing in the earlier literature regarding the stoichiometry of the complex and the mode of binding (Barr and Guo, 2014; Gregory et al., 2006; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Ma et al., 2013).

DGCR8

While Drosha provides the catalytic site for cleavage, DGCR8 anchors substrate pri-miRNAs. A crystal structure of the human DGCR8 core (residues 493–720) showed two double-stranded RNA-binding domains (dsRBDs) arranged with pseudo two-fold symmetry tightly packed against the C-terminal helix (Sohn et al., 2007). Interestingly, DGCR8 uses heme as a co-factor. DGCR8 contains a previously uncharacterized heme-binding motif that is also required for its activity. Heme availability and biosynthesis in HeLa cells positively affect pri-miRNA processing and production of mature miRNAs while heme-binding-deficient DGCR8 mutants are defective in pri-miRNA processing (Barr and Guo, 2014; Barr et al., 2015; Weitz et al., 2014). Pri-miRNA recognition by DGCR8 seems to involve N(6)-methyladenosine mark deposited by the methyltransferase-like 3 (METTL3) (Alarcon et al., 2015). DGCR8 is also regulated by phosphorylation. There are 23 possible phosphorylation sites mapped on the full-length human DGCR8 expressed in insect or mammalian cells (Herbert et al., 2013). Subsequent analysis showed that DGCR8 phosphorylation may increase DGCR8 stability but not processing activity in response to extracellular cues (Herbert et al., 2013). DGCR8 phosphorylation by the tyrosine kinase ABL has been observed after DNA damage stimulating the processing of selective primary miRNAs (Tu et al., 2015).

Additional Microprocessor co-factors

Apart from the ABL kinase, several additional co-factors of the Microprocessor complex were identified. One of them is the RNA-Binding Protein DDX1, which appears to be a regulatory protein promoting expression of a subset of miRNAs, majority of which is induced after DNA damage (Han et al., 2014). A peculiar Microprocessor complex component is

Methyl-CpG-binding protein MECP2 (Cheng et al., 2014; Tsujimura et al., 2015), which is known to stably bind methylated DNA. According to one report, MECP2 promotes the posttranscriptional processing of particular miRNAs including miR-199a, which stimulates mTOR signalling (the key pathway regulating cell metabolism, growth, and survival) by targeting inhibitors of mTOR signalling (Tsujimura et al., 2015). In contrast, Cheng et al reported that MECP2 binds directly to DGCR8 and interferes with the assembly of the Microprocessor complex, thus affecting gene expression posttranscriptionally via relieving repression of miRNA targets (Cheng et al., 2014).

Microprocessor complex localization and function(s)

The Microprocessor complex shows apparent nuclear compartmentalization. While transiently expressed pri-miRNAs accumulate in nuclear foci with splicing factor SC35 and Microprocessor components, Drosha and DGCR8. (Pawlicki and Steitz, 2008), these foci do not appear to be major sites of pri-miRNA processing, which seems to be coupled to transcription (Pawlicki and Steitz, 2009). This is consistent with live-imaging, which revealed that a large fraction of Microprocessor resides with unspliced pri-miRNAs in close proximity to their genes. This analysis also provided a direct visual evidence that DGCR8 and Drosha are targeted to pri-miRNAs as a preformed complex (Bellemer et al., 2012).

Importantly, literature review identified also reports describing additional roles of the Microprocessor complex and its components beyond miRNA biogenesis although Microprocessor expression seems to be tuned according to pri-miRNA substrates (Barad et al., 2012). Non-canonical roles of Microprocessor (or Drosha) include: 1) mRNA cleavage (Chong et al., 2010), exemplified by Drosha-dependent cleavage of *Hoxd4* RNA (Phua et al., 2011) or destabilization of *Neurog2* mRNA, which supports neural stem cell maintenance by blocking accumulation of differentiation and determination factors (Knuckles et al., 2012), 2) processing of long non-coding RNAs restricted to the nucleus (Ganesan and Rao, 2008), 3), ribosomal RNA biogenesis (Liang and Crooke, 2011), and 4) cleavage of viral RNA (Shapiro et al., 2014). While immunoprecipitation of the Microprocessor complex followed by next-generation sequencing showed that precursors of canonical miRNAs and miRNA-like hairpins are the major substrates of the Microprocessor complex (Seong et al., 2014), high-throughput sequencing and cross-linking immunoprecipitation (HITS-CLIP) analysis of RNAs bound to DGCR8 suggest that miRNAs may not be the most abundant targets. DGCR8-bound RNAs also comprised several hundred mRNAs, small nucleolar RNAs (snoRNAs), and long noncoding RNAs (Macias et al., 2012). Interestingly, DGCR8-mediated cleavage of snoRNAs was independent of Drosha, indicating participation of DGCR8 in other RNA processing complexes (Macias et al., 2012). One of such complexes is the exosome (an hRRP6-containing nucleolar form), where DGCR8 is essential for its recruitment to snoRNAs and to the human telomerase RNA component (hTR/TERC) (Macias et al., 2015). Thus, DGCR8 acts as an adaptor recruiting the exosome complex to structured RNAs and inducing their degradation. (Macias et al., 2015).

Microprocessor complex crosstalk with other pathways

In terms of a crosstalk with other pathways, it has been established that some miRNA precursors are edited by ADARs (Alon et al., 2012; Garcia-Lopez et al., 2013; Peng et al., 2012; Tomaselli et al., 2015; Vesely et al., 2014; Vesely et al., 2012; Yang et al., 2006) apparently as early as pri-miRNAs (Bahn et al., 2015; Chen et al., 2015). According to one model, ADAR1 interacts with Drosha and DGCR8 in the nucleus and possibly outcompetes DGCR8 in primary miRNA binding, thus enhancing mature miRNA expression. This appears dependent on ADAR1 editing activity, at least for a subset of targets (Bahn et al., 2015). According to the selective elimination model, miRNAs, such as miR-151, are edited and eliminated by Tudor-SN (a ribonuclease specific to inosine-containing dsRNAs and a reported component of RISC) during mouse preimplantation development (Garcia-Lopez et al., 2013). Similarly, pri-miR-142 editing results in suppression of its processing by Drosha while the edited pri-miR-142 is degraded by Tudor-SN. Consequently, mature miRNA-142 expression substantially increases in ADAR1$^{-/-}$ or ADAR2$^{-/-}$ mice (Yang et al., 2006). According to the stimulation model, exemplified by miR-497, abundant editing event promotes processing by Drosha of the corresponding pri-miRNA (Vesely et al., 2014).

Dicer – cytoplasmic production of miRNA from pre-miRNA

A pre-miRNA produced by the Microprocessor complex is transported to the cytoplasm via Exportin 5 in a RanGTP-dependent manner. The next pre-miRNA processing step is Dicer mediated cleavage, which takes place the cytoplasm.

Structure of Dicer

The full length mammalian Dicer has not been crystallized. The current understanding of the mammalian Dicer structure has thus been inferred from several different sources, which can be divided into four groups:

(I) **Biochemical studies of recombinant Dicer and individual domains** (Ma et al., 2008; Park et al., 2011; Provost et al., 2002; Zhang et al., 2002; Zhang et al., 2004).

(II) **The crystal structure of Giardia intestinalis Dicer** (serving as a comparative scaffold) (MacRae et al., 2007; MacRae et al., 2006b).

(III) **Crystallographic studies on mammalian Dicer fragments** (Du et al., 2008; Wilson et al., 2015) **or on individual domains** (Ma et al., 2004; Takeshita et al., 2007; Tian et al., 2014; Wilson et al., 2015)

(IV) **Cryo-EM studies of human Dicer and its complexes with other proteins** (Lau et al., 2012; Lau et al., 2009; Taylor et al., 2013; Wang et al., 2009; Wilson et al., 2015).

Dicer is an siRNA-producing RNase III enzyme conserved across eukaryotes (Bernstein et al., 2001). Mammalian Dicer proteins are ~220 kDa multidomain proteins, which are composed of domains ordered from the N- to the C-terminus as follows: N-terminal DExD and helicase superfamily C-terminal domains, a domain of unknown function DUF283,

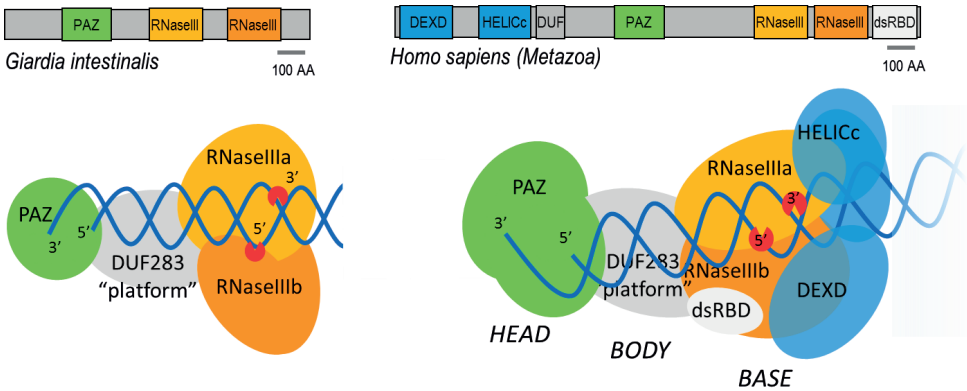

Figure 2 Domain architectures of Dicers from *Giardia* and humans.

a PAZ domain, RNase IIIa and RNase IIIb domains, and the C-terminal dsRBD (Fig. 2) (Nicholson and Nicholson, 2002). In contrast to the simplest RNase III family members (exemplified by E. coli RNase III), which carry only one RNase III domain and dimerize when cleaving dsRNA (Johanson et al., 2013; Lamontagne et al., 2001). Dicer proteins carry two RNase III domains, which form an intramolecular dimer (Zhang et al., 2004).

Giardia Dicer structure revealed spatial organization of the core part of eukaryotic Dicer proteins and explained how Dicer generates small RNAs of specific lengths (MacRae et al., 2006a). This crystal structure then served as a framework for deciphering the structure of other Dicer proteins, including mammalian Dicers. The front view of the *Giardia* Dicer structure resembles an axe. The blade is formed of an intramolecular duplex of two RNase III domains, which are connected by a bridging domain constituting the back end of the blade. The platform domain is adjacent to the RNase IIIa domain and makes up the upper part of the handle. The PAZ domain is connected by a long helix to the RNase IIIa domain and forms the base of the handle (MacRae et al., 2006b). Altogether, the *Giardia* Dicer is formed of three rigid regions, which are linked by flexible hinges. One region is formed by RNase III domains and the bridging domain, the second by the platform domain and the connector helix, and the third by the PAZ domain. These three parts can swing relative to each other and possibly ensure accommodation of Dicer to the structure of its substrate (MacRae et al., 2006a). This conformational flexibility likely enables binding of dsRNAs with non-canonical base pairing as well as imperfect duplexes of pre-miRNAs (MacRae et al., 2006a). In addition, dsRNA binding is presumably stabilized by several positively charged patches on the surface of Giardia Dicer between the processing center and the PAZ domain, which are in contact with dsRNA (MacRae et al., 2006a; MacRae et al., 2007).

Mammalian Dicers are much larger and contain domains absent in the *Giardia* Dicer but follow the same organizational and functional principles (Fig. 2). The crystal structure of *Giardia* Dicer confirmed an earlier biochemical analysis predicting that the two RNase III domains of the human Dicer form an intramolecular dimer resulting in a single processing center placed at a specific distance from the PAZ domain (Zhang et al., 2004).

A structural component defining this distance is an α helix (connector helix), which directly links PAZ and RNase III domains (MacRae et al., 2006b). Thus, the key functional aspect that emerged from Dicer's structural analysis was that it functions as a molecular ruler, measuring the length of the substrate from the PAZ domain to RNase III domains where each domain cleaves one strand. Importantly, the mammalian Dicer (and metazoan Dicers in general) differ from Giardia's in two main aspects. The first is the general topology reflecting the fact that the typical mammalian Dicer product is shorter (21–23 nt). The second one is that the mammalian Dicer contains additional functional domains important for substrate recognition and processing.

As indicated above, the architecture of the human Dicer and positions of its domains and interacting partners have been inferred by cryo-EM of the full length protein and its mutants (Lau et al., 2012; Lau et al., 2009; Taylor et al., 2013; Wang et al., 2009; Wilson et al., 2015). The overall shape of the human Dicer resembles the letter L; the shape is further divided into a head, a body and a base (Fig. 2). The PAZ domain is adjacent to the platform domain in the head of the protein while the RNase IIIb is located in the body. Thus, the head of the human Dicer is a topological equivalent of the base of the handle in *Giardia*'s Dicer. The helicase domain constitutes the base, which has no equivalent in *Giardia*'s Dicer. The position of the processing center relative to the PAZ domains differs between human and *Giardia* Dicers, which explains the fact that the human Dicer produces siRNA about four nucleotides shorter than the Giardia Dicer, which corresponds to ~ one-third of a dsRNA helical turn (Lau et al., 2012). Therefore, the processing center has to access the cleavage site of dsRNA from the different angle relative to the dsRNA helical end in comparison with *Giardia* Dicer (Lau et al., 2012).

For understanding substrate selection and processing, two areas of Dicer's structure deserve special attention: the PAZ and the N-terminal domains, which are described below. The following text represents exhaustive literature survey focused on the structural and functional aspects of the two domains.

The PAZ domain

The PAZ domain found in Dicer and Argonaute proteins is a dsRNA-terminus binding module (Ma et al., 2004; MacRae et al., 2006b). The PAZ domain has a 3' overhang binding pocket but only the PAZ domain of Dicer has an extra loop enriched in basic amino acids, changing electrostatic potential and molecular surface of the pocket. These changes may influence RNA binding by Dicer and handing-off the substrate to other proteins complexes (MacRae et al., 2006b). The PAZ domain of metazoan Dicers also recognizes phosphorylated 5' end of a pre-miRNA. A mutation of the 5' binding pocket leads to dysregulation of miRNA biogenesis *in vivo* (Park et al., 2011). The 5' binding pocket is conserved in *Drosophila* DCR-1 and human Dicer but not in *Giardia* Dicer (Park et al., 2011). Importantly, the 5' binding pocket appears conserved in Dicer proteins functioning in miRNA biogenesis (human Dicer, *Drosophila* DCR-1) but not in Dicer proteins dedicated to long dsRNA processing (*Giardia*, *Schizosaccharomyces*, *Drosophila* DCR-2). Accordingly, simultaneous fixing of 3' and 5' ends emerges as a feature important for fidelity of miRNA biogenesis but not for siRNAs (Park et al., 2011).

The N-terminal helicase domain

The N-terminus of metazoan Dicers harbours a complex helicase structure, which is adjacent to RNase III catalytic domains (Lau et al., 2012). Although the helicase must come into contact with the substrate, its functional significance is still only partially understood. However, it is clear that the N-terminal helicase region is the key for the substrate preference. In mammals (and in most metazoan phyla), a single gene encodes Dicer, which has to process both: miRNA precursors into miRNAs as well as long double-stranded RNAs into small interfering RNAs (siRNAs). Different taxons apparently differ in how much they employ both types of Dicer activities; the mammalian Dicer is mainly dedicated to the miRNA pathway while its natural production is very limited.

The N-terminal helicase belongs to the RIG-I-like helicase family (Zou et al., 2009) and consists of a proximal DExD/H domain and an adjacent helicase superfamily c-terminal domain (Fig. 2). A conventional helicase domain has an ATPase activity. Indeed, invertebrate Dicers bind and hydrolyze ATP (Bernstein et al., 2001; Ketting et al., 2001; Nykanen et al., 2001; Zamore et al., 2000). However, despite the N-terminal helicase with conserved motifs important for ATP binding and hydrolysis is present in mammalian Dicers, there is no evidence of ATP requirement for the human Dicer activity (Provost et al., 2002; Zhang et al., 2002). The human Dicer has the same processing efficiency in the presence or absence of ATP. Moreover, the rate of cleavage is not influenced by addition of other nucleotides, non-cleavable ATP analogues or a mutation in the Walker A motif of ATPase/helicase domain (Provost et al., 2002; Zhang et al., 2002). Notably, these experiments were performed using a long dsRNA substrate with blunt ends, whose processing by invertebrates Dicers is ATP-dependent (Bernstein et al., 2001; Ketting et al., 2001; Nykanen et al., 2001; Zamore et al., 2000; Zhang et al., 2002). Remarkably, deletion of the helicase domain results in high cleavage rate of long dsRNAs by human Dicer *in vitro* (Ma et al., 2008) as well as *in vivo* in murine and human cells (Flemr et al., 2013; Kennedy et al., 2015). Thus, the N-terminal helicase in mammalian Dicers has a different role in substrate recognition and processing than the helicase in invertebrate Dicers although the overall shapes of human and *Drosophila* Dicer proteins are similar (Lau et al., 2012).

The crystal structure of the N-terminal helicase has not been obtained. Thus, based on the cryo-EM-based modelling, the N-terminal helicase is composed of three globular subdomains (HEL1, HEL2, HEL2i) where the DExD/H domain corresponds to HEL1 and the helicase superfamily c terminal domain to HEL2 and HEL2i. All three parts of the helicase form a clamp near the RNase III domain active site. Interestingly, the N-terminal helicase was found in two distinct conformations, with respect to the body of the enzyme (Lau et al., 2012), similar to the RIG-I helicase which was used as a template for modelling (Kowalinski et al., 2011).

Analysis of substrate-specific structural rearrangements proposed that human Dicer exists in three states depending on presence and type of substrate (Taylor et al., 2013). Unbound Dicer existing in "canonical state" rearranges upon substrate binding that involves the PAZ domain as well as the helicase domain. Substrate-bound Dicer exists either in an "open" or closed" state. The open state is cleavage-competent and it is typical for pre-miRNA binding. It is characterized by binding of a pre-miRNA along the platform, bending of the helicase

domain, and access of RNase IIIa and IIIb sites to the substrate (Taylor et al., 2013). The closed state has been observed for a 35 bp A-form RNA duplex, which represents a siRNA precursor. In this state, the substrate is trapped between the PAZ and helicase domains away from the catalytic sites (Taylor et al., 2013). This provides a structural explanation for previous observations that Dicer poorly processes longer perfect duplexes *in vitro* and *in vivo* (Kim et al., 2005; Nejepinska et al., 2012b).

Taken together, it is apparent that miRNA biogenesis has been the preferred role for Dicer during vertebrate evolution. The helicase domain in mammalian Dicers provides a structural basis for substrate specificity, namely distinguishing pre-miRNAs as the preferred substrate. In addition, a natural Dicer isoform has been found in mouse oocytes, which lacks the N-terminal helicase domain, can efficiently generate siRNAs from long dsRNAs, and is sufficient for enhancing RNAi in cultured cells. This isoform is a consequence of a rodent-specific retrotransposon insertion and is present in *Muridae* family (Flemr et al., 2013). This demonstrates that, while the mammalian Dicer primarily dedicated to the miRNA pathway, a small change in a mammalian Dicer gene can restore RNAi activity.

Substrates and their processing by mammalian Dicer proteins

The first *in vitro* studies of recombinant human Dicer showed that substrate cleavage is dependent on Mg2+ but not on ATP presence (Provost et al., 2002; Zhang et al., 2002). Subsequently, it was reported that Dicer can cleave long dsRNAs and pre-miRNAs with different efficiency, which stems from substrate's structural properties (Chakravarthy et al., 2010; Feng et al., 2012; Flores-Jasso et al., 2009; Ma et al., 2008). Therefore, cleavage of miRNA precursors and long dsRNAs will be discussed in separate sections.

Canonical miRNA substrates

Canonical miRNAs of ~22 nt in length (Fig. 3) are the dominant Dicer products in mammalian cells. Dicer mutagenesis showed that inactivation of the RNase IIIA domain results in complete loss of 3p-derived mature miRNAs, but only partial reduction in 5p-derived mature miRNAs (Gurtan et al., 2012). Conversely, inactivation of the RNase IIIB domain by mutation of D1709, a residue mutated in some cancers, produced complete loss of 5p-derived mature miRNAs, but only partial reduction in 3p-derived mature miRNAs (Gurtan et al., 2012). Mutation of the PAZ domain caused global reduction of miRNA processing, while mutation of the Walker A motif in the helicase domain of Dicer did not alter miRNA processing (Gurtan et al., 2012). These results are consistent with the above mentioned structural features of Dicer.

Pre-miRNAs are the most efficiently cleaved Dicer substrates *in vitro*. In contrast to long dsRNA, a canonical pre-miRNA is cleaved only once and releases a single small RNA duplex. Human Dicer alone cleaves pre-miRNAs much faster than pre-siRNA substrates under both single and multiple turnover conditions; with more than 100-fold difference in maximal cleavage rates (Vmax) under multiple turnover conditions (Chakravarthy et al., 2010). This indicates that the mammalian Dicer is optimized for miRNA biogenesis and

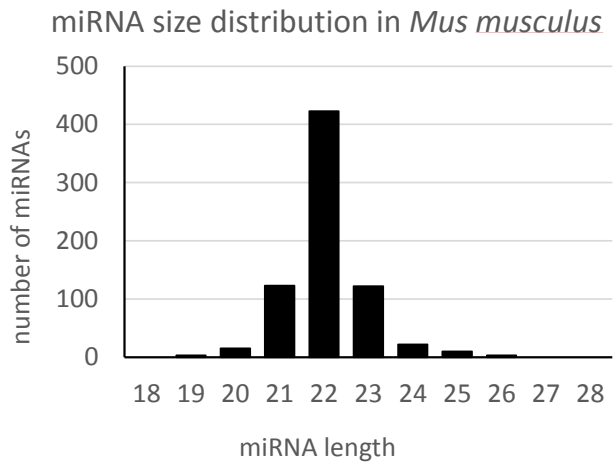

Figure 3 Mammalian miRNA size distribution
Distribution of mature murine miRNA lengths according to miRNA annotations in miRBase (release 21)

several specific structural adaptations discussed below support this notion. Dicer seems to interact directly with the terminal loop region of a pre-miRNA (Feng et al., 2012; Gu et al., 2012b) while a large pre-miRNA terminal loop further enhances pre-miRNA cleavage (Feng et al., 2012). A large-scale *in vitro* analysis and mutagenesis study of 161 human pre-miRNAs showed that human Dicer tolerates remarkable structural variation in pre-miRNA substrates (Feng et al., 2012). The dsRNA structure in the stem region and the 2-nt 3'-overhang structure in a pre-miRNA contribute to binding and cleavage by Dicer (Feng et al., 2012).

A characteristic feature of the pre-miRNA hairpin, which is accessed by the PAZ domain of Dicer, is a 2 nt 3' overhang generated by the nuclear Microprocessor complex (Gregory et al., 2004). Pre-miRNAs with the 2 nt 3' overhang at the 3' terminus are bound by Dicer with higher affinity than pre-miRNAs with different ends (Feng et al., 2012). Moreover, the 2 nt 3'end overhang leads to a higher substrate processing, which was shown on both, pre-miRNAs and perfect duplexes (Feng et al., 2012; Park et al., 2011; Zhang et al., 2004). Such preference is likely conferred by/due to simultaneous binding of pre-miRNA end by both 5' and 3' binding pockets in the PAZ domain (Park et al., 2011). Importantly, fidelity of miRNA biogenesis is critical for miRNA functionality because a single nucleotide shift at the 5' end of a miRNA would redefine its target repertoire. In contrast, RNAi, which typically involves perfect complementarity between a small RNA and its target, would be essentially insensitive to a precise cleavage positioning as long as it would not affect Argonaute loading. Thus, the simultaneous recognition of both strands at the 2 nt 3' overhang terminus by Dicer can be seen as an adaptation driven by miRNA biogenesis (Park et al., 2011).

The second structural adaptation of mammalian Dicer supporting miRNA biogenesis is the N-terminal helicase, which forms a clamp-like structure adjacent to RNase III domains, hence it is positioned to bind the stem loop of a pre-miRNA (Lau et al., 2012). While the loss of the entire N-terminal helicase only slightly increases pre-miRNA processing activity

in vitro (Ma et al., 2008), pre-miRNA-processing by recombinant Dicer *in vitro* is much faster than that of a perfect duplex (Chakravarthy et al., 2010; Ma et al., 2008). *In vivo*, a naturally occurring N-terminally truncated Dicer isoform can rescue miRNA biogenesis in Dicer$^{-/-}$ embryonic stem cells (ESCs) (Flemr et al., 2013). This suggests that the N-terminal helicase domain in mammalian Dicers is not important for miRNA biogenesis per se; it rather provides constrains for substrate selectivity favouring pre-miRNAs.

This is consistent with the model where pre-miRNA binding is associated with the cleavage-competent open conformation. In the open state, a pre-miRNA is bound along the platform, the helicase domain is bent, and RNase IIIa and IIIb sites have access to the substrate (Taylor et al., 2013). It has been proposed that the loop of a pre-miRNA may prevent adoption of the closed conformation by Dicer by interacting with HEL1 and HEL2i domains and possibly stabilizing the open conformation of Dicer (Feng et al., 2012; Lau et al., 2012; Ma et al., 2012). This also indicates that the N-terminal helicase had acquired distinct roles in Dicer function in RNA silencing during evolution. In mammalian cells, the N-terminal helicase has a gatekeeper function where pre-miRNA loops appear to be a key keeping the gate open.

Dicer-dependent non-canonical miRNA substrates

Apart from canonical miRNA substrates mentioned above, Dicer is processing additional miRNA-like substrates, which are independent of the Microprocessor complex (described in a separate section below). Some non-canonical miRNAs are produced by Dicer in a Microprocessor-independent fashion, including mirtrons, which utilize the splicing machinery to bypass the Microprocessor complex. Mirtrons are substantially longer than Microprocessor-generated pre-miRNAs and exhibit 3' uridylation and 5' heterogeneity (Wen et al., 2015). A recent analysis yielded ~500 novel mouse and human introns that generate Dicer-dependent small RNA duplexes (Wen et al., 2015). These represent nearly 1000 loci distributed in four splicing-mediated biogenesis subclasses, with 5'-tailed mirtrons being the dominant subtype (Wen et al., 2015). Another example of non-canonical miRNAs found in the literature are Microprocessor-independent miRNAs which were originally described as small interfering RNAs derived from a unique hairpin formed from short interspersed nuclear elements (SINEs) (Babiarz et al., 2008; Castellano and Stebbing, 2013).

While a typical pre-miRNA is a hairpin RNA with 2-nt 3' overhangs, production of a mature miRNA from an endogenous hairpin RNA with 5' overhangs has also been reported; mouse pre-mir-1982 is a mirtron with an 11 nt tail at the 5' end (Babiarz et al., 2008). A possible mechanism for processing such templates has been provided by an *in vitro* study which showed that Dicer can produce such miRNAs in a two-step cleavage, which releases dsRNAs after the first cleavage and binds them again in the inverse direction for a second cleavage (Ando et al., 2011a).

Long dsRNA substrates

In addition to pre-miRNA, Dicer can process long dsRNAs coming from different sources. Exogenous sources of dsRNA include viral dsRNAs and imply function of RNAi in

eukaryotic antiviral immune response (Vance and Vaucheret, 2001; Wang et al., 2006; Wilkins et al., 2005). Endogenous dsRNAs have variable length and termini, and are generated by transcription of inverted repeats, by convergent transcription or by pairing of complementary RNAs in trans. Importantly, mammals lack an ortholog of RNA-dependent-RNA polymerase (RdRP), which is a conserved component of RNAi-related mechanisms in plants, fungi and invertebrates (see the separate RdRP section). Endogenous RNAi in mouse oocytes, the best documented mammalian endogenous RNAi example, works independently of RdRP activity (Stein et al., 2003).

The human Dicer binds long dsRNA but not siRNAs *in vitro* (Provost et al., 2002). Long dsRNA binding is independent both on Mg^{2+} and ATP. The human Dicer preferentially binds and cleaves long dsRNA from the end, due to inefficient binding of internal regions of dsRNA (Zhang et al., 2002). In comparison to pre-miRNA processing, human Dicer exhibits lower cleavage activity on perfect dsRNA substrates (Ma et al., 2008). An explanation was proposed that a closed conformation of the N-terminal helicase domain disturbs the RNase III catalytic core and inhibits cleavage of perfect dsRNAs (Lau et al., 2012). As it was mentioned, *in vitro* deletion of the N-terminal helicase domain increases cleavage activity of human recombinant Dicer (~65-fold). Authors hypothesize that DExD/H-box domain mainly inhibits the functionality of the Dicer active site, but not RNA binding (Ma et al., 2008). This model is supported by previously mentioned structural data, where Dicer is in a closed state with a 35 bp A-form RNA duplex trapped between PAZ and helicase domains away from the catalytic center (Taylor et al., 2013).

The complexity of the differential substrate processing by Dicer is illustrated by a Dicer mutant carrying an in-frame 43-amino-acid insertion immediately adjacent to the DExH box. This Dicer exhibits defects in the processing of most, but not all, endogenous pre-miRNAs into mature miRNA but enhanced processing efficiency and concomitant RNA interference when thermodynamically stable, long-hairpin RNAs are used (Soifer et al., 2008). This result implies an important function for the helicase domain in the processing of thermodynamically unstable hairpin structures (Soifer et al., 2008).

Dicer-mediated cleavage of dsRNA can be stimulated *in vitro* by TARBP2. However, it is not clear if TARBP2 stimulation could be sufficient to induce endogenous RNAi *in vivo* (Chakravarthy et al., 2010). So far, the evidence for endogenous RNAi (including attempts to induce RNAi with exogenous substrates) is scarce (reviewed in detail in Nejepinska et al., 2012a; Svoboda, 2014). The only tissue type, where abundant endogenous siRNAs are present and where long dsRNA readily induces RNAi are mouse oocytes, which express an oocyte-specific Dicer isoform lacking a part of the N-terminal helicase domain (Flemr et al., 2013), thus mimicking some of the Dicer mutants tested *in vitro* (Ma et al., 2008). Taken together, long dsRNA, the typical endogenous RNAi substrate, is poorly processed by endogenous full-length Dicer. This is due to the gatekeeper role of the N-terminal helicase domain, which does not open upon binding long dsRNA.

Off note is that the human Dicer can bind 21-nt ssRNAs *in vitro*, independent of their sequence and secondary structure. Dicer binds ssRNAs having a 5'-phosphate with greater affinity versus those with a 5'-hydroxyl. (Kini and Walton, 2007).

Dicer-interacting dsRBPs: TARBP2 and PACT

A common Dicer interacting partner found across Metazoa is a dsRBP with tandemly arrayed dsRBDs. Mammals have four dsRBP with tandemly arrayed dsRBDs proteins: trans-activation responsive RNA-binding protein 2 (TARBP2), protein activator of PKR (PACT), Staufen 1 (STAU1), and Staufen 2 (STAU2). However, only TARBP2 (also known as TRBP or TRBP2) and PACT were identified as Dicer binding partners (Chendrimada et al., 2005; Haase et al., 2005).

TARBP2 and PACT are paralogs, which evolved through a gene duplication event in an ancestral chordate (Daniels and Gatignol, 2012). The structure of human TARBP2 has been partially resolved (Benoit and Plevin, 2013). Each protein consists of three dsRBDs, where the first two domains can bind dsRNA (or miRNA) while the third domain has a partial homology to dsRBD and does not bind dsRNA. Instead, it mediates protein-protein interactions and is a part of a larger protein-protein interacting C-terminal region referred to as Medipal domain as it interacts with Merlin, Dicer, and PACT (reviewed in Daniels and Gatignol, 2012). TARBP2 and PACT can also form homodimers and heterodimers through the Medipal domain (Laraki et al., 2008).

The binding site of TARBP2 and PACT on Dicer was recently determined using cryo-EM and crystallography (Wilson et al., 2015). Homology-based modelling showed that Dicer-binding residues are conserved in TARBP2 and PACT, implicating that binding of TARBP2 and PACT to Dicer is mutually exclusive (Wilson et al., 2015).

TARBP has a positive effect on Dicer activity. Human Dicer is much faster at processing a pre-miRNA substrate compared to a pre-siRNA substrate under both single and multiple turnover conditions. Maximal cleavage rates (V_{max}) calculated by Michaelis-Menten analysis differed by more than 100-fold under multiple turnover conditions. TARBP2 was found *in vitro* to stimulate Dicer-mediated cleavage of both, pre-miRNA and pre-siRNA substrates; this stimulation requires the two N-terminal dsRBDs (Chakravarthy et al., 2010). Thus, while the structure of the substrate affects the rate at which Dicer generates small RNAs, TARBP2 stimulates dicing by presumably enhancing the stability of Dicer-substrate complexes (Chakravarthy et al., 2010).

When compared to Dicer and Dicer:TARBP2 complex, PACT inhibits Dicer processing of pre-siRNA substrates (Lee et al., 2013). The two N-terminal dsRBDs contribute to the observed differences in dsRNA substrate recognition and processing behaviour of Dicer:dsRNA-binding protein complexes (Lee et al., 2013). In addition, PACT and TARBP2 have non-redundant effects on the generation of different-sized miRNAs (isomiRs) (Kim et al., 2014; Lee et al., 2013; Wilson et al., 2015). Cells lacking TARBP2 exhibit altered cleavage sites in a subset of miRNAs but no effect on general miRNA abundance or Argonaute loading (Kim et al., 2014). Thus, impact of TARBP2 and PACT on miRNAs biogenesis *in vivo* seems to be relatively minor (Kim et al., 2014; Wilson et al., 2015). However, it should be pointed out that any change in the 5' end position of any miRNA will have a strong effect on its target repertoire. Taken together, TARBP2 and PACT are regulatory factors that contribute to the substrate specificity and cleavage fidelity during miRNA and siRNA production.

Moreover, TARBP2 and PACT have an additional role in a cross-talk of the interferon (IFN) response and small RNA pathways (reviewed in Daniels and Gatignol, 2012). The

IFN response is the major antiviral branch of innate immunity in mammals, which deals with threats associated with long dsRNA. Among the key components sensing dsRNA in the IFN response are protein kinase R (PKR) and helicase RIG-I (reviewed in Gantier and Williams, 2007). The two N-terminal dsRBDs of PACT and TARBP2 bind PKR through the same residues (Wilson et al., 2015) while the (C-terminal) Medipal domain of PACT is needed for PKR activation (Huang et al., 2002). In contrast, the Medipal domain of TARBP2 has an inhibitory effect (Gupta et al., 2003). Furthermore, sequestering of PACT by TARBP2 has negative effect on PKR phosphorylation and activation. PKR inhibition by TARBP2 is released in stress conditions, leading to IFN response activation (Daher et al., 2009). Therefore, absolute and/or relative expression levels of TARBP2 and PACT might be buffering or sensitizing the IFN response to dsRNA. One could envision that suppression of the IFN response might result in increased RNAi. However, there is no evidence, so far that TARBP2 would redirect long dsRNA to Dicer and stimulate RNAi *in vivo* enough to achieve a robust sequence-specific mRNA knock-down.

Taken together, while TARBP2 and PACT are clearly associated with dsRNA binding, Dicer and the miRNA pathway. However, the full extent of biological roles of TARBP2 and PACT in dsRNA response, in the miRNA pathway, and elsewhere is still not fully understood. Since TARBP2 also interacts with and inhibits PKR (Cosentino et al., 1995; Park et al., 1994), it was speculated that TARBP2 could be a component of a network of protein-protein interactions underlying a reciprocal regulation of RNAi/miRNA and IFN-PKR pathway (Haase et al. 2005). This notion is further supported by PACT, a paralog of TARBP2, which exerts the opposite role on PKR. PACT was shown to interact with TARBP2 and Dicer and to facilitate siRNA production (Kok et al., 2007). At the same time, the role of TARB2P in RNA silencing has been studied in cells where the physiological substrate for Dicer processing and RISC loading are miRNA precursors and where long dsRNA readily activates the protein kinase R (PKR) and interferons (IFN). Thus, while the RISC loading role of TARBP2 may be common for miRNA and RNAi pathway, it is not clear if an isoform of TARBP2 plays any specific role in recognition and processing of long dsRNA in the canonical mammalian RNAi pathway. Accordingly, one of the unexplored areas, which deserve further attention is the contribution of different splice variants to TARBP2 biology (Bannwarth et al., 2001). Likewise, it needs to be tested if TARBP2 plays a role in directing long dsRNA into RNAi. Finally, there is RNAi silencing-independent function of TARBP2 to explore. TARBP2 can also bind mRNA hairpins and it has been proposed that TARBP promotes metastasis by destabilizing metastasis suppressor through binding of mRNA structural elements (Goodarzi et al., 2014).

(Absence of) mammalian RNA-dependent RNA polymerase

RNA dependent RNA polymerases were found in RNA silencing in plants an lower invertebrates where they can either produce long dsRNA or short RNAs serving as an initiator or amplifier of the response (reviewed in Maida and Masutomi, 2011). Importantly, these independently discovered RdRPs that act in RNA silencing in plants, fungi and nematodes are homologs. Furthermore, homologs of these RdRPs exist in metazoan phyla, including

Nematoda (e.g. *C. elegans*), *Cnidaria* (hydra), *Chelicerata* (tick), *Hemichordata* (acorn worm), *Urochordata* (sea squirt), and *Cephalochordata* (lancelet) but appear absent in others, including *Platyhelminthes* (planaria), *Hexapoda* (*Drosophila*), and *Craniata* (vertebrates). Phylogenetic analysis suggests that RdRPs in RNA silencing pathways have a monophyletic origin, i.e. evolved once from an ancestral RdRP presumably acting in a common ancestral RNA silencing mechanism (Cerutti and Casas-Mollano, 2006; Murphy et al., 2008). At the same time, mammalian genomes do not contain a homolog of these RdRPs, suggesting that it was lost during evolution and is absent in RNA silencing in mammals. Whether RdRP activity completely disappeared from RNAi in mammals is unclear. One could speculate that RdRP orthologs in RNA silencing in vertebrates could be replaced by another RdRP, for example by exaptation of some viral RdRP.

Our literature search for mammalian RdRPs related to RNA silencing yielded ten original research papers related to mammalian RdRPs that could be put in some context of RNA silencing. Of these, four articles actually dealt with viral RdRPs and silencing of viruses by exogenous siRNAs and not with an RdRP possibly involved in RNAi (Lee et al., 2002; Meng et al., 2006; Moon et al., 2016; Nygardas et al., 2009).

One article reported that ectopic expression of plant RdRP has no effect on RNAi in the mouse model (De Wit et al., 2002). Another report described Aquarius (AQR), a murine protein of unknown function with a weak homology to viral RdRPs expressed in embryos and ESCs (Sam et al., 1998). A follow up search for AQR-related data (28 papers citing the original one and text search by gene name) suggested that Aquarius is an RNA/DNA helicase involved in R-loop processing (Sollier et al., 2014).

An important contribution to RdRP function in mammalian RNAi was provided by a report demonstrating the lack of RdRP activity in RNAi in mouse oocytes (Stein et al., 2003). This report is of a particular importance because mouse oocytes are the only mammalian cell type, with strong endogenous RNAi pathway (reviewed in Svoboda, 2014).

Two reports proposed that two other RdRP activities could generate dsRNA that could be processed by Dicer. An indirect support for a putative RdRP emerged from *Drosophila*, where it was discovered ELP1, a non-canonical RdRP conserved in all eukaryotes, which associates with DCR-2 and its loss results in reduction of endo-siRNAs and upregulation of transposon transcripts (Lipardi and Paterson, 2009). However, a follow up search revealed the lack of data supporting that notion in mammals.

The second proposed mammalian RdRP acting in RNAi is a ribonucleoprotein complex of the human telomerase reverse transcriptase (TERT) and the RNA component of mitochondrial RNA processing endoribonuclease (RMRP). RMRP shows a strong preference for substrates that have 3' fold-back structures and produces dsRNA that can be processed by Dicer yielding self-targeting endogenous siRNAs (Maida et al., 2009). A follow-up analysis of these siRNAs revealed that these off-target effects of these endo-siRNAs would mimic miRNA activities (Maida et al., 2013). The latest report shows that TERT RdRP generates short RNAs that are complementary to template RNAs and have 5'-triphosphorylated ends, which indicates de novo synthesis of the RNAs (Maida et al., 2016).

Taken together, all available data suggest that mammalian RNAi does not employ an RdRP in a canonical way known from plants or nematodes. There are two candidate mechanisms that might involve RdRP in RNA silencing – one of them is based just on existence

of a homolog of ELP1, so further experimental evidence is needed to confirm whether mammalian ELP1 homologs participate in RNAi at all. This would include analysis of small RNAs in mammalian cells lacking *Elp1* and further characterization of complexes containing ELP1. The second candidate mechanism, the TERT RdRP system, seems to produce a unique small population self-targeting endo-siRNAs in a highly localized manner, i.e. does not appear to be an RdRP acting in RNAi *in trans*.

Argonaute proteins

Once a small RNA duplex is produced by Dicer, one of the strands of the duplex is selected and loaded onto an Argonaute protein. However, before summarizing AGO loading and RISC assembly, I will review structure of AGO proteins, their covalent modifications and binding partners.

AGO2 structure and function

Argonaute proteins are the key components of miRNA and RNAi pathways as they bridge the small RNA, its target and the silencing effect. According to the model (Yuan et al., 2005), AGO proteins exist in four basic states according to the bound RNAs: apo (no substrate), pre-RISC (with a passenger and a guide), activated RISC (with a guide), and RISC targeting (with a guide and a target). Transitions between these four states correspond to RISC loading, RISC activation and target recognition, which will be discussed later.

Argonaute proteins are divided into two subfamilies: AGO proteins, which accommodate miRNAs and siRNAs (and are in the focus of this section), and PIWI proteins, which accommodate piRNAs. As piRNAs are produced in the germline in a Dicer-independent manner from single-stranded RNAs, hence they are not systematically covered in this report and neither are PIWI proteins.

Mammalian genomes encode four AGO proteins, where AGO1, 3, and 4 are encoded within one locus on the human chromosome 1 (chromosome 4 in mice) and AGO2 is encoded separately on chromosome 8 (chromosome 15 in mice). independent studies have revealed that mammalian Argonautes were originally discovered through screening of antibodies generated against intracellular membrane fractions from rat pancreas, which yielded a 95-kDa protein that localizing to the Golgi complex or the endoplasmic reticulum (Cikaluk et al., 1999). This initial observation was later refined by analysis of compartmentalization of miRNA- and siRNA-loaded AGO2 populations that co-sediment almost exclusively with the rough endoplasmic reticulum membranes, together with Dicer, TARBP2, and PACT (Stalder et al., 2013). The identified protein of unknown function was originally named GERp95 (Golgi ER protein 95 kDa) and was identified as a member of a family of highly conserved proteins in metazoans which function in the germline (Cikaluk et al., 1999). Apart from the original GERp95 reference, there are three others identified with the GERp95 keyword search. In one of them, GERP95 was found to bind Hsp90 chaperon (Tahbaz et al., 2001) before it was finally linked to RNAi and RISC complex and became classified as an Argonaute family member (Martinez et al., 2002; Thonberg et al., 2004).

AGO2 is the catalytical engine of RNAi and all four AGO proteins operate in the miRNA pathway. The key for understanding functional significance of AGO proteins for RNA silencing and for summarizing the molecular mechanisms mediated by AGO proteins are their structural analyses.

Unlike Dicer, mammalian AGO proteins were successfully crystalized. The core of the structural reports is thus formed by six articles reporting crystal structures of human Argonaute proteins (Elkayam et al., 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014). Four articles describe crystal structures of AGO2 (Elkayam et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014), which is capable of cleaving cognate transcripts and two describe miRNA-pathway dedicated AGO1 (Faehnle et al., 2013; Nakanishi et al., 2013).

There is a number of additional articles, which provided partial insights into AGO functional structure. These could be divided, as in the case of Dicer, into:

(I) **Biochemical studies of recombinant human AGO proteins and individual domains** (Deleavey et al., 2013; Kalia et al., 2016; Lima et al., 2009; Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005; Tan et al., 2009).

(II) **The pioneering AGO crystal structures from archaea *Pyrococcus furiosus*** (Song et al., 2004) **and *Aquifex aeolicus*** (Yuan et al., 2005) that served as a comparative scaffold.

(III) **Crystallographic studies on individual domains of human AGO proteins** (PAZ of human AGO 1 (Ma et al., 2004), or MID domain of human AGO2 (Frank et al., 2011; Frank et al., 2010)) **or modelling** (Deerberg et al., 2013; Gan and Gunsalus, 2015; Jiang et al., 2015; Kandeel and Kitade, 2013; Kinch and Grishin, 2009).

Human AGO1 and AGO2 structures

Among the four mammalian AGO proteins associating with 21–23 nt long small RNAs, only AGO2 was identified to have has the „slicer“ activity (Liu et al., 2004; Meister et al., 2004). The crystal structure of human AGO2 revealed a bilobed molecule with a central cleft for binding guide and target RNAs (Elkayam et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014) (Fig. 4). This domain organization is consistent with that found in archaeal AGO proteins (Song et al., 2004; Yuan et al., 2005). The 3’ end of a short RNA is anchored by the PAZ domain in a sequence-independent manner (Ma et al., 2004). The 5’ phosphate of the siRNA is buried in a pocket at the interface between the MID domain and the PIWI domain. Structural studies of archaeal AGO homologs showed that the PIWI domain has an RNase H-like fold and possess the endonucleolytic “slicer” activity (Song et al., 2004; Yuan et al., 2005). The 5’ end of the base pairing cognate mRNA enters between the N-terminal and PAZ domains and its 3’ end exits between the PAZ and MID domains.

Structural analysis provided a model for recognition of target RNAs, including the first nucleotide in the cognate site, which does not base pair with the loaded RNA. Yet, interaction with the cognate site is enhanced by adenosine in the position 1 of miRNA binding site; the structural analysis revealed that it is recognized indirectly by AGO2 (Schirle et al., 2015). Importantly, N6 adenosine methylation blocks recognition of the adenosine, which

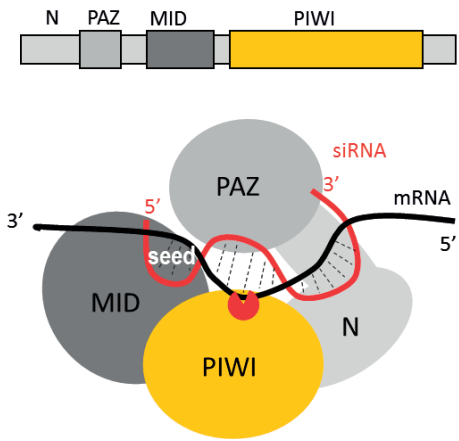

Figure 4 AGO2 structure
The figure shows domain composition and structure of human AGO2. Different colours indicate positions of the central PAZ domain and the c-terminal PIWI domain, which has an RNase H fold (Song et al., 2004). Two additional domains are recognized, the N-terminal domain and the MID domain between PAZ and PIWI domains. The ribbon model was taken from (Schirle et al., 2014).

might reflect a possible mechanism for regulating of miRNA binding through covalent modification of the binding site (Schirle et al., 2015). Nucleotides 2 to 6 of a heterogeneous mixture of guide RNAs are positioned in an A-form conformation for base pairing with target messenger RNAs. Between nucleotides 6 and 7, there is a kink that may function in miRNA target recognition or release of sliced RNA products. (Schirle and MacRae, 2012). Crystallization of loaded human AGO2 in the presence of target RNA sequences suggested a stepwise mechanism for interaction with cognate RNAs. First, AGO2 exposes guide nucleotides (nt) 2 to 5 for initial target pairing, which then promotes conformational changes that expose nt 2 to 8 and 13 to 16 for further target recognition (Schirle et al., 2014). miRNA binding seem to lock otherwise flexible AGO2 enzyme in a stable conformation (Elkayam et al., 2012). The structure of human Ago2 bound to miRNA-20a implies that the miRNA is anchored at both ends by the MID and PAZ domains with several kinks and turns along the binding groove (Elkayam et al., 2012). Spurious slicing of miRNA targets is avoided through an inhibitory coordination of one catalytic magnesium ion (Schirle et al., 2014). Importantly, the PIWI domain contains tandem tryptophan-binding pockets, which function in recruitment of glycine-tryptophan-182 (GW182) or other tryptophan-rich cofactors (Schirle and MacRae, 2012).

Structures of human AGO1 bound to endogenous co-purified RNAs or loaded with let-7 miRNA are strikingly similar to the structures of AGO2 (Faehnle et al., 2013; Nakanishi et al., 2013). Evolutionary changes that rendered hAGO1 inactive included a mutation of a catalytic tetrad residue and mutations on a loop near the actives site (Faehnle et al., 2013; Nakanishi et al., 2013).

Taken together, crystal structures of AGO2 explained the nucleotide-pairing patterns that emerged during previous studies of miRNA sequences, namely analyses of

conservations of miRNA binding sites and biochemical analyses of target recognition, which are discussed later.

Post-translational modifications of AGO proteins

Annotation of Argonaute-related literature yielded a number of reports describing covalent modifications of AGO proteins implicated in post-translational regulations, namely phosphorylation (Horman et al., 2013; Lopez-Orozco et al., 2015; Mazumder et al., 2013; Patranabis and Bhattacharyya, 2016; Rudel et al., 2011; Shen et al., 2013; Zeng et al., 2008), prolyl 4-hydroxylation (Qi et al., 2008; Wu et al., 2011), sumoylation (Josa-Prado et al., 2015), ubiquitination (Bronevetsky et al., 2013; Johnston et al., 2010; Rybak et al., 2009; Smibert et al., 2013), and poly-ADP-ribosylation (Leung et al., 2011; Seo et al., 2013).

Phosphorylation

Phosphorylation of AGO occurs at multiple positions and published data imply that phosphorylation operates through multiple different mechanisms. Phosphorylation has been detected on AGO1 and AGO2 in the PAZ domain (pS253, pT303, pT307), the PIWI domain (pS798), in the L2 linker region (pS387, pY393) and in the MID domain (pY529) (Rudel et al., 2011).

S387 phosphorylation – Serine-387 (S387) was found to be the major Ago2 phosphorylation site *in vivo* (Zeng et al., 2008). Phosphorylation of Ago2 at S387 was significantly induced upon stress in a mitogen-activated protein kinase (MAPK)-dependent manner but, apparently independently of JNK and MEK kinases (Zeng et al., 2008). Another kinase implicated in S387 phosphorylation was AKT3 (Horman et al., 2013). S387A mutation or treatment with a p38 MAPK inhibitor reduced the localization of Ago2 to cytoplasmic P-bodies suggesting a potential regulatory mechanism (Zeng et al., 2008). Conversely, S387 phosphorylation downregulated RNAi-like cleavage and increased miRNA-mediated translational repression (Horman et al., 2013). Furthermore, AGO2 phosphorylation at S387 facilitated interaction with GW182 and localization to P bodies (Horman et al., 2013).

S798 phosphorylation – Serine-798 (S798) phosphorylation has been also associated with P-body localization as mutation analysis of phospho-residues within AGO2 revealed that S798D completely abrogated association of Ago2 with P-bodies and stress granules (Lopez-Orozco et al., 2015).

Y529 phosphorylation – Tyrosine 529 (Y529), which is conserved in all other species that have been analyzed. is located in the small RNA 5'-end-binding pocket of Ago proteins was found to be phosphorylated *in vivo* (Rudel et al., 2011). Y529E phosphomimicking mutation strongly reduced small RNA binding suggesting a potential regulatory role (Rudel et al., 2011). Y529 phosphorylation causing impaired binding miRNAs was subsequently implicated as a mechanisms transiently relieving miRNA repression during macrophage function (Mazumder et al., 2013). Y529 phosphorylation was also proposed as a mechanism relieving Let-7-mediated repression during neuronal differentiation (Patranabis and Bhattacharyya, 2016)

Y393 phosphorylation – Tyrosine 393 (Y393) was implicated in EGFR-mediated repression of miRNA biogenesis during hypoxia (Shen et al., 2013). According to the model, Y393 negatively impacts the interaction between AGO2 and Dicer and inhibits maturation of long-loop pre-miRNAs carrying tumour-suppressor-like miRNAs (Shen et al., 2013).

Prolyl 4-hydroxylation

Prolyl 4- hydroxylation has been implicated in AGO stabilization and increased RNAi. Mass spectrometry analysis hydroxylation of the endogenous AGO2 at proline 700 (P700) and P700A mutation resulted in destabilization of AGO2 (Qi et al., 2008). Prolyl hydroxylation was observed under hypoxic conditions, where it lead to increased AGO2 stability (Wu et al., 2011). AGO2 hydroxylation correlated with increased miRNA levels as well as the endonuclease activity of AGO2 (Wu et al., 2011). Conversely, human cells depleted and mouse embryonic fibroblast cells depleted of a specific prolyl-4-hydroxylase showed reduced stability of AGO2 and impaired RISC activity (Qi et al., 2008). Hydroxylation of AGO2 was required for its association with HSP90 (see further below), which is implicated in the RISC loading with miRNAs and translocation to stress granules (Wu et al., 2011).

SUMOylation

The small ubiquitin-like modifier (SUMO) regulates various cellular processes. AGO2 was identified as a substrate for SUMO E3 ligase PIAS3. AGO2 was SUMOylated in mammalian cells by both SUMO1 and SUMO2 primarily at lysine 402. Mutation of the SUMO consensus site reduced RNAi activity of AGO2, suggesting that SUMOylation might regulate endonucleolytic activity of AGO2 (Josa-Prado et al., 2015)

Ubiquitination

Ubiquitin-proteasome apparently tunes AGO levels to adjust miRNA, AGO and Dicer stoichiometry (Smibert et al., 2013). It was found that levels of AGO1 are adjusted according to miRNA expression in a ubiquitin-proteasome-dependent manner (Smibert et al., 2013). Similarly, lower stability of AGO2 in Dicer-knockout cells could be rescued by proteasome inhibition or Dicer expression (Smibert et al., 2013). AGO and GW182 protein levels also depend on HSP90 availability (Johnston et al., 2010). Two studies show examples of developmentally regulated ubiquitination, which is apparently used to suppress AGO activities during developmental transitions. First, the let-7 target *Lin-41* gene in mice is a stem cell specific E3 ubiquitin ligase targeting AGO1, AGO2, and AGO4 proteins (Rybak et al., 2009). Second, AGO proteins are downregulated in a proteasome-dependent manner during T cell differentiation, presumably as a part of gene expression reprogramming (Bronevetsky et al., 2013).

Poly-ADP-ribosylation

This AGO modifications seems to be linked to suppression of RNA silencing. Poly(ADP-ribose) has been associated with the assembly of stress granules, which accumulate

RNA-binding proteins regulating mRNAs stability and translation upon stress. Stress granule proteins modified by poly(ADP-ribose) include AGO1—4 (Leung et al., 2011). Interestingly, poly-ADP-ribosylation of RISC associated with reduced RISC activity has been observed upon viral infection (Seo et al., 2013). According to the model, poly-ADP-ribosylation after viral infection releases miRNA-mediated repression of interferon-stimulated genes, hence boosting innate antiviral pathways (Seo et al., 2013).

Other Dicer and AGO interacting proteins

Apart from the RISC-loading complex and miRISC components such as GW182/TNRC6 or DDX6 and others mentioned above and elsewhere, a large number of AGO-interacting partners has been identified in the past and reported individually (see further below) or comprehensively (Meister et al., 2005). Here, I provide an overview of those interacting partners.

DDX3 – DEAD-box helicase 3 is one of the helicases sensing viral double-stranded RNAs. DDX3 was also among the P-body components recruited to the West Nile virus replication sites and regulating viral replication (Chahar et al., 2013). DDX3 was also identified by an RNAi screen as an essential factor involved in RNAi pathway (Kasim et al., 2013). DDX3 is co-localized with AGO2 and a dominant negative mutant of DDX3 affected the RNAi activity (Kasim et al., 2013).

CLIMP-63 – The cytoskeleton-linking endoplasmic reticulum (ER) membrane protein of 63 kDa (CLIMP-63) was identified as a novel Dicer-interacting protein through a yeast two-hybrid screening. CLIMP-63 interacts with Dicer to form a high molecular weight complex, which is catalytically active in pre-miRNA processing (Pepin et al., 2012). These results are consistent with analysis of Dicer compartmentalization, which showed that loading of small RNAs into RISC, cognate mRNA binding, and Ago2-mediated mRNA slicing in mammalian cells are nucleated at the rough endoplasmic reticulum (Stalder et al., 2013). While the major RNAi pathway proteins are found in most subcellular compartments, the miRNA- and siRNA-loaded AGO2 populations co-sediment almost exclusively with the rough endoplasmic reticulum membranes, together with Dicer, TARBP2, and PACT (Stalder et al., 2013).

NUP153 – The nuclear pore complex protein NUP1 was found to associate with human Dicer protein. The association was detected mainly in the cytoplasm but was also apparent at the nuclear periphery. Accordingly, it has been suggested that NUP153 plays a role in the nuclear localization of Dicer (Ando et al., 2011b)

FMRP – X mental retardation protein (FMRP) is included in the list despite its questionable role in mammalian RNA silencing. In any case, our literature search revealed a number of articles dealing with mammalian FMRPs because FMMRP is a highly conserved protein and its *Drosophila* ortholog dFXR was implicated in RNAi (Caudy et al., 2002; Ishizuka et al., 2002). According to the available data, FMRP is associated with RNA silencing factors. FMRP co-localized with AGO2 (Goodier et al., 2007) and immunoprecipitation suggested that a portion of Dicer and AGO were associated with each other and with FMRP (Lugli et al., 2005). *In vitro* data using recombinant proteins, suggested

that human FMRP can act as a miRNA acceptor protein for Dicer and facilitate the assembly of miRNAs on specific target RNA sequences (Plante et al., 2006). The requirement of FMRP for efficient RNAi was also supported *in vivo* by reporter assays supporting the role of FMRP in the mammalian RISC (Plante et al., 2006). However, the loss of mammalian FMRP did not reveal any apparent direct impact on RISC function (Didiot et al., 2009; Madsen et al., 2009).

Huntingtin – AGO2 was found as one of the Huntingtin associated proteins by co-immunprecipitation. Furthermore, Huntingtin and AGO2 co-localized in P-bodies and, importantly, depletion of Huntingtin compromised RNA-mediated gene silencing (Savas et al., 2008). However, the molecular mechanism by which Huntingtin would influence RNA silencing remain unknown.

14–3–3 – Cell cycle regulating 14–3–3 proteins were reported to bind the amino terminus of AGO1 and AGO2 (Stoica et al., 2006). Overexpression of the Ago1 amino terminus in yeast resulted in cell cycle delay at the G(2)/M boundary prompting a hypothesis that 14–3–3 proteins contribute to Argonaute protein functions in cell cycle and/or gene-silencing pathways (Stoica et al., 2006).

UPF1 – mRNA surveillance protein appears to provide a nexus between three different mechanisms of RNA metabolism: adenosine deamination, mRNA surveillance (non-sense-mediated decay) and RNA silencing. Both, human ADAR1 and UPF1 were found associated within nuclear RNA-splicing complexes (Agranat et al., 2008). At the same time, UPF1 was connected to RNA silencing (Jin et al., 2009). UPF1 interacts with human AGO1 and AGO2 and co-localizes with them into P-bodies. UPF knockdown yielded upregulation of miRNA targets while its overexpression resulted in their downregulation (Jin et al., 2009). This would suggest that UPF may contribute to RNA silencing, maybe at the level of RISC binding to its targets and accelerating their decay (Jin et al., 2009).

RBM4 – The RNA-binding motif protein 4 (RBM4) plays multiple roles in mRNA metabolism.RBM4 was found during proteomic analysis of AGO-containing miRNPs (i.e. miRISC) and RBM4 knockdown showed that it is required for miRNA-guided gene regulation (Hock et al., 2007). It was also found to co-localize with AGO2 during muscle cell differentiation (Lin and Tarn, 2009). RBM4 interacts directly with AGO2 and may selectively enhance miRISC association with target mRNAs (Lin and Tarn, 2009). RBM4 was also implicated it miRNA-mediated repression in inflammation where inflammation-induced miRNA-146 promotes a feed-forward loop that modifies through phosphorylation the subcellular localization RBM4 and promotes its interaction with AGO2 and, subsequently, tames an excessive acute inflammatory response (Brudecki et al., 2013)

TRIM32 – TRIM-NHL 32 protein regulates protein degradation and miRNA activity in neural progenitor cells to control the balance between differentiating neurons and daughter cells retaining the progenitor fate. TRIM32 was shown to bind AGO1 and increase the activity of specific miRNAs, such as Let-7 (Schwamborn et al., 2009)

QKI-6 – QKI-6 is one of the protein isoforms encoded by the qkI gene in mice. QKI-6 was found to interact with AGO2 and to co-localize with AGO2 into stress granules (Wang et al., 2010). At the same time QKI-6 depletion lead to increased miR-7 expression while QKI-6 presence inhibits processing of pri-miR-7 into miR-7 in glioblastoma cells (Wang

et al., 2013). It has been suggested that OKI-6 mediates selective nuclear retention of pri-miR-7, hence preventing its processing (Wang et al., 2013). Further research is needed to clarify these two seemingly distant activities of OKI-6.

RACK – receptor for activated protein kinase C (RACK1), a constituent of the eukaryotic 40S subunit, was reported to be important for miRNA-mediated gene regulation in *C. elegans* and humans, essentially linking miRISC with the ribosome (Jannot et al., 2011). RACK1 was also identified as a gene necessary for full miRNA function a screen for genes regulating miRNA function (Otsuka et al., 2011). RACK1 interacts with components of the miRISC in nematodes and mammals; the alteration of RACK1 expression alters miRNA function and impairs the association of the miRNA complex with the translating ribosomes (Jannot et al., 2011). Another study found that RACK1 binds to KH-type splicing regulatory protein (KSRP) and is required for the recruitment of mature miRNAs to RISC (Otsuka et al., 2011)

PTB – Polypyrimidine Tract Binding Protein (hnRNP I) was found during a search for proteins involved in let-7 mediated gene regulation. (Engels et al., 2012). PTB interacts with miRNAs and human AGO2 through RNA and there is a population of cellular targets that are co-regulated by PTB and AGO2 (Engels et al., 2012).

LRRK2 – leucine-rich repeat kinase 2 (LRRK2) gain-of-function mutations cause age-dependent degeneration of dopaminergic neurons. the analysis of the molecular mechanism of pathogenesis in *Drosophila* and humans revealed that LRRK2 associates with *Drosophila* AGO1 or human AGO2 (Gehrke et al., 2010) and that the gain-of-function LRRK2 mutant antagonizes let-7, causing derepression of Let-7 targets (Gehrke et al., 2010)

APOBEC3G – the apolipoprotein-B-mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) is cytidine deaminase. APOBEC3G is an antiviral factor is found in P-bodies (Izumi et al., 2013; Wichroski et al., 2006). APOBEC3H also inhibits miRNA-mediated repression of translation (Huang et al., 2007) by competitively inhibiting binding of MOV10 to AGO2, causing either abnormal assembly or abnormal maturation of miRISC (Liu et al., 2012a).

AGO loading and RISC formation

The next important step is formation of RISC, the effector complex of miRNA and RNAi pathways. It involves formation of the RISC Loading Complex (RLC), transfer of a small RNA on an AGO-protein, and RISC activation.

RISC Loading Complex (RLC)

RISC assembly was so far explored more in *Drosophila* (Iwasaki et al., 2010; Pham et al., 2004; Tomari et al., 2004a; Tomari et al., 2004b) than in mammals (Bernard et al., 2015; Gregory et al., 2005; MacRae et al., 2008) perhaps because of the robust *in vitro* system of *Drosophila* embryo lysate. Mammals differ from *Drosophila* because they do not use different Dicer and Argonaute proteins dedicated to RNAi and miRNA pathway although

it is assumed that both pathways use a similar if not the same RLC. Our knowledge of the mammalian RLC comes mainly from cells where RLC normally loads miRNAs or from *in vitro* reconstitution of the RLC with purified proteins. The minimal RLC is composed of Dicer, TARBP2 and AGO2 (Gregory et al., 2005; MacRae et al., 2008). *In vitro* reconstituted mammalian RLC contains one copy of each protein and has dicing, guide-strand selection, loading, and slicing activities (Bernard et al., 2015; Gregory et al., 2005; MacRae et al., 2008; Martinez et al., 2002).

AGO interacts with Dicer through a subregion of the PIWI domain (the PIWI-box), which binds directly to the Dicer RNase III domain. (Tahbaz et al., 2004). Single-particle EM analysis suggested that Dicer's N-terminal DExH/D domain interacts with TARBP2, whereas its C-terminal catalytic domains in the main body are proximal to AGO2 (Wang et al., 2009). Interestingly, binding of AGO to Dicer inhibits dicing activity *in vitro* (Tahbaz et al., 2004). Analysis of individual siRNA positions revealed that RNA sequences at positions 9–12 and 15–18 were associated with TARBP2 while positions 19–21 with AGO. AGO binding was enhanced by positions 15–18 (Takahashi et al., 2014). AGO2 was reported to binds primarily to the 5'- and alternatively, to the 3'-end of pre-miRNAs. (Tan et al., 2011). All four human AGO proteins show remarkably similar structural preferences for small-RNA duplexes: central mismatches promote RISC loading, and seed or 3'-mid (guide position 12–15) mismatches facilitate unwinding. All these features of human AGO proteins are highly reminiscent of fly AGO1 but not fly AGO2. (Yoda et al., 2010). Biochemical and structural analysis suggests that TARBP2 is flexibly bound to the Dicer DExH/D domain (Daniels et al., 2009; Wang et al., 2009). TARBP2 seems to bridge release of the siRNA by Dicer and loading of the duplex onto AGO2. Binding by TARBP2 may allow the siRNA intermediate to stay associated with the RLC after release from Dicer and may also help in orientation of the siRNA for AGO2 loading. Just as in flies, human RISC assembly is uncoupled from dicing (Yoda et al., 2010).

Analysis of miRNA-carrying RISC (miRISC) yielded a similar picture. Since loading of miRNA duplexes to AGO proteins is assisted by HSP70/ HSP90 chaperones (Maniataki and Mourelatos, 2005b; Yoda et al., 2010), HSP90 is sometimes also included as the component of miRLC (Liu et al., 2012b). At the same time, AGO2 and Dicer are sufficient for processing and loading of miRNAs into RISC (Tan et al., 2011).

Combination of *in vivo* studies in Dicer$^{-/-}$ cells reconstituted with wild-type or catalytically inactive Dicer showed that the miRNA loading complex (miRLC) is the primary machinery linking pre-miRNA processing to miRNA loading and lead also to definition of a miRNA Precursor Deposit Complex (miPDC) for Dicer-independent RISC loading exemplified by miR-451 (Liu et al., 2012b). miPDC is formed of AGO, pre-miRNA, and HSP chaperone. It functions in Dicer-independent miRNA biogenesis (e.g. miR-451) and also promotes miRNP assembly of certain Dicer-dependent miRNAs (Liu et al., 2012b).

Earlier studies suggested a difference between fly and human systems because human RISC assembly using immunopurified or reconstituted human RLC containing AGO2, Dicer and TARBP2 did not require ATP hydrolysis, (Gregory et al., 2005; MacRae et al., 2008; Maniataki and Mourelatos, 2005b). Recent data suggest that ATP facilitates also human RISC loading while it is dispensable for unwinding (Yoda et al., 2010).

Accessory RLC factors

Apart from the three established RLC components, several proteins emerged as RLC cofactors, among which stand out HSP70/90 chaperones. Heat shock protein 90 was actually the first identified AGO-associated protein even before AGO was associated with RNA silencing (Tahbaz et al., 2001). Inhibition of HSP90 reduces AGO levels (Johnston et al., 2010; Martinez and Gregory, 2013; Tahbaz et al., 2001) as well as GW182 protein levels and abolishes P-bodies (Johnston et al., 2010). In addition, stable binding between AGO and Dicer is dependent on the activity of Hsp90 (Tahbaz et al., 2004) and association of AGO2 with HSP90 involves prolyl-hydroxylation of AGO2 (Wu et al., 2011). HSP90 activity is not required for association of AGO with intracellular membrane (Tahbaz et al., 2001) but appears to chaperon AGO proteins before binding RNA and may facilitate loading of small RNAs (Johnston et al., 2010). Interestingly, miRNA*s (miRNA* is an equivalent of the passenger strand) with fast turnover exhibited different sensitivity to HSP90 inhibition suggesting differential HSP90 requirements for different miRNA*s (Guo et al., 2015). HSP90 is also a negative regulator of PKR; it is able to bind and inhibit PKR phosphorylation and prevent apoptosis (Donze et al., 2001). Thus, HSP90 provides a factor bridging RNA silencing and innate immunity.

Furthermore, HSP90 co-chaperones FKBP4/5 control AGO2 expression and facilitate RISC assembly (Martinez et al., 2013). FKBP4/5 were identified as AGO2-associated proteins in mouse embryonic stem cells. Inhibition of FKBP4/5 lead to decreased Ago2 protein levels while overexpression stabilized AGO2 expression (Martinez et al., 2013). Another study has found that FKBP4 forms a stable complex with human AGO2 before small RNA loading in the cytoplasm and is required for efficient RNAi (Pare et al., 2013).

Another component reported to function as an RISC-loading factor is RNA helicase A (RHA, also known as DHX9) Dicer (Robb and Rana, 2007). RHA is a conserved protein with two dsRBDs (Nagata et al., 2012) with multiple roles in the gene expression of cellular and viral mRNAs. RHA recognizes highly structured nucleotides and catalytically rearranges the various interactions between RNA, DNA, and protein molecules to provide a platform for the ribonucleoprotein complex. RHA was shown in human cells to function in the RNAi pathway and interact with siRNA, AGO2, TARBP2, and Dicer (Robb and Rana, 2007). RHA-depleted cells, showed reduced RNAi, apparently as a consequence of lower active RISC suggesting that RHA functions in RISC as an siRNA-loading factor (Robb and Rana, 2007). A later structural analysis of dsRBDs showed that both dsRBDs are required for RISC association, and such association is mediated by dsRNA (Fu and Yuan, 2013).

Are mammalian miRNAs sorted?

As mentioned above, of the four AGO proteins that can be loaded with small RNAs equally well (Meister et al., 2004). All four mouse AGO proteins seem to be functionally redundant in the miRNA pathway as shown by rescue experiments in ESCs lacking all four Argonaute genes (Su et al., 2009). Consistent with this, all four AGOs are functionally equivalent when accommodating bulged miRNA duplexes, whereas AGO1 and AGO2 appear to be more effective at utilizing perfectly matched siRNAs (Su et al., 2009). Furthermore, AGO2 can

execute endonucleolytic cleavage of cognate RNAs while all four can mediate translational repression. This raises a question whether small RNAs may undergo some kind of sorting that would result in preferential loading onto specific AGO homologs.

Structural analysis showed that all four human AGO proteins showed similar structural preferences for small-RNA duplexes, which were highly reminiscent of *Drosophila* AGO1 but not of AGO2 (Yoda et al., 2010). Human AGO2 and AGO3 immunoprecipitation and subsequent sequencing of small RNAs revealed that both AGOs were associated with 21–23 nt RNAs, majority of which were miRNAs (Azuma-Mukai et al., 2008). While fifteen miRNAs showed more than 2-fold significant difference in loading onto AGO2 or AGO3, it is not clear whether this discrimination occurs also *in vivo* (Azuma-Mukai et al., 2008).

A detailed analysis of small RNAs associated with all four human AGO proteins revealed approximately equivalent amounts of sequence tags derived from miRNA loci associated with individual AGOs with some exceptions that could be coupled to specific AGOs (Burroughs et al., 2011). However, further analysis suggested existence of some sorting mechanism affecting a subset of distinct isomiRs that seemed to be differentially associated with distinct AGO proteins (Burroughs et al., 2011). This observation contrasts with another cloning and deep sequencing experiment addressing distribution of endogenous miRNAs associated with AGO1–3, which did not find evidence for miRNA sorting in human cells. (Dueck et al., 2012).

It is possible that sorting of small RNAs on AGO proteins may not be a general phenomenon while differential presence of small RNAs on AGO proteins can also emerge from selective mechanisms operating after loading. This can be illustrated on selective progressive 3' shortening of AGO2-bound miRNAs observed in the brain (Juvvuna et al., 2012). Furthermore, Dueck et al also reported that AGO identity appears to influence the length of some miRNAs, while others remain unaffected (Dueck et al., 2012). Taken together, it seems that miRNAs are generally not sorted for loading onto AGO proteins. Notable exceptions include miRNAs with unique biogenesis such as miR-451 whose biogenesis requires AGO2 slicing activity (Dueck et al., 2012).

Loading asymmetry

While both siRNA strands can guide post-transcriptional silencing in mammals (Wei et al., 2009), selection of the loaded strand exhibits a clear and long-known thermodynamic bias where the strand whose 5′-end is less thermodynamically stable is preferentially loaded onto AGO as the guide strand (Khvorova et al., 2003; Schwarz et al., 2003). Selection of the guide strand involves multiple sensors – this includes AGO2 strand selection capability (Noland and Doudna, 2013; Suzuki et al., 2015), which is enhanced in complex with Dicer and TARBP2 or PACT. In addition, strand selection for some miRNAs is enhanced in complexes containing PACT but not TARBP2 (Noland and Doudna, 2013). Notably, TARBP2 was predicted to be a sensor of the thermodynamic stability of 5' siRNA in strand selection during RISC loading, similarly to DCR-2 and R2D2 (a TARBP2 homolog) in *Drosophila* (Wang et al., 2009). However, the supporting evidence is inconclusive (Haase et al., 2005) although some argue that TARBP2 can indeed acts as a sensor (Gredell et al., 2010). Furthermore, while TARBP2 function is similar to that of R2D2, TARBP2 sequence

is more closely related to Loquacious than R2D2 (Murphy et al., 2008). Finally, quantitative analysis of RISC assembly and target silencing activity in the presence or absence of Dicer suggest that the mammalian Dicer is nonessential for asymmetric RISC loading *in vivo* and *in vitro*. (Betancur and Tomari, 2012).

RISC activation

The next step after AGO loading is removal of the passenger strand from the loaded duplex RNA. In some cases, the passenger strand can be eliminated by the slicer activity where the RISC complex uses the guide siRNA to cleave the passenger strand. In other words the first cleavage actually targets the passenger strand of a loaded siRNA duplex to free the guiding strand, so it can base pair to cognate mRNAs (Matranga et al., 2005). The cleavage-assisted mechanism is typical for AGO2-loaded fly and human siRNAs in the RNAi pathway while passenger strand cleavage is not important for loading miRNAs (Matranga et al., 2005).

Slicer-independent mechanism is needed to remove the passenger strands from non-slicing AGO proteins and from miRNA duplexes bound to AGO2 where the passenger strand cannot be cleaved. As slicer-deficient hAGO1, hAGO3, and hAGO4 are able to eject the passenger strand of siRNA duplexes at 37°C, it is apparent that AGO1, 3, and 4 can be readily programmed with siRNAs at the physiological temperature (Park and Shin, 2015). This implies that a slicer-independent mechanism, which relies on the thermal dynamics of the PAZ domain (Gu et al., 2012a; Park and Shin, 2015), is likely a common feature of human AGOs.

Importantly, RISC activation has been associated with additional factors. One of them is C3PO, an endonuclease that activates RISC (Ye et al., 2011). According to the model of RISC activation that integrates the C3PO crystal structure, Ago2 directly binds duplex siRNA and nicks the passenger strand, and then C3PO activates RISC by degrading the Ago2-nicked passenger strand (Ye et al., 2011)> Another factors is La, Sjogren's syndrome antigen B (SSB)/autoantigen, which is acting as an activator of the RISC-mediated mRNA cleavage activity. (Liu et al., 2011). Thus, similarly to C3PO, La is a regulatory factor helping to remove AGO2-cleaved products in order to promote active RISC formation (Liu et al., 2011).

Additional small RNAs associated with AGO proteins

Our literature search revealed a heterogeneous group of publications describing small RNAs loaded on AGO proteins that were clearly distinct from canonical miRNAs – small RNAs generated by the mechanism described above. A canonical miRNA is transcribed by polymerase II, the primary transcript contains a ~ 70 nt short hairpin precursor pre-miRNA, which is released by the Microprocessor complex, transported to the cytoplasm where Dicer cleaves of the loop and one of the strands of the miRNA duplex is loaded onto miRISC. However, next generation sequencing revealed existence of AGO-loaded small RNAs that were apparently generated from different substrates and by molecular mechanisms, which deviated from the canonical pathway. Below is an overview of diversity of AGO-bound RNAs, which emerged from the literature search.

Non-canonical miRNAs can be divided across two axes – (I) according to the RNA precursor and (II) according to the proteins involved in (or omitted from) their biogenesis. Non-canonical miRNAs were discovered during systematic analyses of small RNAs in different model systems, such as disease models (e.g. (Xia et al., 2013) or cultured cells (Babiarz et al., 2011; Babiarz et al., 2008). A good experimental strategy to identify non-canonical miRNAs is a high throughput sequencing analysis of genetic models lacking some of the components of RNA silencing such as Dicer or DGCR8 (Babiarz et al., 2011; Babiarz et al., 2008). Phenotypic difference and differential expression of distinct miRNA-like sequences can indicate biological roles of non-canonical miRNAs while knock-out data offer an insight into the non-canonical biogenesis mechanism

Perhaps the best known non-canonical miRNA class, which comes from unique, Microprocessor-independent precursors, are mirtrons, miRNA-like molecules arising from spliced-out introns, which are Microprocessor-independent (Babiarz et al., 2011; Berezikov et al., 2007; Ladewig et al., 2012; Schamberger et al., 2012; Sibley et al., 2012; Westholm et al., 2012). Interestingly, some predicted mirtron-like miRNAs (miR-1225 and miR-1228) are splicing-independent (simtrons) and their biogenesis involves Drosha but neither DGCR8 nor Dicer (Havens et al., 2012). Other non-canonical substrates can be, for example, 5‘-Capped RNAs (Xie et al., 2013), SINE repeat-derived, (Babiarz et al., 2008; Castellano and Stebbing, 2013), small vault RNA (svtRNA2–1a) (Minones-Moyano et al., 2013), or RNase III transcripts (Maurin et al., 2012) including annotated RNAs such as snoRNAs (Burroughs et al., 2011; Ender et al., 2008; Li et al., 2012), 7SL RNA (Ren et al., 2012), tRNA fragments (Burroughs et al., 2011; Haussecker et al., 2010; Kumar et al., 2014; Li et al., 2012; Maniataki and Mourelatos, 2005a; Maute et al., 2013; Venkatesh et al., 2016). Non-canonical miRNAs can be also produced from viral RNAs (Bogerd et al., 2010; Kincaid et al., 2014; Li et al., 2009; Xu et al., 2009). A non-canonical small RNA class of unclear significance are semi-microRNAs (smiRNAs), which are ~ 12nt short RNA fragments apparently emerging from other miRNAs, such as let-7 or miR-223 (Plante et al., 2012).

Non-canonical miRNAs can be also classified by their biogenesis as Microprocessor, DGCR8-, or Dicer-independent. For instance, the above-mention mirtrons do not require the Microprocessor complex while simtrons require Drosha but neither DGCR8 nor Dicer. Non-canonical miRNAs can be also produced from bona-fide miRNA precursors, which give a rise to a small RNA in a non-canonical way, for example by a dual role of AGO protein (Diederichs and Haber, 2007). A classic example is miR-451, a Dicer-independent miRNA biogenesis pathway that requires Ago catalysis (Cheloufi et al., 2010). A unique type of non-canonical miRNAs are loop-miRs, which are released from the loop region of a pre-miRNA (Okamura et al., 2013; Winter et al., 2013).

Target recognition and modes of silencing

Target recognition

Target recognition by RISC is mediated by base pairing between RISC-loaded small RNAs and cognate RNAs. Consistently with the structural analysis of AGO proteins, target

recognition by siRNAs exhibits a distinct 5' bias. Analysis of miRNA-targeted mRNAs in *Drosophila* and mammals revealed that miRNA bases 2–8 form a distinct „seed", which base pairs perfectly to the target transcript (Enright et al., 2003; Lewis et al., 2003). This is consistent with the fact that the 5' half of a small RNA provides most of the binding energy that tethers RISC to a target RNA (Doench et al., 2003; Haley and Zamore, 2004). Structural features of the target site are only important for RISC binding, while sequence features such as the A/U content of the 3' UTR are important for mRNA degradation. (Hausser et al., 2009). According to analyses of RISC kinetics, small RNAs loaded onto AGO proteins are actually composed of five distinct domains (Fig. 5): the anchor, seed, central, 3' supplementary, and tail (Wee et al., 2012).

Biochemical analysis of target recognition by mammalian RISC showed that the RISC is apparently not systematically scanning transcripts. RISC is unable to unfold structured RNA. Thus, RISC randomly transiently contacts single-stranded RNA and promotes siRNA-target base pairing where the 5'end of the loaded siRNA creates a thermodynamic threshold for stable association of RISC with its target (Ameres et al., 2007).

The fact that 5' and 3' ends of a siRNA are bound by distinct binding pockets and that both ends contribute differently to binding to the target lead to a „two-state model of Argonaute function proposed based on the *Drosophila* model (Tomari et al., 2004b). In this model, the 3' end is bound in the PAZ domain and the 5' end to in a pocket at the interface between the MID and the PIWI domains. The 5' end is pre-organized to interact with the cognate mRNA and, upon binding, the 3' end is dislodged from the binding pocket to allow for base pairing of the 3' end.

Importantly, kinetics of silencing is critical for understanding target recognition and silencing by of small RNAs. A kinetic study of *Drosophila* and mouse AGO2 found that mouse AGO2, which mainly mediates miRNA-directed repression *in vivo*, dissociates rapidly and with similar rates for fully paired and seed-matched targets (Wee et al., 2012). An important conclusion from this study is that low-abundant miRNAs are unlikely to contribute much biologically meaningful regulation because they are present at a concentration less than their KD for seed-matching targets (Wee et al., 2012).

These results were subsequently corroborated by single molecule analysis. Single-molecule fluorescence experiments using a minimal RISC (a small RNA and AGO2) showed that target binding starts at the seed region of the guide RNA (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). AGO2 initially scans for complementarity to nucleotides 2–4 of the miRNA and this interaction propagates into a stable association when target complementarity extends across the seed (Chandradoss et al., 2015). Stable RISC binding is thus efficiently established with the seed match only, providing a potential explanation for the seed-match rule of miRNA target selection (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). Remarkably, mouse AGO2 binds tighter to miRNA targets than its RNAi cleavage product, even though the cleaved product contains more base pairs (Salomon et al., 2015). In contrast, target cleavage required extensive sequence complementarity and accelerated core-RISC dissociation for recycling (Jo et al., 2015b) and sensitively depended on the sequence (Jo et al., 2015a). RISC thus utilizes short RNAs as specificity determinants with thermodynamic and kinetic properties more typical of RNA-binding proteins while a small RNA loaded on AGO no longer follows rules by which sole oligonucleotides

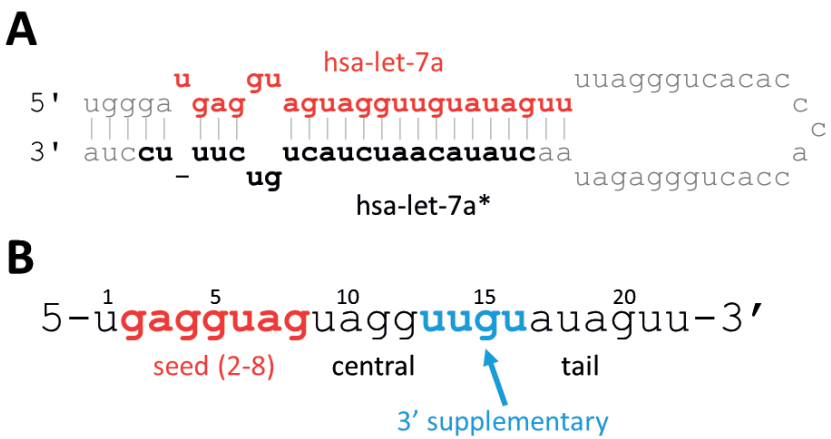

Figure 5 miRNA/siRNA functional domain
(A) miRNA precursor stem loop (exemplified by human Let-7a from miRBase). In red is highlighted sequence of mature miRNA, in black is depicted the passenger (miRNA-star) strand. (B) Mature miRNA can be divided into several functionally distinct regions (Wee et al., 2012).

find, bind, and dissociate from complementary nucleic acid sequences (Salomon et al., 2015). Importantly, target site recognition is coupled to lateral diffusion of RISC along the target RNA, which facilitates recognition of target sites within the cellular transcriptome space (Chandradoss et al., 2015).

To put the aforementioned kinetic data into comparison with laboratory practice, the table below shows on ten randomly selected examples of RNAi experiments that an effective repression by exo-RNAi in cultured cells usually employs siRNA transfection in 20–50 nM range (v 1). Thus, a robust biological effect of a specifically designed perfectly complementary siRNA acting through the AGO2-mediated endonucleolytic cleavage is usually observed with a nanomolar concentration of siRNA. Silencing by direct endonucleolytic cleavage – RNAi-like silencing

Base pairing in the middle of siRNA results in correct positioning of the cognate strand and its cleavage in the active site of the PIWI domain of AGO2 – out of four mammalian AGO proteins, which bind small RNAs, only AGO2 has the „slicer“ activity (Liu et al., 2004; Meister et al., 2004). These simple facts have several important consequences. First, siRNAs loaded on AGO1, AGO3, and AGO4 will repress their perfectly complementary targets by other means (translational repression/deadenylation/decapping) than by cleavage. Conversely, AGO2 loaded miRNA binding perfectly complementary targets will behave as siRNAs and mediate RNAi-like endonucleolytic cleavage. Third, imperfect complementarity in the middle of the base pairing site of AGO-loaded siRNAs will result in miRNA-like effects (Doench et al., 2003). Thus, RISC-loaded siRNAs have additional silencing effects due to 2–8 nucleotide seed matches to other RNAs (which is the molecular foundation of the off-targeting phenomenon).

Table 1 Random selection of RNAi experiments in cultured cells

concentration	method	cells	exposure time	knockdown efficiency	reference – doi
5 nM	transfection	mouse preadipocytes 3T3-L1	48 h	70 %	10.1128/ MCB.01856–08
20 nM	transfection	human HUVEC	0–5 d	60–80 %	10.1016/ j.bone.2014.12.060
20 μM	transfection	human breast carcinoma cell lines	48 h	60 %	10.1038/onc.2014.421
20 μM	transfection	murine breast epithelial cell	48 h	60 %	10.1038/onc.2014.421
25 nM	transfection	mouse kidney	7 d	20 %	10.1152/ ajprenal.00052.2014
20–40 nM	transfection	human LA4	24 h	60–80 %	10.1155/2015/473742
50 nM	transfection	mouse cardiomyocyte	48 h	60 %	10.5582/ bst.2015.01159.
80 nM	transfection	mouse osteoblasts	0–5 d	40–50 %	10.1016/ j.bone.2014.12.060
100 nM	transfection	human endothelial cells	72 h	80%	10.1152/ ajplung.00263.2009
500 nM	electroporation	human monocytic cell	24 h	60–90%	10.1111/jdi.12434

Silencing by translational repression and RNA degradation – common miRNA silencing

In order to understand the molecular mechanism of repression mediated by small RNA-loaded AGO, many AGO-associated proteins were identified in mammalian cells (reviewed in detail in Peters and Meister, 2007). These include MOV10, DDX6 (Rck/p54), DDX20 (Gemin3), TNRC6A (GW182),and many others (Hauptmann et al., 2015; Meister et al., 2005). Over a decade of research focused on understanding how miRNA-guided RISC suppresses gene expression. it became clear that miRNAs provide the guide while the repression is mediated through AGO and associated proteins as shown by suppression occurring upon tethering AGO proteins (Pillai et al., 2004). Building a model of silencing, was a lengthy and convoluted process (which is not over yet) from which emerged GW182 as a key docking factor integrating miRNA-mediated silencing.

GW182 proteins

Mammalian GW182 proteins carry at the N-terminus multiple glycine-tryptophan (GW) repeats, a central ubiquitin-associated (UBA) domain, a glutamine-rich (Q-rich) domain, and an RNA recognition motif (RRM) (Fig. 6). Interestingly, GW182 seems to be largely unstructured protein, including functionally essential domains, such as the N-terminus, which is interacting with AGO2 (Behm-Ansmant et al., 2006; Lazzaretti et al., 2009; Lian

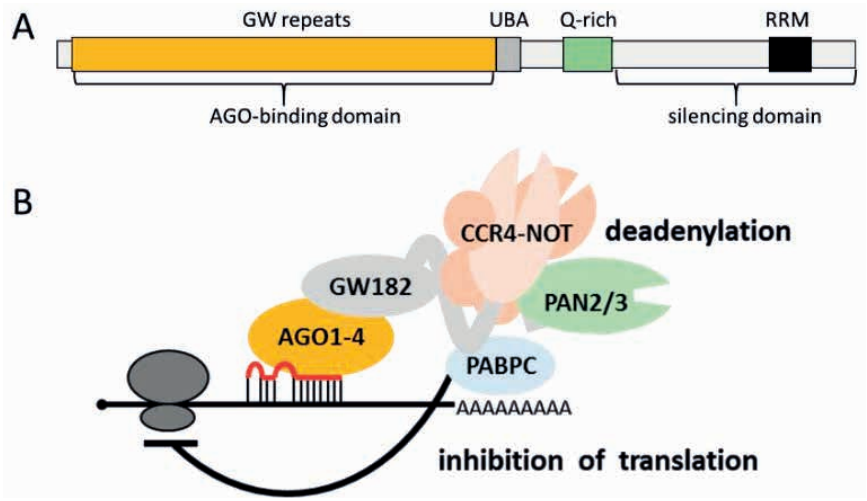

Figure 6 GW182 domain structure and function.
(A) Domain composition: UBA, ubiquitin-associated motif; RRM, RNA recognition motif. (B) A schematic depiction of GW182-mediated bridging of AGO and translational repression and mRNA degradation. See the text below for details.

et al., 2009; Takimoto et al., 2009). Similarly, the silencing domain is also predicted to be disordered (Huntzinger et al., 2010; Lazzaretti et al., 2009; Zipprich et al., 2009).

The N-terminal part of GW182 can interact with any human (and presumably all mammalian) AGO proteins through multiple GW repeats, which bind tryptophan binding pockets on AGO and contribute to the interaction in an additive manner (El-Shami et al., 2007; Jakymiw et al., 2005; Lazzaretti et al., 2009; Lian et al., 2009; Liu et al., 2005a; Schirle and MacRae, 2012; Takimoto et al., 2009; Yao et al., 2011; Zipprich et al., 2009). The role of AGO is most likely to anchor through miRNA-mediated interaction GW182 to the silenced RNA as tethering of GW182 alone or even of (its silencing domain) is sufficient to mediate repression (Chekulaeva et al., 2011). The silencing domain directly binds PolyA Binding protein (PABP) as well as with PAN3 and NOT1 components of PAN2/PAN3 and CCR4–NOT deadenylase complexes, respectively. (Chekulaeva et al., 2011; Fabian et al., 2009; Huntzinger et al., 2010; Jinek et al., 2010; Zekri et al., 2009).

The general, somewhat simplistic model of miRNA-mediated silencing proposes that GW182 interacts with PABP of the cognate RNA and recruit PAN2/3 and CCR4-NOT deadenylases, which would result in translational repression likely occurring at the level of initiation and the repressed RNA would be deadenylated (Braun et al., 2011; Chekulaeva et al., 2011; Christie et al., 2013; Fabian et al., 2011a; Fabian et al., 2011b; Huntzinger et al., 2013; Kuzuoglu-Ozturk et al., 2016; Zekri et al., 2013). Deadenylated RNAs in mammalian cells are generally decapped and degraded by XRN1 exonuclease (Schoenberg and Maquat, 2012).

Several mechanisms have been proposed how the GW182–PABPC interaction contributes to silencing (Fabian et al., 2009; Jinek et al., 2010; Zekri et al., 2009) although it is not

clear how critical this role is as mRNAs lacking polyA tail (i.e. neither circularize nor are deadenylated) are silenced nonetheless (Chekulaeva et al., 2011; Pillai et al., 2005; Zekri et al., 2013). This would suggest for a non-essential role of PABPC/GW182 interaction.

There are additional RISC interactions, which are important for miRNA-mediated silencing, such as DDX6/RCKp54 (Chu and Rana, 2006; Kuzuoglu-Ozturk et al., 2016; Mathys et al., 2014) or LIM domain proteins LIMD1, Ajuba, and WTIP, which are required for miRNA-mediated, but not siRNA-mediated gene silencing (James et al., 2010). According to the model, the LIM proteins facilitate miRNA-mediated gene silencing by creating an inhibitory closed-loop complex where they bridge the translationally inhibited cap structure and AGO1/2 within the miRISC complex bound to the 3'-UTR (James et al., 2010). The list of proteins associated with RISC or the target repression is longer, and includes, for example, also Pumilio/FBF , a miRNA targeting cofactor (Friend et al., 2012) or eIF4GI (Ryu et al., 2013). However, for understanding the miRNA mechanism, their detailed review is would be overreaching.

P-bodies

Studies on localization of miRNA pathways components revealed their presence in distinct cytoplasmic foci, known as P-bodies or GW-bodies (Liu et al., 2005a; Liu et al., 2005b; Pillai et al., 2005; Yu et al., 2005). P-bodies (reviewed in detail by Jain and Parker, 2013) are distinct cytoplasmic foci, which contain proteins associated miRNA-mediated repression (Behm-Ansmant et al., 2006; Chahar et al., 2013; Chu and Rana, 2006; James et al., 2010; Johnston et al., 2010; Liu et al., 2005b; Ozgur and Stoecklin, 2013; Pare et al., 2011; Rehwinkel et al., 2005; Yu et al., 2005; Zhou et al., 2009). P-body association has been observed for the mature RISC components and RNA degradation pathway proteins but not for Dicer or TARBP2, indicating that P-bodies are associated with miRNA-mediated suppression but not biogenesis. This is supported by the presence of miRNA-targeted mRNAs in P-bodies (Liu et al., 2005b; Shih et al., 2011). P-bodies are highly dynamic (Aizer et al., 2014; Kedersha et al., 2005) but not an essential structure for miRNA-mediated repression (Eulalio et al., 2007). P-bodies probably should be seen as aggregating foci, which may facilitate miRNA-mediated repression. Since inhibition of the miRNA pathway prevents P-body formation, it was proposed that aggregation of miRNA pathway factors to P-bodies is not required for miRNA function and mRNA degradation *per se* but rather is a consequence of miRNA activity (Eulalio et al., 2007).

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