Mechanistic Insights into MicroRNA-Mediated Gene Silencing

Thomas F. Duchaine¹ and Marc R. Fabian^{2,3}

¹Department of Biochemistry & Goodman Cancer Research Centre, McGill University, Montreal, Quebec H3G 1Y6, Canada

²Department of Oncology, McGill University, Montreal, Quebec H3G 1Y6, Canada

³Lady Davis Institute, Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada

Correspondence: thomas.duchaine@mcgill.ca; marc.fabian@mcgill.ca

MicroRNAs (miRNAs) posttranscriptionally regulate gene expression by repressing protein synthesis and exert a broad influence over development, physiology, adaptation, and disease. Over the past two decades, great strides have been made toward elucidating how miRNAs go about shutting down messenger RNA (mRNA) translation and promoting mRNA decay.

Here, we review the mechanism of microRNA (miRNA)-mediated gene silencing and describe the most recent transformative discoveries. Focus is placed on miRNA target site recognition, and new insights into the structure of the miRNA-induced silencing complex (miRISC) and miRISC-interacting proteins. Finally, we discuss recent models that have been proposed regarding how the miRISC represses mRNA translation and promotes messenger RNA (mRNA) deadenylation and decay.

THE FUNCTIONS OF miRNAs IN GENE NETWORKS

miRNAs are small, ~21-nucleotide (nt)-long noncoding RNAs that are encoded within the genomes of multicellular organisms and regulate gene expression. At the molecular level, miRNAs base-pair imperfectly with the 3'

untranslated regions (3'UTRs) of targeted mRNAs, from where they engender mRNA translation inhibition and destabilization (Brennecke et al. 2005; Farh et al. 2005; Lim et al. 2005; Stark et al. 2005; Bartel 2009). The functions of miRNAs are thus defined through the identity of their targets. Consequently, the information an miRNA encodes takes on a physiological meaning only when considered as an integral part of gene-regulation networks. The functional impact of miRNAs is both broad and specific; more than 2000 miRNAs have been detected in the human transcriptome, and each miRNA specifically targets one to several dozen mRNAs (Krek et al. 2005; Lall et al. 2006; Baek et al. 2008; Friedman et al. 2009; Fang and Rajewsky 2011). It is estimated that more than 60% of human protein-coding transcripts are targeted by miRNAs (Friedman et al. 2009).

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miRNAs play a dual role in gene-regulation networks, in shaping transcriptomes, and in controlling their output in protein synthesis. To perform these global functions, the expression and abundance of miRNAs, those of their target sites in mRNAs, their affinities, and their functional output through regulation of their effector machineries, have all been precisely tuned through evolution (Farh et al. 2005; Stark et al. 2005; Chen and Rajewsky 2006, 2007; Bartel 2009; Friedman et al. 2009). Variations on these parameters between biological conditions and cell types allow miRNAs to serve a diverse array of biological purposes (Ambros et al. 2003a; Ambros 2004; Chen and Rajewsky 2007; Bartel 2009; Sun and Lai 2013; Alberti and Cochella 2017). First, miRNAs can act as rheostats governing the expression of individual genes, by promoting partial but functionally significant repression. This function in fine-tuning gene expression appears pervasive in somatic cells, based on several genome-wide studies (Farh et al. 2005; Selbach et al. 2008; Guo et al. 2010; Fang and Rajewsky 2011). Second, miRNAs play important roles in making developmental decisions, such as steering a daughter cell away from its parental lineage (reviewed in Alberti and Cochella 2017). Examples include control of epidermal seam cell differentiation through regulation of *lin-41* mRNA by *let-7* miRNA in *Caenorhabditis elegans* (Ecsedi et al. 2015), and the function of *miR-155* in controlling macrophage maturation in mammals (Mann et al. 2010). Third, miRNAs can act in a concerted manner over multiple genes in a genetic cascade by toning down a group of genes in a pathway and/or by acting in feedback or feedforward loops. This can have deep impact at the cell, tissue, and organismal levels (Krek et al. 2005; Pelaez and Carthew 2012; Giri and Carthew 2014; Posadas and Carthew 2014; Alberti and Cochella 2017). A well-characterized example is the multilevel functions of *lin-4* and *let-7* miRNAs in the heterochronic cascade of *C. elegans* (Lee et al. 1993; Pasquinelli et al. 2000; Reinhart et al. 2000; Ambros 2011). In *Drosophila*, *miR-7* provides robustness to developmental programs amid stochastic variations in endogenous and exogenous signals (Stark et al.

2005; Li et al. 2009; Alberti and Cochella 2017). Recent evidence highlights the importance of similar functions for miRNAs in controlling stress and aging (Emde and Hornstein 2014; Inukai et al. 2018). Fourth, miRNAs can drive the rapid turnover of large spans of the transcriptome in developmental or other physiological transitions. This is the case during the maternal-to-zygotic transition (MZT) in the zebrafish early embryo, wherein *miR-430* instigates the rapid deadenylation and decay of hundreds of maternal mRNA targets (Giraldez et al. 2005, 2006).

Perhaps not surprising in light of their importance in gene-regulation networks, miRNA-mRNA networks are often deregulated in human diseases ranging from heart disease (Xin et al. 2013) to various types of cancer (Croce and Calin 2005; He et al. 2005; Calin and Croce 2006). miRNA expression profiles provide signatures that inform on the pathogenic state of cells and tissues, on the origin of tumor outgrowth, and sometimes even on treatment prognosis (Iorio and Croce 2013; Sun and Lai 2013; Iorio and Croce 2017; Romano et al. 2017).

MATURATION AND LOADING OF miRNAs ONTO ARGONAUTE PROTEINS

The canonical biogenesis pathway of miRNAs (Fig. 1) involves a series of steps governed by the RNase III family enzymes Drosha and Dicer and their associated cofactors (see Ha and Kim 2014 for a review). miRNAs are derived from structured RNA polymerase II-generated primary miRNA transcripts (pri-miRNAs), which encode one or several precursor miRNAs (pre-miRNAs). Drosha and its double-stranded RNA (dsRNA)-binding protein cofactor DGCR8/Pasha process pri-miRNAs and release ~60- to 75-nt-long hairpin pre-miRNAs, which are exported into the cytoplasm (Lee et al. 2003; Carmell and Hannon 2004). Dicer and a family of dsRNA-binding protein cofactors further process the pre-miRNA yielding a 18- to 21-nt-long RNA duplex consisting of the passenger strand and the guide strand, which is also the mature miRNA (Bernstein et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al.

Figure 1. MicroRNA (miRNA) biogenesis and miRNA-induced silencing complex (miRISC) maturation. Primary miRNA (pri-miRNA) transcripts are generated by RNA polymerase II (1). Drosha and its double-stranded RNA (dsRNA)-binding protein cofactor DGCR8 bind to a pri-miRNA and release a ~60- to 75-nt-long hairpin precursor (pre)-miRNA (2), which is exported from the nucleus to the cytoplasm (3). Dicer and a family of dsRNA-binding proteins (TRBPs in mammals) cleave the pre-miRNA (4), yielding an 18- to 21-nt-long RNA duplex consisting of the passenger strand (blue), and the guide strand (red), which is also the mature miRNA. The double-stranded miRNA duplex is loaded into an Argonaute protein (5), and the passenger strand is either cleaved by Argonaute slicing activity and released (6) or released independently of slicing when the two strands are mismatched. The mature single-stranded miRNA (red), Argonaute, and the Argonaute-interacting protein GW182 constitute the core components of miRISC (7).

2001; Knight and Bass 2001). Processing by Dicer and loading of the miRNA duplex into an Argonaute protein are closely coordinated processes and occur in a series of transitory (loading) complexes involving both proteins (Gregory et al. 2005; Maniataki and Mourelatos 2005; Kobayashi and Tomari 2016). Within the Argonaute, the

miRNA further matures as the passenger strand is either cleaved through the endonuclease activity of the Argonaute (an activity called slicing) and released, or released independently of slicing when the two strands are mismatched (Gregory et al. 2005; Matranga et al. 2005; Miyoshi et al. 2005; Park and Shin 2015). The fully mature,

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single-stranded miRNA is stably embedded in the Argonaute (Fig. 2) wherein it carries out its function as a guide for silencing activities. Although some species including *Drosophila* and *C. elegans* rely on the sorting of miRNAs into functionally specialized Argonautes (DmAGO1

and ALG-1/2, respectively), mammalian species do not sort miRNAs into a specific Argonaute. miRNAs are thus found in association with HsAGO1-4, and HsAGO2 is shared between its siRNA and miRNA functions (Czech et al. 2009; Czech and Hannon 2011).

Figure 2. Structure and RNA interactions of Argonautes. (*Top*) The domain distribution and linkers 1 and 2 in the HsAGO2 primary structure are represented. Key interactions with the guide (the microRNA [miRNA]) and passenger (the target messenger RNA [mRNA site]) strands are indicated. The extensive interactions of linkers 1 and 2 (L1/L2) with strand backbones in the central cleft are not indicated. Lines are connected when the interaction occurs at the interface between two domains (PIWI and MID for the guide 5'-phosphate, and MID and L2 for the target t1 nucleotide). Positioning of helix 7 (encoded in L2) in HsAGO2 and its blocking of nucleotides g6–8 of the seed are indicated (yellow arrow and dashed line) (Klum et al. 2017). (*Middle*) Linear representation of the base-pairing of an archetypal miRNA/mRNA pair. Proposed positioning of Seed, Center, 3' Supplementary, and Tail nucleotides (Wee et al. 2012) are indicated. (*Bottom*) Structure of HsAGO2 bound to both guide miRNA (red strand), target RNA (blue strand) (*Right* model), and without RNA (*Left* model). Note that only the dark blue positions of the target RNA were observed in the structure. The path of the target strand in light blue is extrapolated. Target sequences opposite to Center nucleotides are bulged because of clashes within the cleft of HsAGO2 in those positions. See text for more details. (The structure [PDB 4W5O] was published in Schirle et al. 2014 and Sheu-Gruttadauria and MacRae 2017.)

THE miRNA-INDUCED SILENCING COMPLEX

The term RNA-induced silencing complex (RISC) was originally used to describe the assembly of the guide RNA with an Argonaute, the minimal components required to exert the slicing activity associated with RNA interference (RNAi) (Hammond et al. 2000; Hammond et al. 2001; Liu et al. 2004). In miRNA-mediated silencing, the physiological forms of RISC involve more components. The two core proteins of mammalian miRISC are one of the four Argonaute proteins (AGO1–4) and GW182 (glycine-tryptophan [GW] repeat-containing protein of 182 kDa), which directly binds to Argonaute and is essential for recruiting effectors of miRNA-mediated mRNA silencing (Fig. 1). For clarity, herein we distinguish the core components of miRISC (the miRNA, the Argonaute, and GW182 proteins) from proteins and complexes that effect silencing (the translation repression, deadenylation, decapping, and decay cofactors). The amino terminus of mammalian, zebrafish, and *Drosophila* GW182 proteins house numerous conserved GW, WG, or GWG repeats, which form AGO-interacting motifs that bind to two W-binding pockets in AGO proteins (Behm-Ansmant et al. 2006a; El-Shami et al. 2007; Lian et al. 2009; Takimoto et al. 2009; Yao et al. 2011; Pfaff et al. 2013). A recent structural study showed that human GW182 contains three distinct AGO-interacting motifs (motif 1, motif 2, and the Argonaute hook) that bind with high affinity to both HsAGO1 and AGO2 (Elkayam et al. 2017), suggesting that multiple mRNA-bound AGO proteins may recruit a single GW182 molecule to engender mRNA silencing.

miRNA-ARGONAUTE INTERACTIONS IN TARGET RECOGNITION

The functions of miRNAs are inseparable from the structure and interactions of Argonaute proteins (Fig. 2) (Wee et al. 2012; Chandradoss et al. 2015; Salomon et al. 2016; Sheu-Gruttaduria and MacRae 2017). An Argonaute protein folds in a bilobal structure (Song et al. 2004). Two lobes are formed by four distinct domains (amino terminal [N]/PAZ and MID/PIWI) and are

separated by a central cleft wherein the guide RNA (the mature miRNA) and the target strand base-pair (Wang et al. 2008b). The guide RNA is held by the Argonaute in an extended conformation. The MID domain binds the 5' terminal nucleotide of the miRNA (Boland et al. 2011; Frank et al. 2011), and the PAZ domain interacts with the very last (3' end) nucleotides (Lingel et al. 2003, 2004; Song et al. 2003; Yan et al. 2003; Kidner and Martienssen 2004; Ma et al. 2004). Two linkers (L1 and L2) connect the lobes of the Argonaute and cradle the sugar-phosphate backbone of both guide and target RNAs (Wang et al. 2008a,b; Elkayam et al. 2012; Schirle and MacRae 2012). Argonaute shows sequence preferences for a U or C in the 5'-most nucleotide of the miRNA (position g1, see Fig. 2) and for an A in target sequences facing this position (t1). Binding to nucleotide recognition pockets of the MID domain prevents these nucleotides from base-pairing on target recognition (Schirle et al. 2015). The PIWI domain adopts an RNase H fold within Argonaute (Liu et al. 2004; Song et al. 2004) and carries the catalytic residues for the slicing activity, which is only permitted with extensive or complete base-pairing between the guide and the target RNAs. However, for the vast majority of animal miRNA-binding sites, one or more central residues of the miRNA (g9–g12) are mispaired (Fig. 2). Furthermore, genomic surveys indicate that pairing in these positions is not correlated with miRNA-mediated repression (Grimson et al. 2007). Slicing thus seems primarily to serve a biogenesis purpose in the miRNA pathway, in the cleavage and release of the passenger strand (Bouasker and Simard 2012; Jee et al. 2018). The PIWI domain also carries two tryptophan pockets in its outer structure, which are the binding sites for GW182 (Eulalio et al. 2008, 2009a; Schirle and MacRae 2012; Elkayam et al. 2017).

From within Argonaute, an miRNA is not free to hybridize with an mRNA the way a naked 21-nt RNA molecule would. Argonaute interactions profoundly modify miRNA hybridization kinetics by compartmentalizing the miRNA sequence into subdomains, namely, the 5' anchor (g1), the seed (g2–g8), the center (g9–g12), the 3' supplementary (g14–g16), and tail sequences

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(the 3'-most nucleotides) (Fig. 2) (Wee et al. 2012; Schirle et al. 2014; Salomon et al. 2016). Perhaps the most striking consequence of Argonaute interactions with the miRNA is the unique contribution of the seed nucleotides to target specificity (Lai 2002; Lewis et al. 2003, 2005; Brennecke et al. 2005; Farh et al. 2005). The importance of seed nucleotides became clear early on based on their conservation between paralogous and orthologous miRNA genes (Lagos-Quintana et al. 2001, 2003; Lau et al. 2001; Lee and Ambros 2001; Ambros et al. 2003b; Lim et al. 2003a,b). The functional significance of the seed has since been extensively supported with a conjuncture of conservation, unbiased forward genetics, in vivo and in vitro reporter assays, and structural biology (Lai 2002; Haley and Zamore 2004; Brennecke et al. 2005; Farh et al. 2005; Krek et al. 2005; Lewis et al. 2005; Lim et al. 2005). The positioning of the seed within Argonaute exposes the seed nucleotides (g2–g8) in the central cleft of the protein and enables their base-pairing with mRNAs. Argonaute–miRNA interactions benefit target recognition by preordering the nucleotides of the seed to defray the entropic cost of stacking bases in a readily hybridizable conformation (Nakanishi et al. 2012; Schirle and MacRae 2012; Schirle et al. 2014; Salomon et al. 2016). This accelerates base-pairing of seed nucleotides by >20-fold in comparison to free, disordered nucleic acid oligomers of the same length, to reach rates nearing the limits set by diffusion (Wee et al. 2012; Salomon et al. 2016). Seed pairing with a target site is a two-step process and involves the sequential base-pairing of nucleotides g2–g5 and g6–g8 (Wee et al. 2012; Schirle et al. 2014; Chandradoss et al. 2015; Sheu-Gruttadauria and MacRae 2017). Upon favorable pairing of g2–g5, the helix 7 of Argonaute (formed by L2 residues) is docked away from the guide RNA to give way and enable pairing of nucleotides g6–g8 (Klum et al. 2017). Binding of preordered g2–g5 is rate limiting for target recognition, whereas base-pairing of g6–g8 slows down release from a favorable target site (a pause called *dwelling*).

Another important impact of Argonaute structure on target site binding involves the interactions of the 3' supplementary nucleotides

with the N and PAZ domains. In target-free Argonaute, nucleotides g14–g18 of the miRNA thread through a narrow channel formed by the N and PAZ domains, wherein they are excluded from base-pairing (Schirle et al. 2014; Sheu-Gruttadauria and MacRae 2017). Recent structures suggest a widening of the N-PAZ channel upon seed pairing to the target, possibly through conformational propagation involving helix 7 (Klum et al. 2017). Widening of the channel repositions the 3' end of the miRNA and enables base-pairing of g14–g16, which is thought to nucleate an outward helix propagation and contribute to miRISC/target stabilization. Early genomic studies suggested that only about 5% of miRNA targets involve 3' supplementary pairing of functional significance (Grimson et al. 2007), but this has remained a matter of debate. The most recent structures provide a conceptual framework to interpret studies which suggest that certain miRNAs may rely more on 3' supplementary nucleotides for target recognition than others (Broughton et al. 2016; Wolter et al. 2017).

FUNCTIONAL INTERACTIONS OF miRNAs AND miRISC IN 3'UTRs

Within 3'UTRs, miRNAs interact with a broad diversity of factors and mechanisms, which determine an mRNA's localization, stability, and translatability (see Mayr 2017 for a recent review). The regulatory functions of a 3'UTR are dictated through the sequences it encodes, its folding structure, and its interactions with miRISC and RNA-binding proteins (RBPs). miRNA target recognition is sensitive to steric hindrance by 3'UTR structures. Sites located near the stop codon (but outside the ribosome footprint) or closer to the poly(A) tail tend to have a more potent effect on silencing, presumably because they are positioned outside of the bulk structures of the 3'UTR. Furthermore, miRNA-binding sites located near A/U-rich regions of 3'UTRs are on average more potent, possibly because of the lower stability of the structures in such regions (Farh et al. 2005; Grimson et al. 2007).

Cooperative interactions between miRNA-binding sites in 3'UTRs are also a major determinant of miRNA-mediated silencing.

Genome-wide studies in mammals revealed early on that miRNA-binding sites that are located in close proximity to each other in a 3'UTR elicit more potent silencing than sites separated by more than 50 nt (Grimson et al. 2007; Saetrom et al. 2007). In addition, tandem repeats of miRNA-binding sites located in 3'UTRs of reporter mRNAs greatly sensitize artificial miRNA reporter assays (Pillai et al. 2005; Mathonnet et al. 2007; Fabian et al. 2009; Mayya and Duchaine 2015). miRNA-binding site cooperation appears to be a strict requirement in certain biological contexts such as the *C. elegans* embryo (Wu et al. 2010). Two non-mutually exclusive mechanisms have been described to explain miRNA-binding site cooperativity in 3'UTRs. First, cooperative interactions between nearby binding sites can stabilize miRISC binding to the target mRNA (Broderick et al. 2011). Second, cooperation of multiple miRNA-binding sites can promote assembly of the effector machinery, through the recruitment of the CCR4-NOT deadenylase complex to target mRNAs (Flamand et al. 2017; Wu et al. 2017). A deeper understanding of the functional cooperation between miRNA-binding sites warrants further investigation.

Cooperative and competitive interactions between RBPs and miRISC in 3'UTRs also profoundly modulate miRNA-mediated silencing (Bhattacharyya et al. 2006; Huang et al. 2007; Kedde et al. 2007; Elcheva et al. 2009; Takeda et al. 2009; Jafarifar et al. 2011; Kundu et al. 2012; Jens and Rajewsky 2015). For example, the Pumilio RBP can enable miRNA-mediated silencing by improving the accessibility of nearby miRNA-binding sites, presumably by destabilizing local RNA folding structures (Kedde et al. 2010). In an opposite scenario, the RBP Dead end 1 (Dnd1) binds to elements in uridine-rich sequences of 3'UTRs located in close proximity to miRNA-binding sites and inhibits the association of miRISC (Kedde et al. 2007). Finally, several RBPs interact and recruit some of the same effector machineries to mRNAs, in particular, the CCR4-NOT complex. Clarifying how these RBPs functionally interact with miRISC should help to reliably predict the functional output of miRNA-binding sites.

Recently, an elegant single-molecule imaging system has been used to examine the dynamics of miRNA/Argonaute interactions on a 3'UTR mimic (Chandradoss et al. 2015). The results suggested that miRISC could slide along the 3'UTR RNA between miRNA-binding sites. This sliding may represent a *scanning* activity of miRISC that could accelerate target recognition by reducing the collisions to a one-dimensional search along individual 3'UTRs. Even though enticing, the association rates observed were slower than the random-collision, near-diffusion rates observed with other assays (Wee et al. 2012; Salomon et al. 2016). It will be interesting to address the physiological implications of this phenomenon, and how sliding cooperates with or is counteracted by RBPs and 3'UTR folding structures.

GW182-INTERACTING PROTEINS

The carboxy-terminal region of GW182 proteins, termed the silencing domain, is required for GW182 to silence target mRNAs (Figs. 3 and 4). The silencing domain can be subdivided into a number of regions that are predicted to be unstructured, including the middle (Mid) and carboxy-terminal subdomains. These regions flank an RNA-recognition motif (RRM)-like domain, which does not bind RNA because of the presence of an additional α helix at its carboxyl terminus (Eulalio et al. 2009b). The Mid domain can be further subdivided into the M1 and M2 regions, which flank a poly(A)-binding protein (PABP)-interacting motif 2 (PAM2) (Roy et al. 2002). Although miRNA-loaded AGOs require GW182 to silence targeted mRNAs, GW182 alone is not sufficient. It is now well established that GW182 proteins engender miRNA-mediated gene silencing by interacting with a number of proteins, including PABP, the poly(A) nuclease (PAN)2-PAN3 and CCR4-NOT deadenylase complexes, and mRNA decapping factors.

PABP

In addition to binding to the mRNA 3' poly(A) tail, PABP also interacts with a number of pro-

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Figure 3. Schematic diagrams of glycine-tryptophan protein of 182 kDa (GW182), poly(A)-binding protein (PABP), and GRB10-interacting GYF protein 2 (GIGYF2). The amino-terminal region of GW182 (the Argonaute-binding domain) contains a number of glycine-tryptophan (GW) repeats, several of which form regions that bind AGO proteins (motif 1, motif 2, and the AGO hook). This region, in addition to the ubiquitin-associated (UBA) and glutamine-rich (Q-rich) domains, is responsible for targeting GW182 to processing (P) bodies. The carboxy-terminal region of mammalian and insect GW182 proteins encompasses the silencing domain, which is the major effector domain by which GW182 engenders miRNA-mediated silencing. It is comprised of the M1 and M2 regions, the PABP association motif 2 (PAM2), the proline-rich PG-L motif, an RNA-recognition motif-like domain (RRM) that cannot bind RNA, and a carboxy-terminal domain. PABP domains include four functional RRM and a conserved carboxy-terminal MLE domain that directly binds the mammalian and *Drosophila* GW182 silencing domain via its PAM2 motif. Mammalian GIGYF2 interacts with the PG-L motif in the silencing domain of human GW182 via its GYF domain.

teins that regulate both mRNA translation and mRNA turnover (Derry et al. 2006; Smith et al. 2014). PABP stimulates cap-dependent mRNA translation via its interactions with the 5' cap-associated translation factor eukaryotic initiation factor (eIF)4G (Kahvejian et al. 2005). In contrast, PABP also associates with PAIP2, which inhibits translation by displacing PABP from the mRNA poly(A) tail (Khaleghpour et al. 2001). PABP can also regulate mRNA decay by directly interacting with the PAN3 subunit of the PAN2–PAN3 deadenylase complex, or the transducer of ERBB2 (Tob), which in turn recruits the CCR4–NOT deadenylase complex to target mRNAs (Siddiqui et al. 2007; Mauxion et al. 2009). PABP also directly binds to GW182 proteins via a stretch of residues in the GW182 carboxy-terminal silencing domain (Fig. 3). Originally referred to as a domain of unknown

function (DUF), this region in the silencing domain is now classified as PABP-interacting motif 2 (PAM2) (Fabian et al. 2009; Jinek et al. 2010; Kozlov et al. 2010). Structurally, mammalian GW182 proteins bind PABP in a manner similar to that of other PABP-interacting proteins (i.e., PAN3, PAIP2, eRF3, and Tob) in that they interact with a PABP carboxy-terminal motif termed the MLE domain (Jinek et al. 2010; Kozlov et al. 2010). The GW182 PAM2 motif is evolutionarily conserved in mammals, as well as in *Drosophila* where it also binds PABP. In contrast to mammalian and insect GW182 proteins, the *C. elegans* GW182 ortholog AIN-1 lacks a PAM2 motif; nevertheless, it still interacts with PABP, albeit via its amino-terminal RRM (Kuzuoğlu-Öztürk et al. 2012). Several groups have reported that PABP enhances miRNA-mediated deadenylation in vitro (Fabi-

Figure 4. Schematic diagram of the human glycine-tryptophan protein of 182 KDa (GW182) silencing domain and its interactions with the CCR4-NOT and PAN2-PAN3 deadenylase complexes. The GW182 silencing domain reportedly uses two CCR4-interaction motifs (CIM-1 and -2) and a number of conserved tryptophan residues in the M2 region and carboxy-terminal domain to interact with the CCR4-NOT complex. The silencing domain physically contacts both the CNOT1 subunit as well as CNOT9, the latter containing two tryptophan-binding pockets (denoted by W). The PAN3 subunit contains two tryptophan-binding pockets that directly bind to conserved tryptophan residues in the M2 region of the silencing domain. Conserved tryptophans in the silencing domain that have been reported to interact with PAN3 and CNOT subunits are denoted by red asterisks.

an et al. 2009; Flamand et al. 2016) and miRNA-mediated silencing in cells (Huntzinger et al. 2010); however, not all groups have observed the PABP-GW182 interaction being required to promote miRNA-mediated repression (Fukaya and Tomari 2011). PABP and the poly(A) tail promote the association of the miRISC with targeted mRNAs (Moretti et al. 2012), suggesting that the PABP-GW182 interaction promotes GW182 recruitment to AGO-bound, miRNA-targeted mRNAs. Thus, it is possible that the PABP-GW182 interaction plays an important role in promoting miRNA-mediated silencing of mRNAs with long 3'UTRs (Flamand et al. 2016), or with certain miRNA target sites that require PABP to enhance miRISC recruitment.

CCR4-NOT and PAN2-PAN3 Deadenylase Complexes

miRNAs promote the deadenylation of target mRNAs in a number of systems, including human and *Drosophila* cultured cells, zebrafish embryos, as well as mammalian, *Drosophila*, and *C. elegans* cell-free systems (Behm-Ansmant et al. 2006b; Giraldez et al. 2006; Wu et al. 2006; 2010; Wakiyama et al. 2007). miRNA-mediated deadenylation occurs as a biphasic event, which is mediated primarily by the CCR4-NOT deadenylase complex, as well as by the PAN2-PAN3 deadenylase complex (Chen et al. 2009). An array of studies indicates that GW182 proteins act as evolutionarily conserved scaffolds

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that recruit the CCR4–NOT and PAN2–PAN3 deadenylation complexes to miRNA-targeted mRNAs (Fig. 4) (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011; Kuzuoğlu-Öztürk et al. 2012; Christie et al. 2013; Mauri et al. 2017; Wu et al. 2017). Structural and biochemical studies revealed that human GW182 proteins contact several CCR4–NOT complex subunits (Braun et al. 2011; Fabian et al. 2011; Chen et al. 2014; Mathys et al. 2014). The CNOT9 subunit uses two tryptophan-binding pockets to directly bind conserved tryptophans in the GW182 silencing domain (Chen et al. 2014; Mathys et al. 2014). In addition to binding CNOT9, the human GW182 silencing domain directly interacts with the CNOT1 subunit of the CCR4–NOT complex (Fabian et al. 2011). Thus, the GW182 silencing domain most likely makes multiple contacts with the CCR4–NOT complex, by contacting both the CNOT1 and CNOT9 subunits. The GW182 silencing domain also recruits the PAN2–PAN3 heterodimeric complex by interacting with PAN3. This has been reported to occur indirectly via PABP (Chekulaeva et al. 2011; Fabian et al. 2011) or direct binding through a tryptophan-containing pocket in the PAN3 kinase-like domain interacting with the M2 region of the GW182 silencing domain (Braun et al. 2011; Chekulaeva et al. 2011; Christie et al. 2013).

GIGYF2

PABP, the CCR4–NOT and PAN2–PAN3 deadenylase complexes are not the only proteins to directly interact with GW182. The GRB10-interacting GYF protein 2 (GIGYF2) was recently identified as a novel GW182 silencing domain-interacting protein by using a novel proximal biotinylation approach (Split-BioID) to search for miRISC-associating proteins in human cells (Schopp et al. 2017). Coimmunoprecipitation experiments and in vitro pull-downs using recombinant proteins showed that GIGYF2 directly binds to a conserved proline-rich P-GL motif in the Mid region of the GW182 silencing domain (Fig. 3). Knocking down GIGYF2 partially derepressed a tetracycline-inducible miRNA-targeted reporter in HeLa cells. As this derepression oc-

curred at an early time point, before deadenylation (Bethune et al. 2012), GIGYF2 may play a role in translational repression. In keeping with these data, the P-GL motif contributes to tethered GW182-mediated repression in zebrafish embryos (Mishima et al. 2012). Interestingly, GIGYF2 directly binds to the 5' cap-binding protein 4EHP/eIF4E2, which plays a role in translationally repressing select mRNAs (Rom et al. 1998; Cho et al. 2005; Morita et al. 2012; Peter et al. 2017). Moreover, GIGYF2 can silence a luciferase reporter mRNA when artificially tethered to its 3'UTR (Morita et al. 2012). Nevertheless, whereas these data suggest that GIGYF2 interacts with GW182, its exact role in miRNA-mediated repression has yet to be established.

Mechanistic Insights into miRNA-Mediated Translational Repression

Translation initiation begins with eIF4F complex binding to the mRNA 5' cap structure. This complex is comprised of the cap-binding protein eIF4E, the eIF4A RNA helicase, and the scaffolding protein eIF4G, which interact with both eIF4E and eIF4A. Together, these translation initiation factors help recruit the 43S pre-initiation complex (PIC) to the mRNA 5' terminus (Gingras et al. 1999; Merrick and Pavitt 2018). eIF4A helicase activity promotes ribosome subunit scanning by unwinding highly structured regions within mRNA 5'UTRs.

A number of early studies suggested that miRNAs translationally repress targeted mRNAs by inhibiting cap-dependent translation at the initiation step, which is currently the most widely supported model (Fig. 5) (Humphreys et al. 2005; Pillai et al. 2005; Mathonnet et al. 2007; Thermann and Hentze 2007). miRNAs were also reported to repress translation postinitiation (i.e., by inhibiting ribosome elongation) (Olsen and Ambros 1999; Nottrott et al. 2006; Petersen et al. 2006). However, transcriptome-wide ribosome profiling experiments, which measure translation efficiencies, do not detect any changes in ribosome elongation rates on miRNA-targeted mRNAs (Guo et al. 2010). Early reports that miRNAs inhibit translation are supported by investigations of the kinetics of

Figure 5. Model of microRNA (miRNA)-mediated gene silencing. Step 1, repression of cap-dependent translation via the CCR4–NOT complex: miRNA-induced silencing complex (miRISC) inhibits translation at the initiation step in a deadenylation-independent manner. Step 2, messenger RNA (mRNA) deadenylation: deadenylation is mediated by the CCR4–NOT complex and, to a lesser extent, the PAN2–PAN3 complex. Step 3, mRNA decapping: following deadenylation, the 5' cap moiety, denoted by a black circle, is removed by the DCP1–DCP2 decapping complex. Step 4, mRNA decay: once the mRNA 5' cap is hydrolyzed, the mRNA is degraded by the XRN1 5'–3' exonuclease.

translational repression and mRNA deadenylation. Studies using fly and mouse cell-free extracts show that translational inhibition can be observed before detectable mRNA deadenylation (Fabian et al. 2009; Zdanowicz et al. 2009). miRNA-mediated translational repression before mRNA deadenylation was also reported in cells (Bethune et al. 2012; Djuranovic et al. 2012) and zebrafish embryos (Bazzini et al. 2012). Although these studies indicate that miRNAs repress mRNA translation, several global studies report that the majority of miRNA-mediated repression can be attributed to mRNA decay (Guo et al. 2010; Eichhorn et al. 2014). How miRNAs facilitate pure translational repression before mRNA destabilization remains a topic of current study, and several groups gained key insights in this field.

miRNA-Mediated Dissociation of Translation Initiation Factors from Target mRNAs

In keeping with repression of translation at the initiation step, several groups have reported that

miRNA-mediated silencing leads to the dissociation of translation initiation factors from targeted mRNAs. RNA pulldown experiments for miRNA- and GW182-targeted reporter mRNAs expressed in *Drosophila* S2 cells showed a decreased association of eIF4E, eIF4G, and PABP with miRNA-targeted mRNAs (Zekri et al. 2013). Similar results were observed in RNA immunoprecipitation experiments performed for miRNA targets in human embryonic kidney (HEK) 293 cells (Rissland et al. 2017) and cell-free extracts that recapitulate miRNA-mediated silencing in vitro (Moretti et al. 2012; Fukao et al. 2014). However, whereas deadenylation should remove PABP from a mRNA poly(A) tail, PABP is still displaced from deadenylation-resistant reporters that are targeted by either miRNAs or tethered GW182 (Zekri et al. 2013; Fukao et al. 2014). Nevertheless, miRNAs efficiently silenced a mRNA that lacked a poly(A) tail but instead had PABP artificially tethered to the mRNA 3' end (Nishihara et al. 2013). Taken together, these data suggest that whereas miRNA-mediated dissociation of PABP may assist in translational

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repression, it is not the only mode by which miRNAs repress translation. Interestingly, knocking down the decapping factors DCP1 and EDC4 in tandem rescued the association of eIF4E with an miRNA-targeted reporter, even though eIF4G and PABP remained dissociated (Zekri et al. 2013). Thus, the miRNA-mediated silencing machinery may engender the dissociation of PABP and eIF4G before mRNA decapping, whereas eIF4E may be displaced as a result of 5' cap hydrolysis. The linkages between mRNA translation initiation factors, decapping, and decay are further reviewed in this collection (Heck and Wilusz 2018).

DDX6, 4E-T, and 4EHP

Although the CCR4–NOT complex engenders deadenylation of miRNA targets, several studies showed that it can repress target mRNAs independent of its deadenylase activity (Cooke et al. 2010; Braun et al. 2011; Chekulaeva et al. 2011). In addition, tethering the CCR4–NOT complex can translationally repress deadenylation-resistant reporter mRNAs that contain a stretch of non-A nucleotides following the 3' poly(A) tail, which suppresses mRNA deadenylation and decay (Kuzuoğlu-Öztürk et al. 2016). How does the CCR4–NOT complex repress mRNA translation? Several early studies showed that knocking down the translational repressor and decapping enhancer protein DDX6 in human cells, or its homolog in flies (Me31B), impairs miRNA-mediated silencing (Chu and Rana 2006; Eulalio et al. 2007). In addition, DDX6 directly binds the MIF4G (middle of eIF4G) domain of CNOT1, the central subunit of the CCR4–NOT complex (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014). Moreover, the interaction between DDX6 and CNOT1 enhances miRNA-mediated silencing (Chen et al. 2014; Mathys et al. 2014; Rouya et al. 2014), including silencing of miRNA-targeted reporters where deadenylation is prevented (Mathys et al. 2014; Kuzuoğlu-Öztürk et al. 2016). All in all, these data suggest that DDX6 binding to CNOT1 plays an important role in miRNA-mediated translational repression. Using *in vitro* ATPase assays, Mathys et al. (2014) showed that DDX6 ATPase

is activated upon binding to CNOT1. Mutational analyses suggest that this plays a role in miRNA-mediated silencing, albeit via an unknown mechanism.

In addition to binding CNOT1, DDX6 directly interacts with several proteins that inhibit translation and/or enhance mRNA decapping and decay. These include EDC3, LSM14, PATL1, and the eIF4E-binding protein, 4E-T, all of which bind to the DDX6 RecA-C domain in a mutually exclusive manner (Tritschler et al. 2009; Jonas and Izaurralde 2013; Sharif et al. 2013; Nishimura et al. 2015; Ozgur et al. 2015; Waghay et al. 2015). The crystal structure of the CNOT1/DDX6/4E-T complex (Ozgur et al. 2015) shows that DDX6, when directly bound to CNOT1, interacts with 4E-T but cannot bind to other binding partners, including EDC3 and PATL1. Notwithstanding that PATL1 cannot directly interact with DDX6 when bound to CNOT1, PATL1 does interact with the CCR4–NOT complex in a DDX6-independent manner (Haas et al. 2010; Ozgur et al. 2010).

How does 4E-T enhance miRNA-mediated gene silencing? 4E-T contains an amino-terminal eIF4E-binding motif, as well as other regions that interact with DDX6, LSM14, PATL1, and UNR (Dostie et al. 2000; Nishimura et al. 2015; Kamenska et al. 2016). Knocking down 4E-T in human cell lines impairs miRNA-mediated silencing of reporter mRNAs (Kamenska et al. 2016; Chapat et al. 2017). Mechanistically, 4E-T translationally represses reporter mRNAs when tethered to their 3'UTRs in human cells and *Xenopus* oocytes (Kamenska et al. 2014; Waghay et al. 2015). Despite the ability of 4E-T to compete with eIF4G for binding to eIF4E, tethered 4E-T represses mRNA translation in an eIF4E-independent manner (Kamenska et al. 2014; Waghay et al. 2015). Moreover, 4E-T can also interact with the eIF4E homolog 4EHP (also known as eIF4E2), which represses mRNA translation. Recently, 4EHP has been implicated in miRNA-mediated translational repression, as knocking down 4EHP in mammalian cells partially derepressed miRNA-mediated silencing (Chapat et al. 2017; Chen and Gao 2017). 4EHP has also been reported to be essential in miR-145-mediated silencing of DUSP6

mRNA, a repressor of ERK signaling (Jafarnejad et al. 2018). These data led to a model where a 4E-T/4EHP complex, recruited by CCR4–NOT, represses translation by competing with eIF4E for the 5' cap (Fig. 6A). However, as 4E-T can bind either eIF4E or 4EHP, it may translationally repress mRNAs whose 5'-cap structures are bound by either eIF4E or 4EHP (Fig. 6B). Although these studies suggest that 4E-T assists in miRNA-mediated translational repression, 4E-T has also been linked to enhancing mRNA decay of CCR4–NOT targets, including the let-7 miRNA target HMGA2 and reporter mRNAs targeted by the AU-rich element-binding protein tristetraprolin (TTP) (Ferraiuolo et al. 2005; Nishimura et al. 2015). Importantly, a 4E-T mutant that cannot interact with eIF4E was defective in promoting the decay of TTP- and miRNA-targeted mRNAs in HeLa cells (Nishimura et al. 2015). Thus, 4E-T may play a role in promoting both early translational repression, as well as the decay of miRNA targets by bringing the CCR4–NOT complex and the decapping machinery into the vicinity of the mRNA 5' terminus via its interaction with eIF4E or 4EHP.

A Link between the CCR4–NOT Complex and eIF4A

As previously mentioned, the eIF4A translation initiation factor is an ATP-dependent RNA helicase that assists 43S ribosomal scanning by unwinding secondary structures in the mRNA 5'UTR (Sonenberg and Hinnebusch 2009; Merrick and Pavitt 2018). The CCR4–NOT complex was reported to recruit eIF4A2, a vertebrate-specific paralog of eIF4A, and this interaction translationally represses miRNA targets by impairing 43S ribosomal subunit scanning (Meijer et al. 2013). However, these data have been contested in several follow-up studies (Galicía-Vázquez et al. 2015; Kuzuoğlu-Öztürk et al. 2016), one reporting that a human cell line where eIF4A2 has been knocked out displays no noticeable defects in silencing by miRNAs. Although a model wherein miRISC specifically interacts with eIF4A2 to silence target mRNAs has been challenged, two independent studies

reported that miRNA-mediated translational repression targets eIF4A (Fukao et al. 2014; Fukaya et al. 2014). Knocking down eIF4A in *D. melanogaster* S2 cells impairs miRNA-mediated translational repression. Moreover, RNA pulldown and chemical cross-linking experiments in human and fly cell-free extracts that recapitulate miRNA-mediated silencing show that eIF4A is displaced from miRNA-targeted reporter mRNAs (Fukao et al. 2014; Fukaya et al. 2014). Furthermore, the addition of HuD, an eIF4A-binding protein that stimulates translation, to a cell-free extract impairs miRNA-mediated translational repression, whereas a HuD mutant that cannot bind eIF4A does not (Fukao et al. 2014). Taken together, these data suggest that the miRNA machinery interfaces with eIF4A (either directly or indirectly) to repress translation.

In keeping with a link between miRNA-mediated silencing and eIF4A helicase activity, it has been reported that miRNA reporters harboring unstructured 5'UTRs are refractory to miRNA-mediated silencing in a rabbit reticulocyte in vitro translation system (Ricci et al. 2013). However, a recent study (Kuzuoğlu-Öztürk et al. 2016) did not come to the same conclusion. Efficient miRNA-mediated translational repression was observed with luciferase reporters containing short (<10 nt) 5'UTRs, including the translation initiator of short 5'UTR (TISU) that directs cap-dependent translation via a scanning-independent mechanism (Elfakess et al. 2011; Kwan and Thompson 2018). Both the miRISC and the CCR4–NOT complex efficiently translationally repressed these reporters, suggesting that miRNAs inhibit translation initiation at a step other than 43S ribosomal subunit scanning. In addition, varying the 5' secondary structure had no noticeable impact on translational repression, again demonstrating that miRNA-mediated silencing does not seem to require eIF4A helicase activity (Kuzuoğlu-Öztürk et al. 2016). Although these data indicate that eIF4A helicase activity is not the major target of the miRNA machinery, they do suggest that eIF4A plays an important role in miRNA-mediated translational repression. One possibility is that miRISC-associated factors, including

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Figure 6. Models for microRNA (miRNA)-mediated translational repression. It is important to note that these models are not mutually exclusive. (A) The CCR4-NOT/DDX6 complex recruits 4E-T and 4EHP, the latter functioning to displace eukaryotic initiation factor (eIF)4E from the cap structure and associated translation factors (i.e., eIF4G and eIF4A). (B) The CCR4-NOT/DDX6 complex recruits 4E-T, which binds to eIF4E thereby displacing eIF4G. (C) The CCR4-NOT/DDX6 complex, potentially with unknown binding partners (denoted by ???), interacts with eIF4A leading to inhibition of translation initiation.

DDX6, may inhibit translation initiation by physically interacting with eIF4A, rather than by specifically impairing eIF4A helicase activity (Fig. 6C). However, this model remains to be directly tested.

CONCLUSIONS AND FUTURE PERSPECTIVES

These are exciting times in the field of miRNA-mediated silencing. Progress is being made with respect to how the miRISC identifies and interacts with target sites, and on how its interactions direct mRNA translation repression, deadenylation, and decay. Parameters such as cellular miRNA concentrations, their stoichiometric ratio with their mRNA targets, and affinities for target sites are being examined in a quantitative manner. Genome-wide studies have yielded critical information regarding miRNA-mRNA 3'UTR networks. The recent development of libraries capturing the poly(A) tail status in transcriptomes is particularly promising (Subtelny et al. 2014; Rabani et al. 2017; Rissland et al. 2017; Yartseva et al. 2017). Emerging quantitative single-molecule imaging methods consolidate previous models and unveil properties of miRISC that had been missed through batch experiments. Several key aspects of miRNA-mediated silencing remain to be elucidated, however. miRISC recruits the CCR4-NOT deadenylase complex to repress mRNA translation before initiating mRNA deadenylation and decay, but it remains to be established how the CCR4-NOT complex and associated factors bring this about. In addition, whereas several studies indicate that 5' cap-associated translation initiation factors such as eIF4G and eIF4A are displaced from miRNA-targeted mRNAs before, or in the absence of, mRNA destabilization, the mechanistic underpinnings of this observation remain unknown. Cooperative and/or competitive interplay between RBPs such as TTP and Pumilio, miRISC, and the CCR4-NOT complex also warrants further resolution. Perhaps progress on those topics will explain some of the lingering disparities on the relative contributions of miRNA in driving mRNA translation repression, deadenylation, decapping, and decay.

Mechanisms of miRNA-Mediated Gene Silencing

These topics remain at the front line of current miRNA biology.

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Mechanisms of miRNA-Mediated Gene Silencing

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Thomas F. Duchaine and Marc R. Fabian

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