Selective effects of miRNA effector protein Argonaute on miRNA processing

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Selective effects of miRNA effector protein Argonaute on miRNA processing

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Abbreviations

3' UTR	3' untranslated region
ac-pre-miRNA	Ago-cleaved precursor miRNA
Ago	Argonaute
ATP	Adenosine triphosphate
Dcr	Dicer
DGCR8	DiGeorge Syndrome Critical Region 8
EGFP	Enhanced green fluorescent protein
Exp-5	Exportin-5
HEK293T	Human embryonic kidney 293T
Loqs	Loquacious
miRNA	MicroRNA
mRNA	Messenger RNA
N2a	Neuro 2a
PAR-CLIP	Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation
PDC	Precursor deposit complex
Pol II / RNAP II	RNA polymerase II
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA transcript
nt	Nucleotide
RISC	RNA induced silencing complex
RLC	RISC loading complex
TRBP	Transactivation response element RNA-binding protein

1 Abstract

Since the discovery of miRNAs, studies have been focused on understanding the mechanisms of miRNA biogenesis. However, the molecular/mechanistic function of some miRNA biogenesis factors remain elusive. This project examines a novel role for Argonaute proteins as a factor in regulation of the pre-mir-124 hairpin. Pre-miR-124, but not other pre-miRNAs, was reduced in response to Ago overexpression. This phenomenon was reproducible in mouse and fly cells, suggesting that the mechanism is broadly conserved. Ago-dependent decrease of pre-miR-124 was shown not to be caused by secondary effects of miR-124 target regulation. Experiments using Dicer knockout cells revealed that Ago-induced reduction of pre-mir-124 is a Dicer-dependent phenomenon. Overexpression of Ago2 appeared to result in an increase in the average neurite length of N2a cells. These observations suggest previously unknown roles for the core miRNA biogenesis factors.

2 Introduction

2.1 Background

MicroRNAs (miRNAs) are small noncoding RNAs 19-25 nucleotides (nt) in length which are generated from short RNA precursors, folded back onto themselves to form a hairpin structure (Lagos-Quintana, Rauhut et al. 2001, Lau, Lim et al. 2001, Lee and Ambros 2001). Unlike other endogenous small RNA species such as endogenous-small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs), sequences of many miRNAs are highly conserved among eukaryotes (Tanzer and Stadler 2004). Studies have shown that miRNAs are involved in many biologically important pathways such as cell proliferation (Brennecke, Hipfner et al. 2003), differentiation (Chen, Li et al. 2004, Lim, Lau et al. 2005) and apoptosis (Cimmino, Calin et al. 2005, Johnson, Grosshans et al. 2005). Expression of miRNAs is spatially and temporally regulated (Lagos-Quintana, Rauhut et al. 2001, Lau, Lim et al. 2001, Lee and Ambros 2001, Lagos-Quintana, Rauhut et al. 2002, Wienholds, Kloosterman et al. 2005), hence achieving cell type-specific (Poy, Eliasson et al. 2004) post-transcriptional regulation of their target genes. More than half of protein-coding genes in the human genome have evolutionarily conserved miRNA target sites, further highlighting the importance of regulation by miRNAs (Friedman, Farh et al. 2009). Undoubtedly, dysregulation of miRNA pathways are implicated in many diseases such as cancer (Lu, Getz et al. 2005), heart disease (Son, Kumar et al. 2013) and mental illnesses (Beveridge, Gardiner et al. 2010), among others (reviewed in (Mockenhaupt, Schurmann et al. 2011)).

2.2 Canonical microRNA biogenesis

While there exists a variety of non-canonical biogenesis pathways, the biogenesis of most miRNAs follow the canonical pathway, whereby miRNAs are transcribed from the miRNA gene locus and processed stepwise by a series of processing factors to generate the mature miRNA which is loaded into the Ago protein, the effector protein for miRNA function. Canonical miRNA biogenesis involves Drosha and Dicer-dependent maturation of the miRNA strand. After transcription of the miRNA gene, the pre-miRNA hairpin is first excised by Drosha in the nucleus. The pre-miRNA is then exported from the nucleus into the cytoplasm by Exp-5 and then processed by Dicer to form the miRNA duplex. One of the strands is then selected to be loaded into the Ago protein to form the mature RISC complex for post-transcriptional gene silencing (figure 2.2.1). The following subsections will describe the canonical miRNA biogenesis pathway machinery in greater detail.

Figure 2.2.1: Canonical miRNA biogenesis pathway.

2.2.1 Transcription by Pol II

In the canonical miRNA processing pathway, miRNA biogenesis begins in the nucleus when miRNA genes containing the miRNA hairpin are transcribed by RNA polymerase II to form a primary miRNA transcript (pri-miRNA). As miRNA genes are often located in the introns of mRNA transcripts or mRNA-like non-coding RNA transcripts, they are often co-transcribed by RNA polymerase II (Pol II) together with their host genes and are often regulated together with their host genes at a transcriptional level (Rodriguez, Griffiths-Jones et al. 2004, Baskerville and Bartel 2005), although some miRNAs also possess their own promoters (Monteys, Spengler et al. 2010). miRNA genes also tend to be clustered together (Lagos-Quintana, Rauhut et al. 2001, Lau, Lim et al. 2001, Mourelatos, Dostie et al. 2002) and are often transcribed as a polycistronic unit (Baskerville and Bartel 2005). Like pre-mRNAs transcribed by Pol II, pri-miRNAs have 5' 7-methyl guanylate (m7G) caps and a 3' poly(A) tails (Cai, Hagedorn et al. 2004, Lee, Kim et al. 2004) and can function as both pri-miRNAs and pre-mRNAs (Cai, Hagedorn et al. 2004).

2.2.2 Drosha cleavage

Following pri-miRNA transcription, the pri-miRNA transcript is processed in the nucleus by the RNase III endonuclease Drosha and its partner, the RNA binding protein DGCR8 (DiGeorge Syndrome Critical Region 8), also known as Pasha in flies (Denli, Tops et al. 2004, Gregory, Yan et al. 2004, Landthaler, Yalcin et al. 2004). Nuclear localization of Drosha is facilitated by the N-terminal region of Drosha (Tang, Zhang et al. 2010). Drosha contains a dsRNA binding domain and two RNase III catalytic domains at the C terminus which dimerize together to allow processing of substrates (Denli, Tops et al. 2004). The first and second RNase III catalytic domains cleave the 3' and 5' strands of the pri-miRNA stem, respectively (Blaszczyk, Tropea et al. 2001, Denli, Tops et al. 2004, Zhang, Kolb et al. 2004) at a position which is about 11 nt away from the basal junction and about 22nt (two helical turns) away from the apical junction (Zeng, Yi et al. 2005, Han, Lee et al. 2006). Binding of pri-miRNA substrates requires both Drosha and also

the dsRNA binding activity of DGCR8 (Denli, Tops et al. 2004). DGCR8 contains two dsRNA binding domains for pre-miRNA interaction and a C-terminal domain for Drosha interaction (Han, Lee et al. 2006). DGCR8 contains a heme binding domain (Faller, Matsunaga et al. 2007) which is required for efficient pri-miRNA processing (Barr, Smith et al. 2012, Weitz, Gong et al. 2014, Partin, Ngo et al. 2017). The microprocessor complex is comprised of Drosha and DGCR8 in a 1:2 molecular ratio (Denli, Tops et al. 2004, Gregory, Yan et al. 2004, Landthaler, Yalcin et al. 2004, Nguyen, Jo et al. 2015, Herbert, Sarkar et al. 2016).

The microprocessor complex has a general preference for hairpin structures with ~3 helical turns (~33nt) in the length which contain deformable structures one helical turn away from the cleavage site (Quarles, Sahu et al. 2013), but additional sequence motifs are known to enhance the binding of the microprocessor complex (figure 2.2.2). The basal UG motif and apical UGU motifs are recognized by Drosha and DGCR8, respectively, and facilitate binding of the microprocessor complex to substrate RNA molecules (Auyeung, Ulitsky et al. 2013, Nguyen, Jo et al. 2015). Helical distortions are preferred on the miRNA hairpin at the site of Drosha cleavage (Warf, Johnson et al. 2011). Upon binding to pri-miRNA, Drosha cleaves within the stem-loop structure to produce a short (~65 nucleotides long) RNA hairpin with a 2nt overhang at the 3' end called a pre-miRNA hairpin (Blaszczyk, Tropea et al. 2001, Lee, Ahn et al. 2003, Denli, Tops et al. 2004, Zhang, Kolb et al. 2004). The splicing regulatory protein KSRP also aids in the association of Drosha to a subset of pri-miRNAs by binding to the loop of these pri-miRNAs (Trabucchi, Briata et al. 2009).

For intronic miRNAs, Drosha processing of the miRNA hairpins are performed before intron splicing occurs (Kim and Kim 2007, Kataoka, Fujita et al. 2009). miRNAs situated in exonic regions can cause destabilization of the host mRNA transcript (Han, Pedersen et al. 2009, Sundaram, Common et al. 2013). Drosha cleavage of miRNAs coded within lncRNAs allows polyadenylation-independent transcriptional termination of lncRNA transcripts (Dhir, Dhir et al. 2015). The processing efficiency of Drosha is thought to be a major determinant of mature miRNA levels (Conrad, Marsico et al. 2014). Expression

of DGCR8 and Drosha is crossregulated by the destabilization of DGCR8 mRNA by Drosha processing of regulatory hairpins in the exon of DGCR8 mRNA and protein-protein stabilization of Drosha and DGCR8 (Han, Pedersen et al. 2009).

2.2.3 Nuclear export

The pre-miRNA hairpin produced by Drosha processing is then exported from the nucleus to the cytoplasm by the nuclear export factor Exportin-5 (Exp-5), which belongs to the karyopherin β family (Yi, Qin et al. 2003, Bohnsack, Czapinski et al. 2004, Lund, Guttinger et al. 2004). Exp-5 consists of 20 HEAT repeats arranged in a structure similar to a baseball mitt with a channel, allowing binding of the pre-miRNA stem. It also has a pocket at the base of the channel to accommodate the 2-nt overhang of the pre-miRNA at the 3' end (Okada, Yamashita et al. 2009). Exp-5 binds to pre-miRNAs and Ran-GTP to form Exp-5:Ran:pre-miRNA complex in the nucleus. This complex translocates through the nuclear pore complex into the cytoplasm, where the hydrolysis of GTP by Ran leads to the dissociation of the complex (Brownawell and Macara 2002). Aside from its role of shuttling pre-miRNAs from the nucleus into the cytoplasm, Exp-5 binding of pre-miRNAs may also protect pre-miRNAs from exonuclease degradation (Yi, Qin et al. 2003, Zeng and Cullen 2004).

2.2.4 Dicer cleavage

Once the pre-miRNA hairpin is exported to the cytoplasm, it is recognized and cleaved by the RNase III endoribonuclease Dicer (Bernstein, Caudy et al. 2001, Grishok, Pasquinelli et al. 2001, Hutvagner, McLachlan et al. 2001, Ketting, Fischer et al. 2001, Knight and Bass 2001). Besides pre-miRNAs, Dicer has also been shown to process long dsRNA substrates into siRNAs (Bernstein, Caudy et al. 2001, Grishok, Pasquinelli et al. 2001). Mammals encode one Dicer gene which processes both pre-miRNAs and dsRNAs into siRNAs, whereas flies encode two Dicer genes, Dcr-1 and Dcr-2, which process pre-miRNAs and dsRNAs into siRNAs, respectively (Lee, Nakahara et al. 2004). Dicer is composed of an N-terminal helicase domain which recognizes the loop

structure of the pre-miRNA (Tsutsumi, Kawamata et al. 2011) and a PAZ domain which selectively binds the 5'-monophosphate and 2-nt 3'-overhang of the pre-miRNA (Macrae, Zhou et al. 2006, Park, Heo et al. 2011), signatures of Drosha cleavage products (Zhang, Kolb et al. 2004). The two RNase III domains at the C terminus of Dicer dimerize together to form a catalytic site for miRNA processing (Zhang, Kolb et al. 2004). The length of the connector helix between the PAZ domain and the RNase III domain allows Dicer to act as a molecular ruler (Macrae, Zhou et al. 2006), measuring from the 5' and 3' ends of the pre-miRNA hairpin (Vermeulen, Behlen et al. 2005, MacRae, Zhou et al. 2007, Park, Heo et al. 2011). In various species, the Dicer cleavage site is determined by the distance from the 3' end (3'-counting rule) (Zhang, Kolb et al. 2002, Vermeulen, Behlen et al. 2005), whereas mammalian and fly Dicer also use a 5'-counting rule whereby Dicer cuts 22nt from the 5' phosphorylated end of the pre-miRNA, if the end is thermodynamically unstable (Park, Heo et al. 2011). Cleavage by Dicer releases small RNA duplexes, of which the strand derived from the 5' and 3' arms of the hairpin are called 5p and 3p miRNA species, respectively (Griffiths-Jones, Grocock et al. 2006).

Like Drosha, Dicer also functions together with a dsRNA binding protein partner for substrate processing. In mammals, Dicer functions in a complex with the dsRNA-binding proteins Transactivation response element RNA-binding protein (TRBP) (Chendrimada, Gregory et al. 2005, Haase, Jaskiewicz et al. 2005) or PACT (Lee, Hur et al. 2006) as dicing cofactors, although neither cofactor is strictly necessary for Dicer processing of pre-miRNAs (Lee, Hur et al. 2006). Both TRBP and PACT contain three dsRNA binding domains (Chendrimada, Gregory et al. 2005, Haase, Jaskiewicz et al. 2005), of which the third dsRNA binding domain of both proteins interacts with the helicase domain of Dicer (Lee, Hur et al. 2006). TRBP interaction with Dicer helps to increase the kinetics of pre-miRNA processing by Dicer (Chakravarthy, Sternberg et al. 2010, Lee and Doudna 2012), can aid in generating longer sequence isoforms of miRNAs, termed isomiRs from Dicer processing (Lee and Doudna 2012), and also confers substrate specificity of Dicer in the presence of pre-miRNA-like competitors (Fareh, Yeom et al. 2016). In comparison, PACT interaction with Dicer preserves the

isomiR generation profile of Dicer and reduces the affinity of the Dicer-PACT complex to dsRNA in comparison to Dicer-TRBP complex (Lee, Zhou et al. 2013). Just as for Drosha, the splicing regulatory protein KSRP also aids in the interaction of Dicer to a subset of pri-miRNAs by binding to the loop of these pri-miRNAs (Trabucchi, Briata et al. 2009).

In *Drosophila*, Dcr-1 interacts with either loqs-PA or loqs-PB, which are structurally homologous to TRBP and PACT (Forstemann, Tomari et al. 2005, Jiang, Ye et al. 2005, Saito, Ishizuka et al. 2005). Unlike mammalian Dicer, loqs-PA and loqs-PB are needed for the processing of some pre-miRNAs, with a subset of miRNAs dependant on loqs-PB for their generation (Liu, Park et al. 2007, Park, Liu et al. 2007), especially pre-miRNAs with unstable base-pairing at the dicing site (Lim, Ng et al. 2016). Like TRBP, loqs-PB can also tune the Dcr-1 cleavage site on pre-miRNAs and hence alter isomiR generation and subsequent target specificity (Fukunaga, Han et al. 2012). In contrast, Dcr-2 interacts with loqs-PD, another loquacious homolog, during dsRNA processing (Hartig, Esslinger et al. 2009, Zhou, Czech et al. 2009, Miyoshi, Miyoshi et al. 2010). Interaction of Dcr-2 with loqs-PD enhances Dcr-2 processing of dsRNA substrates (Miyoshi, Miyoshi et al. 2010).

Figure 2.2.2: Primary miRNA transcript with miRNA hairpin showing Drosha cleavage site (green), Dicer cleavage site (blue), and 5p and 3p arms of the miRNA hairpin.

2.2.5 Loading and strand selection

Following Dicer cleavage, the miRNA duplex is loaded into the Argonaute (Ago) protein and subsequently unwound to dissociate the passenger strand, leaving the single-stranded guide RNA strand bound to Ago to form the RNA-induced silencing complex (RISC) (Hammond, Boettcher et al. 2001, Nykanen, Haley et al. 2001, Mourelatos, Dostie et al. 2002). Ago family proteins are highly conserved proteins which have PAZ (PIWI, Argonaute and Zwiller), MID and PIWI domains. The 5' monophosphate binding pocket in the MID domain binds the phosphate group at the 5' end of the guide RNA (Frank, Sonenberg et al. 2010, Elkayam, Kuhn et al. 2012, Nakanishi, Weinberg et al. 2012, Schirle and MacRae 2012), a binding loop on the MID domain interacts with the first nucleotide on the 5' end of the guide strand, with a preference for A or U (Frank, Sonenberg et al. 2010), and the PAZ domain of the Ago protein binds to the last two nucleotides at the 3' end of the guide RNA (Lingel, Simon et al. 2004). The guide RNA is situated within a channel spanning from between the MID domain and PIWI domain to the PAZ domain of the Ago protein (Wang, Juranek et al. 2008, Wang, Sheng et al. 2008), with the seed region corresponding the 2nd to 7th nucleotides of the guide RNA lying in an A-form helical structure which allows efficient recognition of target mRNAs (Elkayam, Kuhn et al. 2012, Nakanishi, Weinberg et al. 2012, Schirle and MacRae 2012). The C-terminus of Ago contains a PIWI domain, similar to the RNase H domain, which functions to cleave mRNA targets between the 10th and 11th nucleotide position of the guide strand, in an action known as slicing (Song, Smith et al. 2004).

In flies, miRNA duplexes and siRNA duplexes are sorted to Ago1 and Ago2, respectively (Okamura, Ishizuka et al. 2004, Forstemann, Horwich et al. 2007, Tomari, Du et al. 2007). Mismatches at position 9-10 of the guide strand of the miRNA duplex directs it to Ago1, whereas perfectly complementary siRNA duplexes are loaded onto Ago2 by the R2D2-Dcr-2 complex (Czech, Zhou et al. 2009, Okamura, Liu et al. 2009, Ghildiyal, Xu et al. 2010). Also, Ago1 binds guide RNAs with a preference for 5' U, whereas Ago2 binds guide RNAs with a preference for 5' C (Ghildiyal, Xu et al. 2010). In contrast to insect small RNA pathways, equivalent populations of miRNAs, preferably

duplexes with mismatches in the central region (nucleotides 8-11) (Meister, Landthaler et al. 2004, Yoda, Kawamata et al. 2010), are loaded onto the four human Ago proteins (AGO1, AGO2, AGO3, and AGO4) (Liu, Carmell et al. 2004, Meister, Landthaler et al. 2004, Su, Trombly et al. 2009). Loading of mature miRNAs into Ago proteins consists of two steps: ATP-dependent loading of the miRNA duplex into the Ago protein and ATP-independent removal of the passenger strand to leave the guide strand within the Ago protein to form the mature RISC complex (Kawamata, Seitz et al. 2009, Yoda, Kawamata et al. 2010). ATP-dependent loading of miRNA duplexes into Ago proteins is mediated by the activity of the Hsc70/Hsp90 chaperone machinery (Iwasaki, Kobayashi et al. 2010). Ago proteins possessing the ability to effect target RNA cleavage during RNAi (slicer activity) such as fly Ago2 and human AGO2 and are loaded with duplexes which are base-paired at the central region can perform cleavage of the passenger strand (Matranga, Tomari et al. 2005, Rand, Petersen et al. 2005, Leuschner, Ameres et al. 2006, Diederichs and Haber 2007). The cleaved passenger strand is then degraded by the endoribonuclease C3PO (Liu, Ye et al. 2009). The choice of guide strand is usually determined with the strand containing the more thermodynamically unstable 5' end being selected as the guide strand (Khvorova, Reynolds et al. 2003, Schwarz, Hutvagner et al. 2003). Additionally, Ago proteins also select for guide strands with a 5' U (Czech, Zhou et al. 2009, Hu, Yan et al. 2009, Okamura, Liu et al. 2009, Ghildiyal, Xu et al. 2010).

2.2.6 miRNA target regulation

As the effector complex of miRNAs, RISC functions in post-transcriptional gene silencing of target mRNAs by base pairing of guide miRNA to the target mRNA (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993). Often, miRNA-target interactions are not perfect matches. However, for effective silencing, a perfect base-pairing at the seed sequence at the 2-7 nt position of the guide miRNA with its target is required, with minor contribution of base-pairing at the 3' region of the miRNA to miRNA binding stability (Lai 2002, Lewis, Shih et al. 2003, Brennecke, Stark et al. 2005, Krek, Grun et al. 2005, Lewis, Burge et al. 2005). The vast majority of known miRNA-target mRNA interactions

contain mismatches at the central region which prevents slicing of target mRNAs but allows stable binding of the RISC complex to target mRNA (Martinez and Tuschl 2004, Chandradoss, Schirle et al. 2015, Salomon, Jolly et al. 2015). Of the four Ago proteins in humans, only AGO2 has retained Slicer activity and can cleave target mRNAs with perfect complementarity to the miRNA (Liu, Carmell et al. 2004, Meister, Landthaler et al. 2004), although all human Argonaute proteins can repress expression of target mRNAs via a slicer-independent mechanism (Pillai, Artus et al. 2004, Liu, Rivas et al. 2005). RISC complex can also repress translation of target mRNAs by preventing cap-dependent translational initiation (Humphreys, Westman et al. 2005, Pillai, Bhattacharyya et al. 2005), inhibit translational elongation by causing premature termination (Nottrott, Simard et al. 2006, Petersen, Bordeleau et al. 2006) and ribosome drop-off (Petersen, Bordeleau et al. 2006). miRNA-mediated repression requires GW182 proteins (TNRC6A-C in mammals) which act downstream of Ago proteins. GW182 binds to the PAZ domain of Ago proteins via multiple GW repeats at its N-terminal region (Till, Lejeune et al. 2007, Lazzaretti, Tournier et al. 2009, Lian, Li et al. 2009, Takimoto, Wakiyama et al. 2009) and represses protein translation through its C-terminal silencing domain (Baillat and Shiekhata 2009, Chekulaeva, Filipowicz et al. 2009, Eulalio, Helms et al. 2009, Lazzaretti, Tournier et al. 2009, Zipprich, Bhattacharyya et al. 2009). GW182 proteins also sequester target mRNAs into P-bodies, where decapping enzymes and RNases are enriched while ribosomes are depleted (Hsu and Stevens 1993, Mukherjee, Gao et al. 2002, Jakymiw, Lian et al. 2005, Liu, Rivas et al. 2005, Liu, Valencia-Sanchez et al. 2005, Behm-Ansmant, Rehwinkel et al. 2006).

2.3 Non-canonical microRNA biogenesis pathways

While the vast majority of miRNAs follow the canonical miRNA biogenesis pathway which is dependent on both Drosha and Dicer, there exists other miRNA species which do not follow the canonical miRNA processing steps. Such miRNAs employ Drosha-independent or Dicer-independent processing pathways to produce mature miRNA species. Several Drosha-independent miRNA biogenesis mechanisms have previously been reported. One of the first identified classes of non-canonical miRNAs is mirtrons, which utilize a splicing step for the production of pre-miRNAs, making their biogenesis independent of Drosha (Berezikov, Chung et al. 2007, Okamura, Hagen et al. 2007, Ruby, Jan et al. 2007). Furthermore, the biogenesis of simtrons, another class of mirtron-like miRNAs, are independent of splicing and do not appear to follow any of the conventional miRNA biogenesis steps (Havens, Reich et al. 2012). Atypical endogenous shRNA-type miRNAs also bypass Drosha processing and Exportin-5 export for their biogenesis (Babiarz, Ruby et al. 2008, Chong, Zhang et al. 2010, Xie, Li et al. 2013). Biogenesis of two miRNAs have shown to be dependent on the slicing activity of Ago2 (Cheloufi, Dos Santos et al. 2010, Cifuentes, Xue et al. 2010, Yang, Maurin et al. 2010). Finally, Argonautes have also been shown to load single-stranded RNA species (Okamura, Ladewig et al. 2013, Winter, Link et al. 2013). They will be described in detail in the following subsections.

2.3.1 Mirtrons

Mirtrons are Drosha-independent miRNAs which are found in introns of spliced transcripts. Like normal miRNAs, they also form secondary structures which fold back onto themselves to form a hairpin structure. However, unlike canonical miRNAs, which has an extended base-pairing region forming a slightly longer hairpin beyond the Drosha cleavage site called the basal stem region (figure 2.2.2), mirtrons do not contain the extended base-pairing region extending beyond the edges of the pre-miRNA hairpin which are necessary for pri-miRNA processing by Drosha (Han, Lee et al. 2006). Instead, mirtrons are flanked by exon sequences at the 5' and/or 3' end, with the ends of the pre-miRNA hairpin of the mirtron corresponding to 5' and/or 3' splice sites (Berezikov, Chung et al. 2007, Okamura, Hagen et al. 2007, Ruby, Jan et al. 2007). These indicate that mirtrons bypass the Drosha processing step by utilizing the splicing mechanism for excision of the mirtron pre-miRNA hairpin.

Like all spliced transcripts, mirtron-containing host gene transcripts are transcribed by RNA polymerase II and then spliced in the nucleus after transcription by the spliceosome. As for normal introns, the mirtron-containing intron is excised in two S_N2 -type transesterification reactions. The first step involves branching, whereby the 2'-OH of an adenosine in the branch point sequence performs nucleophilic attack on the guanine nucleotide at its 5' end at the 5' splice site of the intron, forming a free 5' exon end and an intron lariat-3' exon intermediate. The second step involves a nucleophilic attack by the 5' exon end on the first intron of the 3' exon, creating a 5' exon-3' exon spliced product and the release of the intron lariat as a product (Konarska, Grabowski et al. 1985). The branched structure of the intron lariat consists of a 2',5' phosphodiester linkage between the 5' guanine nucleotide and the adenosine at the branch point sequence close to the end of the intron (Grabowski, Padgett et al. 1984, Padgett, Konarska et al. 1984, Ruskin, Krainer et al. 1984), which is then cleaved by the lariat debranching enzyme to produce a linear RNA molecule (Ruskin and Green 1985). The debranched intron comprising of the mirtron then folds itself into a hairpin structure similar to a pre-miRNA hairpin. As such, due to their reliance on the splicing step for

their biogenesis, mirtrons usually begin with the 5' splice site consensus motif GURAGU and end with the 3' splice site consensus motif CAG (Mount, Burks et al. 1992, Zhang 1998). In the case of tailed mirtrons which are not flanked at both 5' and 3' end by exons, splicing and debranching of the mirtron-containing intron followed by hairpin refolding results in a mirtron hairpin containing extra sequences at the 5' end or 3' end. These tail regions are then trimmed by the exosome to form the pre-miRNA mirtron hairpin (Flynt, Greimann et al. 2010) (figure 2.3.1.1).

The refolded mirtron hairpin can then rejoin the conventional miRNA biogenesis pathway at the miRNA export step to be exported out of the nucleus by Exp-5, and proceed to be processed by Dicer and loaded into Ago proteins (Berezikov, Chung et al. 2007, Okamura, Hagen et al. 2007, Ruby, Jan et al. 2007). Since the 5' end of the pre-miRNA hairpin begins with the G from the 5' splice site consensus motif, whereas 5' U or A nucleotides are strongly preferred in mature miRNAs (Frank, Sonenberg et al. 2010), mature miRNA strand selection usually occurs on the 3' end, which would have less constraints on the 5' nucleotide (Ruby, Jan et al. 2007). Mirtrons also appear to be more frequently uridylated on the terminal nucleotide compared to conventional miRNAs (Westholm, Ladewig et al. 2012).

Figure 2.3.1.1: Mirtron biogenesis pathway.

2.3.2 Simtrons

While mirtrons are dependent on splicing for their biogenesis, Recently, a subset of annotated mirtrons (miR-1225 and miR-1228) were shown to be expressed independently of splicing in their host genes and hence were termed splicing independent mirtron-like miRNAs (simtrons) (Havens, Reich et al. 2012). Most intriguingly, expression of these simtrons did not require Exp-5 or Dicer, indicating that they bypass conventional pre-miRNA export mechanisms and conventional pre-miRNA processing by Dicer. Moreover, unlike the Dicer-independent miRNA miR-451, it does not require the slicer activity of Ago2 for maturation. Instead, biogenesis of simtrons appear to require Drosha, but not DGCR8, indicating that although simtron biogenesis is Drosha dependent, it is not processed in the same way as conventional microprocessor-mediated processing of pri-miRNAs, since conventional microprocessor-mediated processing of miRNAs is both Drosha and DGCR8-dependent. Despite their atypical biogenesis pathway, simtrons are able to be bound by Ago proteins to undergo posttranscriptional gene silencing.

2.3.3 Drosha-independent endogenous short hairpin RNAs

Besides mirtrons, another class of Drosha-independent class of small RNAs have been recently described, termed endogenous short hairpin RNAs (shRNAs). While both mirtrons and endogenous shRNAs are also Drosha-independent and Dicer-dependent, endogenous shRNAs are not dependent on splicing as a substitute for Drosha processing, unlike mirtrons. Endogenous shRNAs lack canonical splicing signals and tend not to map to introns (Babiarz, Ruby et al. 2008), instead, they tend to have their own independent transcriptional units (Chong, Zhang et al. 2010). They tend to fold into extended hairpin structures which have stem structures which are typically longer than the ~33nt stem lengths of conventional miRNA hairpins (Chong, Zhang et al. 2010).

A few endogenous shRNAs have been described, one of which is miR-320. These endogenous shRNAs have their own independent transcriptional units. As these endogenous shRNA hairpins are transcribed by RNAP II, the transcript is capped with a m7G cap at the 5' end, a feature of RNAP II transcripts, and undergoes transcriptional termination at the 3' end due to the presence of an A-rich tract downstream of the endogenous shRNA hairpin sequence. As such, these endogenous shRNAs have been termed as m7G-capped pre-miRNAs. Unlike conventional miRNAs which do not contain the m7G cap at the 5' end and are exported from the nucleus by Exp-5, these m7G-capped pre-miRNAs are exported out of the nucleus by Exportin-1 (Xpo1). They are then processed by Dicer and loaded into the Ago protein. Only the 3p strand from the m7G-capped duplex miRNA is loaded into Ago, because the 5p strand is selected against Ago due to the presence of the m7G cap at the 5p strand (Xie, Li et al. 2013) (figure 2.3.3.1).

Figure 2.3.3.1: m7G- capped pre-miRNA biogenesis pathway.

2.3.4 Slicer-dependent miRNAs

Ago proteins have a PIWI domain which has the ability to undergo catalytic activity called slicing by cleaving mRNA targets between the 10th and 11th nucleotide position of the guide strand when the guide strand of the miRNA base-pairs with target mRNA with perfect complementarity (Song, Smith et al. 2004). In mammals, only Ago2 retains slicer activity (Liu, Carmell et al. 2004, Meister, Landthaler et al. 2004), however Ago3 has also been reported to undergo slicing under specific conditions (Park, Phan et al. 2017). Ago2 is exclusively expressed in mammalian developing and mature erythrocytes, suggesting an important role of Ago2 slicer activity in mammalian systems (Jee, Yang et al. 2018).

Slicer-competent human AGO2 has previously been shown to have the ability to load some canonical 5p-coded pre-miRNAs and cleave them at the middle of the 3p arm of the pre-miRNA hairpin to produce Ago-cleaved pre-miRNA (ac-pre-miRNA) species which are shorter than the pre-miRNA hairpin but longer than the mature miRNA species (Diederichs and Haber 2007). ac-pre-miRNA generation by Ago2 slicer activity has shown to be important for the biogenesis of the Dicer-independent miR-451. miR-451 is an erythropoietic miRNA which is transcribed and processed by Drosha to produce an unusually short pre-miRNA hairpin with a stem region about 18bp in length. However, this short pre-miRNA hairpin is unable to be processed by Dicer and instead loaded into Ago2. Using its slicer function, Ago2 cleaves the middle part of the 3p arm of pre-miR-451 to generate an ac-pre-miRNA species (Cheloufi, Dos Santos et al. 2010, Cifuentes, Xue et al. 2010, Yang, Maurin et al. 2010). The 3' end of the ac-pre-miR-451 is then trimmed by Poly(A)-specific ribonuclease (PARN) to form mature miR-451 (Yoda, Cifuentes et al. 2013) (figure 2.3.4.1).

Additionally, miR-486, another erythrocyte-specific miRNA, is dependent on Ago2 slicing activity for its maturation. Unlike miR-451 which is completely Dicer-independent, miR-486 requires Dicer for its biogenesis. miR-486 forms a miRNA hairpin with a perfectly complementary stem region, similar to that of siRNA duplexes. miR-486

follows the conventional miRNA biogenesis pathway in which it is processed by both Drosha and Dicer, and the duplex is then loaded into Ago2. However, unlike conventional miRNAs which utilize a slicer-independent mechanism to remove the passenger strand, the perfectly base-paired duplex of miR-486 requires Ago2 slicer activity for removal of its passenger strand miR-486-3p for mature RISC formation (Jee, Yang et al. 2018) (figure 2.3.4.2).

Figure 2.3.4.1: Biogenesis pathway of miR-451.

Figure 2.3.4.2: Biogenesis pathway of miR-486.

2.4 Ago loading of single-stranded RNAs

While most small RNA biogenesis pathways include generation of a small RNA duplex and loading of the duplex into Ago proteins, recombinant analysis of Ago proteins show that they have the ability to bind both duplex and single-stranded small RNAs without the help of other loading cofactors, and that single-stranded small RNAs (ssRNA) are preferentially bound under this condition (Meister, Landthaler et al. 2005, Lima, Wu et al. 2009, Hauptmann, Dueck et al. 2013). Hence, in an in vitro system, mature RISC can be generated by through the loading of single-stranded small RNAs, although the significance of ssRNA loading is debatable in vivo, since the duplex loading cofactor Hsc70/Hsp90 chaperone machinery usually aids in the loading of Ago proteins in vivo in an ATP-dependent manner (Iwasaki, Kobayashi et al. 2010), and ssRNAs are much more easily degraded in vivo compared to duplex RNAs which are comparatively more stable, due to the abundance of single-stranded RNases in the cells.

Recently, two groups observed that endogenous miRNA hairpin loops can be loaded into Ago proteins (Okamura, Ladewig et al. 2013, Winter, Link et al. 2013), a surprising finding given that the biogenesis of most endogenous small RNAs which are loaded into Ago proteins usually goes through a duplex RNA intermediate step, and showed a function for miRNA hairpin loops which were previously expected to be degraded after Dicer processing and were thought to be just a byproduct of miRNA biogenesis. Differential sorting of miRNA loops for miR-34 and miR-317 in *Drosophila* Ago1 and Ago2 suggests that sequence specificity underlies the loading of different miRNA loops to either Ago1 or Ago2 (Okamura, Ladewig et al. 2013). Indeed, it was recently found that ssRNA species which were loaded into Ago1 show a preference for the GAC motif at the 5' end (Goh and Okamura, unpublished).

2.5 piRNA biogenesis

Besides miRNAs, Piwi-interacting RNAs (piRNAs) are another class of small RNAs which mediate RNA-mediated gene silencing of transposable elements in the germline. piRNAs are 23-30nt long, usually have a 5' uracil and a 2'-O-methylated sugar at the 3' end (Vagin, Sigova et al. 2006, Kirino and Mourelatos 2007, Saito, Sakaguchi et al. 2007). Similar to miRNAs which interact with the Ago subclade of the Argonaute protein family for its function, piRNAs interact with another subclade of the Argonaute protein family, known as Piwi proteins. piRNAs are functionally active after loading into Piwi proteins to form a Piwi-piRNA complex called the piRISC.

Primary piRNA production begins by transcription of RNAP II from truncated transposons-rich sequences at pericentromeric and telomeric regions, known as piRNA clusters (Brennecke, Aravin et al. 2007). Like all RNAP II transcripts, the single-stranded piRNA transcripts undergo 5' capping, 3' polyadenylation and splicing (Goriaux, Desset et al. 2014). Primary piRNA transcripts are then exported from the nucleus and processed in a perinuclear structure in the cytoplasm called the Yb body (Olivieri, Sykora et al. 2010, Saito, Ishizu et al. 2010, Qi, Watanabe et al. 2011), also termed the nuage in the germline. The 5' and 3' ends of piRNAs are determined by Zucchini (Zuc) (Ipsaro, Haase et al. 2012, Nishimasu, Ishizu et al. 2012) and Nibbler (Kawaoka, Izumi et al. 2011, Vourekas, Zheng et al. 2012, Hayashi, Schnabl et al. 2016), respectively. piRNAs are then 2'-O-methylated (Horwich, Li et al. 2007, Saito, Sakaguchi et al. 2007) and loaded into Piwi and Aub in *Drosophila* (Brennecke, Aravin et al. 2007, Gunawardane, Saito et al. 2007). Piwi piRISCs mediate transcriptional gene silencing of transposon loci in the nucleus, whereas Aub piRISCs mediate post-transcriptional gene silencing of transposons in the cytoplasm in a Slicer-dependent manner.

Besides primary piRNA production, secondary piRNAs can be produced from the ping-pong cycle, whereby targets of Aub slicing produce cleavage products which are processed into piRNAs and loaded into Ago3. Slicer activity of Ago3 then cleaves antisense transposon transcripts to generate antisense piRNAs which are loaded into

Aub, leading to piRNA amplification. Aub-associated piRNAs are antisense to transposon transcripts, whereas Ago3-associated piRNAs are sense to transposon transcripts (Brennecke, Aravin et al. 2007, Malone, Brennecke et al. 2009). Ping-pong signatures are displayed by Aub-bound piRNAs having a strong bias for 1U and Ago3-bound piRNAs which are complementary to Aub piRNAs 10nt from the 5' end having a bias for 10A (Brennecke, Aravin et al. 2007, Gunawardane, Saito et al. 2007). Secondary piRNAs from Ago3 piRISC can also produce 3'-directed phased piRNAs from target RNA. The first piRNA produced from Ago3 piRISC target slicing from the ping-pong cycle is loaded into Aub and leads to production of successive phased piRNAs of 27nt in length from the same RNA transcript by Zuc endonuclease activity (Homolka, Pandey et al. 2015, Mohn, Handler et al. 2015, Xu, Zhang et al. 2015).

piRNAs which are loaded into Piwi proteins to form piRISC function to suppress transposon transcripts (Aravin, Naumova et al. 2001, Vagin, Sigova et al. 2006). Piwi piRISC localizes to the nucleus to undergo transcriptional silencing of transposons by promoting trimethylation of H3K9, leading to heterochromatin formation at the targeted loci (Klenov, Sokolova et al. 2011, Wang and Elgin 2011, Sienski, Donertas et al. 2012, Huang, Yin et al. 2013, Le Thomas, Rogers et al. 2013, Rozhkov, Hammell et al. 2013, Klenov, Lavrov et al. 2014). Aub and Ago3 remain in the cytoplasm where they cleave transposon transcripts complementary to the piRNA guide strand using their slicer activity (Brennecke, Aravin et al. 2007, Gunawardane, Saito et al. 2007, Huang, Li et al. 2014).

Figure 2.5.1: piRNA biogenesis pathway.

2.6 Known substrates of Exp-5

Exp-5 is the major miRNA nuclear export factor and recognizes structural features of pre-miRNA hairpins. Within the nucleus, Exp-5 binds to Ran-GTP and pre-miRNAs and translocates through the nuclear pore complex into the cytoplasm, where hydrolysis of GTP by Ran occurs, resulting in dissociation of the Exp-5:Ran:pre-miRNA complex (Brownawell and Macara 2002). Exp-5 consists of 20 HEAT repeats arranged in a structure similar to a baseball mitt with a channel allowing binding of the pre-miRNA stem and a pocket at the base of the channel to accommodate the 2nt overhang of the pre-miRNA at the 3' end (Okada, Yamashita et al. 2009). In line with its structure, Exp-5 prefers pre-miRNA substrates with a stem length of at least 14nt and blunt or 3' overhangs of up to 8nt (Gwizdek, Ossareh-Nazari et al. 2003, Zeng and Cullen 2004). Aside from its role of shuttling pre-miRNAs from the nucleus into the cytoplasm, Exp-5 binding of pre-miRNAs may also protect pre-miRNAs from exonuclease degradation (Yi, Qin et al. 2003, Zeng and Cullen 2004).

Besides being an important export factor for pre-miRNAs, Exp-5 can also bind tRNAs (Bohnsack, Regener et al. 2002, Calado, Treichel et al. 2002). However, in most organisms, tRNAs are exported by a tRNA-specific export factor, called Exportin-t (Exp-t) in mammals (Arts, Fornerod et al. 1998, Arts, Kuersten et al. 1998, Hellmuth, Lau et al. 1998, Kutay, Lipowsky et al. 1998, Lipowsky, Bischoff et al. 1999), and hence Exp-5 is not a major export factor for tRNAs. In some arthropods such as *Drosophila*, the ortholog of Exp-t does not exist (Lippai, Tirian et al. 2000). In these organisms lacking Exp-t orthologs, Exp-5 appears to function as the export factor for both pre-miRNAs and tRNAs, whereas Exp-5 in organisms which have Exp-t orthologs mainly export pre-miRNAs (Shibata, Sasaki et al. 2006).

Besides being involved in the transport of pre-miRNAs out of the nucleus, Exp-5 also exports other minihelix-containing RNAs and their associated RNA-binding proteins by using RNA as an adaptor for RBP transport (Bohnsack, Regener et al. 2002, Calado, Treichel et al. 2002, Gwizdek, Ossareh-Nazari et al. 2004). Exp-5 has been found to

export tRNA alongside Exportin-t and the tRNA-associated eukaryotic elongation factor 1A (eEF1A) (Bohnsack, Regener et al. 2002, Calado, Treichel et al. 2002). Exp-5 also binds to and exports the minihelix motif-containing adenovirus VA1 RNA (Gwizdek, Ossareh-Nazari et al. 2003), and also exports the dsRBP ILF3 and JAZ in a minihelix RNA-dependent manner (Chen, Brownawell et al. 2004, Gwizdek, Ossareh-Nazari et al. 2004). Interestingly, Exp-5 has been identified besides Crm1 as an export factor for human 60S ribosomal subunit in human (Wild, Horvath et al. 2010) and the RNA editing enzyme ADAR1 (Fritz, Strehblow et al. 2009). Although signal recognition particle RNA (7SL RNA) is exported using the Crm1 pathway in *Saccharomyces cerevisiae*, export of 7SL RNA relies on Exp-5 in *Xenopus* (Takeiwa, Taniguchi et al. 2015).

Besides being the integral export factor for pre-miRNAs, Exp-5 also performs an integral role in the RNAi pathway as the export factor for Dicer mRNA. Production of Dicer protein can thus be regulated through the export of Dicer mRNA by Exp-5 and can be affected through competition with pre-miRNAs and viral miRNAs to control miRNA levels (Bennasser, Chable-Bessia et al. 2011). Exp-5 knockout also resulted in decreased miRNA production, however the extent of miRNA decrease indicates that although Exp-5 is the major export factor for pre-miRNAs, other export pathways may also mediate the nuclear export of some pre-miRNAs, or pre-miRNA processing can be performed in the nucleus by nuclear Dicer (Kim, Kim et al. 2016).

Although Exp-5 substrates resemble Drosha processing products, not all hairpin structures produced by Drosha are exported from the nucleus. The two hairpins produced through Drosha cleavage of DGCR8 mRNA during regulation of DGCR8 protein levels appear to be contained in the nucleus, as the hairpins can only be detected in the nuclear, but not cytoplasmic fraction (Han, Pedersen et al. 2009). Furthermore, Drosha also produces a cleavage product from the mrhl lncRNA which is not exported but is localized in the nucleus (Ganesan and Rao 2008). These examples suggest that there could be Drosha cleavage products which may not be recognized by Exp-5, or that they are prevented from being recognized by Exp-5 through unknown mechanisms. Because current research cannot accurately predict substrates of Exp-5, it

is necessary to investigate the structural features of Exp-5 substrates to determine the specificity of Exp-5 in substrate export.

2.7 Functional interaction of Dicer and Argonaute proteins

According to current knowledge on the canonical miRNA biogenesis pathway, Dicer cleavage of the pre-miRNA hairpin occurs before the binding of the substrate by Ago. Consistent with the current established model, the pre-miRNA processing factor Dicer, TRBP and Ago form a complex called the RISC loading complex (RLC) that exhibits both dicing and silencing activities (Gregory, Chendrimada et al. 2005, Maniataki and Mourelatos 2005), suggesting that dicing of pre-miRNA and loading of the RNA duplex into Ago may be coupled. However, other studies have shown that Dicer is not required for RISC loading (Yoda, Kawamata et al. 2010, Betancur and Tomari 2012). It has also been proposed that the interaction of Dicer and TRBP with Ago2 enhances slicing of targets by Ago2 (Chendrimada, Gregory et al. 2005, Gregory, Chendrimada et al. 2005). Hence, the reason for the functional association of the Dicer-TRBP complex and Ago is still under debate.

Slicer-competent human AGO2 has previously been shown to have the ability to generate ac-pre-miRNA species by cleaving in the middle of the miRNA 3p arm (Diederichs and Haber 2007). The mechanism of ac-pre-miRNA formation and published examples of ac-pre-miRNA-generating species suggests that ac-pre-miRNA production is more important for 5p-coded miRNAs compared to 3p-coded miRNAs. Moreover, the precursors of Dicer-independent miRNA miR-451 and some Dicer-dependent miRNAs have been reported to be loaded into a Hsp70-dependent Ago2-containing miPDC complex (Liu, Jin et al. 2012). These observations suggest that RNA-Ago binding may precede processing by Dicer.

Additionally, phosphorylation of Ago2 by EGFR during hypoxia has been shown to reduce Ago2 binding to Dicer and TRBP, which in turn resulted in an increase of pre-miRNAs and corresponding decrease of mature miRNAs for a group of EGFR-sensitive miRNAs termed mHESM (miRNAs regulated by hypoxia-dependent EGFR-suppressed maturation), indicating that the interaction of Ago2 to Dicer and TRBP may affect pre-miRNA processing for a subset of miRNAs (Shen, Xia et al. 2013). Furthermore, Ago

knockdown appears to affect miRNA processing via an unknown mechanism (Grishok, Pasquinelli et al. 2001, Bouasker and Simard 2012). Finally, biogenesis of a subset of miRNAs during hematopoiesis appears to be dependent on Ago2, but not Ago1, 3 and 4, and this role of Ago2 is independent of Ago2 slicer activity (O'Carroll, Mecklenbrauker et al. 2007). These observations collectively indicate that Ago proteins may function not just as downstream effectors of the RNAi pathway, but may also have other functions in upstream steps of miRNA biogenesis.

The Makeyev laboratory previously described differential miR-124 trimming when loaded to different Ago proteins (Juvvuna, Khandelia et al. 2012). From one of the described experiments and subsequent experiments, I previously observed that overexpression of miR-124 together with either of the Ago proteins in mammalian cells resulted in a significant decrease of pre-miR-124 compared to control or Dicer-overexpressing cells (Lee and Makeyev unpublished data; NTU FYP thesis 2012). Current knowledge is unable to explain the decrease of pre-miR-124 upon Ago overexpression since Ago proteins are not known to affect pre-miRNAs, except through generation of ac-pre-miRNA species. However, ac-pre-miRNA does not appear to be the cause of pre-miR-124 decrease during Ago overexpression due to three reasons: firstly, there is no lower molecular weight ac-pre-miRNA band, secondly, the decrease of pre-miR-124 occurs for all Ago proteins, including those without slicer activity, and thirdly, miR-124 is a predominantly 3p miRNA which cannot be produced through the proposed ac-pre-miRNA mechanism. We therefore propose that this phenomenon may be due to a novel effect of Ago proteins.

2.8 Biology of miR-124

miR-124 is a highly conserved miRNA which is highly expressed in neuronal cells, accounting for 25-48% of all brain miRNAs (Lagos-Quintana, Rauhut et al. 2002). miR-124 is encoded at three distinct loci in human and mouse (miR-124-1, miR-124-2 and miR-124-3), each containing identical stem sequence and structure but different loop sizes (Conaco, Otto et al. 2006, Deo, Yu et al. 2006). miR-124 is expressed specifically in neurons but not astrocytes (Smirnova, Grafe et al. 2005), and appears to increase in abundance in neuronal cells during development (Krichevsky, King et al. 2003, Miska, Alvarez-Saavedra et al. 2004, Smirnova, Grafe et al. 2005). Expression of miR-124 in non-neuronal cells causes a shift towards a neuronal gene profile (Lim, Lau et al. 2005). Disruption of miR-124 results in neural abnormalities, indicating that miR-124 is important for the maturation and survival of dentate gyrus neurons and retinal cones (Sanuki, Onishi et al. 2011). Overexpression of miR-124 promotes neuronal differentiation in a variety of neural progenitor cells, including mesenchymal, brain tumor and neuronal stem cells (Silber, Lim et al. 2008, Zou, Chen et al. 2014, Mondanizadeh, Arefian et al. 2015, Jiang, Du et al. 2016, Jiao, Liu et al. 2017, Sharif, Ghahremani et al. 2017), and overexpression of both miR-124 and miR-9 were sufficient to induce differentiation of neural progenitor cells and fibroblasts into neurons (Krichevsky, Sonntag et al. 2006, Yoo, Sun et al. 2011). miR-124 has been shown to be an important neuronal fate determinant for the neuronal stem cells in the subventricular zone (SVZ), the largest neurogenic region in the adult mammalian brain (Cheng, Pastrana et al. 2009, Akerblom, Sachdeva et al. 2012). miR-124 is also important in neurite outgrowth and synaptogenesis (Yu, Chung et al. 2008, Franke, Otto et al. 2012, Xue, Yu et al. 2016, Li and Ling 2017). Taken together, miR-124 appears to be important in neural fate determination and neurogenesis as well as neuronal outgrowth in mammals. However, the function of miR-124 appears to be divergent in different organisms, as in *Drosophila melanogaster*, miR-124 is expressed in proliferating neural precursor cells and differentiating postmitotic neurons, and knockout of miR-124 in flies results in almost normal behavior (Weng and Cohen 2012).

In mammals, miR-124 regulates a large number of target genes in different pathways (Lim, Lau et al. 2005). One of the pathways targeted by miR-124 is the REST pathway. REST (RE1 silencing transcription factor) represses neural genes in non-neuronal cells by recruitment of the corepressor complex containing histone deacetylases (Andres, Burger et al. 1999, Huang, Myers et al. 1999, Roopra, Sharling et al. 2000, Ballas, Grunseich et al. 2005) and acts in a reciprocal manner with miR-124, repressing miR-124 in non-neuronal cells via the RE-1 element at the miR-124 loci but does not bind to the miR-124 loci in neuronal cells, hence allowing miR-124 expression (Conaco, Otto et al. 2006). Additionally, REST is a direct target of miR-124, creating a double-negative feedback loop between REST and miR-124 (Wu and Xie 2006). Moreover, SCP1 (Small C-terminal domain phosphatase 1) which is recruited by REST to RE-1 element to function as a silencer for neuronal genes in non-neuronal cells (Yeo, Lee et al. 2005), is directly targeted by miR-124 on its 3' UTR during neuronal differentiation (Visvanathan, Lee et al. 2007). Additionally, miR-124 also regulates neuronal differentiation through the Notch signaling pathway via repression of the Notch ligands Jag1 (Jagged1) (Cheng, Pastrana et al. 2009, Liu, Chopp et al. 2011) and DLL4 (Delta-like 4) (Jiao, Liu et al. 2017), allowing exit from stem cell self-renewal and differentiation into neuronal cell fate. Besides the Notch signaling pathway, miR-124 also controls the switch between neuronal stem cell fate by downregulation of the stem cell transcription factor Sox9 (Cheng, Pastrana et al. 2009, Farrell, Power et al. 2011, Akerblom, Sachdeva et al. 2012). Furthermore, miR-124 has been described to be involved in promoting neuronal differentiation via a third pathway via downregulation of PTBP1. PTBP1 is a repressor of alternative splicing in non-neuronal cells which is downregulated by miR-124, leading to an increase in correctly spliced PTBP2 mRNA and consequently PTBP2 protein, allowing a shift to neuronal splicing patterns (Makeyev, Zhang et al. 2007). miR-124 also regulates the exit from the cell cycle by targeting cell cycle-related genes, including inhibitor of apoptosis-stimulating protein of p53 (iASPP) and cyclin-dependent kinases (CDK) such as CDK4, CDK6 and cyclin D1 (Liu, Kang et al. 2018). miR-124 also acts as a key regulatory switch between neuronal and astrocyte differentiation pathways through targeting of polycomb group protein Ezh2, which epigenetically represses a wide range of neuronal-specific target genes (Neo, Yap et al. 2014). Hence, miR-124 is

involved in regulation of target genes for multiple pathways leading to neuronal differentiation.

Besides being a neuronal fate determinant, miR-124 is also involved in neurite outgrowth and synaptogenesis. Neurite outgrowth by miR-124 occurs through the targeting of RhoG, which has been shown to inhibit axonal and dendritic branching by a Rac1 signaling pathway and a cdc42-dependent pathway (Yu, Chung et al. 2008, Franke, Otto et al. 2012). Besides RhoG, miR-124 also enhances neurite outgrowth by targeting inhibitors of neurite elongation such as the small GTPase Rho kinase ROCK1 (Gu, Meng et al. 2014), oxysterol-binding protein (OSBP) (Gu, Li et al. 2016) and HDAC5 (Gu, Fu et al. 2018). miR-124 is also involved in axon formation of hippocampal neurons and survival of retinal cone neurons by targeting LHX2 which is required for hippocampal formation (Sanuki, Onishi et al. 2011). miR-124 is also involved in neural development via direct repression of BAF53a and miR-124-mediated repression of REST leading to transcriptional repression of BAF53a. The repression of BAF53a, a subunit of the npBAF chromatin remodeling complex essential for neural progenitor proliferation, allows the switch to BAF53b and nBAF chromatin remodeling complex which are required for post-mitotic neural development and dendritic outgrowth (Yoo, Staahl et al. 2009). miR-124 also targets CoREST which in turn represses NRP1 encoding a Sema3A receptor, thus altering the sensitivity of retinal ganglion cell (RGC) growth cones to Sema3A which is important for axon guidance (Baudet, Zivraj et al. 2011). Finally, gene ontology studies show that miR-124 functions mainly as a repressor for negative regulators of neurogenesis, positive regulators of neuron projection development and neuron projection morphogenesis (Li and Ling 2017). Hence, miR-124 also plays a key role in the regulation of key factors contributing to neurite outgrowth.

2.9 Scope of project

While we have gained much knowledge on miRNA biogenesis, many questions remain unanswered on how miRNA biogenesis factors recognize their substrates as well as non-canonical small RNA biogenesis pathways. This is especially so for Exp-5, which even though is an important export factor for pre-miRNA, little is known about Exp-5 substrate recognition and binding. This project aims to investigate the substrate specificities of Exp-5 as well as study non-canonical behaviors of miRNAs during biogenesis. The DGCR8 hairpin which has a pre-miRNA-like structure has been recently identified as a substrate of Drosha, but does not appear to be exported from the nucleus (Han, Pedersen et al. 2009). The project aims to study the substrate recognition of Exp-5 by performing mutagenesis of DGCR8 hairpin to make it a better substrate of Exp-5, and in doing so, identify key features of Exp-5 substrates. The project also aims to study endogenous Exp-5 substrates through the use of Exp-5 PAR-CLIP in S2 cells to identify endogenous bound substrates of Exp-5.

My previous observation that overexpression of miR-124 together with either of the Ago proteins in mammalian cells results in a significant decrease of pre-miR-124 compared to control or Dicer-overexpressing cells (Lee and Makeyev unpublished data; NTU FYP thesis 2012) suggests that this phenomenon may be due to a novel effect of Ago proteins. We aim to study how pre-miR-124 levels decrease during Ago overexpression and the structural features which predispose miR-124 hairpin to this effect. The phenomenon of pre-miRNA decrease for miR-124 will be characterized in human HEK293T, mouse N2a and fly S2 cells using in vitro overexpression experiments and northern blotting, with an emphasis on the human HEK293T cell line where this phenomenon was first observed. We aim to design in vitro assays to examine the role of Ago in decreasing amounts of pre-miR-124. Mutagenesis of the miR-124 hairpin will also be performed to examine the structural features which give rise to this phenomenon of pre-miRNA decrease.

3 Materials and methods

3.1 Subcellular fractionation

Cells were washed twice with ice-cold PBS and resuspended in 150ul of ice cold buffer A (10mM HEPES pH 7.9, 10mM KCl, 1mM DTT and 0.1mM EDTA pH 8.0) and incubated for 25 min on ice. 1.5 ul of 10% NP-40 was then added and incubated for 2 min on ice. Nuclei were precipitated by centrifugation at 5000 rpm for 3 min at 4°C. Supernatant was removed and kept as the cytoplasmic fraction. The nuclei pellet was then washed twice with buffer A at 5000 rpm for 3 min and used as the nuclear fraction. RNA from both the nuclear and cytoplasmic fraction was extracted with TRIzol reagent (Ambion).

3.2 Site-directed mutagenesis

Site-directed mutagenesis was performed using PCR by using complementary PCR primers containing the mutation at the middle of the primer. The PCR product was then purified and subjected to DpnI digestion (NEB) overnight at 37°C to remove parental wild type plasmid. The resulting product was then transformed into competent cells, plated and incubated at 37°C overnight. Colonies were picked and sequenced to obtain correctly mutated plasmids containing the correct sequence.

3.3 Protein purification

Bacteria were grown to 0.6 OD and induced with 0.4mM IPTG, then cultured overnight at 25°C. Cells were collected by centrifugation at 2000 rpm for 20 min at 4°C. Cells were then resuspended in 4ml lysis buffer (20mM NaPO₄, 0.5M NaCl pH 7.4) supplemented with 0.1mM PMSF and 1mg/ml lysozyme per gram of pellet. Cells were sonicated on ice at for 10 min at 30 sec intervals until the solution became translucent. The solution was then centrifuged at 10,000g for 30 min at 4°C. Supernatant was then used for protein purification.

250µl of GST beads slurry was used for every 5ml of protein lysate supernatant. GST beads were added into the column and washed thrice with ice-cold PBS. GST beads were then washed thrice with lysis buffer and allowed to drip out completely. Column was loaded with protein lysate supernatant from sonication and incubated on a shaker for 1 h at 4°C. Column was then allowed to stand to allow beads to settle to the bottom of the column. Protein lysate was then eluted out of the column and the column was washed thrice with lysis buffer at 4°C. GST-tagged protein was then eluted out and collected into 7 fractions using elution buffer (50mM Tris pH 8.0, 50mM NaCl, 10mM glutathione). Elution was confirmed by coomassie blue staining.

3.4 PAR-CLIP

PAR-CLIP protocol was adapted from (Hafner, Landthaler et al. 2010). S2R+ cells containing a FLAG-Exp-5 construct cloned in pMK33 plasmid backbone under the control of metallothionein promoter were grown in 20 15cm cell culture dishes in 20ml of Schneider's Drosophila Medium containing 10% FBS and 1% penstrep to confluency. Induction was performed by the addition of CuSO₄ and 4-thiouridine to 1mM CuSO₄ and 100µM 4-thiouridine respectively and incubated for 48 h at 25°C. The medium was then discarded and the cells were crosslinked using a 0.15J/cm² 365nm UV crosslinker. Cells were then collected and washed twice with ice-cold PBS, then resuspended in RIPA buffer (1x PBS, 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40 with Complete EDTA-free protease inhibitor (Roche)) and incubated on ice for 10 min. The lysate was then passed through a 25G needle four times and precleared by centrifugation at 13,000g for 10 min at 4°C. 1µl of 1000U/µl RNase T1 was added per 1ml of lysate and incubated for 15 min in a 22°C water bath, then cooled on ice for 5 min. Per 1ml of lysate, 675µl of magnetic bead slurry was washed thrice using citrate-phosphate buffer (0.47% citric acid, 0.92% Na₂HPO₄, pH 5.0), then bound with 180µg of FLAG antibody (Wako) for 40 min on a rotator at room temperature. Antibody-bound beads were then washed thrice using citrate-phosphate buffer and twice using RIPA buffer, then incubated with 1ml lysate for 1 h at 4°C on a rotator. Beads were washed thrice using IP wash buffer

(50mM Hepes-KOH pH 7.5, 300mM KCl, 2mM EDTA, 1mM NaF, 0.05% NP-40, 0.5mM DTT with Complete EDTA-free protease inhibitor (Roche)) and resuspended in 1 volume of IP buffer, then 1µl of 1000U/µl RNase T1 was added per µl of IP buffer and incubated for 15 min on a 22°C water bath, then cooled on ice for 5 min. The beads were washed thrice using high salt wash buffer (50mM Hepes-KOH pH 7.5, 500mM KCl, 2mM EDTA, 1mM NaF, 0.05% NP-40, 0.5mM DTT with Complete EDTA-free protease inhibitor (Roche)) and once using dephosphorylation buffer (100mM NaCl, 50mM Tris-HCl, 10mM MgCl₂, 1mM DTT, pH 7.9). The beads were then resuspended in 60µl of dephosphorylation buffer and 3µl of CIP was added and incubated for 10 min at 37°C. The beads were washed twice with phosphatase wash buffer (50mM Tris-HCl pH 7.5, 20mM EGTA, 0.5% NP-40) and twice with 1x PNK buffer (50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM MgCl₂, 5mM DTT). Beads were resuspended in 80µl 1x PNK buffer and labeled with 1µl 6000Ci/mol γ-ATP and 2µl PNK for 30 min at 37°C. 100µM ATP was then added and incubated at 37°C for 5 min, then beads were washed for 5 times with 1x PNK buffer and resuspended in 70µl SDS-PAGE buffer (4% SDS, 250mM Tris pH 6.8, 16% β-mercaptoethanol, 30% glycerol, 0.006% bromophenol blue). Samples were boiled for 5 min at 95°C, then the sample was loaded onto a 6% SDS-PAGE gel after removal of magnetic beads and run at 120V for 30min. The radioactive Exp-5 band was excised and placed in a dialysis tube, and RNAs from the gel piece was eluted for 1 h at 100V in a submarine gel box. Proteinase K buffer was added to the eluate to 1x concentration and 1.2mg/ml proteinase K was added and incubated for 30 min at 55°C. RNA was extracted using phenol-chloroform extraction followed by ethanol precipitation and used for library cloning.

3.5 Library cloning

RNA was mixed with equal volumes of 2x RNA loading buffer (Ambion) and loaded together with ³²P-labeled 19bp and 24bp RNA marker onto a 10% denaturing polyacrylamide urea gel. The gel was run in 0.5x TBE buffer at 300V for 1.5 h and the gel fragment corresponding to the marker size range was excised. The gel fragment was crushed using a pipette tip and RNA was eluted from the gel fragment in 400µl of

0.4M NaCl overnight at room temperature on a rotator. After the eluted RNA was precipitated and resuspended in water, 3' sequencing linker (RA3) was ligated to the 3' end of the RNA and RNA marker using T4 RNA ligase 2, truncated K227Q at room temperature for 4 h. Ligated RNA was then size-separated on a 10% denaturing polyacrylamide urea gel and the gel fragments corresponding to the molecular weight of the ligated RNA marker was excised and eluted. After precipitation and resuspension of the ligated product, 5' sequencing linker (RA5) was ligated to the 5' ligated RNA product and RNA marker using T4 RNA ligase at 37°C for 2 h. Ligated RNA was then size-separated on a 6% denaturing polyacrylamide urea gel and the gel fragment corresponding to the molecular weight of the 5' and 3' ligated RNA marker was excised from the gel. The ligated product was then eluted and reverse transcribed with RT primer and Superscript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. PCR amplification was performed on the reverse transcribed DNA using RP1 forward primer annealing onto the RA5 linker and barcoded RP reverse primer. The 140nt PCR product was extracted using phenol-chloroform and gel-purified on a 6% native TBE PAGE gel and then sent for sequencing.

3.6 Bioinformatics analysis

The Exp-5 PAR-CLIP library (D041) was preprocessed using a previously described pipeline (Chak, Mohammed et al. 2015), whereby adaptor sequences trimmed and reads without adaptors were removed using FASTX Toolkit. Reads with lengths ranging from 20-50nt were first mapped using Bowtie1 (Langmead, Trapnell et al. 2009) to the pMK33 plasmid backbone and FLAG-Exp-5 sequence with the parameters $-a -v 2$ to remove construct-derived reads, and unmapped reads were then mapped to the *Drosophila melanogaster* genome (dm3 assembly) using Bowtie1 with the same mapping parameters. The bowtie output file was processed using PARalyzer (Corcoran, Georgiev et al. 2011) with default parameters except for the following changes: minimum read count per cluster = 1, minimum read count for KDE = 1, minimum read count for cluster inclusion = 1, minimum read length = 20, maximum number of non-conversion mismatches = 2, additional nucleotides beyond signal = 0. Exp-5 PAR-CLIP

mapping results were visualized in UCSC genome browser alongside a S2R+ RNA seq library (SRR1197280) mapped to dm3 using STAR (Dobin, Davis et al. 2013).

3.7 Genomic DNA extraction

Cells from a T25 flask were washed twice with PBS and pelleted by centrifugation at 300 rcf for 5 min. 200µl of proteinase K buffer (80mM NaCl, 100mM Tris pH 8.5, 0.5% SDS, 50mM EDTA) was added with 20µg proteinase K to the pellet and resuspended, then incubated at 55°C on a rotor machine for 1h. 400µl phenol-chloroform-isoamylalcohol (25:24:1) was then added and mixed by inverting tube and centrifuged at max speed at 25°C for 10 min. The upper aqueous phase was transferred to a new tube. gDNA was precipitated by the addition of 40µl 3M NaOAc and 1000µl EtOH and mixed well, then centrifuged at max speed for 20 min at 25°C. The pellet was washed with 70% EtOH and air-dried, then resuspended in water.

3.8 Molecular cloning

Primer sets for hsa-miR-16, 28 and 30a and dme-miR-124 were designed flanking the gene of interest. PCR was performed on genomic DNA template using Kapa Hifi PCR kit (KAPA Biosystems) according to manufacturer's instructions, using 55°C for the annealing temperature. The size of the PCR product was checked on a 2% agarose gel. For dme-miR-124 cloning using restriction enzyme cloning, both the purified PCR product and the plasmid vector were then incubated with the appropriate restriction enzymes (NEB) for 3 h. The reaction was then run on a 2% agarose gel and the correct band was excised using a blade. DNA fragments were extracted from the gel using a gel extraction kit (Geneaid) according to manufacturer's instructions. Vector and insert were ligated in a 1:3 ratio using T4 DNA ligase (NEB) at 4°C overnight. For hsa-miR-16, 28 and 30a, Taq polymerase was added directly to the completed PCR reaction and incubated at 72°C for 10 min to generate single A-overhangs for TOPO cloning. The PCR products were then run on a 2% agarose gel and the correct band was excised using a blade. DNA fragments were extracted from the gel using a gel extraction kit

(Geneaid) according to manufacturer's instructions. TOPO cloning was performed with pcDNA6.2-EmGFP-TOPO cloning plasmid (Invitrogen) according to manufacturer's instructions. Competent *E. coli* cells were transformed with ligated plasmid at 42°C for 30 sec, allowed to recover in LB (250mM NaCl, 1% tryptone, 0.5% yeast extract, pH 7.0) for 1 h and plated on LB agar plates with appropriate antibiotic and incubated at 37°C overnight. Colonies were picked and cultured in 3ml LB overnight. Miniprep was performed on the cultures using a plasmid miniprep kit (Invitrogen) to purify plasmid DNA and sequenced to confirm for the correct DNA sequence before use.

3.9 Transfection

One day before transfection, 1.5×10^6 HEK293T cells were seeded per well in DMEM (Gibco) containing 10% FBS without antibiotics in a 6-well plate. On the day of transfection, 4µg of DNA (2µg of miRNA plasmid and 2µg of Ago-overexpressing plasmid) was transfected per well using 10µl of Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The cells were collected after incubation at 37°C for 24 h for protein and RNA extraction.

3.10 Northern blotting

Total RNA was extracted from cells using TRIzol reagent (Ambion). 10µg of total RNA was mixed with equal volumes of 2x RNA loading buffer (Ambion), denatured by heating to 95°C for 5 min and loaded per well on a 15% denaturing polyacrylamide urea gel alongside radioactively labelled Decade marker (Ambion) or a pre-stained small RNA ladder (BioDynamics) after pre-running the gel for 30 min. The gel was ran in 0.5x TBE (44.5mM Tris-borate, 44.5mM boric acid, 1mM EDTA) at 300V for 3 h. The gel was then transferred onto a Hybond-N+ nylon membrane (Amersham) in a semi-dry transfer apparatus at 0.30mA for 1 h. The membrane was crosslinked in a 312nm UV crosslinker and prehybridized with hybridization buffer (3x SSC (900mM NaCl, 90mM sodium citrate), 1mM EDTA, 2x Denhardt's solution (0.04% Ficoll 400, 0.04% polyvinylpyrrolidone, 0.04% BSA), 1% SDS, 2% dextran sulfate, 30µg/ml ssDNA) at

45°C for 1 h. DNA oligo probe was radioactively labelled with 6000Ci/mol γ -ATP using T4 PNK at 37°C for 1 h, then cleaned using a G-25 spin column (GE healthcare). The labelled probe was then added into the hybridization buffer and incubated overnight at 45°C. The membrane was then washed thrice with wash buffer (2x SSC (600mM NaCl, 60mM sodium citrate), 0.1% SDS) at 45°C, then exposed to a phosphorimaging screen and scanned using a phosphorimager.

3.11 Western blotting

After washing twice in ice-cold 1x PBS (10mM Na₂HPO₄, 1.8mM KH₂PO₄, 137mM NaCl, 2.7mM KCl), cells were resuspended in ice-cold 1x RIPA buffer (1x PBS, 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40 with Complete EDTA-free protease inhibitor (Roche)) and incubated on ice for 30 min. Cells were lysed by passing the solution through a 25G needle 4 times, then cleared by centrifugation at top speed on a tabletop centrifuge for 10 min at 4°C. Protein concentration was measured using Bradford assay. 10 μ g of protein was mixed with 5x protein loading dye (4% SDS, 250mM Tris pH 6.8, 16% β -mercaptoethanol, 30% glycerol, 0.006% bromophenol blue), denatured at 95°C for 10 min and loaded per well together with prestained protein marker (Bio-rad) on a 6% separating polyacrylamide gel with 4% stacking gel. The gel was run in SDS-PAGE running buffer (3% Tris base, 14.4% glycine, 1% SDS) at 120V for 1 h until the dye front reached the bottom of the plate. The gel was then transferred onto a methanol-activated PVDF membrane in CAPS buffer (10mM CAPS pH 11.0, 0.05% SDS, 20% methanol) in a wet transfer apparatus at 300mA for 1.5 h at 4°C. The membrane was then blocked in 5% skim milk for 1 h, washed thrice with 1x TBST (20mM Tris pH 7.4, 0.138M NaCl, 0.1% Tween 20) for 15 min each, then incubated with primary antibody (anti-myc, Wako; anti-FLAG, Wako) at 1:1000 dilution in 1x TBST at 4°C overnight on a rotator. The membrane was then washed thrice with 1x TBST for 15 min each and incubated with secondary HRP antibody at 1:5000 dilution in 5% skim milk for 1 h at room temperature. The membrane was then rinsed thrice with 1x TBST for 15 min each and exposed with ECL western blotting detection reagent (GE healthcare).

3.12 Immunostaining

Cells on coverslips were washed once with PBS and fixed with 5.3% PFA for 15 min, then washed thrice with PBX (1x PBS, 0.2% Triton X-100). Fixed cells were then blocked with 5% Normal Goat Serum (NGS; Sigma) in PBX for 30 min and washed once with PBX, followed by incubation with rabbit anti-myc primary antibody (Abcam) with 1:500 dilution in 5% NGS in PBX for 1 h at room temperature. Cells were washed thrice with PBX and then incubated with Alexa Fluor 488 anti-rabbit secondary antibody with 1:500 dilution in 5% NGS in PBX for 1 h at room temperature. Cells were washed thrice using PBX, stained with DAPI with 1:500 dilution in PBX for 3 min, washed once with PBX and twice with PBS, then mounted onto glass slides using vectashield.

3.13 Neurite outgrowth assay

DsRed empty vector or dsRed miR-124 vector and EGFP or myc-mAgo2 vector was transfected in N2a cells as previously described. Following transfection, cells were incubated for 6 h, then dissociated and plated onto glass coverslips coated with poly-L-lysine with a 1:10 ratio and incubated for 48 h after transfection. Cells were then washed with PBS and immunostained with rabbit anti-myc antibodies (Abcam). Coverslips were mounted onto glass slides and imaged on a confocal microscope (Olympus FV3000). Images were processed using Imaris filament tracer function on 570nm (dsRed) channel. Average total neurite length per cell was calculated by summation of all drawn filaments on the field, divided by the number of dsRed-expressing cells detected on the 570nm channel using DAPI staining as an indication of nuclei number.

3.14 Plasmids used in the study

Name	Vector description	Citation
pcDNA-DGCR8hp	CMV promoter-driven DGCR8hp expression (derived from TOPO cloning of DGCR8 hp into pcDNA6.2-EmGFP-TOPO (Invitrogen)) (constructed by Zhou Li)	
pcDNA-DGCR8hp mut 1	CMV promoter-driven DGCR8hp mut 1 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut1_F and DGCR8_mut1_R primers)	
pcDNA-DGCR8hp mut 2	CMV promoter-driven DGCR8hp mut 2 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut2_F and DGCR8_mut2_R primers)	
pcDNA-DGCR8hp mut 3	CMV promoter-driven DGCR8hp mut 3 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut3_F and DGCR8_mut3_R primers)	
pcDNA-DGCR8hp mut 4	CMV promoter-driven DGCR8hp mut 4 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut4_F and DGCR8_mut4_R primers)	
pcDNA-DGCR8hp mut 5	CMV promoter-driven DGCR8hp mut 5 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut5_F and DGCR8_mut5_R	

	primers)	
pcDNA-DGCR8hp mut 6	CMV promoter-driven DGCR8hp mut 6 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut6_F and DGCR8_mut6_R primers)	
pcDNA-DGCR8hp mut 7	CMV promoter-driven DGCR8hp mut 7 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut7_F and DGCR8_mut7_R primers)	
pcDNA-DGCR8hp mut 8	CMV promoter-driven DGCR8hp mut 8 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut8_F and DGCR8_mut8_R primers)	
pcDNA-DGCR8hp mut 9	CMV promoter-driven DGCR8hp mut 9 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut9_F and DGCR8_mut9_R primers)	
pcDNA-DGCR8hp mut 10	CMV promoter-driven DGCR8hp mut 10 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut10_F and DGCR8_mut10_R primers)	
pcDNA-DGCR8hp mut 11	CMV promoter-driven DGCR8hp mut 11 expression (derived from site-directed mutagenesis of pcDNA-DGCR8hp using DGCR8_mut11_F and DGCR8_mut11_R primers)	

pcDNA-Pashahp(490bp)	CMV promoter-driven Pashahp expression (derived from TOPO cloning of a 490bp fragment containing Pasha hp into pcDNA6.2-EmGFP-TOPO (Invitrogen)) (constructed by Zhou Li)	
pcDNA-Pashahp(540bp)	CMV promoter-driven Pashahp expression (derived from TOPO cloning of a 540bp fragment containing Pasha hp into pcDNA6.2-EmGFP-TOPO (Invitrogen)) (constructed by Zhou Li)	
pUAS-dsRed-DGCR8hp	UAS promoter-driven DGCR8hp expression (derived from cloning of DGCR8 hp into pUAS-dsRed) (constructed by Zhou Li)	
pUAS-dsRed-Pashahp(490bp)	UAS promoter-driven Pashahp expression (derived from cloning of a 490bp fragment containing Pasha hp into pUAS-dsRed) (constructed by Zhou Li)	
pUAS-dsRed-Pashahp(540bp)	UAS promoter-driven Pashahp expression (derived from cloning of a 490bp fragment containing Pasha hp into pUAS-dsRed) (constructed by Zhou Li)	
pET-Dest-Exp5	IPTG-inducible expression of 6xHis-tagged dme-Exp-5 (derived from cloning of dme-Exp-5 into pET-DEST42 (Life Technologies)) (constructed by Zhou Li)	
pET-RanQ(69)L	IPTG-inducible expression of GST-tagged dme-RanQ(69)L (derived from cloning of dme-RanQ(69)L into pDEST15 (Life Technologies)) (constructed by Zhou Li)	
pMirlib let-7a-1	CMV promoter-driven hsa-let-7a-1 expression (Origene)	

pMirlib miR-1	CMV promoter-driven hsa-miR-1 expression (Origene)	
pMirlib miR-19a	CMV promoter-driven hsa-miR-19a expression (Origene)	
pMirlib miR-26a-1	CMV promoter-driven hsa-miR-26a-1 expression (Origene)	
pMirlib miR-27b	CMV promoter-driven hsa-miR-27b expression (Origene)	
pMirlib miR-34a	CMV promoter-driven hsa-miR-34a expression (Origene)	
pMirlib miR-92a	CMV promoter-driven hsa-miR-92 expression (Origene)	
pMirlib miR-133a	CMV promoter-driven hsa-miR-133a expression (Origene)	
pMirlib miR-206	CMV promoter-driven hsa-miR-206 expression (Origene)	
pcDNA6-miR-16	CMV promoter-driven hsa-miR-16 expression (derived from TOPO cloning of miR-16 amplified by hsa_miR-16_F1 and hsa_miR-16_R1 primers into TOPO cloning site of pcDNA6.2-EmGFP-TOPO (Invitrogen))	
pcDNA6-miR-28	CMV promoter-driven hsa-miR-28 expression (derived from TOPO cloning of miR-28 amplified by hsa_miR-28_F1 and hsa_miR-28_R1 primers into TOPO cloning site of pcDNA6.2-EmGFP-TOPO (Invitrogen))	
pcDNA6-miR-30a	CMV promoter-driven hsa-miR-30a expression (derived from TOPO cloning of miR-30a amplified by hsa_miR-30a_F2 and	

	hsa_miR-30a_R1 cloned into pcDNA6.2-EmGFP-TOPO (Invitrogen))	
RI vector	CMV promoter-driven dsRed2 expression	(Makeyev, Zhang et al. 2007)
RIPmiR-9	CMV promoter-driven dsRed2 and hsa-miR-9-2 expression, with miR-9 inserted into intron of dsRed2 gene	(Makeyev, Zhang et al. 2007)
RIPmiR-124	CMV promoter-driven dsRed2 and hsa-miR-124-2 expression, with miR-124 inserted into intron of dsRed2 gene	(Makeyev, Zhang et al. 2007)
pEM590	CMV promoter-driven 3x myc-tagged hAGO1	(Juvvuna, Khandelia et al. 2012)
pcDNA3-3xMyc-AGO2	CMV promoter-driven 3x myc-tagged hAGO2	(Karginov, Conaco et al. 2007)
pEM694	CMV promoter-driven 3x myc-tagged hAGO2D597A (hAgo2 slicer mutant)	(Juvvuna, Khandelia et al. 2012)
pEM1125	CAG promoter-driven 3x myc-tagged hAGO3	
pEM697	CAG promoter-driven 3x myc-tagged hAGO4	(Juvvuna, Khandelia et al. 2012)
pEM1143	CAG promoter-driven 3x myc-tagged hDicer	
pEM899	CMV promoter-driven 3x myc-tagged mAgo1	(Juvvuna, Khandelia et al. 2012)
pEM900	CMV promoter-driven 3x myc-tagged mAgo2	(Juvvuna, Khandelia et al. 2012)
pEM957	CMV promoter-driven 3x myc-tagged mAgo3	(Juvvuna, Khandelia et al. 2012)
pEM960	CMV promoter-driven 3x myc-tagged mAgo4	(Juvvuna, Khandelia et al. 2012)
pcDNA6-EGFP	CMV promoter-driven expression of EmGFP (derived from circularization of pcDNA6.2-	

	EmGFP-TOPO cloning plasmid (Invitrogen))	
pUb-Gal4	Ubiquitin promoter-driven Gal4 expression as driver for expression from UAS promoter	
pUAS-dsRed	UAS promoter-driven dsRed expression	(Brennecke, Hipfner et al. 2003)
pUAS-miR-6	UAS promoter-driven dme-miR-6 expression	
pUAS-miR-10	UAS promoter-driven dme-miR-10 expression	
pUAS-miR-124	UAS promoter-driven dme-miR-124 expression (derived from cloning of miR-124 amplified by dme_miR-124_F1 and dme_miR-124_R1 primers into pUAS-dsRed using NotI and XhoI site)	
pRH 3xFLAG EGFP	MT promoter-driven Cu ²⁺ inducible expression of EGFP	(Saito, Ishizuka et al. 2005)
pRH 3xFLAG Ago1wt	MT promoter-driven Cu ²⁺ inducible expression of wild type dme-Ago1	(Miyoshi, Okada et al. 2009)
pRH 3xFLAG Ago1F2V2	MT promoter-driven Cu ²⁺ inducible expression of dme-Ago1 MID domain mutant	(Miyoshi, Okada et al. 2009)
pRH 3xFLAG Ago1PAZ	MT promoter-driven Cu ²⁺ inducible expression of dme-Ago1 PAZ domain mutant	(Miyoshi, Okada et al. 2009)
pRmHa-FLAG-Dcr-1	MT promoter-driven Cu ²⁺ inducible expression of dme-Dcr1	(Saito, Ishizuka et al. 2005)

Table 3.14: Plasmids used in the study

3.15 Oligos used in the study

Oligo name	Sequence 5' → 3'
<u>DNA northern probes</u>	
anti_miR-124a	TGGCATTACACGCGTGCCTTAA
anti_let-7a-1-5p	AACTATACAACCTACTACCTCA
anti_miR-1-3p	ATACATACTTCTTTACATTCCA
anti_miR-9-5p	TCATACAGCTAGATAACCAAAGA
anti_miR-15a-5p	CACAAACCATTATGTGCTGCTA
anti_miR-16-1-5p	CGCCAATATTTACGTGCTGCTA
anti_miR-17-5p	CTACCTGCACTGTAAGCACTTG
anti_miR-19a-3p	TCAGTTTTCATAGATTTCACACA
anti_miR-26a-5p	AGCCTATCCTGGATTACTTGAA
anti_miR-27b-5p	GTTACCAATCAGCTAAGCTCT
anti_miR-27b-3p	GCAGAACTTAGCCACTGTGAA
anti_miR-28-5p	CTCAATAGACTGTGAGCTCCTT
anti_miR-28-3p	TCCAGGAGCTCACAATCTAGTG
anti_miR-30a-5p	CTTCCAGTCGAGGATGTTTACA
anti_miR-30a-3p	GCTGCAAACATCCGACTGAAAG
anti_miR-34a-5p	TGGCAGTGCTTAGCTGGTTGT
anti_miR-92a-3p	ACAGGCCGGGACAAGTGCAATA
anti_miR-133a-3p	CAGCTGGTTGAAGGGGACCAAA
anti_miR-206-3p	CCACACACTTCCTTACATTCCA
anti_U6	GCCATGCTAATCTTCTCTGTATC
<u>LNA northern probes</u>	
anti_dme_miR-6	AAAAAGAACAGCCACTGTGATA
anti_dme_miR-10	AAACCTCTCTAGAACCGAATTTG
anti_dme_miR-124a	TGGCATTACACGCGTGCCTTAA
<u>Cloning primers</u>	
hsa_miR-16_F1	ACAAGGATCTGATCTTCTGAAG
hsa_miR-16_R1	AGACTATCAATAAAACTGAAAACACA
hsa_miR-28_F1	TGTCTATTTCAGCAAGCACTTATTAAG
hsa_miR-28_R1	TCAGAGCAGTTAACAGATGTGA
hsa_miR-30a_F2	CACCGCATACACAGAGCACCTCCT
hsa_miR-30a_R1	TGCCTGCACATCTTGGAAC
dme_miR-124_F1	AAAGCGCCGCCGTTTCACTTTCGTTCCGGT
dme_miR-124_R1	AAACTCGAGTGTTACAATGATTCCACATAAGT
<u>Hairpin sequencing</u>	
RA3	rAppNNTGGAATTCTCGGGTGCCAAGGddC
RA5	GUUCAGAGUUCUACAGUCCGACGAUCNN
RTP	GCCTTGGCACCCGAGAATTCCA
RA5_F	G TTCAGAGTTCTACAGTCCGACGATC
<u>Mutagenesis primers</u>	
DGCR8_mut1_F	GCTGTCTACATTAATGGAAATCGCAATGTGGCCAGCTTG
DGCR8_mut1_R	CAAGCTGGCCACATTGCGATTTCCATTAATGTAGACAGC
DGCR8_mut2_F	GGTTCTAAAAGCTGTCTACATTATTGGAAAGAGCAATGTGGCCAGCTTG
DGCR8_mut2_R	CAAGCTGGCCACATTGCTCTTTCCAATAATGTAGACAGCTTTTAGAACC
DGCR8_mut3_F	CTAAAAGCTGTCTACATTATTGAAATCGCAATGTGGCCAGCTTG
DGCR8_mut3_R	CAAGCTGGCCACATTGCGATTTTCAATAATGTAGACAGCTTTTAG
DGCR8_mut4_F	GGTTCTAAAAGCTGTCTCAATTAATGGAAAGAGCAATGTGGCCAGCTTG
DGCR8_mut4_R	CAAGCTGGCCACATTGCTCTTTCCATTAATGTAGACAGCTTTTAGAACC
DGCR8_mut5_F	GGTTCTAAAAGCTGTCTCAATTAATGGAAATCGCAATGTGGCCAGCTTG

DGCR8_mut5_R	CAAGCTGGCCACATTGCGATTTTCATTAATTGAGACAGCTTTTAGAACC
DGCR8_mut6_F	CTAAAAGCTGTCTACATTATTGGAAATCGCAATGTGGCCAGCTTG
DGCR8_mut6_R	CAAGCTGGCCACATTGCGATTTCCAATAATGTAGACAGCTTTTAG
DGCR8_mut7_F	GGTTCTAAAAGCTGTCTCAATTATTGGAAATCGCAATGTGGCCAGCTTG
DGCR8_mut7_R	CAAGCTGGCCACATTGCGATTTCCAATAATGTAGACAGCTTTTAGAACC
DGCR8_mut8_F	GTCTACATTAATGAAAATCGCAATGTGGCCAGCTTG
DGCR8_mut8_R	CAAGCTGGCCACATTGCGATTTTCATTAATGTAGAC
DGCR8_mut9_F	CTAAAAGCTGTCTACATTAATGGAAAAGAGCAATGTGGCCAGCTTG
DGCR8_mut9_R	CAAGCTGGCCACATTGCTCTTTCCATTAATGTAGACAGCTTTTAG
DGCR8_mut10_F	CTAAAAGCTGTCTACATTATTGAAAAGAGCAATGTGGC
DGCR8_mut10_R	GCCACATTGCTCTTTTCAATAATGTAGACAGCTTTTAG
DGCR8_mut11_F	GTGGTTCTAAAAGCTGTCTCAATTAATGAAAAGAGCAATG
DGCR8_mut11_R	CATTGCTCTTTTCATTAATTGAGACAGCTTTTAGAACCAC
In vitro dicing assay	
hsa_miR-9-2_hp	UCUUUGGUUAUCUAGCUGUAUGAGUGUAUUGGUCUUCAUAAAAGCUAGAUAA CCGAAAAGUA
hsa-miR-26a-1	UUCAAGUAAUCCAGGAUAGGCUGUGCAGGUCCCAAUGGGCCUUAUUCUUGGU UACUUGCACG
hsa-miR-30a_hp	UGUAAACAUCUCGACUGGAAGCUGUGAAGCCACAGAUGGGCUUUCAGUCG GAUGUUUGCAGC
Hsa_miR-124-2- 5p+loop+2nt	CGUGUUCACAGCGGACCUUGAUUUAAUGUCAUACAAUUA
Hsa_miR-124-2-3p-2nt_B	AGGCACGCGGUGAAUGCCAA
Hsa_miR-124-2_bridge_B	TTGGCATTACCGCGTGCCTTAATTGTATGACATTAAATCAAGGTCCGCTG TGAACACG
hsa_miR-124_mut1	GUCAUUCACUGAGUGCCUUAUUAAUAAUGUCCAUACGUUAAGGCACGCGG UGAAUGCCAA
hsa-miR-124_mut2_A	CGUGUUCACAGCGGACCUUGAUGUGCAGGUCCCAAUGAUUA
hsa_miR-124_mut2_bridge	TTGGCATTACCGCGTGCCTTAATCATTGGGACCTGCACATCAAGGTCCGC TGTGAACACG
hsa_miR-124_mut3	CGUGAGUACAGCGGACCUUGAUUUAAUGUCAUACAAUUAAGGCACGCGGUA CUUGCACG
hsa_miR-124_mut4	CGUGUUCAAGCAAACCCUUGAUUUAAUGUCAUACAAUUAAGGUCUUGGUUG AAUGCCAA
hsa_miR-124_mut5	CGUGUUCACAGCGGAAAUAGGCCUAAUGUCAUACGGCUUAUUCACGCGGUG AAUGCCAA

Table 3.15: Oligo sequences used in the study

4 Results

4.1 Exp-5 substrate recognition project

4.1.1 The 60nt Drosha cleavage fragment from DGCR8 hairpin A is detectable in the nuclear fraction of HeLa cells

In order to study Exp-5 recognition of pre-miRNA hairpins, we decided to utilize a reported pre-miRNA-like hairpin which did not appear to be recognized and exported by Exp-5. The mRNA of DGCR8 has been reported to contain two pre-miRNA-like hairpin structures in its 5' UTR and near the start codon, named hairpin A and hairpin B, respectively (Pedersen, Bejerano et al. 2006, Han, Pedersen et al. 2009) (figure 4.1.1.1) which can be cleaved from the DGCR8 mRNA transcript by Drosha (Han, Pedersen et al. 2009). In vitro Drosha cleavage of DGCR8 hairpin A occurs at two distinct cleavage sites on the stem of hairpin A, generating a 76nt and 60nt fragment of hairpin A, whereas Drosha cleavage of DGCR8 hairpin B occurs at a single cleavage site on the stem of hairpin B, generating a 60nt fragment of hairpin B. Experimentally, only the 60nt Drosha cleavage fragment from DGCR8 hairpin A is detectable by northern blotting, and has been shown to be confined in the nuclear fraction but not the cytoplasmic fraction. Drosha cleavage of these two hairpins in DGCR8 mRNA has been reported to lead to destabilization of the DGCR8 hairpin transcript, leading to decreased DGCR8 protein production. The decrease in DGCR8 protein leads to decreased DGCR8 protein available to undergo stabilizing protein-protein interactions with Drosha protein, leading to crossregulation of Microprocessor components. Additionally, these hairpin structures also appear to be present in Pasha, the Drosophila homolog of DGCR8, although the sequences do not appear to be conserved (Han, Pedersen et al. 2009).

Figure 4.1.1.1: Position of DGCR8 hairpin A and B on DGCR8 mRNA.

DGCR8 hairpin A and hairpin B, as well as Pasha hairpin A and hairpin B, were cloned into fly and mammalian expression vectors. S2R+ and HeLa cells were then transfected with plasmids expressing DGCR8 hairpin A and hairpin B, as well as Pasha hairpin A and hairpin B. The nuclear and cytoplasmic fractions of transfected S2R+ and HeLa cells were then obtained by subcellular fractionation. Neither DGCR8 hairpin A nor DGCR8 hairpin B could be detected in S2R+ cells (figure 4.1.1.2A and B). Similar to Han et al.'s previously published study (Han, Pedersen et al. 2009), the 60nt Drosha cleavage product of DGCR8 hairpin A, but not the 76nt Drosha cleavage product of DGCR8 hairpin A, could be detected in HeLa cells by northern blotting, and this 60nt Drosha cleavage product of DGCR8 hairpin A was present in the nuclear fraction but not the cytoplasmic fraction (figure 4.1.1.2A). In comparison, pre-miRNA of miR-34a could be detected in both the cytoplasmic and nuclear fraction of HeLa cells (figure 4.1.1.2E), suggesting that while conventional pre-miRNA is recognized and exported from the nucleus into the cytoplasm by Exp-5, the 60nt Drosha cleavage fragment of DGCR8 hairpin A is not recognized and exported from the nucleus into the cytoplasm by Exp-5 and hence is contained in the nuclear fraction, or is recognized and exported by Exp-5 into the cytoplasm but is rapidly degraded and hence undetectable in the cytoplasmic fraction by northern blotting. The expected 60nt Drosha cleavage product of DGCR8 hairpin B could not be detected in HeLa cells (figure 4.1.1.2B), possibly due to it being a poorer substrate of Drosha, or due to it being unstable in the cell, hence is undetectable by northern blotting.

Pasha hairpin A and hairpin B were also expressed in S2R⁺ cells and HeLa cells. Neither Pasha hairpin A nor hairpin B were able to be properly expressed in HeLa cells. However, in S2R⁺ cells, probing with Pasha hairpin A and Pasha hairpin B did not yield a clear 60nt band which was the expected product from Drosha cleavage of the Pasha hairpin (figure 4.1.1.2C and D). Like DGCR8 hairpin B, the Drosha cleavage product from Pasha hairpin A and hairpin B cleavage could be unstable and hence could not be detected by northern blotting.

Figure 4.1.1.2: Expression of DGCR8 hairpin A and B, Pasha hairpin A and B and miR-34a in S2R+ and HeLa cells. (A) Northern blot showing subcellular fractionation of expressed DGCR8 hairpin A construct in S2R+ and HeLa cells. (B) Northern blot showing subcellular fractionation of expressed DGCR8 hairpin B construct in S2R+ and HeLa cells. (C) Northern blot showing subcellular fractionation of expressed Pasha hairpin A construct in S2R+ and HeLa cells. (D) Northern blot showing subcellular fractionation of expressed Pasha hairpin B construct in S2R+ and HeLa cells. (E) Northern blot showing subcellular fractionation of expressed miR-34a construct in S2R+ and HeLa cells.

4.1.2 Design of DGCR8 hairpin A mutants with removed bulge regions for the study of Exp-5 substrate recognition

Since the 60nt Drosha cleavage product of DGCR8 hairpin A is detectable only in the nuclear fraction of cells, it was hypothesized that the cleaved hairpin was either quickly degraded to undetectable levels in the cytoplasm, or not recognized and exported by Exp-5 into the cytoplasm. Assuming that the stability of DGCR8 hairpin A is similar in both the nucleus and cytoplasm, it is intriguing that DGCR8 hairpin A is not recognized and exported into the cytoplasm by Exp-5 despite being a Drosha cleavage product, since the preferred substrates of Exp-5 have been described to resemble Drosha processing products, with an optimal hairpin stem length similar to that of pre-miRNAs (Lund, Guttinger et al. 2004, Zeng and Cullen 2004).

We decided to investigate the characteristics of DGCR8 hairpin A which led to its inability to be recognized and exported by Exp-5 by creating DGCR8 hairpin A mutants. Comparing the hairpin structure of DGCR8 hairpin A to that of other conventional miRNAs showed that wild type DGCR8 hairpin A contained 4 mismatch or bulge regions along the stem region of the hairpin, more than the mismatch or bulge regions on other conventional miRNAs. We hypothesized that the higher number of mismatches in DGCR8 hairpin A could be a reason for its inability to be recognized and exported by Exp-5. We decided to remove some of the bulge regions on the DGCR8 hairpin by

mutating the nucleotides on the 3p arm of DGCR8 hairpin A to change the bulge regions into complementary base-paired regions by site-directed mutagenesis. Bulges to be converted into complementary base-paired regions were counted from the Drosha cleavage site on the wild type DGCR8 hairpin A. DGCR8 hairpin A mutants 1-5 had two bulge regions converted into complementary base-paired regions. DGCR8 hairpin A mutant 1 had the first and second bulges converted into complementary base-pairs, mutant 2 had the second and third bulges converted into complementary base-pairs, mutant 3 had the first and third bulges converted into complementary base-pairs, mutant 4 had the second and fourth bulges converted into complementary base-pairs, and mutant 5 had the first and fourth bulges converted into complementary base-pairs. This was made to mimic different pre-miRNA hairpins which had two bulge regions along the length of the stem region of the hairpin. DGCR8 hairpin A mutant 6 had the first, second and third bulges converted to complementary base-pairs, leaving one bulge on the stem of the hairpin, whereas mutant 7 had all bulges converted into complementary base-pairs, creating a perfectly complementary stem-loop structure with no mismatches (figure 4.1.2.1). We hypothesized that DGCR8 hairpin A mutants 6 and 7 would be most easily recognized and exported by Exp-5 into the cytoplasm.

To test which of the mismatched positions on DGCR8 hairpin A are important for Exp-5 recognition and export, four other DGCR8 hairpin A mutants were created by converting the first, second, third and fourth bulge regions into complementary base-pairs to leave three bulge regions on the stem region of the hairpin, called mutants 7-11 respectively (figure 4.1.2.1).

DGCR Hairpin A wild type										DGCR Hairpin A mut 6									
5'-ac	AG	GA	C	A	UG	GGUU				5'-ac	AG			UG	GGUU				
	uUAAGCUG	UGCAUUGU	UUU	CA	UAAU	AGGCAGU	C				uUAAGCUG	UGCAUUGU	GAUUUCCA	UAAU	AGGCAGU	C			
	aGUUCGAC	GUGUAACG	AAA	GU	AUUA	UCUGUCG	U				aGUUCGAC	GUGUAACG	CUAAAGGU	UAUUA	UCUGUCG	U			
3'-uc	CG	AG	A	A	CA	AAAA				3'-uc	CG			CA	AAAA				
DGCR8 Hairpin A mut 1										DGCR Hairpin A mut 7									
5'-ac	AG			A	UG	GGUU				5'-ac	AG				GGUU				
	uUAAGCUG	UGCAUUGU	GAUUUCCA	UAAU	AGGCAGU	C					uUAAGCUG	UGCAUUGU	GAUUUCCA	UAAU	AGGCAGU	C			
	aGUUCGAC	GUGUAACG	CUAAAGGU	AUUA	UCUGUCG	U					aGUUCGAC	GUGUAACG	CUAAAGGU	UAUUA	UCUGUCG	U			
3'-uc	CG			A	CA	AAAA				3'-uc	CG				AAAA				
DGCR Hairpin A mut 2										DGCR8 Hairpin A mut 8									
5'-ac	AG	GA			UG	GGUU				5'-ac	AG		C	A	UG	GGUU			
	uUAAGCUG	UGCAUUGU	UUUCCA	UAAU	AGGCAGU	C					uUAAGCUG	UGCAUUGU	GAUUU	CA	UAAU	AGGCAGU	C		
	aGUUCGAC	GUGUAACG	AAAGGU	UAUUA	UCUGUCG	U					aGUUCGAC	GUGUAACG	CUAAA	GU	AUUA	UCUGUCG	U		
3'-uc	CG	AG			CA	AAAA				3'-uc	CG			A	A	CA	AAAA		
DGCR Hairpin A mut 3										DGCR Hairpin A mut 9									
5'-ac	AG		C		UG	GGUU				5'-ac	AG	GA		A	UG	GGUU			
	uUAAGCUG	UGCAUUGU	GAUUU	CAUAAU	AGGCAGU	C					uUAAGCUG	UGCAUUGU	UUUCCA	UAAU	AGGCAGU	C			
	aGUUCGAC	GUGUAACG	CUAAA	GUUAUUA	UCUGUCG	U					aGUUCGAC	GUGUAACG	AAAGGU	AUUA	UCUGUCG	U			
3'-uc	CG		A		CA	AAAA				3'-uc	CG	AG		A	CA	AAAA			
DGCR Hairpin A mut 4										DGCR Hairpin A mut 10									
5'-ac	AG	GA		A		GGUU				5'-ac	AG	GA	C		UG	GGUU			
	uUAAGCUG	UGCAUUGU	UUUCCA	UAAU	UGAGGCAGU	C					uUAAGCUG	UGCAUUGU	UUU	CAUAAU	AGGCAGU	C			
	aGUUCGAC	GUGUAACG	AAAGGU	AUUA	ACUCUGUCG	U					aGUUCGAC	GUGUAACG	AAA	GUUAUUA	UCUGUCG	U			
3'-uc	CG	AG		A		AAAA				3'-uc	CG	AG	A		CA	AAAA			
DGCR Hairpin A mut 5										DGCR Hairpin A mut 11									
5'-ac	AG		C	A		GGUU				5'-ac	AG	GA	C	A		GGUU			
	uUAAGCUG	UGCAUUGU	GAUUU	CA	UAAU	UGAGGCAGU	C				uUAAGCUG	UGCAUUGU	UUU	CA	UAAU	UGAGGCAGU	C		
	aGUUCGAC	GUGUAACG	CUAAA	GU	AUUA	ACUCUGUCG	U				aGUUCGAC	GUGUAACG	AAA	GU	AUUA	ACUCUGUCG	U		
3'-uc	CG		A	A		AAAA				3'-uc	CG	AG	A	A		AAAA			

Figure 4.1.2.1: Wild type hairpin sequence and structure of DGCR8 hairpin A and mutant hairpin sequence and structure for DGCR8 hairpin A mutants 1-11. Black nucleotides indicate unmutated nucleotides from wild type DGCR8 hairpin A. Green nucleotides indicate mutated nucleotides in DGCR8 hairpin A mutants.

HeLa cells were transfected with DGCR8 hairpin A wild type and mutant 1-11 plasmids and the cells were then fractionated to yield nuclear and cytoplasmic fractions. Northern blotting was then performed with a probe complementary to the 5p arm of DGCR8 hairpin A. As observed previously, wild type DGCR8 hairpin A produces a 60nt Drosha cleavage product which can be detected only in the nuclear fraction but not the cytoplasmic fraction.

Of the four DGCR8 hairpin A mutants with only one bulge region converted into a complementary base-paired region (mutants 8-11), only mutant 10 which had the bulge region at position 10 from the expected Drosha cleavage site appeared to retain the wild type DGCR8 hairpin A Drosha cleavage site and also showed detectable DGCR8 hairpin cleavage product in both the nuclear fraction and the cytoplasmic fraction (figure 4.1.2.2C). Hence, it could be that the mismatches at the central region corresponding to 9-12nt from the Drosha cleavage site of the DGCR8 hairpin A could not only decrease the efficiency of Drosha processing during Drosha limiting conditions (Sperber, Beem et al. 2014), it could also be inhibitory for Exp-5 recognition and export into the cytoplasm.

As more of the bulge regions on the DGCR8 hairpin A were converted into complementary base-pairing regions, there appeared to be a less distinct cleavage product. Instead, there appeared to be multiple products of varying sizes from 40nt to 80nt which were detectable in the nuclear fraction which could be seen for mutants 2 to 7 (figure 4.1.2.2A and B). It could be possible that the decrease in the number of bulge regions on the stem of the hairpin could lead to the different-sized products formed from Drosha cleavage. Although current consensus on Drosha processing states that the Drosha cleavage site is determined by the basal ssRNA, terminal loop and internal loop regions (Zeng, Yi et al. 2005, Han, Lee et al. 2006, Zhang and Zeng 2010, Auyeung, Ulitsky et al. 2013, Burke, Kelenis et al. 2014), this does not explain how the removal of bulge regions on the stem of the hairpin can cause Drosha to cut at multiple positions on the DGCR8 hairpin A mutant stem region to generate multiple cleavage products of varying sizes. The presence of mismatches at positions 5 and 9-12 from the Drosha cleavage site has been reported to decrease the efficiency of Drosha processing during

Drosha limiting conditions (Sperber, Beem et al. 2014), hence it is expected that the removal of the central bulge regions on DGCR8 hairpin A may aid to increase the efficiency of Drosha cleavage, although it is unknown what effect it has on Drosha cleavage site selection. Another possible reason could be that the Drosha cleavage product could be unstable in the cell and cleaved by other ribonucleases, generating the multiple DGCR8 hairpin A products with different sizes.

Additionally, the DGCR8 hairpin A mutants with two or more bulge regions converted into complementary base-pairing regions appeared to show a ~20nt product formation which was detected by northern blotting (figure 4.1.2.2A and B). This ~20nt product was not observed for DGCR8 hairpin A mutants with only one bulge region converted into a complementary base-paired region (figure 4.1.2.2C). We hypothesize that this ~20nt product could be the mature miRNA-like product which was formed after the export of the DGCR8 hairpin A Drosha cleavage fragment by Exp-5 from the nucleus into the cytoplasm, and then underwent subsequent Dicer processing to generate a ~20nt small RNA product similar to mature miRNAs. Hence, the formation of the ~20nt miRNA-like product could be an indication of the recognition and export of the DGCR8 hairpin A mutant hairpins by Exp-5. If so, it could be an indication that DGCR8 hairpin A could contain too many bulge regions for Exp-5 recognition and export into the cytoplasm, and could mean that Exp-5 may not be able to recognize pre-miRNA-like hairpins with a large amount of mismatch regions.

We found that a slightly longer form of DGCR8 hairpin A of about ~64nt was clearly in some mutants, clearly seen in DGCR8 hairpin A mutants 1, 3 and 8 (figure 4.1.2.2A and C). These mutants all had the first bulged region converted into complementary base-paired regions. Coincidentally, the Drosha cleavage site on the wild type DGCR8 hairpin A is situated at the first bulged region on the stem of the hairpin. Since deformable sites such as bulges and mismatches have been reported to be important in defining the Drosha cleavage site (Quarles, Sahu et al. 2013), it is likely that by mutating the first bulge region into complementary base-paired region, the Drosha cleavage site on the original wild type DGCR8 hairpin A had shifted from the first bulge region to another site

which was closer to the base of the hairpin. In the case of DGCR8 hairpin A mutant 8, this larger Drosha cleavage fragment appeared to be more efficiently exported from the nucleus into the cytoplasm by Exp-5 as compared to the 60nt wild type DGCR8 hairpin A Drosha cleavage fragment.

To more accurately determine the cleavage site for the DGCR8 hairpin A mutants, we attempted to perform an in vitro Drosha assay of the DGCR8 hairpin A. We first performed in vitro transcription of the transcript which contained the DGCR8 hairpin A and radiolabeled the transcript with ^{32}P - α -UTP. We then incubated the radiolabeled transcript with nuclear extract to examine Drosha cleavage of DGCR8 hairpin A. Unfortunately, we did not manage to successfully perform the Drosha cleavage assay as we did not obtain the expected Drosha cleavage products from the DGCR8 hairpin A transcript (figure not shown).

Figure 4.1.2.2: Expression of DGCR8 hairpin A wild type and mutants 1-11 in HeLa cells. (A) Northern blot showing subcellular fractionation of expressed DGCR8 hairpin A wild type and mutant 1-3 construct in HeLa cells. (B) Northern blot showing subcellular fractionation of expressed DGCR8 hairpin A mutant 4-7 construct in HeLa cells. (C) Northern blot showing subcellular fractionation of expressed DGCR8 hairpin A wild type and mutant 8-11 construct in HeLa cells.

4.1.3 Recombinant expression of His-tagged Exp-5 and GST-tagged Ran in *E. coli* for use in Electrophoretic Mobility Shift Assay (EMSA)

In order to study the pre-miRNA hairpin structures which allow Exp-5 substrate recognition independently from the prior hairpin processing by Drosha in vivo, we planned to perform an electrophoretic mobility shift assay (EMSA). By incubating different structural mutants of the cleaved DGCR8 hairpin A product with purified Exp-5 and Ran protein and allowing Exp-5 binding with Ran-GTP and the DGCR8 hairpin A mutants followed by addition of a competitor hairpin, the efficiency of binding between the DGCR8 hairpin A mutant substrates and Exp-5 protein complex can be investigated by performing gel electrophoresis to find out the proportion of bound and unbound Exp-5 complexes with the DGCR8 hairpin A.

The Exp-5 protein coding sequence was cloned into an inducible *E. coli* expression plasmid with a 6x His-tag and the Ran protein coding sequence was cloned into an inducible *E. coli* expression plasmid with a GST-tag. The two plasmids were then transformed into BL21 cells and grown in culture. Recombinant protein expression was then induced with IPTG and the cells sonicated and fractionated to separate them into a soluble supernatant fraction and a pellet fraction containing inclusion bodies. Before induction with IPTG, there was no expression of His-tagged Exp-5 in the *E. coli* cells. After induction with IPTG, a ~150kDa protein began to get expressed, corresponding to the size of 6x His-tagged Exp-5 (figure 4.1.3.1A). This ~150kDa protein was mainly found in the pelleted fraction, indicating that it was being aggregated in the inclusion

body in an insoluble form, which occurs during a majority of recombinant protein expression when the recombinant protein is expressed at a very high rate, saturating the cellular degradation system (Marston 1986). In comparison, induction with IPTG of GST-tagged Ran for 3 h showed accumulation of a ~50kDa protein in the pelleted fraction corresponding to the size of GST-tagged Ran protein, indicating that at this timepoint, the expressed recombinant protein was accumulated in the inclusion body in an insoluble form. When GST-Ran was further induced by IPTG for 6 h, the ~50kDa protein also appeared in the supernatant fraction (figure 4.1.3.1B), indicating that GST-Ran was also being produced in both a soluble form and an insoluble form in the *E. coli* cells.

GST-Ran expressing *E. coli* cells were induced with IPTG and sonicated, and the supernatant containing the soluble fraction was obtained. The GST-tagged Ran was then column purified using the GST-tag using glutathione beads, and the resultant purified protein eluted into seven fractions. Most of the GST-tagged Ran protein was eluted in fractions 2 to 4. Surprisingly, there was an accompanying 60nt band which was also eluted from the column (figure 4.1.3.1C).

For His-tagged Exp-5, due to the protein being accumulated in the inclusion body, the inclusion body needs to be purified and the recombinant protein subsequently refolded to obtain the correctly folded purified Exp-5 protein. Due to time constraints, we decided to drop the Exp-5 experiments in favour of the miR-124 Ago overexpression project.

Figure 4.1.3.1: Expression and purification of His-tagged Exp-5 and GST-tagged Ran in BL21 cells. (A) Expression of His-tagged Exp-5 in BL21 cells upon IPTG induction. (B) Expression of GST-tagged Ran in BL21 cells upon IPTG induction. (C) GST protein purification of GST-tagged Ran expressed in BL21 cells.

4.1.4 Determining Drosha cleavage sites of DGCR8 hairpin A and hairpin B

In order to perform EMSA with recombinant purified Exp-5 and Ran protein to study the structures of pre-miRNA-like hairpins which would allow Exp-5 substrate recognition and binding, the precise Drosha cleavage sites for DGCR8 hairpin A and hairpin B need to be correctly sequenced so that mutant hairpin structures can be correctly designed and produced. To determine the Drosha cleavage sites on these two hairpins, the DGCR8 hairpin A and hairpin B construct was expressed in HeLa cells. RNA was then extracted and RNAs of size 50-100nt was then purified after size separation by gel electrophoresis. 5' and 3' adaptor sequences were then ligated to the hairpins and gene-specific PCR was performed with a primer complementary to a site on the DGCR8 hairpin A or hairpin B and on the 5' or 3' adaptor sequence. The PCR products were cloned into vectors and sequenced to determine the primary Drosha cleavage fragment for DGCR8 hairpin A and hairpin B.

A total of 30 clones were picked for both the 5' and 3' ends of DGCR8 hairpin A and 20 clones for both the 5' and 3' ends of DGCR8 hairpin B. In the case of DGCR8 hairpin A, 21 out of 23 clones which mapped onto the DGCR8 hairpin A for the 5' end mapping were mapped to the previously published Drosha cleavage site on the 5p arm of the DGCR8 hairpin A, before the first GA bulge region (figure 4.1.4.1A), validating the previously published Drosha cleavage site mapping data (Han, Pedersen et al. 2009). In comparison, 2 out of 7 clones for the 3' end mapping of the Drosha cleavage site was mapped onto a position on the 3p arm of the DGCR8 hairpin A 2nt from the Drosha cleavage site on the 5p arm (figure 4.1.4.1A), producing a product with 2nt overhang at

the 3' end characteristic of cleavage by an RNaseIII enzyme. Interestingly, the 3p cleavage site on the DGCR8 hairpin A which was previously described was 1nt offset from our mapped 3p Drosha cleavage site, which would be expected to produce a product with a 3nt overhang at the 3' end (Han, Pedersen et al. 2009).

We also attempted to map the Drosha cleavage sites for the DGCR8 hairpin B, but did not find any clear cleavage sites by Drosha cleavage unlike that of DGCR8 hairpin A. Of the 5 clones which were picked for the 5p end and the 12 clones which were picked for the 3p end, no major cleavage site for Drosha could be determined and none of the clones validated the previously annotated Drosha cleavage site on DGCR8 hairpin B.

A DGCR8 Hairpin A

B DGCR8 Hairpin B

Figure 4.1.4.1: Determination of Drosha cleavage sites for DGCR8 hairpin A and hairpin B. (A) Drosha cleavage site mapping of DGCR8 hairpin A showing number of clones with cleavage site at that position. Number of clones is shown as a fraction whereby numerator corresponds to total number of clones at that cleavage site and denominator corresponds to total number of sequenced clones. The site marked by a box

corresponds to the primary 60nt cleavage site which was previously described (Han, Pedersen et al. 2009). (B) Drosha cleavage site mapping of DGCR8 hairpin B showing number of clones with cleavage site at that position. Number of clones is shown as a fraction whereby numerator corresponds to total number of clones at that cleavage site and denominator corresponds to total number of sequenced clones.

4.1.5 Exp-5 PAR-CLIP analysis for Exp-5 substrate identification

As an alternative strategy to identify endogenous substrates *in vivo* which could be bound by Exp-5, Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed as a highly effective method to elucidate transcriptome-wide binding sites of RNA-binding proteins at high resolution (Hafner, Landthaler et al. 2010). The technique involves the incorporation of 4-thiouridine into newly synthesized RNA in cells and UV crosslinking of the 4-thiouridine-labelled RNA to covalently bind them to associated RNA-binding proteins. The covalently crosslinked protein-RNA complexes are immunoprecipitated using an antibody against the RNA binding protein and further purified by size-fractionation using SDS-PAGE. Co-purified RNA molecules are extracted and cloned for deep-sequencing analysis to identify the RNA species directly bound by the RNA binding protein. This method allows high-resolution analysis because 4-thiouridines that are covalently crosslinked with peptides induce errors at reverse transcription, typically incorporating a G instead of an A at the position complementary to the crosslinked 4sU in the cDNA molecule (Hafner, Landthaler et al. 2010). Therefore, the characteristic T-to-C transitions in the cDNA sequence can be considered as evidence for crosslinking events.

This method was applied to the poorly studied miRNA biogenesis factor Exp-5. S2R+ cells stably transfected with a FLAG-tagged Exp-5 construct under the control of a metallothionein promoter, and the PAR-CLIP experiment was performed using FLAG antibody to create a library consisting of RNAs binding to Exp-5 (library constructed by

Katsutomo Okamura and Zhou Li). Crosslinked reads were identified with a T to C transition at the site of crosslinking. The library was preprocessed by trimming adaptor sequences and removing reads without adaptors using FASTX Toolkit (library preprocessing performed by Chak Li Ling). Because of the presence of a large number of reads arising from the recombinant FLAG-tagged Exp-5 overexpression construct, reads arising from the recombinant FLAG-tagged Exp-5 construct were removed, and the remaining reads were then mapped to the dm3 genome using Bowtie1 (Langmead, Trapnell et al. 2009), allowing two mismatches. We obtained ~3.7 million reads mapping to the genome, of which 38.74% of reads contained at least one T-to-C transition. The frequency of T-to-C transition was much higher than those of other potential nucleotide transitions, indicating the specificity of crosslinking signals (figure 4.1.5.1). The mapped reads were then searched for high-confidence binding sites using PARalyzer, a computational pipeline that detects T-to-C transition signals higher than the estimated background level (Corcoran, Georgiev et al. 2011).

PARalyzer identified multiple high-confidence binding sites based on the read densities of reads containing T to C transitions. Using the pipeline, we could identify 186,025 potential target sites within known Exp-5 target species as well as novel target RNA species. The list of high-confidence groups was ranked according to number of T to C transitions. Of the top ranked results, there were novel predicted substrates as well as several known substrates of Exp-5 which indicated the reliability of the library dataset. Generally, the presence of high T to C transition count and higher T to C transition position counts were indicative of Exp-5 binding. Additionally, high read counts were also indicative of Exp-5 binding due to enrichment of Exp-5-binding RNAs due to the immunoprecipitation and gel excision step, but were also affected by endogenous expression levels of RNAs. There was a general correlation of PAR-CLIP read density and RNA-seq read density (RNA seq STAR alignment performed by Zhou Li).

Minihelix-containing RNAs are known substrates of Exp-5, including pre-miRNAs and tRNAs. Notably, as *Drosophila* does not have an Exp-t ortholog, Exp-5 is a key export factor in the export of both pre-miRNAs and tRNAs (Shibata, Sasaki et al. 2006). In

terms of known Exp-5 binding substrates, many tRNAs were among the most highly-ranked. This was unsurprising due to the high expression of most tRNA genes which are needed for protein translation. Generally, expression of tRNAs were much higher than most miRNA genes as observed from the RNA seq dataset. Additionally, the other class of known Exp-5 substrates which appeared in the list of predicted Exp-5 binding substrates are pre-miRNAs (figure 4.1.5.1) which have been known to depend on Exp-5 for transport out of the nucleus. Due to the lower expression of miRNAs as seen in the RNA seq dataset compared to general protein-coding genes and tRNAs, they were less highly ranked in the predicted substrates list compared to more abundantly-expressed substrates such as tRNAs.

Exp-5 is also proposed to act as an export factor of specific mRNAs but the pervasiveness of Exp-5 mediated mRNA export remains unknown (Bennasser, Chable-Bessia et al. 2011). We identified 7364 protein coding genes with Exp-5 binding sites (about 50% of genes that were expressed in S2 cells). Identification of the large number of protein-coding genes from the Exp-5 PAR-CLIP library was unexpected, given that most mRNAs are known to be exported from the nucleus via the Nxf1-Nxt1 pathway (Segref, Sharma et al. 1997, Herold, Suyama et al. 2000), with a subset of mRNAs being exported via the Crm1 pathway (Stade, Ford et al. 1997).

Figure 4.1.5.1: Known Exp-5 substrate bantam showing PAR-CLIP reads mapping to the bantam pre-miRNA. USCS snapshot shows the first track showing small RNA-seq library reads, second track showing total Exp-5 PAR-CLIP reads, third track showing Exp-5 PAR-CLIP T to C reads, fourth track showing Exp-5 PAR-CLIP T to C transitions and Flybase bantam pre-miRNA annotation.

4.2 miR-124 Ago expression project

4.2.1 Levels of pre-miR-124 decrease upon overexpression of Ago proteins together with miR-124 in HEK293T cells

The neuron-specific microRNA miR-124 is a 3p-coded miRNA which functions as a key regulator in neuronal differentiation (Yoo, Staahl et al. 2009). My previous work in the Makeyev lab showed that levels of pre-miR-124 were decreased upon overexpression of Ago proteins together with miR-124 in HEK293T cells (Lee and Makeyev unpublished data; NTU FYP thesis 2012). To ensure that this observation was reproducible, HEK293T cells were transfected with miR-124-expressing vector together with a control vector or myc-tagged human AGO1, AGO2, AGO2 slicer mutant (Ago2D597A), AGO3, AGO4 or Dicer, and pre-miR-124 and mature miR-124 were detected by northern blotting (figure 4.2.1.1A). Because AGO3 and AGO4 is usually expressed poorly due to non-optimal codon usage (Valdmanis, Gu et al. 2012), a much stronger CAG promoter instead of CMV promoter used for AGO1, AGO2 and AGO2D597A was used for AGO3 and AGO4 to achieve similar expression levels to the other Ago proteins. Western blotting analysis of the tagged AGO1-4 and Dicer confirmed successful transfection, and showed that all AGO proteins were overexpressed at similar levels (figure 4.2.1.1C). Experiments were performed in triplicate. Consistent with my previous results, levels of pre-miR-124 decreased when AGO1-4 was co-expressed (figure 4.2.1A and B). Overexpression of AGO1-4 also resulted in an increase of mature miR-124 similar to what was seen with many miRNAs, potentially due to loaded mature miRNAs in Ago proteins being protected from ribonuclease degradation (Diederichs and Haber 2007). However, unlike other miRNAs, levels of pre-miR-124 were decreased in a greater extent in Ago1-4 overexpressing cells compared to Dicer overexpressing cells, indicating a significant role of Ago protein in decreasing amounts of pre-miR-124 compared to control. Moreover, levels of pre-miR-124 were also similarly decreased when the Ago2D597A slicer mutant was overexpressed, indicating that the effect of Argonaute on decreasing pre-miR-124 levels is slicer-independent.

Figure 4.2.1.1: Levels of pre-miR-124 decrease upon AGO1-4 overexpression. (A) Northern blot showing pre-miR-124 and mature miR-124 following expression of miR-124 and AGO1-4 or Dicer encoded plasmids in HEK293T cells. (B) Quantification of pre-miR-124, normalized to U6 and EGFP control. (C) Western blot showing expression of AGO1-4 and Dicer proteins in HEK293T cells transfected with AGO1-4 and Dicer-expressing plasmids.

4.2.2 Levels of other pre-miRNAs do not change upon overexpression of Ago proteins

To test if the pre-miRNAs of other miRNAs change upon overexpression of Ago proteins, HEK293T cells were transfected with vectors expressing various miRNAs together with AGO1-4 expressing vectors and probed for the predominantly-loaded guide strand. miRNAs with the 5p strand as the guide strand included let-7a, miR-26a-1, miR-34a and miR-9 (figure 4.2.2.1A-D), miRNAs with the 3p strand as the guide strand included miR-1, miR-19a, miR-92a, miR-133a and miR-206 (figure 4.2.2.2A-E), whereas miRNAs which had almost equivalent loading efficiencies for both strands included miR-27b and miR-30a (figure 4.2.2.3A-D). Experiments for let-7a and miR-26a-1 were done in triplicate, whereas miR-34a, miR-9, miR-1, miR-19a, miR-92a, miR-133a, miR-206, miR-27b and miR-30a were done once. However, the levels of pre-miRNA for the tested miRNAs did not appear to reliably decrease during Ago overexpression regardless of the arm the major miRNA species is derived from, with the exception of miR-9. Although the levels of pre-miR-9 did not change when AGO1, 3 or 4 were overexpressed as compared to control, when AGO2 was overexpressed together with miR-9, a large smear at the pre-miRNA region was observed along with a band of slightly lower molecular weight, similar to the production of ac-pre-miRNA as described. Additionally, overexpression of let-7a-1 together with AGO1-4 resulted in an increase in pre-let-7a-1, suggesting that AGO1-4 stabilizes the pre-let-7a-1 hairpin, possibly by loading of pre-let-7a-1 into AGO1-4 as part of the PDC (Liu, Jin et al. 2012). From the miRNAs tested, the phenomenon of Ago-dependent pre-miRNA decrease appears to be an isolated observation specific to miR-124 and is not a general phenomenon for mature miRNA derived from either arm.

Figure 4.2.2.1: Levels of pre-miRNAs for 5p-miRNAs do not change upon overexpression of AGO1-4 or Dicer. Northern blots showing overexpression of miRNA and AGO1-4 or Dicer plasmid in HEK293T cells and quantification of pre-miRNA, normalized to U6 and EGFP control for (A) let-7a-5p (B) miR-26a-5p (C) miR-34a-5p (D) miR-9-5p.

Figure 4.2.2.2: Levels of pre-miRNAs for 3p-miRNAs do not change upon overexpression of AGO1-4 or Dicer. Northern blots showing overexpression of miRNA and AGO1-4 or Dicer plasmid in HEK293T cells and quantification of pre-miRNA, normalized to U6 and EGFP control for (A) miR-1-3p (B) miR-19a-3p (C) miR-92a-3p (D) miR-133a-3p (E) miR-206-3p.

Figure 4.2.2.3: Levels of pre-miRNAs for 5p- and 3p-expressing miRNAs do not change upon overexpression of AGO1-4 or Dicer. Northern blots showing overexpression of miRNA and AGO1-4 or Dicer plasmid in HEK293T cells and quantification of pre-miRNA, normalized to U6 and EGFP control for (A) miR-27b-5p (B) miR-27b-3p (C) miR-30a-5p (D) miR-30a-3p.

4.2.3 Levels of endogenous pre-miRNAs do not change upon overexpression of Ago proteins in HEK293T cells

To test if the precursor hairpins of endogenous miRNAs change upon overexpression of Ago proteins, HEK293T cells were transfected with AGO1-4 expressing vectors and total RNA was extracted and probed for endogenously-expressed miRNAs. The experiment was done once. Of the probed miRNAs, miR-15a, miR-16, miR-17a and let-7a (figure 4.2.3.1A, C, E and G) had visible pre-miRNA bands in addition to mature miRNA bands. None of the miRNAs showed a clear change in pre-miRNA levels in response to AGO1-4 overexpression (figure 4.2.3.1A-H), indicating that Ago overexpression does not have an effect on endogenous pre-miRNAs. Since neither of the tested overexpressed and endogenously expressed pre-miRNAs except miR-124 responded to Ago overexpression, this further indicates that the decrease in the precursor level upon Ago overexpression may be specific to miR-124.

Figure 4.2.3.1: AGO1-4 or Dicer overexpression does not affect pre-miRNA levels of endogenously expressed miRNAs. (A) Northern blot showing overexpression of AGO1-

4 or Dicer in HEK293T cells and probed for endogenously-expressed miR-15a. (B) Quantification of pre-miR-15a, normalized to U6 and EGFP control. (C) Northern blot showing overexpression of AGO1-4 or Dicer in HEK293T cells and probed for endogenously-expressed miR-16. (D) Quantification of pre-miR-16, normalized to U6 and EGFP control. (E) Northern blot showing overexpression of AGO1-4 or Dicer in HEK293T cells and probed for endogenously-expressed miR-17a. (F) Quantification of pre-miR-17a, normalized to U6 and EGFP control. (G) Northern blot showing overexpression of AGO1-4 or Dicer in HEK293T cells and probed for endogenously-expressed let-7a. (H) Quantification of pre-let-7a, normalized to U6 and EGFP control.

4.2.4 Levels of pre-miR-124 decrease upon overexpression of Ago proteins together with miR-124 in mouse and *Drosophila* cells

The mature miR-124 sequence and its expression in neuronal cells is extremely well-conserved in many organisms (Lagos-Quintana, Rauhut et al. 2002, Conaco, Otto et al. 2006). Human and mouse possess three miR-124 paralogs (miR-124-1, miR-124-2 and miR-124-3) which have perfectly conserved 5p and 3p sequences and differing loop size, whereas *Drosophila* contains a miR-124 gene with perfectly conserved 3p (mature) strand sequence, but has a less conserved 5p sequence and loop sequence generating a slightly different pre-miR-124 structure as compared to mammalian miR-124 (figure 4.2.4.1). I asked whether the phenomenon of Ago-dependent pre-miR-124 decrease was conserved in other organisms. To test this, I first used the mouse neuroblastoma cell line Neuro2a (N2a). Mouse N2a cells were transfected with miR-124 expressing plasmid and myc-tagged control, mouse Ago1, Ago2, Ago3 or Ago4, and pre-miR-124 and mature miR-124 were detected by northern blotting (figure 4.2.4.2A). The experiment was done in triplicate. Western blotting showed expression of mouse Ago1-3 (figure 4.2.4.2B). In mammalian systems, Ago3 and Ago4 show dramatically lower expression levels compared to Ago1 and Ago2 due to the presence of non-optimized codon sequences in Ago3 and Ago4 which interferes with protein expression (Valdmanis, Gu et al. 2012). Since the same promoter was used for all mouse Ago1-4

constructs, Ago1 and Ago2, but not Ago3 and Ago4, were able to be expressed well under the control of the CMV promoter, mimicking endogenous Ago 1-4 expression profiles. Similar to HEK293T cells, levels of pre-miR-124 were decreased in samples with overexpressed Ago1-4 compared to control in N2a cells, suggesting that decrease of pre-miR-124 in response to Ago protein overexpression may be a common phenomenon in mammalian cells.

	5p strand	loop	3p strand
cel-mir-124	GGCAUGCACCCUAGUGACUUUAGUGGACAUCUAAGUCUUCCAACUAAGGCACGCGGUGAAUGCCAC		
dme-mir-124	GGUAUCCACUGUAG-G-CCUAUAUGUA-UUU-CC-CC-A-UAAGGCACGCGGUGAAUGCCAA		
hsa-mir-124-1	CGUGUUCACAGCGG-ACCUGAUUUAAUGU-CCAUAACAAUUAAGGCACGCGGUGAAUGCCAA		
mmu-mir-124-1	CGUGUUCACAGCGG-ACCUGAUUUAAUGU-CCAUAACAAUUAAGGCACGCGGUGAAUGCCAA		
hsa-mir-124-2	CGUGUUCACAGCGG-ACCUGAUUUA-AUGU-C-AUACAAUUAAGGCACGCGGUGAAUGCCAA		
mmu-mir-124-2	CGUGUUCACAGCGG-ACCUGAUUUA-AUGU-C-AUACAAUUAAGGCACGCGGUGAAUGCCAA		
hsa-mir-124-3	CGUGUUCACAGCGG-ACCUGAUUUA-AUGU-CUAUACAAUUAAGGCACGCGGUGAAUGCCAA		
mmu-mir-124-3	CGUGUUCACAGCGG-ACCUGAUUUA-AUGU-CUAUACAAUUAAGGCACGCGGUGAAUGCCAA		
	* * * * *	* * * * *	* * * * * *

Figure 4.2.4.2: Levels of pre-miR-124 decrease upon Ago1-4 overexpression in N2a and S2R+ cells. (A) Northern blot showing pre-miRNA-124 and mature miR-124 following expression of miR-124 and Ago1-4 or Dicer plasmids in N2a cells. (B) Quantification of pre-miR-124 in N2a cells, normalized to U6 and EGFP control. (C)

Northern blot showing pre-miR-124 and mature miR-124 following expression of miR-124 and Ago1 or Dcr1 encoded plasmids in S2R+ cells. (D) Quantification of pre-miR-124 in S2R+ cells, normalized to U6 and EGFP control. (E) Western blot showing expression of Ago1-4 proteins in N2a cells. (F) Western blot showing expression of Ago1 and Dcr1 protein in S2R+ cells.

4.2.5 Decrease in pre-miR-124 is not due to general secondary effects caused by miR-124 overexpression

Expression levels of miRNA processing factors are tightly regulated by many mechanisms including regulation by miRNAs, with an example being the regulation of Dicer mRNA by let-7 miRNA (Forman, Legesse-Miller et al. 2008). Although the results above suggested an effect specific to the mir-124 hairpin, it was formally possible that processing of all miRNAs was enhanced in cells overexpressing mir-124 and Argonautes if mir-124 overexpression could alter general miRNA processing activity in the cell. Hence, to find out if the miRNA biogenesis pathway was affected due to overexpression of miR-124 which led to changes in the amount of pre-miRNA, 293T cells were transfected with both miR-1 and miR-124-expressing plasmids together with AGO1-4 expressing plasmids. The experiment was done in triplicate. After probing for miR-124, the characteristic decrease in pre-miR-124 was observed in the samples where AGO1-4 protein was co-expressed (figure 4.2.5.1B and D). However, after probing for miR-1, there was no visible change in the levels of pre-miR-1 in the samples expressing only miR-1 miRNA as well as the samples expressing both miR-1 and miR-124 (figure 4.2.5.1A and C). Hence, this indicates that the decrease in the levels of pre-miR-124 upon Ago overexpression is not due to miR-124 regulatory behavior of miRNA biogenesis factors but is in fact an intrinsic property of miR-124 pre-miRNA.

Figure 4.2.5.1: Decrease in pre-miRNA levels is not due to general secondary effects of miR-124 overexpression. miR-124 and/or miR-1 was overexpressed together with AGO1-4 or Dicer in HEK293T cells and probed with (A) miR-124 probe (B) miR-1 probe.

Quantification of (C) pre-miR-1 and (D) pre-miR-124, normalized to U6 and EGFP control.

4.2.6 Effect of pre-miR-124 decrease upon Ago overexpression does not correlate with miRLC and miPDC formation

Pre-miRNAs assemble with Dicer, TRBP and Ago protein to form a complex with pre-miRNA processing and miRNA loading functions termed the miRNA loading complex (miRLC) (Gregory, Chendrimada et al. 2005, Maniataki and Mourelatos 2005, MacRae, Ma et al. 2008). Recently, it was found that pre-miRNA of Dicer-independent miR-451 is loaded into Ago2 in a Hsp70-dependent manner to form a complex distinct from the previously described miRLC, termed the miPDC (miRNA Precursor Deposit Complex), and that certain other Dicer-dependent miRNAs such as miR-16 can also form the miPDC (Liu, Zheng et al. 2014). To examine if miRLC or miPDC assembly could give rise to the observed phenomenon of pre-miR-124 decrease during Ago protein overexpression, the previously described canonical miRLC-forming miR-28 and the miPDC-forming miR-16 was overexpressed together with AGO1-4 or Dicer in HEK293T cells to see if the pre-miRNA of either miRNA would decrease similarly to pre-miR-124 during Ago overexpression. The experiment was done once. Neither miR-16 (figure 4.2.6.1A) nor miR-28 (figure 4.2.6.1B) showed any change in the levels of pre-miRNA during Ago coexpression, indicating that the decrease of pre-miR-124 during Ago overexpression does not correlate to differential assembly of miRLC or miPDC, since the representative miRNAs for miRLC and miPDC formation did not display a similar response to miR-124 upon Ago1-4 overexpression.

Figure 4.2.6.1: Effect of pre-miR-124 decrease upon Ago overexpression does not correlate with miRLC or miPDC formation. Northern blot showing overexpression of AGO1-4 or Dicer in HEK293T cells with (A) miR-16 or (B) miR-28, probed for the corresponding mature miRNA.

4.2.7 Decrease of pre-miR-124 during Ago overexpression is Dicer-dependent

The Ago protein interacts with Dicer, TRBP and Ago protein to form the miRLC complex which can process pre-miRNA (Gregory, Chendrimada et al. 2005, Maniataki and Mourelatos 2005, MacRae, Ma et al. 2008). Hence, to examine if Dicer could be involved in the decrease in pre-miR-124 during Ago overexpression, miR-124 and miR-26a-1 were overexpressed together with control, AGO1-4, or Dicer in both HEK293T wild type and Dicer knockout (4-25 nodice) cells (Bogerd, Whisnant et al. 2014) and probed for the corresponding miRNAs. The experiment was done in triplicate. Levels of pre-miR-26a-1 appeared unchanged upon overexpression of AGO1-4 in both wild type and Dicer knockout cells, with the pre-miR-26a-1 levels showing more variable levels in Dicer knockout cells (figure 4.2.7.1B and D). Levels of pre-miR-124 decreased during AGO1-4 overexpression compared to control in wild type cells as previously observed, but this decrease in pre-miR-124 during AGO1-4 overexpression was not observed in Dicer knockout cells, conversely, pre-miR-124 increased when AGO1-4 was overexpressed in Dicer knockout cells (figure 4.2.7.1A and C). Western blots showed similar expression of myc-tagged AGO1-4 and Dicer in both wild type and Dicer knockout HEK293T cells for both miR-124 and miR-26a-1 samples (figure 4.2.7.1E). Hence, Dicer may be involved in the decrease of pre-miR-124 during Ago overexpression.

Figure 4.2.7.1: Decrease in pre-miR-124 during Ago overexpression does not occur in Dicer knockout cells. Northern blot showing overexpression of control, AGO1-4 or Dicer with (A) miR-124 or (B) miR-26a-1 in HEK293T wild type and Dicer knockout cells, probed for the corresponding mature miRNA. Quantification of (C) pre-miR-124 and (D) pre-miR-26a-1, normalized to U6 and EGFP control. (E) Western blot showing the expression of myc-tagged AGO1-4 and Dicer in HEK293T wild type and Dicer knockout cells.

Since Dicer was suggested to be involved in the decrease of pre-miR-124 during coexpression with Ago1-4 from the overexpression experiments in Dicer mutant cells, we tested the combined effect of Dicer and Ago1-4 overexpression in the processing of pre-miR-124 and pre-miR-26a-1. miR-124 and miR-26a-1 was coexpressed together with myc-tagged Dicer and Ago1-4 plasmids in HEK293T wild type cells and northern blotting performed on total RNA extracted from transfected cells. Quantification of pre-miRNA for miR-124 and miR-26a-1 was performed and normalized to U6 loading control. Western blotting confirmed the expression of Ago1-4 and Dicer in each sample (figure 4.2.7.2E). For miR-124, similar to previously observed (figure 4.2.1.1), amounts of pre-miR-124 decreased to about 10% of pre-miR-124 levels in the miR-124+EGFP control when overexpressed together with Ago1-4. Conversely, pre-miR-124 levels decreased to about 30% of pre-miR-124 levels in the miR-124+EGFP control when overexpressed together with Dicer (figure 4.2.7.2A and C). This suggests that Dicer may be limiting in the processing of pre-miR-124, since overexpression of Dicer can decrease pre-miR-124 levels. However, the effect of Ago1-4 in decreasing the levels of pre-miR-124 was much more pronounced than Dicer overexpression. Co-overexpression of miR-124, Ago1-4 and Dicer did not drastically alter levels of pre-miR-124 from that of miR-124 and Ago1-4 overexpression, although the levels of pre-miR-124 decreased by a very small amount with both Ago1-4 and Dicer co-overexpression compared to Ago1-4 without Dicer co-overexpression. This suggests that while Dicer overexpression may aid in further processing of a small amount of pre-miR-124 substrate when both Dicer and Ago1-4 are overexpressed, the decrease in pre-miR-124

may be saturated upon Ago1-4 overexpression such that further Dicer overexpression together with Ago1-4 has a minimal effect in the decrease of pre-miR-124.

For miR-26a-1, Ago1-4 overexpression conversely increased the amount of pre-miR-26a-1 compared to the miR-26a-1+EGFP sample, suggesting that Ago1-4 overexpression could act to stabilize pre-miR-26a-1 by loading of the pre-miR-26a-1 into Ago1-4 as part of the PDC (Liu, Jin et al. 2012). Interestingly, overexpression of Dicer with miR-26a-1 also increased the levels of pre-miR-26a-1 compared to miR-26a-1+EGFP (figure 4.2.7.2B and D), suggesting that in Dicer is not a limiting factor for pre-miR-26a-1 processing. Meanwhile, co-overexpression of miR-26a-1 with Ago1-4 and Dicer decreased pre-miR-26a-1 levels very slightly compared to that of miR-26a-1+Ago1-4 without Dicer overexpression, suggesting that with Ago1-4 overexpression, Dicer can process pre-miR-26a-1 slightly more efficiently compared to miR-26a-1+Dicer sample. However, the increase of Dicer processing during Ago1-4 overexpression does not appear to offset the pre-miR-26a-1 stabilization effect caused by Ago1-4 overexpression. Taken together, the results suggest that Ago1-4 overexpression together with Dicer may appear to confer slightly increased Dicer processing of pre-miRNAs.

Figure 4.2.7.2: Co-overexpression of miR-124 and miR-26a-1 with Ago1-4 and Dicer does not appear to drastically alter levels of pre-miR-124 and pre-miR-26a-1 compared to Ago1-4 overexpression without Dicer. Northern blot showing overexpression of (A) miR-124 or (B) miR-26a-1 with Ago1-4 with or without Dicer overexpression in 293T cells. Quantification of (C) pre-miR-124 and (D) pre-miR-26a-1, normalized to U6 and

EGFP control. (E) Western blot showing overexpression of myc-tagged Ago1-4 and Dicer in each sample in HEK293T cells.

4.2.8 The pre-miRNA regulatory function of Ago can be separated from loading function of Ago

Loading of the mature strand into the Ago protein to form the active RISC complex remains the most established role of Ago proteins in miRNA biogenesis. Hence, to investigate whether the loading functions of Ago proteins could be separated from the pre-miRNA regulatory function of Ago proteins, S2R⁺ cells were transfected with miR-6, miR-10 and miR-124 together with wild type fly Ago1 and mutant fly Ago1 MID domain (F2V2) and PAZ domain (PAZ) mutants (Miyoshi, Okada et al. 2009) lacking the ability to load mature miRNAs, and pre-miRNA and mature miRNA levels were analyzed by northern blotting. Experiments were performed in triplicate. Since Ago1 F2V2 and PAZ mutants cannot load mature miRNAs from Dicer processing but can interact using their PIWI domain to the RNaseIII domain of Dicer (Tahbaz, Kolb et al. 2004), they could in theory still perform pre-miRNA regulatory functions without being able to load mature miRNAs. We observed expression of FLAG-tagged EGFP, Ago1wt, Ago1F2V2, Ago1PAZ and Dcr1 in S2R⁺ for miR-6, miR-10 and miR-124 samples, with the Ago1 F2V2 and PAZ domain mutants more highly expressed compared to wild type Ago1 (figure 4.2.8.2). The levels of pre-miR-6 and pre-miR-10 did not change significantly when wild type Ago1, Ago1 F2V2 mutant or Ago1 PAZ mutant were overexpressed, compared to control EGFP overexpression (figure 4.2.8.1A-B), whereas levels of pre-miR-124 were decreased upon wild type Ago1, Ago1F2V2 mutant and Ago1 PAZ mutant overexpression compared to control EGFP overexpression (figure 4.2.8.1C), further supporting that Ago has pre-miRNA regulatory functions for miR-124. However, the expression of Ago1 F2V2 and PAZ domain mutants were expressed at much higher levels compared to wild type Ago1. Although the dose dependence of the effects of Ago1 overexpression was not determined in this study, we do not exclude the possibility that mutant AGO1 proteins could act as less efficiently as pre-miRNA regulators for

miR-124. The levels of mature miR-6, miR-10 and miR-124 appeared to be more variable. Although we expected to see the highest level of mature miRNAs in Ago1 wt overexpression due to increased loading into Ago1 and protection from RNases (Diederichs and Haber 2007), we also observed unexpectedly high levels of mature miRNAs in both Ago1 F2V2 and Ago1 PAZ domain mutant overexpression samples. We observed trimming of all three mature miRNA species in the Ago1 PAZ domain mutant. Mature miRNA which is loaded into Ago has its 5' end bound by the MID domain and its 3' end bound by the PAZ domain, respectively (Ma, Ye et al. 2004, Frank, Sonenberg et al. 2010), and 3' miRNA isoforms formed by trimming and tailing appear after miRNA release from Ago proteins (Ameres, Horwich et al. 2010, de la Mata, Gaidatzis et al. 2015). This suggests that the Ago1 PAZ domain mutant can form mature RISC for trimming of mature miRNAs to occur, since mature miRNAs can be trimmed only after loading into Ago1 and removal of the passenger strand (Ameres, Horwich et al. 2010, Han, Hung et al. 2011). The levels of mature miRNAs in cells expressing Ago1 F2V2 mutant tended to be lower than the Ago1 wild type overexpression sample, suggesting that Ago1 F2V2 mutant could also load mature miRNAs, although loading into Ago1 F2V2 mutant was much less compared to wild type Ago1. Thus, the ability of Ago to regulate pre-miRNA levels can be functionally separated from the mature miRNA loading functions of Ago.

Figure 4.2.8.1: Ago pre-miR-124 regulatory function can be functionally separated from Ago function in loading mature miRNAs. miR-6, miR-10 or miR-124 were overexpressed together with an EGFP, wild type Ago1, Ago1 F2V2 mutant, Ago1 PAZ mutant or Dcr1 mutant in S2R+ cells and pre-miRNA and mature miRNA were analyzed by northern blotting. Northern blot shows pre-miRNA and mature miRNA coexpressed with EGFP, wild type Ago1, Ago1 F2V2 and PAZ mutants and Dcr1. Graphs show quantification of pre-miRNA and mature miRNA from northern blot, normalized to intensity of miRNA+EGFP band for both pre-miRNA and mature miRNA. (A) Northern blot and quantification for miR-6. (B) Northern blot and quantification for miR-10. (C) Northern blot and quantification for miR-124.

Figure 4.2.8.2: Ago pre-miR-124 regulatory function can be functionally separated from Ago function in loading mature miRNAs. Western blot showing the expression of FLAG-tagged EGFP, Ago1wt, Ago1F2V2, Ago1PAZ and Dcr1 in S2R+ cells.

4.2.9 Coexpression of both miR-124 and Ago2 in N2a cells induces longer neurite outgrowth in N2a cells

Increased expression levels of Ago protein could be hypothesized to be a mechanism for the efficient expression of miR-124 through a two-step process involving the enhancement of dicing of pre-miR-124 by Ago and the stabilization of mature miR-124 by loading onto Ago (Diederichs and Haber 2007). Previously, miR-124 expression was shown to induce neurite outgrowth in N2a and CAD cells (Makeyev, Zhang et al. 2007). To examine the effect of increased Ago expression on miR-124 expression in biological systems, a neurite outgrowth assay was utilized using a mouse N2a neuronal cell culture-based system which used neurite outgrowth as an output for miR-124 expression. N2a cells were transfected with dsRed empty vector or miR-124-expressing vector and EGFP or myc-Ago2-expressing vector and then immunostained for myc-tagged Ago2 (figure 4.2.14.1A). For each field, neurite length of dsRed-expressing cells were measured and averaged by the total number of dsRed-expressing cells. The assay was performed in triplicate, and the average of the total number of counted dsRed-expressing cells in each triplicate (n) and the average of the average total neurite length per cell of the dsRed-expressing cells in each triplicate (mean length) is shown (figure 4.2.9.1C). The average total neurite length per cell for each field from all triplicates were pooled together and plotted (figure 4.2.9.1B). Total RNA and total protein was also extracted from all four samples and northern blotting for miR-124 (figure 4.2.9.1E) and western blotting for myc-tagged Ago2 (figure 4.2.9.1D) was also performed to test for miR-124 expression and myc-Ago2 expression, respectively.

Nearly all cells expressing dsRed also showed expression of myc-tagged Ago2 or EGFP, indicating coexpression of both plasmids within the same cell. Expression of dsRed, EGFP and myc-Ago2 was distributed throughout the cell body and neurites. The control+EGFP sample and control+Ago2 sample had few cells displaying neurites, with most of the neurites having short lengths. In comparison, the miR-124+EGFP and miR-124+Ago2 sample contained significantly more cells which displayed neurites of longer length, resulting in longer average neurite length per cell in each field in both the miR-

124+EGFP and miR-124+Ago2 sample ($p < 0.001$, Kruskal-Wallis rank sum test) (figure 4.2.9.1A and B), consistent with previous results that miR-124 expression in N2a cells was sufficient to induce neurite outgrowth in a fraction of N2a cells (Makeyev, Zhang et al. 2007). The average neurite length per cell in each field of the miR-124+EGFP sample was about 50 μ m compared to about 7 μ m for the control+EGFP and control+Ago2 samples ($p < 0.001$, Wilcoxon rank sum test) (figure 4.2.9.1C), consistent with previous observations which suggest that miR-124 overexpression in N2a cells results in neurite outgrowth and development of a neuronal phenotype in a fraction of N2a cells. In comparison, the miR-124+Ago2 sample displayed a mean total neurite length per cell of about 100 μ m, suggesting more significant outgrowth of neurites compared to the miR-124+EGFP sample ($p < 0.001$, Wilcoxon rank sum test) (figure 4.2.9.1C). The increased mean total neurite length per cell in the miR-124+Ago2 samples compared to miR-124+EGFP samples could be due to the increased dicing of pre-miR-124 due to Ago2 performing as a dicing factor and stabilization of mature miR-124 by Ago2 loading in the cells which express both miR-124 and Ago2 (figure 4.2.9.1C), allowing increased mature miR-124 accumulation which resulted in further progression into neuronal differentiation pathway and adoption of a neuronal gene profile (Lim, Lau et al. 2005, Xue, Ouyang et al. 2013). Hence, it could be the case that biological systems could utilize higher expression levels of Ago proteins to enhance miR-124 processing for effective miR-124 expression.

C

Sample	n	Mean length (μm)
Control+EGFP	124	7.77
Control+Ago2	157.3	6.72
miR-124+EGFP	123.3	48.00
miR-124+Ago2	136	105.86

Figure 4.2.9.1: Co-expression of miR-124 and Ago2 causes increase in neurite length in N2a cells. (A) Neurite growth assay showing expression of control+EGFP, control+Ago2, miR-124+EGFP and miR-124+Ago2 plasmids in N2a cells. Red, green and blue channels show dsRed (control or miR-124 expressing), EGFP or myc-tagged Ago2, and DAPI channels, respectively. (B) Quantification of mean total neurite length per cell for each field for control+EGFP, control+Ago2, miR-124+EGFP and miR-124+Ago2 samples respectively. (C) Average of three replicates of mean total neurite length per cell per field calculated for control+EGFP, control+Ago2, miR-124+EGFP and miR-124+Ago2, calculated for $n > 100$ cells. (D) Western blot showing the expression of myc-tagged Ago2 in each sample. (E) Northern blot from control+EGFP, control+Ago2, miR-124+EGFP and miR-124+Ago2 samples showing expression of pre-miR-124 and mature miR-124 in N2a cells.

5 Discussion

5.1 Exp-5 substrate recognition project

5.1.1 DGCR8 hairpins as a tool to study Exp-5 substrate recognition

Conventional knowledge suggests that products of Drosha processing, which have hairpin-like structures and have a 2nt overhang just like pre-miRNAs, have properties which are predicted to be recognized and exported by Exp-5 according to currently known information about Exp-5 substrate recognition and binding. However, the DGCR8 hairpin A is a pre-miRNA-like hairpin structure which is clearly cleaved by Drosha in the cell but is only localized in the nuclear fraction, likely due to its inability to be recognized and exported into the cytoplasm by Exp-5. It is likely that DGCR8 hairpin A is a unique pre-miRNA-like hairpin which can be processed by Drosha but cannot be recognized and exported by Exp-5. Hence, it is an important tool for the study of Exp-5 substrate recognition and binding.

Common consensus states that the structural properties of Drosha substrates are very similar to the characteristics of Exp-5 substrates. Through mutagenesis of DGCR8 hairpin A and in vitro expression experiments coupled with cellular subfractionation, it appears that some structural changes in the DGCR8 hairpin A may be able to drastically alter Drosha cleavage and other subsequent processing steps. Hence, our choice of in vitro expression experiments coupled with cellular subfractionation may not be a good choice to study Exp-5 binding and export against the backdrop of many other cellular processes such as Drosha processing, Dicer processing and degradation of hairpin RNAs. It would be more efficient to use in vitro systems which test binding of substrate hairpin structures to Exp-5 such as EMSA, to decrease the influence of other processing steps on result interpretation and output.

The removal of bulge regions on the stem region of the DGCR8 hairpin appears to be able to affect different processing steps besides Exp-5 binding and export. Given the

inability of DGCR8 hairpin A to be exported by Exp-5 possibly due to the increased number of bulge regions, it could also be interesting to study whether downstream processes such as dicing are also impeded by this unusual structure of DGCR8 hairpin A.

Structural analysis of the 60nt Drosha cleavage product of wild type DGCR8 hairpin A suggests that both sequence and structure of the wild type DGCR8 hairpin A may be involved in causing wild type DGCR8 hairpin A to be contained in the nuclear fraction after Drosha cleavage. When the pre-miRNA hairpin-like sequence of the 60nt DGCR8 hairpin A was folded in both RNA structural prediction programs RNAfold and Mfold, an extremely high thermodynamic free energy for the hairpin structure was obtained, ranging from -9.80 kcal/mol from RNAfold to -9.50 kcal/mol from Mfold. In comparison, conventional pre-miRNA hairpins usually have a thermodynamic free energy of about -30 kcal/mol to -20 kcal/mol. Closer examination of the predicted folded structure in both RNAfold and Mfold revealed that there was unstable base-pairing at the Drosha cleavage site due to the weak hydrogen bonding between the three U-A base-pairs, leaving the end of the hairpin likely to form single-stranded RNA compared to a duplex structure. The propensity of the end of the wild type DGCR8 hairpin A to unpair and form ssRNA structures at the base of the hairpin structure is a likely reason for the inability of Exp-5 to recognize and bind to wild type DGCR8 hairpin A for export into the cytoplasm. This problem would likely not affect Drosha cleavage, due to the presence of 16nt of compensatory base-pairing interactions at the basal stem region of the DGCR8 hairpin A which helps to stabilize the hairpin structure and unstable base-pairing interactions at the Drosha cleavage site for Drosha processing.

5.1.2 PAR-CLIP analysis

PAR-CLIP allowed the genome-wide detection of known and novel interactions between Exp-5 and its target RNAs. Our preliminary analysis indicated high specificity of this assay judged by the frequency of T-to-C transitions. The high resolution of PAR-CLIP results provided further molecular insights by successfully mapping the target regions within tRNAs and miRNAs. In addition to signals from known substrates, PAR-CLIP identified many novel candidates including mRNAs, ncRNAs and transposon transcripts. Although it is assumed that Exp-5 binds to specific regions of the transcript, given the widespread presence of secondary structures in ssRNA, Exp-5 binding of transcripts may be more common than originally thought and may encompass many other yet unidentified non-miRNA substrates. The Exp-5 PAR-CLIP library is unique in that it would be uninformative to apply the traditional workflow of motif identification after identification of predicted Exp-5 binding targets. Unlike most RNA-binding proteins which bind to a specific sequence motif on the RNA, Exp-5 recognizes and binds to double-stranded regions of about 22nt in length. Hence, motif identification based on sequence is unlikely to yield useful results. Instead, a search of double-stranded secondary structures of 20-25nt in length based on thermodynamic stability would be more likely to pinpoint Exp-5 binding regions.

The PAR-CLIP library showed a major enrichment in the mature miRNA sequence compared to the passenger strand sequence, which was surprising considering that Exp-5 is a well-established export factor for pre-miRNAs and hence would be expected to bind preferentially to the stem region of pre-miRNAs, leading to an equal proportion of mature miRNA sequence reads and passenger strand sequence reads from the miRNA hairpin region. Thus, it appears that the PAR-CLIP library has mature miRNA contaminants during library preparation, as it is difficult to remove all the small RNAs from the library during 20-50nt size selection. It could be possible to remove the small RNA contaminants which range from 20nt to 30nt by performing size selection of reads from 30nt to 50nt, but due to current structural models of Exp-5 binding of pre-miRNA which describe the stem of pre-miRNA being bound in the channel of Exp-5 and the

loop region of the pre-miRNA exposed outside the Exp-5 protein, with the current PAR-CLIP protocol using RNase T1 to cleave exposed ssRNA into smaller fragments, it could be possible that most of the PAR-CLIP signals would be around the 20-30nt range as it corresponds to the length of the binding pocket of Exp-5. Hence, it may be difficult to completely remove the small RNA contaminating reads without suffering a decrease in PAR-CLIP signal. A possible approach would be to use a size-matched input such as those used in eCLIP. The size-matched input would contain cell lysate which has not undergone immunoprecipitation but crosslinked and undergone subsequent sample preparation steps in the same manner as the PAR-CLIP sample, giving rise to a control which contains the same crosslinking, fragmentation, ligation and amplification biases as the PAR-CLIP sample. Highly abundant RNAs which contaminate the PAR-CLIP sample library could be identified from the size-matched input sample and the enrichment score based on IP relative to input could be calculated to identify enriched binding in the PAR-CLIP sample (Wheeler, Van Nostrand et al. 2018).

5.2 miR-124 Ago expression project

5.2.1 Biological relevance of elevated Ago expression

The observation that Ago protein overexpression aids in the regulation of pre-miR-124 leads to the question of biological relevance of Ago protein overexpression. While protein expression and transcript expression data is unclear for Ago1-4 in a temporal and cell type-specific manner for cells differentiating from neural stem cells into a neuronal fate, several papers have suggested that Ago proteins may be more highly expressed in neuronal cells and tissues compared to other tissues. qRT-PCR of Ago1-4 from human tissues showed that Ago1-4 were the most highly expressed in fetal brain tissue compared to other tissues tested, and Ago1-4 in the adult brain showed slightly lower expression compared to fetal brain tissue, but were still highly expressed compared to the other tissues tested (Voller, Linck et al. 2016). This trend was mirrored in *Drosophila*, whereby Ago1 mRNA showed very high expression levels in larval and pupal stages, and high expression in the head of adult flies (Brown, Boley et al. 2014). Moreover, Drosha, Dicer, Ago2 and Ago3 mRNAs were found to be significantly highly expressed in brain tissue compared to other tested somatic tissues in mouse (Gonzalez-Gonzalez, Lopez-Casas et al. 2008). However, stable protein expression levels of Ago1-4 in neuronal and other somatic tissues have been less characterized. The significantly high mRNA transcript levels of Ago1-4 in neuronal tissues compared to other somatic tissues suggest that total Ago protein levels are much higher in neuronal tissues compared to other somatic tissues, which would provide a high Ago environment allowing Ago1-4 to perform as a dicing cofactor for efficient miR-124 expression. Interestingly, qRT-PCR of Ago1-4 in SK-N-BE(2)-C human neuroblastoma cells during neuronal induction showed that expression of Dicer, Ago1, 3 and 4 seemed to increase during neuronal differentiation whereas Ago2 decreased during neuronal differentiation, although expression levels of Dicer and Ago1-4 were higher after neuronal induction compared to induction into Schwann cells performed in parallel (Potenza, Papa et al. 2009). It will be useful to know the protein expression levels of Ago1-4 in differentiating neuronal cells in parallel with miR-124 expression, although

taken together seems to suggest that neuronal cells support a cellular environment with higher Ago expression which can aid in miR-124 expression.

5.2.2 A novel method for fine-tuning of miR-124 expression

Mammalian miR-124 is expressed from three different genomic loci (Conaco, Otto et al. 2006, Deo, Yu et al. 2006). In human, both miR-124-1 and miR-124-2 are situated on chromosome 8 (chr8), whereas miR-124-3 is situated on chr20. The miR-124-1 gene partially overlaps with the long intergenic non-protein coding RNA (lincRNA) linc599 which is highly expressed in the cerebellum. On the other hand, the miR-124-2 gene is situated within the intron of a gene named miR-124-2 host gene (miR-124-2hg), which is abundantly expressed in the brain, especially in the cortex (RNA-seq expression data from GTEx taken from UCSC genome browser). While miR-124 is ubiquitously expressed in all progenitor and mature neuronal cells, the final miR-124 population can arise from the differential transcription of the three miR-124 genes, which in turn is influenced by the transcription of the pri-miRNA transcript, which could be co-transcribed together with surrounding host genes. This is especially so with the case of miR-124-2 and its host gene miR-124-2hg. Hence, regulation of the differing efficiencies of downstream processing steps are instrumental in order to obtain differential levels of host gene and miRNA from equal amounts of the same transcript. Hence, the effect of Ago on the regulation of miR-124 allows an additional mechanism with which different miR-124 levels can be achieved with the same starting transcript.

Additionally, differential expression of Ago proteins in different cell types can also contribute to differential accumulation of miR-124 in the cell. While an increased expression of Ago would be expected to increase the entire population of miRNAs proportionately due to stabilization of the mature miRNA after loading (Diederichs and Haber 2007), the ability of Ago to selectively regulate miR-124 could allow differential expression of miR-124 compared to the other miRNAs, allowing the cellular system to quickly accumulate mature miR-124. Hence Ago expression levels could be an important switch for the spatial and temporal expression of miR-124.

5.2.3 Clinical relevance of Ago as a regulatory factor for miR-124

In normal development, miR-124 has been shown to be very important for neuronal differentiation and development (Krichevsky, Sonntag et al. 2006, Silber, Lim et al. 2008, Yoo, Sun et al. 2011, Zou, Chen et al. 2014, Mondanizadeh, Arefian et al. 2015, Jiang, Du et al. 2016, Jiao, Liu et al. 2017, Sharif, Ghahremani et al. 2017), indicating that miR-124 dysregulation would underlie many neural disease conditions.

The function of miR-124 as a pro-neural differentiation factor indicates an important function as a tumor suppressor miRNA. miR-124 appears to be downregulated in different types of brain cancer such as glioma, glioblastoma multiforme (GBM) and astrocytoma (Silber, Lim et al. 2008, Xia, Cheung et al. 2012, Lv and Yang 2013, Shi, Chen et al. 2014, Chen, Wang et al. 2015, Cai, Qi et al. 2016, Qiao, Guo et al. 2017, Zhang, Gong et al. 2017, Liu, Kang et al. 2018, Zhang, Chen et al. 2018). miR-124 overexpression appeared to inhibit tumor cell proliferation and invasion (Silber, Lim et al. 2008, Xia, Cheung et al. 2012, An, Liu et al. 2013, Lv and Yang 2013, Silber, Hashizume et al. 2013, Lu, Jiang et al. 2014, Cai, Qi et al. 2016, Qiao, Guo et al. 2017, Zhang, Gong et al. 2017, Zhang, Chen et al. 2018) and conferred radiosensitivity (Deng, Ma et al. 2013) and chemosensitivity to tumor cells (Shi, Chen et al. 2014, Qiao, Guo et al. 2017). Unlike that of miR-124, consensus on the effect of Ago proteins and other miRNA biogenesis factors in cancer has been mixed. It is believed that the effect of Ago protein upregulation or downregulation helps to stabilize both tumor suppressor miRNAs and oncogenic miRNAs, leading to differing observations on the effect of Ago in cancer. In brain cancer, Ago2 expression was reported to be decreased, promoting glioma invasiveness and increased chemoresistance and radioresistance (Kim, Jin et al. 2015), whereas Ago2 has also been reported to be upregulated in high-grade gliomas (Feng, Hu et al. 2014). This indicates that Ago can act as an important signal amplifier for the effect of miRNAs. Hence, miR-124 can be utilized as a tumor suppressor gene, with Ago as an amplifier for the effect of miR-124, both of which can be exogenously introduced using gene delivery systems for long-term expression. miR-124 can be

differentially regulated in the presence of Ago and thus could be accumulated differentially from other miRNAs.

Besides the role of miR-124 in cancer, miR-124 has also shown to be implicated in neurodegenerative diseases. miR-124 has been shown to be downregulated in Alzheimer's disease (Lukiw 2007) as well as models of Alzheimer's disease (Kong, Wu et al. 2015, Kang, Xiang et al. 2017). miR-124 has been implicated in targeting of β -site APP cleaving enzyme 1 (BACE1) which is important for β -amyloid production (Fang, Wang et al. 2012) as well as prevent cell apoptosis and Tau phosphorylation by targeting caveolin-1 in Alzheimer's disease (Kang, Xiang et al. 2017). None of the studies above has examined the roles of Ago levels in neurodegeneration. Hence, miR-124 can be supplemented together with Ago to augment miR-124 effect in the treatment of neurodegenerative diseases.

Although decreased expression of miR-124 has been described in some major disease conditions, unregulated expression of miR-124 may also underlie other disease conditions. Upregulation of miR-124 was found to be involved in major depressive disorder and depression-like behavior, and inhibition of miR-124 was shown to alleviate this effect (Bahi, Chandrasekar et al. 2014, Roy, Dunbar et al. 2017, Wang, Mu et al. 2017). Most interestingly, inhibition of hippocampal miR-124 but not cortical miR-124 was shown to display antidepressant-like effects (Bahi, Chandrasekar et al. 2014). Treatment of depression could be performed by targeted inhibition of miR-124 levels using antagomirs to miR-124 together with Ago knockdown in the hippocampus.

Finally, miR-124 provides a protective effect in neuroinflammation during brain injury. miR-124 has been shown to reduce the production of pro-inflammatory cytokines in microglia (Ponomarev, Veremeyko et al. 2011, Sun, Li et al. 2013). Exogenous transfer of miR-124 into damaged neurons decreased neuronal inflammation and promoted neurogenesis and neurite outgrowth (Willemen, Huo et al. 2012, Doeppner, Doebring et al. 2013, Yang, Zhang et al. 2017, Yu, Zhang et al. 2017, Huang, Ge et al. 2018).

Hence, this neuroprotective effect of miR-124 could be enhanced with Ago to increase the effect of miR-124 to treat neuronal inflammation.

The implication of miR-124 in various neural disorders is beginning to be understood. Clearly, differential fine-tuning of miR-124 expression occurs across the many brain regions, and thorough understanding of how miR-124 expression varies spatiotemporally across brain regions is very important for designing therapeutic uses of miR-124 clinically. Current studies commonly rely on the exogenous delivery of chemically modified miRNA mimics in an attempt to regulate the levels of miR-124 (Baumann and Winkler 2014). Chemical modifications such as phosphorothioate backbone modifications and 2'-alkylated nucleotides are commonly used to increase stability and uptake (Bramsen, Laursen et al. 2009), however, the number of modifications that can be used while still allowing RISC loading is limited. Moreover, activity of miRNA mimics is limited by its relative stability. Hence, a longer-term solution appears to be the introduction of shRNA-like constructs of pre-miR-124 driven under a continuous promoter as part of a gene therapy approach to upregulate levels of miR-124 in tissues which require it. Expression of miR-124 from the construct could be fine-tuned spatially using Ago expression levels. Further study will be needed in order to design therapeutic miR-124 agents for clinical use.

5.2.4 Technological applications of Ago as a regulator for miR-124

This study has shown that miR-124 is regulated by Ago levels and that Ago can act as a dicing cofactor for efficient processing. miR-124 is an extremely important neuronal miRNA which is necessary for proper neuronal development, and dysregulation of this miRNA is implicated in many neural disease conditions. By understanding the properties which makes pre-miR-124 but not other pre-miRNAs regulated by Ago as a dicing cofactor, miR-124-based shRNAs could be designed which can also be differentially regulated by Ago for Dicer processing. This would provide a mechanism for fine-tuning of the expression of that shRNA construct, allowing greater flexibility on controlling expression levels other than strength of promoter expression.

Conversely, by understanding the properties of pre-miR-124 which makes it sensitive to Ago overexpression, we could design miR-124 shRNAs which could be less dependent on the expression of Ago, which could then be used as an efficient means of miR-124 supplementation for clinical use without its reliance on Ago expression levels.

6 Future work

6.1 Exp-5 substrate recognition project

6.1.1 Studying Exp-5 substrate recognition and binding using EMSA

Due to time constraints, the Exp-5 project was dropped in favour of the miR-124 Argonaute overexpression project. Hence, the planned experiments were not performed. Several difficulties were encountered while planning for the Exp-5 EMSA. Firstly, the expressed His-tagged Exp-5 in *E. coli* was present only in the pellet fraction of the cell, indicating that the protein was completely sequestered in the inclusion bodies of the *E. coli* cells. Hence, the purification of His-tagged Exp-5 from *E. coli* requires purification of the inclusion bodies containing the His-tagged Exp-5, followed by refolding of the His-tagged Exp-5 into its native conformation before use in EMSA. The purified and refolded His-tagged Exp-5 can then be used in conjunction with GST-tagged Ran protein and synthesized RNA oligos of different DGCR8 hairpin mutants to perform EMSA, to investigate the substrate recognition and binding of Exp-5 for different DGCR8 hairpin A mutants.

Additionally, besides the DGCR8 hairpin A bulge mutants, other mutants such as DGCR8 hairpin A structural mutants and sequence mutants could also be designed and tested in order to probe the sequence and structural specificity of Exp-5 substrate recognition and binding. Preliminary testing of DGCR8 hairpin A mutants with one or more bulges removed using in vitro expression experiments indicated that there was more export and processing of the DGCR8 hairpin when more bulges were removed. Hence, it would be interesting to test these DGCR8 hairpin A bulge mutants in Exp-5 substrate recognition and binding. Furthermore, sequences of DGCR8 hairpin A could be mutated without altering the structure of DGCR8 hairpin A to investigate whether sequence plays a part in Exp-5 substrate recognition and binding. Additionally, it will be of note to generate sequence mutants of DGCR8 hairpin A whereby the sequence at the base of the hairpin is mutated into C-G pairing instead of A-U pairing to test whether

the hypothesis that DGCR8 hairpin A cannot be recognized and exported by Exp-5 due to the unstable base-pairing at the base of the stem is true.

6.1.2 PAR-CLIP validation

Using the novel high-confidence targets identified by PAR-CLIP analysis, validation of those high-confidence targets could be performed by doing RNA-IP to see if those high-confidence targets bind to Exp-5. Preliminary RNA-IP experiments were performed for three highly-ranked mRNA targets which were predicted to bind to Exp-5, and two tRNAs and two miRNAs which are known to be bound by Exp-5 and were also ranked highly on the PAR-CLIP analysis. However, we did not achieve efficient pulldown of FLAG-tagged Exp-5, and when we managed to pull down FLAG-tagged Exp-5, there was no significant enrichment of the selected targets after Exp-5 IP compared to input even for our tRNA or pre-miRNA positive controls. Additionally, RT-qPCR is more difficult on tRNAs and pre-miRNAs due to their short length, which makes them less likely to be reverse transcribed to its full length even with the use of random primers and thus also less detectable due to inability of qPCR primers to anneal due to secondary structure and incomplete reverse transcription.

Another method of validation would be to perform Exp-5 knockdown by RNAi or Exp-5 knockout and to perform RT-qPCR of target genes on both nuclear and cytoplasmic fractions. If there is decreased detection of target RNA in the cytoplasmic fraction during Exp-5 knockdown or knockout, then the target is likely to be a substrate for Exp-5. However, it is possible that the cell may use different export factors for the export of the predicted Exp-5 substrates if Exp-5 is being knocked down or knocked out, so results could possibly be more difficult to interpret.

6.2 miR-124 Ago expression project

6.2.1 Identification of other miRNAs which are regulated by Ago

Using our current study, we identified miR-124 as a miRNA that is regulated by Ago overexpression. Besides miR-124, it could be possible that there exist other miRNAs which may rely on Ago expression levels for their own biogenesis. However, there did not appear to be any candidate miRNAs which appear to rely on Ago similar to miR-124 among the other miRNAs which we tested. Other miRNAs which may utilize Ago as a regulatory factor may not have been tested in our study.

To identify other miRNAs which are also regulated by Ago, pre-miRNA libraries (Liu, Zheng et al. 2014) could be constructed from wild type and Ago overexpression samples and normalized with a spike-in to compare between the two samples. miRNAs which are sensitive to Ago levels could be identified by decreased pre-miRNA amounts in the Ago overexpression samples relative to wild type libraries. These miRNAs would then be studied on how Ago regulates their pre-miRNA levels. miRNAs whose pre-miRNA levels are upregulated upon Ago overexpression could be binding to Ago to form a pre-miRNA-Ago-Dicer complex before Dicer processing (Liu, Jin et al. 2012), whereas miRNAs whose pre-miRNAs decrease during Ago overexpression may be regulated in the same manner by Ago as that of miR-124. It would be interesting to examine how such miRNAs are regulated by Ago.

6.2.2 Mechanisms of Ago regulation of miR-124

In vitro overexpression experiments of miR-124 and Ago1-4 shows that upon Ago overexpression, the levels of pre-miR-124 are decreased by about 90% of pre-miR-124 levels without Ago overexpression. No other miRNA exhibits such a significant decrease in the levels of pre-miRNA upon Ago overexpression. Clearly, Ago regulates the levels of pre-miR-124 in a manner different from other pre-miRNAs. A possible mechanism for Ago regulation of pre-miR-124 could be the dicing step, since Ago has been shown to interact with Dicer (Gregory, Chendrimada et al. 2005, Maniataki and Mourelatos 2005). Dicing assays could be designed to investigate whether the regulation of pre-miR-124 by Ago occurs in the dicing step. Using recombinant proteins of both Dicer, TRBP and Ago and association of Dicer and TRBP with or without Ago, a comparison of pre-miR-124 dicing kinetics could be done between two samples of Dicer and TRBP complex only and Dicer, TRBP and Ago complex (RLC). If pre-miR-124 displays increased dicing kinetics with the Dicer, TRBP and Ago complex sample compared to the Dicer and TRBP complex sample, this would indicate that Ago association with Dicer directly regulates the dicing of pre-miR-124.

Additionally, it could be the case that Ago interaction with Dicer does not directly regulate pre-miR-124 levels, but instead requires the presence of other unknown cofactors in the regulation of pre-miR-124. To identify such cofactors which are needed in pre-miR-124 regulation, a mass spectrometry approach could be used whereby tagged Ago protein could be overexpressed with and without miR-124, and the tagged Ago protein and interacting proteins forming a complex could be purified with or without crosslinking. Mass spectrometry analysis would then be performed on both samples with and without miR-124 overexpression. By comparison of the interacting proteins in the samples with and without miR-124 overexpression, unknown cofactor candidates could be identified which appear in the miR-124 overexpression sample but not the sample without miR-124 overexpression. After identification of potential cofactor candidates, in vitro knockdown experiments could be performed to see whether knockdown of these cofactors can abolish the Ago-dependent decrease in pre-miR-124

levels, which would confirm the involvement of these cofactors in pre-miR-124 regulation by Ago. Subsequently, these cofactors could then be examined in dicing assays, whereby pre-miR-124 dicing could be examined with four different samples: Dicer and TRBP only; Dicer, TRBP and Ago only; Dicer, TRBP and cofactor only or Dicer, TRBP, Ago and cofactor sample. This could then show the regulatory effect of Ago and cofactors on the dicing of pre-miR-124.

If Ago acts as an additional dicing cofactor for pre-miR-124 dicing, it will be of interest to know how the dicing of pre-miR-124 with Ago changes in the presence of different dicing cofactors. Dicer has two other known cofactors, TRBP (Chendrimada, Gregory et al. 2005, Haase, Jaskiewicz et al. 2005) and PACT (Lee, Hur et al. 2006), both which alters dicing of pre-miRNAs in different ways (Chakravarthy, Sternberg et al. 2010, Lee and Doudna 2012, Lee, Zhou et al. 2013). Since binding of TRBP to Dicer increases the kinetics of pre-miRNA processing by Dicer (Chakravarthy, Sternberg et al. 2010, Lee and Doudna 2012), it will be of interest to investigate the dicing activity of Dicer and Ago2 in the presence and absence of TRBP for pre-miR-124 dicing. Since Dicer, TRBP and Ago2 interact to form a complex, it could be possible that the combination of the two dicing cofactors could provide an additive effect on the dicing of pre-miR-124. Dicing assays on pre-miR-124 could be performed comparing the dicing kinetics of pre-miR-124 with Dicer+Ago2, Dicer+TRBP and Dicer+TRBP+Ago2 complexes.

Other possible mechanisms for Ago regulation of pre-miR-124 levels may be Ago regulation of upstream miRNA biogenesis steps. Decreased pre-miR-124 levels could instead be due to an Ago-dependent decrease in Drosha processing of miR-124. While Drosha mainly processes pri-miRNAs to form pre-miRNAs in the nucleus and Ago is present mainly in the cytoplasm, there exists a population of nuclear Ago (Robb, Brown et al. 2005, Weinmann, Hock et al. 2009, Huang, Zheng et al. 2013) which could be interacting with Drosha to decrease processing of pre-miR-124. Firstly, Ago localization experiments could be performed using GFP-labelled Ago or immunostaining of Ago using fluorophore-labelled Ago antibody and examination of cells under confocal microscopy, using cells with and without overexpression in miR-124 to examine whether

there is miR-124-dependent localization of Ago in the nucleus. In vitro Drosha assays could then be performed with two samples, using recombinant or purified Drosha and DGCR8 complex or Drosha, DGCR8 and Ago complex to see whether Ago might inhibit Drosha processing of pri-miR-124 to produce less pre-miR-124. If so, then Ago may function upstream on the Drosha processing step to regulate processing of pre-miR-124 from pri-miR-124.

Another viable mechanism for Ago regulation of pre-miR-124 could be the regulation of pre-miR-124 degradation through an Ago-dependent mechanism. Besides changes in miR-124 processing, increased degradation of pre-miR-124 could also lead to the decrease in pre-miR-124 levels during Ago overexpression. A list of candidate RNases could be obtained and screened for Ago-dependent pre-miR-124 degradation by comparing RNAi knockdown of each RNase with and without Ago overexpression and scrambled RNAi knockdown with and without Ago overexpression and northern blotting of RNA samples. If pre-miR-124 levels in the Ago overexpression and RNAi knockdown of RNase sample shows a return to pre-miR-124 levels in the scrambled RNAi without Ago overexpression sample, it could indicate that Ago might interact with the RNase to trigger pre-miR-124 degradation. Additionally, using a mass spectrometry approach outlined earlier with tagged Ago protein overexpressed with and without miR-124, and purification of the tagged Ago protein and interacting proteins forming a complex, associating RNases could be identified which might lead to the selective degradation of pre-miR-124 during Ago overexpression. Association of Ago and the identified RNase during miR-124 expression could then be confirmed using coimmunoprecipitation of tagged Ago with and without miR-124 expression and western blotting using the antibody for the identified RNase.

6.2.3 Correlation of endogenous expression levels of miR-124 and Ago1-4 during neural development

As previously mentioned, there is a lack of information of tissue-specific and developmental stage-specific Ago expression at the protein level. Hence, to find out the biological significance of high Ago expression levels, it will be of interest to find out the protein expression levels of Ago1-4 correlated with miR-124 expression in differentiating neuronal cells and at different developmental stages and time windows. This could be done by making transgenic animals by tagging differentiating neuronal cells with GFP driven under an early neuronal gene promoter. Neuronal cells of interest at different developmental stages could be mechanically dissociated from tissues and separated from non-neural cells using FACS, then the cell populations tested for Ago1-4 protein expression through mass spectrometry and miR-124 levels by small RNA-seq.

7 Conclusion

This project describes the regulation of pre-miR-124 by Ago overexpression and aims to identify the mechanisms of Ago-mediated pre-miR-124 regulation. So far, we identified miR-124 as the only miRNA whose pre-miRNA hairpin displays such a phenomenon. Co-overexpression of miR-124 and Ago2 appears to increase average neurite length in N2a cells.

Figure 7.1: Graphical abstract.

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