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Investigation in the mechanisms underlying activation-induced microRNA and Argonaute downregulation in helper T cells

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Investigation into the mechanisms underlying activation-induced microRNA and Argonaute downregulation in helper T cells

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CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

Chapter 2 of this dissertation is in press at the Journal of Experimental Medicine as "T cell activation induces proteasomal degradation of Argonaute and rapid remodeling of the microRNA repertoire." The co-authors on this publication were Alejandro V. Villarino[2], Christopher J. Eisley[3,4], Rebecca Barbeau[3], Andrea J. Barczak[3], Gitta A. Heinz[5], Elisabeth Kremmer[5], Vigo Heissmeyer[5], Michael T. McManus[6], David J. Erle[3], and Anjana Rao[7]. K. Mark Ansel[1] supervised the work.

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Investigation into the mechanisms underlying activation-induced miRNA and Argonaute downregulation in helper T cells

Yelena Bronevetsky

ABSTRACT

Helper T cells play a critical role in orchestrating and maintaining a healthy immune system. During an immune response, antigen-specific $CD4^+$ T cells proliferate and differentiate into effector T cells that act to coordinate the adaptive immune response by selective cytokine production. Thus, naïve T cells transition from a state of quiescence in which they express markers required for trafficking between blood and lymphoid organs, to an effector T cell fate, in which they actively divide, secrete effector molecules, and traffic to sites of infection. Major changes in gene expression characterize this transition, and microRNAs (miRNAs) play a critical role in this process. MiRNA expression also changes dramatically during T cell differentiation and in this dissertation, we strove to characterize these expression changes and investigate the underlying mechanism.

We used quantitative analyses to determine that T cell activation induces global post-transcriptional miRNA downregulation in vitro and in vivo. Argonaute (Ago) proteins, the core effector proteins of the miRNA-induced silencing complex (miRISC) are post-transcriptionally downregulated during T cell activation. We found that Ago2 is inducibly ubiquitinated in activated T cells, and its downregulation is inhibited by the proteasome inhibitor MG132. We hypothesize that activation-induced miRNA

downregulation likely occurs at the level of miRISC turnover. We also identified an additional layer of activation-induced miRNA-specific transcriptional regulation. Thus, transcriptional and post-transcriptional mechanisms cooperate to rapidly reprogram the miRNA repertoire in differentiating T cells. Altering Ago2 expression in T cells revealed that Ago proteins are limiting factors that determine miRNA abundance. Naïve T cells with reduced Ago2 and miRNA expression differentiate more readily into cytokine-producing helper T cells, suggesting that activation-induced miRNA downregulation promotes acquisition of helper T cell effector functions by relaxing the repression of genes that direct T cell differentiation.

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CHAPTER I: INTRODUCTION

Part A: MicroRNAs in CD4$^+$ T cell differentiation and function

Helper T cell differentiation and function

CD4$^+$ helper T cells play crucial roles in the coordination and orchestration of host defense and immune-mediated disease (Nakayamada et al., 2012). Based on the type of pathogen and environmental stimulus, helper T cells (Th) differentiate into specialized subsets with distinct immunological functions, restricted patterns of cytokine secretion and specific expression of transcription factors. The process by which a naïve, uncommitted CD4$^+$ T cell develops into a mature, differentiated Th cell is both a useful model of developmentally regulated gene expression, and crucial to the promotion of effective immune responses.

During immune responses, antigen-specific CD4$^+$ T cells transition from a state of relative quiescence in which they express markers required for trafficking between blood and lymphoid organs, to an effector T cell fate, in which they actively divide, secrete effector molecules, and traffic to sites of infection. Signals from the T cell receptor, co-stimulation from antigen presenting cells, and signals from the local cytokine milieu drive unique differentiation programs. Initially the fates of Th cells were thought to be limited to Th1 and Th2 cells (Mosmann and Coffman, 1989). Th1 cells express the transcription factor T-bet and secrete the cytokine Interferon-γ (IFN-γ) in response to IL-12. Th1 cells protect against intracellular infections including viruses, *Leishmania* species and *Toxoplasma gondii* (Murphy and Reiner, 2002). Th2 cells express GATA-3 and

secrete interleukin-4 (IL-4), IL-5, and IL-13 in response to IL-4. These cells protect against helminth infection. More recently, Th17 cells have also been described; these cells express the transcription factor RORγt and secrete IL-17, IL-21 and IL-22 to combat extracellular bacteria and fungi. Regulatory T cells (Tregs) exhibit essential immunosuppressive functions and express the transcription factor FoxP3. Finally, follicular helper T cells (Tfh) express Bcl6 and secrete IL-21 and IL-6. These cells provide help to B cells both to generate and maintain germinal centers and to differentiate into memory B cells and plasma cells (Hirahara et al., 2011). As well as their role in promoting host defenses, all of these subsets have been implicated in pathological responses. Both Th1 and Th17 cells can mediate organ-specific autoimmunity, while Th2 cells have been implicated in the pathogenesis of asthma and allergy. The absence of Tregs, due to mutations in Foxp3, leads to severe, systemic autoimmunity, while Tfh cells have been implicated in autoimmunity and lymphoma (Shekhar and Yang, 2012). Thus the final composition of the Th cell response to antigen can determine whether the outcome of infectious, inflammatory, and autoimmune responses is favorable or unfavorable to the host.

T cell receptor signaling

Engagement of T cell receptor (TCR) signaling in response to antigen recognition leads to downstream transcriptional responses mediated by a complex signaling network. Early TCR signals include phosphorylation of the TCR complex by the Src kinase Lck and subsequent assembly of a multimolecular complex including several signaling proteins, such as PLCγ (Abraham and Weiss, 2004; Huse, 2009). PLCγ transduces TCR

signals by hydrolyzing PIP2 to yield DAG, a membrane-associated lipid, and IP3, a diffusible second messenger. DAG induces MAPK signaling pathways by recruiting RasGRP to the membrane, leading to activation of transcription factors, including AP1, a key factor required at the IL-2 gene. DAG also activates PKCθ, leading to nuclear translocation of NF-κB, another important transcription factor. IP3 stimulates the opening of Ca^{2+}-permeable ion channels in the endoplasmic reticulum. Increased intracellular Ca^{2+} leads to dephosphorylation and nuclear import of NFAT, a third factor required for transcription of the IL-2 gene, mediated by the phosphatase calcineurin.

A key factor that regulates cell growth and metabolism downstream of TCR stimulation is the mammalian target of rapamycin (mTOR) (Chi, 2012). Antigenic stimulation, engagement of the CD28 coreceptor and signaling through the cytokines IL-2 and IL-4 lead to activation of the mTORC1 complex via the PI3 Kinase (PI3K) pathway. Other environmental cues can also activate mTOR activity, including decreases in ATP:AMP ratio, low oxygen concentration and amino acids (Powell et al., 2012). MTOR promotes translation initiation and protein synthesis by phosphorylating the ribosomal protein S6K1 and eIF4E-binding proteins (4E-BP1). MTOR also targets regulatory proteins with roles in cell signaling, metabolism, and autophagy. Tuberous sclerosis 1 (TSC1)-mediated control of mTOR signaling maintains the quiescence of naïve T cells by controlling cell size, cell cycle entry and metabolism (Chi, 2012). MTOR is also required for the differentiation of effector T cells, as mTOR-deficient T cells fail to differentiate into Th1, Th2, or Th17 cells and instead upregulate FoxP3 and develop into Tregs.

MicroRNA biogenesis and function

MicroRNAs (miRNAs) are processed from long pri-miR transcripts that may contain one or several miRNA stem loop structures (Fig. 1) (Kim et al., 2009b). Pri-miRs are cleaved into 60-80 nt hairpin intermediates (pre-miRNAs) by the nuclear microprocessor complex, comprised of the RNase III Drosha and the DiGeorge syndrome critical region gene (DGCR8). This pre-miRNA is exported from the nucleus by the nuclear transport receptor, exportin 5, and processed into an 18-22 nucleotide double stranded miRNA duplex by the cytoplasmic RNase III Dicer and its cofactor TRBP. The duplex is separated and usually one strand is selected as the mature miRNA, whereas the other strand is degraded. Mature miRNAs are incorporated into Argonaute (Ago) protein-containing effector complexes, known as 'miRNP' (miRNA-containing ribonucleoprotein complex) or miRISC (miRNA-containing RNA-induced silencing complex). In mammals, the miRISC generally recognizes a six-nucleotide target sequence in the 3' untranslated region of its target mRNA with imperfect complementarity to a seed sequence within the miRNA. MiRNA binding to target mRNA leads to mRNA destabilization, degradation and/or translational repression.

MicroRNAs in helper T cell function

Since the first descriptions of miRNAs, more than 20 years of research has identified a role for miRNAs in the regulation of diverse biological processes ranging from cell survival and apoptosis to organ development and immunity (Arasu et al., 1991; Kim et al., 2009b; Lee et al., 1993; O'Connell et al., 2010b). A key role for miRNAs in helper T cell development and function was identified in conditions of Dicer deletion in

the T cell lineage (Chong et al., 2008; Cobb et al., 2006; Muljo et al., 2005). These T-cell specific miRNA-deficient mice have many fewer T cells in the thymus and periphery. Dicer-deficient T cells exhibit decreased proliferation, increased apoptosis and dysregulated cytokine production, while mice with specific deletion of Dicer from Tregs develop severe autoimmunity, characterized by decreased numbers of mature Tregs in the periphery, inappropriate activation, and impaired maturation (Liston et al., 2008; Zhou et al., 2008).

Several individual miRNAs with crucial roles in helper T cell function have also been identified. MiR-181a acts to 'tune' TCR signal strength by targeting several protein tyrosine phosphatases, including SHP-2, PTPN22, DUSP5, and DUSP6, leading to enhanced basal activation of Lck and Erk (Li et al., 2007). MiR-29a regulates IFN-γ production by T cells by targeting two transcription factors, T-bet and Eomes, which promote IFN-γ production (Steiner et al., 2011b). MiR-155 is rapidly induced in T cells upon antigen stimulation and miR-155-deficient T cells have a Th2 differentiation bias (Haasch et al., 2002; Rodriguez et al., 2007). The transcription factor c-maf has been been identified as a direct miR-155 target that is partially responsible for the observed increase in IL-4 production in these T cells. MiR-155 also regulates Treg proliferation and survival via its target SOCS1 and promotes inflammatory T cell development through effects on T cells and dendritic cells (Lu et al., 2009; O'Connell et al., 2010a).

MiR-146a is another miRNA with confirmed function in helper T cells. It is one of the few miRNAs with differential expression between Th1 and Th2 cells, and is the most highly expressed miRNA in Tregs (Cobb et al., 2006; Monticelli et al., 2005). T cells from miR-146a-deficient mice display an activated, effector status and contribute to

the autoimmune disorder in these mice (Boldin et al., 2011). Further, miR-146a-deficient Tregs exhibit impaired regulatory activity and increased IFN-γ production, which appears to result from dysregulation of the miR-146a target gene, STAT1 (Lu et al., 2010).

The miR-17-92 cluster comprises several miRNAs expressed as a polycistronic transcript and contains four miRNA seed families that are highly expressed in T cells. Transgenic mice overexpressing these miRNAs develop lymphoid hyperplasia and autoimmunity, with substantial increase in the proliferation and survival of $CD4^+$ T cells (Xiao et al., 2008). Functional dissection of the cluster has revealed that miR-17 and miR-19b are the key players that promote T cell proliferation, survival, IFN-γ production and suppression of inducible Treg differentiation (Jiang et al., 2011). Members of this miRNA cluster target genes including the anti-apoptotic factor Bim, the tumor suppressor PTEN, the cell-cycle regulator p21, and the TGFβ receptor II.

Part B: Regulation of miRNA biogenesis and turnover in the immune system

MicroRNA transcriptional regulation

A key feature of animal development is the precise control of gene expression, such that transcripts are maintained at correct concentrations in space and time. Much of the complexity of transcriptional control involves the combinatorial interplay of transcriptional activators and repressors, leading to some transcriptional output. One mechanism that has evolved to maintain fidelity in gene expression is autoregulation, in which a transcriptional product feeds back to increase or inhibit its own production. MiRNAs are particularly suited to participate in feedback circuits to directly repress

mRNAs that encode factors involved in the transcription or biogenesis of the same miRNAs (Fig. 2) (Davis and Hata, 2009).

Due to limitations in methodology available to investigate primary miRNA transcripts along with a paucity of promoter characterization, transcription of mammalian miRNA genes is poorly understood. One study addressed this issue by combining nucleosome mapping with promoter chromatin signatures to identify the proximal promoters of many human miRNAs. Specifically they combined data about nucleosome-depleted regions, high evolutionary conservation, CpG islands, transcription factor motifs within regions containing trimethylation of Lys 4 of histone 3 (H3K4me3) or acetylation of Lys 9/14 of histone 3 (H3K9/14Ac), and RNA Polymerase II or III binding to determine the transcription initiation regions of 175 miRNAs (Ozsolak et al., 2008). This study and others found that many miRNAs use their own transcription initiation regions, whether they are located between gene clusters (intergenic) or within introns of coding genes (intronic) (Schanen and Li, 2011).

The identity of the RNA Polymerase that transcribes miRNA genes has also been difficult to define. It was first presumed that transcription of miRNA genes would be similar to that of other small RNAs, such as tRNA, and would require RNA Polymerase III (Pol III). However, several studies revealed that many miRNA transcripts contain features typical of RNA Polymerase II (Pol II), including polyadenylation and splicing (Lee et al., 2004). More recent studies have determined that some miRNA genes, especially those interspersed among Alu repeats, are transcribed by Pol III (Borchert et al., 2006). These findings have led to a model in which intragenic miRNAs (within introns or exons of protein-coding genes) on the same strand as their host gene are co-

transcribed by Pol II, while intergenic miRNAs are transcribed from their own Pol II or Pol III promoter (Schanen and Li, 2011).

C-myc. A number of key transcriptional regulators in the immune system have been found to also target miRNA genes. One of earliest identified regulators is the oncogenic transcription factor c-Myc, whose dysregulated expression or function frequently occurs in human malignancies. C-Myc also blocks differentiation of hematopoietic cell models in culture, and controls the balance between hematopoietic stem cell self-renewal and differentiation (Delgado and Leon, 2010). C-Myc is further required to promote proliferation of lymphocytes and mutations and translocations in c-Myc have been found in numerous human lymphomas and leukemias, including Burkitt lymphoma and acute myeloid leukemia (Delgado and Leon, 2010). O'Donnell et al. determined that c-Myc binds directly to the miR-17-92 locus on human chromosome 13 and transactivates expression of these miRNAs in the human B cell line P493-6 (O'Donnell et al., 2005). The miR-17-92 cluster has also been shown to have oncogenic potential, as its overexpression in lymphocytes leads to lymphoproliferative disease and premature death (Xiao et al., 2008). This same research group also found that c-Myc regulates a much broader set of miRNAs (Chang et al., 2008). Using human and mouse B cell lines, they showed c-Myc binding to the promoters of 12 miRNA genes, with the predominant consequence being repression of miRNA expression. Enforced expression of repressed miRNAs diminished the tumorigenic potential of lymphoma cells. Though not through direct transcriptional repression, mature let-7 is a miRNA decreased in response to c-Myc (Chang et al., 2009). Interestingly, let-7, in complex with the RNA-

binding protein HuR, can repress c-Myc transcript, thus regulating its own expression (Kim et al., 2009a).

NF-κB. Nuclear factor κB (NF-κB) is a key transcriptional regulator linking inflammation and tumorigenesis (Ma et al., 2011). Activation of this pathway in numerous cell types leads to the production of cytokines and chemokines, such as TNF-α, IL-1, IL-6, and IL-8, which are associated with tumor development and progression. NF-κB can be activated by many oncogenes, and mutations in the NF-κB pathway are implicated in activated B-cell (ABC)-like diffuse large B-cell lymphoma and multiple myeloma. Taganov et al. showed that exposure of human monocytic THP-1 cells to lipopolysaccharides (LPS), TNF-α, and IL-1β leads to rapid induction of miR-146a and 146b, probably mediated by NF-κB sites in the promoter (Taganov et al., 2006). Two key adapter molecules in the NF-κB pathway, TRAF6 and IRAK1, were identified as direct targets of miR-146, suggesting a negative feedback loop in which NF-κB activation induces miR-146 expression, which downregulates TRAF6 and IRAK1 to reduce NF-κB activity. The NF-κB complex can also transactivate miR-155 transcription in Epstein-Barr virus-immortalized B cells (Gatto et al., 2008). MiR-155 is an oncogenic miRNA critical for B-cell maturation and antibody production. It is highly expressed in many human B cell lymphomas and is induced in innate cells in response to inflammatory cytokines (Ma et al., 2011). NF-κB signaling also indirectly increases the transcription of several other miRNAs, including miR-21, -181a, and -9. Interestingly, miR-9 can directly repress the NF-κB1 transcript (Bazzoni et al., 2009).

The *c-myb* proto-oncogene encodes a hematopoietic cell transcription factor, which when mutated, is associated with marrow megakaryocyte hyperplasia and

thrombocytosis (Zhao et al., 2009). C-myb expression is highest in hematopoietic progenitors and its down-regulation is required for lineage differentiation. Interestingly, miR-15a can target the c-myb transcript in K562 human erythroleukemia cells and this interaction is important in limiting cell-cycle progression. C-myb itself binds to elements in the miR-15a promoter, transactivating its expression. Another regulator of hematopoiesis, Runx1, participates in a similar regulatory loop. During megakaryocytic differentiation, Runx1 binds a region upstream of miR-27a and induces its expression (Ben-Ami et al., 2009). Concurrently, miR-27a, in addition to several other miRNAs, can repress the Runx1 mRNA. Finally, human granulopoiesis is controlled by a regulatory circuit involving miR-223 and the transcription factors NFI-A and C/EBPα (Fazi et al., 2005). Both factors compete for binding to the miR-223 promoter, with NFI-A repressing miR-223 transcription while C/EBPα induces it in response to retinoic acid-induced differentiation. MiR-223 can target NFI-A mRNA, thus promoting its own expression during granulocyte differentiation.

Regulation of miRNA processing

It has been realized for some time that mature miRNA levels are not determined solely by their transcription. Thomson et al. observed a lack of correlation between many pri-miRs and their corresponding mature miRNA in developing mouse embryos and extended this observation to many tumor samples with global miRNA reduction (Thomson et al., 2006). This observation was extended to developing B cells, where only 25% of the fluctuations in miRNA expression could be attributed to differential transcription (Kuchen et al., 2010). While the mechanism by which transcription factors

confer specificity in their regulation of miRNA levels is fairly straightforward, it is less obvious how regulation of the miRNA processing pathway can lead to effects on individual miRNAs and not others. Many studies have concluded that accessory proteins impart specificity by interacting with Dicer or Drosha, or with miRNA precursors. The immune system contains several examples in which a signaling molecule is recruited to a processing complex where it modulates the processing of a single miRNA or set of miRNAs (Fig. 3).

SMAD. Davis et al. identified one of the first examples of a signal transduction pathway leading to changes in miRNA biogenesis when they showed that the TGF-β and BMP pathways promote processing of pri-miR-21 in smooth muscle cells (Davis et al., 2008). Both of these pathways control gene expression through the Smad proteins, which transduce extracellular signals from TGF-β ligands to changes in transcription of target genes (Schiffer et al., 2000). Interestingly the effect of Smads on miR-21 processing was posttranscriptional and required an interaction between R-Smads and the DEAD-box RNA helicase p68, a critical subunit of the Drosha microprocessor complex (Fukuda et al., 2007). This finding was expanded to include 20 TGF-β/BMP-regulated miRNAs, many of which contain a consensus sequence within the stem region of their pri-miRs (Davis et al., 2010). Smads were demonstrated to directly bind this sequence, and mutation of the sequence abrogated TGF-β/BMP-induced recruitment of Smads, Drosha and DGCR8 to the pri-miR, impairing its processing. Introduction of this sequence to unregulated pri-miRs was sufficient to recruit Smads and allow regulation of processing by TGF-β/BMP. Thus in this example, specificity for miRNA processing is determined by the interaction between a miRNA-specific sequence and a signal transduction protein.

This mechanism is of particular relevance to the immune system, where the TGF-β pathway plays a critical role in suppressing inflammation. Specifically TGF-β induces differentiation of naïve T cells into Foxp3+ regulatory T cells (Tregs) in the peripheral immune system and TGF-β in concert with IL-6 drives T helper 17 (Th17) differentiation (Yoshimura et al., 2010). Interestingly miR-21 is more highly expressed in induced Tregs (iTregs) and natural Tregs (nTregs) than in Th1 or Th2 cells, implying that TGF-β signaling could increase processing of this miRNA in T cells (Kuchen et al., 2010). Recent work has shown that Smads can also regulate transcription of the miR-302-367 gene cluster, indicating that this pathway can modulate miRNA expression at multiple levels (Kang et al., 2012).

P53. Another protein that regulates miRNA levels at multiple steps in their biogenesis is the tumor suppressor p53. Mouse models of p53 deficiency predominantly exhibit malignant lymphomas, and 19.3% of human lymphoid malignancies and 11.1% of myeloid malignancies exhibit mutations in the p53 coding sequence (Xu-Monette et al., 2012). P53 directly activates transcription of miRNAs like miR-34, -200, -15a and -16 that mediate much of p53's tumor suppressive activity (Hermeking, 2012). Ectopic expression of miR-34 arrests cells in the G1 phase and this miRNA directly targets numerous cell cycle regulators like cyclin E2 and c-myc. Further, the *DLEU2* locus containing miR-15a and miR-16-1 is commonly deleted in human chronic lymphocytic leukemia, exemplifying the important role p53-induced miRNAs play in preventing unrestrained cell proliferation. MiR-29, a key miRNA involved in T cell differentiation, is also induced by p53 and participates in a positive feedback loop to reinforce p53

expression by targeting two negative regulators of p53, CDC42 and the p85α-regulatory subunit of PI3K (Park et al., 2009; Steiner et al., 2011a).

Besides regulating the expression of miRNA genes, p53 also affects the processing of specific miRNAs. P53 associates with Drosha, in a process mediated by a direct interaction between p53 and p68 (Suzuki et al., 2009). This interaction increases the processing of several pri-miRs, like pri-miR-16-1, -143, -145 and -206, into mature miRNAs. This capacity of p53 to promote the processing of specific pri-miRs is mediated by its DNA-binding domain, as tumor-derived transcriptionally inactive mutant p53 suppresses the interaction between Drosha and p68, resulting in decreased levels of mature miR-16-1, -143, and -206.

P53 can further function downstream of defective miRNA processing. Dicer can act as a haploinsufficient tumor suppressor, such that partial loss of Dicer function can be tumorigenic (Kumar et al., 2009). Dicer deficiency, and therefore incomplete miRNA maturation, induces p53 expression, which leads to reduced proliferation and premature senescence in mouse embryonic fibroblasts (MEFs) (Mudhasani et al., 2008). This effect is dependent on p53, as deletion of p53 prevents premature senescence induced by Dicer deletion. Furthermore, p63, a p53 family member, can directly transactive Dicer transcription (Su et al., 2010).

Recently another mechanism by which p53 regulates miRNA processing and function was identified. The RNA-binding protein, RNA-binding motif protein 38 (RBM38), is induced by p53 and is required for optimal induction of G1 cell cycle arrest following DNA damage (Leveille et al., 2011). RBM38 functions by shielding the 3' UTRs of several p53 targets, such as *CDKN1A* (which encodes p21) from repression by

miRNAs. Taken together these studies illustrate the importance of control of miRNA biogenesis and function for p53's tumor suppressive activity.

Lin28. The let-7 family constitutes one of the most abundant miRNA clusters in mammals, with high expression in virtually all adult tissues. In contrast, embryonic stem cells and hematopoietic progenitor cells from fetal origin express significantly less let-7. Unexpectedly, pri-let-7 expression remains constant throughout development, indicating that a post-transcriptional mechanism of regulation exists (Thomson et al., 2006). By immunoprecipitating proteins that associate with pre-let-7, the RNA-binding protein Lin28 (and its homologue Lin28b) was identified as a factor that blocks let-7 maturation in embryonic and progenitor cells (Viswanathan et al., 2008). Lin28 represses both Drosha and Dicer processing of let-7 in vitro by binding to conserved sequences in the precursor loop. Interestingly, pri-let-7 and pre-let-7 are not found to accumulate in embryonic cells, suggesting that Lin28 functions through a turnover mechanism. In fact, Lin28 directly recruits a poly(U) polymerase, terminal (U) transferase (TUT4), to pre-let-7 and oligo-uridylation of the precursor triggers degradation (Heo et al., 2009). In another example of feedback regulation, Lin28 is itself a target of let-7, with Lin28 expressed in a pattern reciprocal to that of mature let-7 during embryonic development (Reinhart et al., 2000; Rybak et al., 2008).

Recent work has highlighted the key role of the Lin28-Let-7 axis in hematopoiesis. The immune system develops in waves during ontogeny, such that it is first populated by cells generated from fetal hematopoietic stem cells (HSCs) and later by cells derived from adult HSCs. Yuan et al. found that Lin28b is specifically expressed in mouse and human fetal liver and thymus, but not adult bone marrow or thymus (Yuan et

al., 2012). Further, ectopic expression of Lin28 reprograms hematopoietic stem and progenitor cells from adult bone marrow, allowing them to mediate multilineage reconstitution that mirrors fetal lymphopoiesis. This includes increased development of B-1a B cells, marginal zone B cells, gamma/delta T cells, and natural killer T cells. This is also in line with data that Lin28, in concert with several ES cell-specific transcription factors, can reprogram somatic cells into induced pluripotent stem cells (Yu et al., 2007).

Estrogen is a key steroid hormone that mediates sex differences. Estrogen also affects numerous cell of the immune system, including T and B cells, and monocytes, and may contribute to the increased frequency of autoimmune disorders in females as compared to males (Oertelt-Prigione, 2012). Interestingly, signaling through the estrogen receptor α (ERα) inhibits processing of certain miRNAs, including miR-16 and -125a (Yamagata et al., 2009). In the presence of its ligand estradiol (E_2), ERα is recruited to the Drosha complex, leading to dissociation of Drosha from ERα-targeted pri-miRNAs.

KH-type splicing regulatory protein (KSRP) is an RNA-binding protein that destabilizes mRNAs via adenylate/uridylate-rich elements (ARE) in the 3'UTR region of transcripts. KSRP regulates inflammation in the central nervous system by negatively regulating transcripts including TNF-α and IL-1β in astrocytes (Li et al., 2012). Interestingly KSRP also associates with Dicer and Drosha and increases the processing of several miRNAs including let-7 and miR-21 (Trabucchi et al., 2009). This protein binds specifically to 5' guanosine-rich regions in the loop regions of several miRNA precursors and recruits Drosha and Dicer to the pri- and pre-miRNA, respectively.

Another protein that employs a similar mechanism of regulation is hnRNP A1, a protein implicated in many RNA processing and transport pathways (He and Smith,

2009). Guil et al. determined that hnRNP A1 can also enhance Drosha-mediated processing of pri-miR-18a (Guil and Caceres, 2007). HnRNP A1 binds to the loop of pri-miR-18a and induces relaxation of the stem region, promoting cleavage of this precursor by Drosha (Michlewski et al., 2008). Though miR-18a is part of the polycistronic miR-17-92 cluster, which has key functions in immune cell development and function (O'Connell et al., 2010b), the other miRNAs in this cluster are unaffected by hnRNP A1. MiR-18b, which is part of the homologous primary cluster miR106a~18b~20b, is also processed independently of hnRNP A1, highlighting the specificity of this regulation. Interestingly, approximately 14% of all pri-miRNAs have highly conserved loop regions, suggesting the existence of other auxiliary factors for processing of specific miRNAs (Michlewski et al., 2008).

Modulation of mature miRNA homeostasis

The last stage at which miRNA levels are regulated is at the level of the mature miRNA; RISC loading, targeting of mRNA targets, and stability of mature miRNAs have all been shown to influence mature miRNA homeostasis. One observation that has stemmed from this research is that cell differentiation and/or cell fate changes are often marked by dramatic changes in mature miRNAs. These changes will be discussed in the context of the immune system

Argonaute. In contrast to the *Drosophila* system in which dAgo1 is primarily dedicated to the miRNA pathway while dAgo2 functions in RNAi, the four vertebrate Ago protein appear to have largely overlapping roles in miRNA-mediated repression with little miRNA sorting preferences between the individual proteins (Azuma-Mukai et al.,

2008; Hafner et al., 2010). This is despite Ago2's unique role in endonucleolytically cleaving perfectly complementary RNA targets. Several studies have used Argonaute deficiency, knockdown, or overexpression to implicate Argonautes as limiting factors for miRNA expression. Ago2 is required for normal hematopoiesis and through the use of bone marrow chimeras, O'Carroll et al. determined that Ago2, independent of its "slicer" activity, is required for the maturation of B cell and erythroid precursors (O'Carroll et al., 2007). Ago2-deficient erythroblasts, fibroblasts and hepatocytes display a global reduction in miRNAs. MicroRNA expression is also reduced in Ago2-deficient MEFs and in Ago-deficient *Xenopus* embryos during early development (Diederichs and Haber, 2007; Lund et al., 2011). Conversely, overexpression of Ago2, but not other proteins in the miRNA biogenesis pathway, increases miRNA expression in HEK293 cells. Thus changes in Argonaute stability can have dramatic effects on mature miRNA levels within a cell.

Several post-translational modifications have been identified which regulate Ago2 localization, function and stability. In response to stress signals like sodium arsenite or anisomycin, phosphorylation of Ago2 at serine-387 was observed in HEK293T cells (Zeng et al., 2008). This phosphorylation was crucial for localization of Ago2 to P-bodies, as mutating the residue abrogated this localization. These authors further determined that this phosphorylation was dependent on p38 MAPK. Rüdel et al. identified tyrosine-529 as a crucial residue required for binding of the 5' end of small RNAs, and that phosphorylation of this residue abrogates small RNA binding (Rudel et al., 2011). It was further determined that prolyl hydroxylation of proline-700 is important for the stability of Ago2, with mutation of this residue or knockdown of type I collagen

prolyl-4-hydroxylase, the enzyme required for this modification, resulting in destabilization of Ago2 (Qi et al., 2008b). Adams et al. determined that signaling through Epidermal Growth Factor (EGF) receptor/MAPK signaling pathway enhanced Ago2 stability in human breast cancer cell lines (Adams et al., 2009). Interestingly they also found that Ago2 turnover could be blocked by treatment with the proteasome inhibitor, MG132, indicating that Ago2 may be ubiquitinated. In fact, Rybak et al. determined that Ago2 is ubiquitinated by the let-7 target mLin41 (aka Trim71) in several mouse stem cell niches, leading to Ago2 proteasomal degradation and loss of miRNA-mediated repression (Rybak et al., 2009). However studies in mouse neural progenitors and mouse ES cells found that loss of mLin41 did not lead to changes in Ago2 stability, arguing against a role for mLin41 as the ubiquitin ligase for Ago2 (Chang et al., 2012; Chen et al., 2012).

Global miRNA downregulation has been observed in numerous human tumors, as compared to normal tissue (Gaur et al., 2007; Lu et al., 2005). Further, poorly differentiated tumors have lower global levels of miRNA expression compared with more differentiated samples. This data is consistent with the idea that miRNA expression is closely linked to differentiation. Further, global repression of miRNA maturation promotes transformation and tumorigenesis, and Dicer1 can function as a haploinsufficient tumor suppressor (Kumar et al., 2007; Kumar et al., 2009). It will be interesting to determine if Ago has similar tumorigenic properties.

Exosome. A growing literature suggests that RISC components interact with intracellular membranes associated with vesicle transport, including endosomes and multivesicular bodies (MVBs) (Gibbings et al., 2009; Lee et al., 2009). Blocking MVB formation by depleting components of the endosomal sorting complex required for

transport (ESCRT) results in impaired miRNA silencing in human and *Drosophila* cells, indicating a required role for this physical association. Further, RISC components and miRNAs have also been found in exosomes (Fig. 4) (Valadi et al., 2007). Exosomes are small vesicles (~50nm in diameter), which form by invagination and budding from the limiting membrane of late endosomes, leading to the secretion of internal vesicles into the extracellular environment (Thery et al., 2002). Exosomes have been isolated from the culture supernatant of many hematopoietic cells, including cytotoxic T lymphocytes, mast cells, and dendritic cells (DC), and DC-derived exosomes have been shown to stimulate $CD4^+$ T cell activation and induce tolerance (Zitvogel et al., 1998).

Zhang et al. determined that stimulation of THP-1 human monocytes with LPS, oleic acid/palmitic acid, advanced glycation end products and H_2O_2 led to dramatic changes in miRNA expression both in cells and in vesicles isolated from culture supernatants (Zhang et al., 2010). These vesicles contained both Ago2 and miRNAs including miR-150, -21, and -26b; vesicle-derived miR-150 could be delivered to recipient HMEC-1 human endothelial cells and repress a corresponding mRNA transcript. Subsequent studies have suggested that vitamin D_3- and LPS-treated DCs release miRNA-containing exosomes that can be captured by other DCs during co-culture (Montecalvo et al., 2012). *Staphylococcus* enterotoxin superantigen-E-pulsed Raji B cells were also shown to acquire exosome-derived J77 T cell-expressed miRNAs, which could then repress mRNA targets in the recipient cells (Mittelbrunn et al., 2011).

Recent studies have investigated the role of these extracellular miRNAs in numerous human fluids. Circulating miRNAs have been studied in patient samples and animal models in the context of cardiovascular disease, liver injury, sepsis, cancer, and

various other physiological and pathophysiological states (Cortez et al., 2011). Tumor-specific miRNAs were first discovered in the serum of patients with diffuse large B-cell lymphoma, where high levels of miR-21 correlated with improved relapse-free survival (Lawrie et al., 2008). Since then more than 200 studies have assessed the use of serum or plasma miRNAs as biomarkers in different types of cancers and between patients with different prognoses. For example, a specific profile of plasma miRNAs was identified in patients with chronic lymphocytic lymphoma (CLL), as compared to those with multiple myeloma, hairy-cell leukemia and healthy controls (Moussay et al., 2011). This study indicated that circulating miRNAs could be correlated with the prognosis marker ZAP-70 status and could be used to stratify patients with CLL.

The origin of serum or plasma miRNAs is still poorly understood. Correlations between miRNAs highly expressed in solid tumors and those same miRNAs found in high levels in the circulation of patients with those tumor types have hinted that these miRNAs could be released by tumors themselves (Reid et al., 2011). However Pritchard et al. recently reported that blood cells are a major contributor to circulating miRNAs and that alterations in blood cell counts and hemolysis can change plasma miRNA biomarker levels by up to 50-fold (Pritchard et al., 2012). It also remains unclear whether circulating miRNAs must be contained within exosome vesicles. A recent study found that two populations of circulating miRNAs exist in human plasma: those encapsulated in vesicles and those that are not (Arroyo et al., 2011). These authors determined that the non-vesicle-associated plasma miRNAs could be immunoprecipitated with Ago2, indicating that circulating Ago2 complexes could be a mechanism responsible for the stability of plasma miRNAs. Another study found that some plasma miRNAs are associated with

high-density lipoprotein (HDL) and these HDL-miRNA complexes could deliver miRNAs to recipient cells (Vickers et al., 2011).

Turnover. As compared to the regulation of miRNA transcription and processing, relatively little is known about the regulation of mature miRNA turnover. In *Arabidopsis* a 3'-to-5' exonuclease SDN1 degrades mature miRNAs, while a 2'-O-methyl modification on the 3'-terminal ribose of miRNAs, mediated by the Hen1 methyltransferase, protects them from degradation (Ramachandran and Chen, 2008), In *Caenorhabditis elegans,* a 5'-to-3' exonuclease XRN2 degrades mature miRNAs; introduction of target mRNA protects its cognate miRNA from degradation (Zitvogel et al., 1998). In vertebrate miRNA turnover, a theme has recently emerged in which the interaction between a small RNA and its target can in some cases, usually in the context of extensive base pairing, result in degradation of the miRNA. This is often accompanied by 3'-tailing and trimming of the miRNA (Ameres et al., 2010). In *Drosophila*, extensive complementarity between a target RNA and Ago1-bound miRNA triggers miRNA tailing and 3'-to-5' trimming. Like in *Arabidopsis,* Hen1-mediated 2'-O-methylation of the 3'-terminus protects miRNAs from tailing and trimming.

Sequence-specific degradation of miRNAs following addition of RNA targets has also been observed in mammalian cells. MiRNA "antagomirs" and "sponges", two technologies used to specifically knock down miRNA expression, both rely on high miRNA-to-target complementarity leading to miRNA degradation (Ebert et al., 2007; Krutzfeldt et al., 2005). In HeLa cells, transfection of antagomirs or incubation with target mRNAs triggers 3' tailing and degradation of the corresponding miRNA (Ameres et al., 2010). Addition of mRNA targets into A70 proB cells also leads to depletion of the

cognate miRNA (Kuchen et al., 2010). Several recent studies have also identified mechanisms by which viruses hijack this process in infected cells. During infection of Jurkat T cells with Herpesvirus saimiri, miR-27 abundance is dramatically decreased. This decrease is dependent on two virally-encoded transcripts that contain several miR-27 binding sites (Cazalla et al., 2010). MiR-27 is similarly regulated by a transcript encoded by lytic murine cytomegalovirus (MCMV) in infected fibroblasts (Libri et al., 2012; Marcinowski et al., 2012). Finally, during poxvirus infection of mouse fibroblasts, a virally encoded poly(A) polymerase mediates 3' polyadenylation of host miRNAs, leading to their degradation (Backes et al., 2012)

The identity of the mammalian exoribonuclease that degrades miRNAs remains unknown, though our lab has studied the role of Eri1 in this process. Eri1 is a 3'-to-5' exoribonuclease with a deeply conserved role in 5.8S rRNA 3' end processing (Ansel et al., 2008). We determined that Eri1 also negatively regulates miRNA levels in $CD4^+$ T cells and Natural Killer (NK) cells (Thomas et al., 2012). Eri1-deficient mice have a 50% reduction in NK cell numbers and the remaining NK cells exhibit an immature phenotype and fail to expand sufficiently during MCMV infection. These data suggest that Eri1 is required to promote NK cell homeostasis and immune function. It remains to be determined if Eri1 can directly degrade miRNAs. A recent study determined that IRE1α, an endoplasmic reticulum (ER) transmembrane RNase activated in response to ER stress, degrades miR-17, -34a, -96, and -125b (Upton et al., 2012). This mechanism relieves repression of Caspase-2 mRNA, which is an early apoptotic switch upstream of the mitochondria. Interestingly, ER stress contributes to inflammation by both NFκB-dependent and –independent mechanisms, and ER stress has been implicated in chronic

inflammatory diseases, such as diabetes, arthritis, and inflammatory bowel disease (Hasnain et al., 2012). Further work will be required to determine the role of these miRNAs in inflammation.

Figures

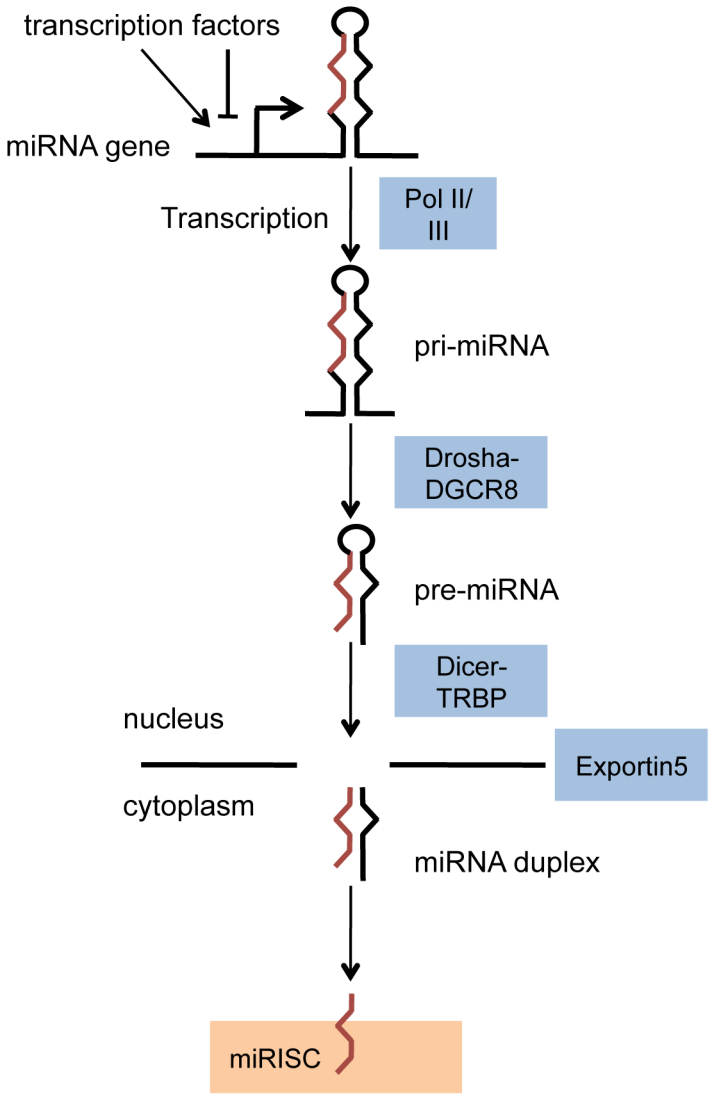

Figure 1. MicroRNA biogenesis. miRNAs are transcribed by RNA Polymerase II or III and processed from long primary transcripts (pri-miRs) which are cleaved into 60-80 nt hairpin intermediates (pre-miRNAs) by the nuclear microprocessor complex, comprised

of Drosha and DGCR8. This pre-miRNA is exported from the nucleus by the nuclear transport receptor, exportin 5, and processed into an 18-22 nucleotide double stranded miRNA duplex by Dicer and its cofactor TRBP. The duplex is separated and one strand is selected as the mature miRNA, whereas the other strand is degraded. Mature miRNAs are incorporated into Argonaute (Ago) protein-containing effector complexes, known as miRISC (miRNA-containing RNA-induced silencing complex). In mammals, the miRISC generally recognizes an imperfectly matched target sequence in the 3' untranslated region of its target mRNA. MiRNA binding to target mRNA leads to mRNA degradation and/or translational repression.

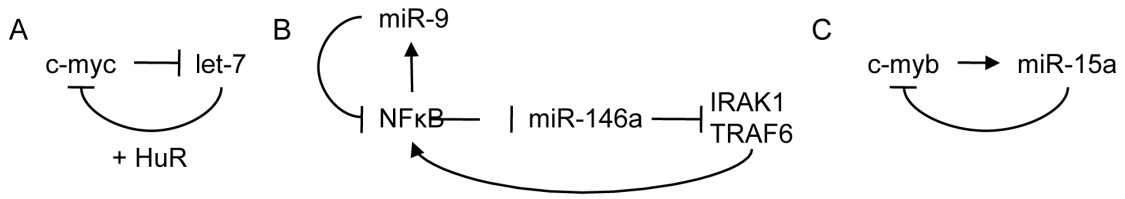

Figure 2. MicroRNA transcriptional feedback loops. (A) C-myc is a repressor of pri-let-7 transcription (Chang et al., 2008). In turn, c-myc mRNA is repressed by let-7, in an HuR-dependent mechanism (Kim et al., 2009a). (B) NF-κB is a transcriptional activator of miR-146a and -146b (Taganov et al., 2006). MiR-146 targets TRAF6 and IRAK1, two adapter molecules in the NF-κB pathway, which leads to decreased NF-κB activity. NF-κB also induces miR-9 transcript (Bazzoni et al., 2009) and miR-9 targets the *NF-κB1* gene. (C) C-myb is an activator of miR-15a transcription and miR-15a represses c-myb mRNA (Zhao et al., 2009).

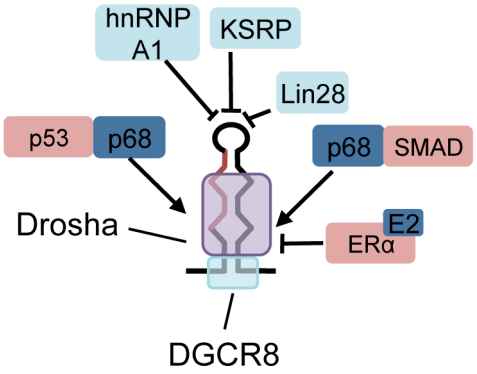

Figure 3. Regulation of miRNA processing. Many regulators of miRNA processing interact with Drosha, p68 or miRNA precursor loops. P53 associates with Drosha, via p68, and increases the processing of several pri-miRs, like pri-miR-16-1, -143, -145, and -206, into mature miRNAs (Suzuki et al., 2009). HnRNP A1 binds a conserved region of the pri-miR-18a loop and promotes its cleavage by Drosha (Michlewski et al., 2008). KSRP binds sequences in the loop region of several miRNA precursors, including let-7 and miR-21. KSRP recruits Drosha and Dicer to the pri- and pre-miRNA, respectively, and increases processing (Trabucchi et al., 2009). Lin28 represses both Drosha and Dicer processing of let-7 by binding conserved sequences in the precursor loop (Viswanathan et al., 2008). Lin28 recruits TUT4 to pre-let 7 and oligo-uridylation of the precursor triggers degradation (Heo et al., 2009). Smad proteins, downstream of TGF signaling, bind a consensus sequence within the stem region of numerous miRNAs, including miR-21 (Davis et al., 2010). This interaction recruits Drosha and increases processing of targeted miRNAs. In the presence of its ligand, estradiol (E_2), estrogen receptor α (ERα) is recruited to the Drosha complex, which causes dissociation of Drosha from ER α-targeted pri-miRs (Yamagata et al., 2009). This inhibits processing of several miRNAs, including miR-16 and -125a.

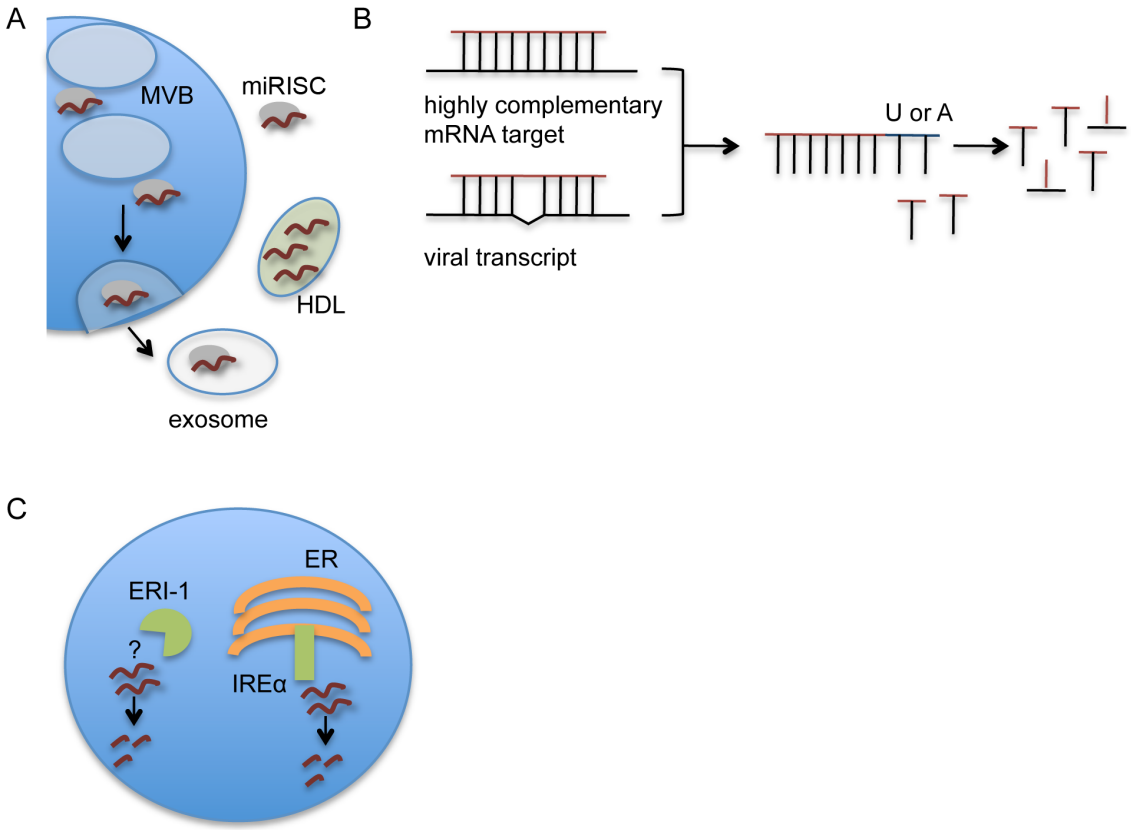

Figure 4. Mechanisms of miRNA turnover. (A) RISC components interact with intracellular membranes associated with vesicle transport, including endosomes and multivesicular bodies (MVBs) (Gibbings et al., 2009; Lee et al., 2009). RISC components and miRNAs have also been found in exosomes (Thery et al., 2002; Valadi et al., 2007). MiRNA-containing exosomes have been isolated from activated monocytes, dendritic cells, and B cells (Mittelbrunn et al., 2011; Montecalvo et al., 2012; Zhang et al., 2010). Extracellular miRNAs have been identified in numerous human fluids, including serum and plasma (Cortez et al., 2011). Circulating miRNAs are found within exosomes, non-vesicle-associated Ago2 complexes, and in association with high-density lipoprotein (HDL) (Arroyo et al., 2011; Vickers et al., 2011) (B) Sequence-specific degradation of miRNAs following addition of highly complementary RNA targets has been observed in

mammalian cells. (Ameres et al., 2010; Ebert et al., 2007; Krutzfeldt et al., 2005; Kuchen et al., 2010). Several viruses encode transcripts with miRNA binding sites, which lead to degradation of the cognate miRNA (Backes et al., 2012; Cazalla et al., 2010; Libri et al., 2012; Marcinowski et al., 2012) (C) Eri1 is a 3'-to-5' exoribonuclease with a deeply conserved role in 5.8S rRNA 3' end processing (Ansel et al., 2008). Eri1 also negatively regulates miRNA levels in $CD4^+$ T cells and Natural Killer (NK) cells, but it remains to be determined if Eri1 can directly degrade miRNAs (Thomas et al., 2012). IRE1α, an endoplasmic reticulum (ER) transmembrane RNase activated in response to ER stress, degrades miR-17, -34a, -96, and -125b (Upton et al., 2012).

CHAPTER II: T CELL ACTIVATION INDUCES PROTEASOMAL DEGRADATION OF ARGONAUTE AND RAPID REMODELING OF THE MICRORNA REPERTOIRE

Abstract

Activation induces extensive changes in the gene expression program of naive $CD4^+$ T cells, promoting their differentiation into helper T cells that coordinate immune responses. MicroRNAs (miRNAs) play a critical role in this process, and miRNA expression also changes dramatically during T cell differentiation. Quantitative analyses revealed that T cell activation induces global post-transcriptional miRNA downregulation in vitro and in vivo. Argonaute (Ago) proteins, the core effector proteins of the miRNA-induced silencing complex (miRISC), were also post-transcriptionally downregulated

during T cell activation. Ago2 was inducibly ubiquitinated in activated T cells, and its downregulation was inhibited by the proteasome inhibitor MG132. Therefore, activation-induced miRNA downregulation likely occurs at the level of miRISC turnover. Measurements of miRNA processing intermediates uncovered an additional layer of activation-induced miRNA-specific transcriptional regulation. Thus, transcriptional and post-transcriptional mechanisms cooperate to rapidly reprogram the miRNA repertoire in differentiating T cells. Altering Ago2 expression in T cells revealed that Ago proteins are limiting factors that determine miRNA abundance. Naïve T cells with reduced Ago2 and miRNA expression differentiated more readily into cytokine-producing helper T cells, suggesting that activation-induced miRNA downregulation promotes acquisition of helper T cell effector functions by relaxing the repression of genes that direct T cell differentiation.

Introduction

During immune responses, antigen-specific $CD4^+$ T cells undergo clonal expansion and differentiate into effector helper T cells that coordinate the immune response. Activation through T cell receptor (TCR) and costimulatory signals increases cellular metabolism to allow sufficient RNA and protein production to support cell growth, proliferation, and effector functions (Frauwirth and Thompson, 2004). Activated cells also become sensitive to signals that induce them to differentiate into distinct subsets of effector helper T cells, which perform specific immune functions through the selective production of cytokines (Zhu and Paul, 2010). For example, Th1 cells mediate immunity against intracellular infections by secreting Interferon-γ (IFN-γ), whereas Th2

cells utilize Interleukin (IL)-4, IL-5, and IL-13 to orchestrate barrier immunity to control extracellular parasites (Stetson et al., 2004; Szabo et al., 2003). Lineage-restricted transcription factors, chromatin remodeling, and post-transcriptional regulation all contribute to the major changes in gene expression that characterize T cell activation and differentiation (Ansel et al., 2006; Wilson et al., 2009).

MicroRNAs are ~22 nucleotide (nt) single stranded RNAs that direct post-transcriptional repression of many mRNAs, and thereby regulate diverse biological processes from cell proliferation and apoptosis to organ development and immunity (Bartel, 2009; Hoefig and Heissmeyer, 2008; Kim et al., 2009b; O'Connell et al., 2010b). MicroRNA genes are transcribed by RNA Polymerase II, and the resulting primary miRNAs (pri-miRNAs) are processed by the Drosha-DGCR8 complex to produce ~60-80 nt hairpin pre-miRNAs. A second complex consisting of Dicer and TRBP cleaves pre-miRNAs to form small dsRNA duplexes, one strand of which becomes the mature miRNA upon loading into the miRNA-induced silencing complex (miRISC). Argonaute (Ago) proteins directly interact with miRNAs and are key factors in the assembly and function of the miRISC. MicroRNAs guide the miRISC to target mRNAs through direct base-pairing, leading to mRNA degradation and repression of protein expression.

T cells deficient in Dicer, Dgcr8, or Drosha, and thus lacking all miRNAs, exhibit decreased proliferation and survival and a propensity to rapidly differentiate into IFN-γ-producing effectors (Chong et al., 2008; Cobb et al., 2006; Liston et al., 2008; Muljo et al., 2005; Steiner et al., 2011a; Zhou et al., 2008). Fully differentiated Th1 and Th2 cells express similar miRNA repertoires that are very distinct from that of naïve T cells (Barski et al., 2009; Kuchen et al., 2011; Monticelli et al., 2005). Among the many miRNAs

whose expression changes are several that regulate T cell clonal expansion or differentiation (Banerjee et al., 2010; Lu et al.; Monticelli et al., 2005; Rodriguez et al., 2007; Rossi et al., 2011; Steiner et al., 2011a; Stittrich et al., 2010; Thai et al., 2007; Xiao et al., 2008) Therefore, it is important to understand the mechanisms by which miRNA expression is regulated during T cell activation.

Some miRNA genes of importance in T cells are transcriptionally regulated by activation-induced transcription factors (Chang et al., 2008; Haasch et al., 2002; Taganov et al., 2006; Thai et al., 2007). However, discrepancies between pri-miRNA and mature miRNA abundance suggest that widespread post-transcriptional events also shape miRNA expression patterns in human lymphoma cell lines and mouse primary lymphocytes (Kuchen et al., 2011; Thomson et al., 2006). RNA binding proteins can promote or repress processing of specific pri-miRNAs (Davis et al., 2008; Trabucchi et al., 2009). In addition, the stability and activity of the Drosha-DGCR8, Dicer-TRBP, and miRNA-induced silencing (miRISC) complexes are subject to regulation (Ghodgaonkar et al., 2009; Han et al., 2009; Paroo et al., 2009). Ago proteins in particular can undergo a variety of post-translational modifications that affect their stability (Rudel et al., 2011) (Adams et al., 2009; Qi et al., 2008a; Rybak et al., 2009). This is important because they can be a limiting factor for global miRNA expression levels (Diederichs and Haber, 2007; Lund et al., 2011; O'Carroll et al., 2007).

Here, we show that miRNAs are globally downregulated shortly after T cell activation. Through measuring miRNA precursors, we found that individual miRNAs are transcriptionally regulated upon T cell activation and that pri-miRNAs are efficiently processed into pre-miRNAs. We also measured miRNA biogenesis factors and

determined that post-transcriptional downregulation of Ago proteins occurs, mediated by ubiquitination and proteasomal degradation. This degradation depends on continuous signaling through the mTOR pathway. Ago2 is a limiting factor in T cell miRNA homeostasis and Ago2-deficient T cells exhibit increased differentiation into cytokine producing effectors. Thus miRNA expression in activated T cells is globally regulated at the post-transcriptional level, likely through increased miRISC turnover. This, together with miRNA gene-specific transcriptional changes rapidly resets the miRNA repertoire during T cell activation.

Materials and Methods

Mice. All mice were housed and bred in specific pathogen-free conditions in the Animal Barrier Facility at the University of California, San Francisco. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. C57BL/6NCr were obtained from National Cancer Institute-Frederick. Ago2 conditional mutant mice (*Eif2c*$^{tm1.1Tara}$) have been described previously (O'Carroll et al., 2007). *CD4-cre* transgenic mice (*Tg(Cd4-cre)1Cwi)* were purchased (Taconic). For in vivo T cell transfer experiments, donors were DO11 TCR transgenics (*Tg(DO11.10)10Dlo*) crossed with Rag2 deficient mice (*Rag2*^{tm1Cgn}; Jackson Laboratories). For recipients, WT Balb/c mice were purchased from Jackson Labs. sOva-transgenic mice (*Tg(Mt1-OVA)#Akab*) were generated as described (Villarino et al., 2011)

T cell stimulation and culture. $CD4^+$ T cells from spleen and lymph nodes of young mice (4-7 weeks old) were isolated by magnetic bead selection (Dynal). Purified $CD4^+$ T cells were stimulated with hamster anti-mouse CD3 (clone 2C11, 0.25 μg/mL) and anti-mouse CD28 (clone 37.51, 1 μg/mL) on plates coated with goat anti-hamster IgG (0.3 mg/mL in PBS for 2 hours at room temperature; MP Biomedicals) for 60-68 hours at an initial cell density of 0.7-1 x 10^6 cells/mL. Following stimulation, cells were expanded in media with 20 units/mL of recombinant IL-2 (National Cancer Institute). ThN (non-polarizing, no exogenous cytokines or blocking antibodies), Low IL-4 (10units/mL IL-4), low IL-12 (10pg/ml IL-12), Th1 (10ng/ml IL-12, 10ug/ml anti-IL-4) or Th2 (500 units/mL IL-4, 5 μg/mL anti-IFN-γ clone XMG1.2) conditions were maintained throughout stimulation and expansion. The resulting cultures were free of $CD8^+$ T cells (<1%) when analyzed by flow cytometry 5 d after activation. For experiments involving carboxyfluorescein succinimidy ester (CFSE), cells were labeled for eight minutes with 5 uM CFSE, quenched with an equal volume of fetal bovine serum (FBS), and washed two times in 10% FBS prior to stimulation and culture. For restimulation experiments, cells were stimulated as described above and expanded in IL-2-containing media until 6 d after activation. Cells were harvested and restimulated with anti-mouse CD3 and anti-mouse CD28 on plates coated with goat anti-hamster IgG for times indicated. For experiments involving inhibitors, cells were restimulated for 16-19.5h and inhibitors were added for the last 0.5-4h of restimulation. Inhibitors used were 20μM PP2, 10μM LY294002, 100nM rapamycin, 1μM Cyclosporin A, 2.5mg/mL Actinomycin D, 10μg/mL Cycloheximide, 100μM U0126, and 10μM MG-132 (Sigma-Aldrich). All T cell culture was in DMEM high glucose media supplemented with 10% FBS, pyruvate, nonessential

amino acids, MEM vitamins, L-arginine, L-asparagine, L-glutamine, folic acid, beta mercaptoethanol, penicillin, and streptomycin.

Retroviral transduction. HA-tagged Ago2 cDNA was amplified from RA802B-1_mAGO2_RFP lentiviral plasmid (System Biosciences) and was subcloned into pRV-IRES-GFP (addgene.org: plasmid 13249). $CD4^+$ cells were stimulated as described for 48 hours and transduced with retrovirus produced by Phoenix-E packaging cells transfected with retroviral plasmids. Following 5 hours of incubation with virus and 5 μg/mL polybrene, media was replaced and cells were cultured and expanded for analysis. A control plasmid was used that lacks HA-tagged Ago2 cDNA.

Transfection and miRNA sensor generation. MiRNA sensor constructs were generated by subcloning 4 perfectly complementary miRNA binding sites into psiCHECK-2 dual luciferase reporter construct immediately downstream of a renilla luciferase coding gene with each miRNA binding site separated by 4 base pairs. $CD4^+$ T cells were stimulated *in vitro* for indicated times and transfected with miRNA sensor constructs. Cells were transfected using the Neon electroporation transfection system (Invitrogen) with an optimized version of the manufacturers recommended protocol. Briefly, transfections were performed using 4×10^7 cells in 10 μL "R buffer" (Invitrogen) with 333 ng of plasmid DNA. Optimized Neon transfection system setting was 1550 V with three 10 ms pulses. For transfection, cells were removed from plates, transfected, and returned to fresh plate-bound stimulation (anti-CD3, anti-CD28). Luciferase activity was measured 18h after transfection using the Dual Luciferase Reporter Assay System (Promega) and a

FLUOstar Optima plate reader (BMG Labtech).

Adoptive transfers and immunizations. LNs and spleens were pooled from 4-6 week-old DO11 *Rag2*$^{-/-}$ mice and CD4^{+} cells purified (>96% purity) by positive selection using magnetic beads (Dynal). 2-5 x 10^5 CD4^{+} cells were then intravenously injected into age and sex-matched recipients (in 400 ul PBS). For immunizations, donor T cells were transferred into WT Balb/c mice and, 24 hours later, these were immunized (IV) with 1-5 x 10^5 bone marrow-derived dendritic cells (BM-DCs) that were pre-activated with LPS and loaded with OVA peptide (1 g/ml each; Sigma).

Ex vivo T cell monitoring. CD4^{+} cells were enriched from pooled LNs and spleens of recipient mice (days 4-5 post-transfer) and stained directly *ex vivo* with fluorochrome-labeled anti-CD4, anti-CD44 and anti-DO11.10 antibodies (eBiosciences). High speed cell sorting was then used to purify CD4^{+} DO11.10^{+} CD44^{hi} cells (<95% purity). For all experiments, naive controls were from LNs and/or spleen of DO11.10 mice (no adoptive transfer) and defined as CD4^{+} DO11.10^{+} CD44^{lo} (>99% purity). For ELISAs, purified T cells were stimulated over-night with LPS/Ova-pulsed BM-DCs (5:1 T cell to DC ratio) and cytokine concentrations measured in supernatants using standard methodologies (antibody pairs from eBioscience).

MicroRNA Arrays. For in vitro analyses, total RNA was extracted from naïve and in vitro activated mouse and human T cells. For ex vivo analyses, 0.5 x 10^6 donor T cells (CD4^{+} D011.10^{+} CD44^{hi} cells from sOVA and immunized hosts) and 1 x 10^6 naive T

controls ($CD4^+$ $D011.10^+$ $CD25^-$ $CD44^{lo}$ cells from DO11.10 *Rag2*$^{+/+}$mice) were purified by high-speed cell sorting. Quality control was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies), and RNA was labeled with Cy3-CTP using the miRCURY LNA microRNA power labeling kit (Exiqon, Inc), according to manufacturers protocol. Labeled RNA (250 ng per well) was then hybridized to Agilent custom UCSF miRNA v3.3 multi-species 8x15K Ink-jet arrays. Hybridizations were performed for 16 hrs, according to the manufacturers protocol. Arrays were scanned using the Agilent microarray scanner and raw signal intensities were extracted with Feature Extraction software.

Data Analysis. For in vitro microarray analyses, no background subtraction was performed, and the median feature pixel intensity was used as the raw signal before quantile normalization. A filter was used to remove probes with a max log2 signal across arrays less than 6. This filter prevents the prevalence of so many low-intensity probes, which tend to have smaller variance, from underestimating global and per-gene estimates of variance. Each array's signal was normalized to the median interpolated tRNA signal. First, the miRNA probes were quantile normalized without the tRNA probes. Then a linear interpolation function between the raw miRNA signal and the normalized data was created. This function was evaluated with the raw tRNA signal values to produce the interpolated tRNA probe values. Within each array, the median tRNA value across tRNA probes was used as the basis of comparison for all miRNA probes on that array.

For ex vivo analyses, tRNA probes were not present and data was normalized using the quantile normalization method with a filter to remove all probes where the max log2

signal across arrays was less than 6 (Bolstad et al., 2003). Microarray data were submitted to the Gene Expression Omnibus database (Accession numbers GSE36606 and GSE36607).

RNA isolation and qPCR. For in vitro activated T cells, total RNA was isolated with Trizol LS reagent (Invitrogen) from naïve and stimulated T cells. Sequences of all primers used for PCR are provided in Table S1. For mRNA expression, total RNA was oligo(dT)-primed for first strand cDNA synthesis (Superscript III Kit, Invitrogen). For mature miRNA expression analysis, total RNA was subjected to polyA addition and cDNA synthesis (Ncode kit, Invitrogen). qPCR was performed using one microRNA-specific primer and one constant primer (corresponding to the modified oligo-dT) with SYBR green master mix (Roche) and a Realplex2 thermocycler (Eppendorf). For pre-miRNA and pri-miRNA expression analysis, total RNA was fractionated into a large and small fraction (miRNeasy kit, Qiagen) and random hexamer-primed for first strand cDNA synthesis. pri-miRNA qPCR was performed using primers spanning one arm of the stem loop and the flanking sequence on cDNA synthesized from the large RNA fraction. Pre-miRNA qPCR was performed using primers within the stem loop on cDNA synthesized from the small RNA fraction. Dilution series were performed to confirm linear performance of these qPCR assays. Pri-miRNAs were below the limit of detection in all small RNA fraction samples. For in vivo activated T cells, PCR Amplification was performed with SYBR green master mix (5-10 ng cDNA per reaction; Applied Biosystems) using an iQ5 Real-Time PCR thermal cycler (BioRad). Reactions were performed in duplicate and threshold cycle (Ct) values normalized to 5.8S or 18S rRNA

for mature miRNAs, 5S for pre-miRNAs, 28S for pri-miRNAs, and β-actin for mRNAs. Trim71 qPCR was performed Taqman Gene Expression Assay Mm01341471_m1 (ABI).

Intracellular staining and antibodies. For intracellular cytokine analysis, cells were restimulated for four hours with 10 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin in the presence of 5 μg/mL brefeldin A to allow intracellular cytokine accumulation. For all cytokine stains, cells were fixed with 4% formaldahyde and subsequently permeabilized and stained in PBS containing 0.5% saponin, 1% bovine serum albumin, and 0.1% sodium azide. For intracellular T-bet stains, cells were fixed and stained with the Foxp3 Staining Buffer Set (Ebioscience). Fluorophore-conjugated antibodies including eFluro450-IFN-γ, allophycocyanin (APC)-IL-4, PE-IL-13 and APC-T-bet were from eBioscience. Stained cells were analyzed with a LSRII and FACSDiva software (BD Biosciences) as well as Flowjo analysis software.

Cell extracts and immunoblot analysis. Cells were lysed in 0.5% Nonidet P40, 150 mM NaCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml of aprotinin, 25 μg/ml of leupeptin, 10 mM NaF, 8 mM β-glycerophosphate for 15 minutes. Lysates were cleared by centrifugation, resolved on 4-15% gradient SDS-PAGE gels, and transferred onto nitrocellulose membranes (Schleicher and Schuell). Membranes were blocked with 5% powdered milk in TBS (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl), incubated with antibodies diluted in blocking solution, and washed with TBS containing 0.1% Tween-20. Antibodies used were anti-β-actin (A5441, Sigma),

HRP donkey anti-mouse IgG (Jackson ImmunoResearch), HRP Donkey anti-mouse IgM (Jackson ImmunoResearch), HRP Goat anti-rabbit IgG (Jackson Immunoresearch), anti-TRBP (72110, Abcam), HRP Goat anti-rat IgG (Jackson ImmunoResearch), anti-Drosha (12286, Abcam), anti-Ago2 (Cell Signaling), anti-Ago1 (4B8, gift of G. Meister, Max-Planck-Institute of Biochemistry), rat anti-mouse pan-Ago (11G1) for immunoblot analysis. Anti-Ago2 (Wako Chemicals USA), anti-Trim71 (R&D), anti-HA (clone 3F10, Roche) and mouse IgG (Abcam) were used for immunoprecipitations.

Ubiquitination assays and immunoprecipitations. For endogenous Ago2 ubiquitination assays, cells were taken off stimulation at 72 hours and expanded in culture until d6. Cells were returned to fresh plate-bound stimulation (anti-CD3, anti-CD28) for 16h, at which point 10μM MG-132 or DMSO control was added for 4 h. Alternatively cells were returned to fresh IL-2 containing media for 16h and MG-132 was added for 4 h more. For overexpressed HA-Ago2 ubiquitination assays, cells were taken off stimulation at d2 following 5 h incubation with retrovirus, expanded in culture until d5, and restimulated as described above. Cells were lysed in preboiled lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% SDS, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml of aprotinin, 25 μg/ml of leupeptin, 10 mM NaF, 8 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM dithithreitol, 10μM MG-132, and 5 mM NEM for 15 minutes) and incubated for 5 min at 95°C to disrupt non-covalent interactions. Lysates were diluted 1:10 with NP-40 buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml of aprotinin, 25 μg/ml of leupeptin, 10 mM NaF, 8 mM β-glycerophosphate, 1 mM

sodium vanadate, 1 mM dithithreitol, 10μM MG-132, and 5 mM NEM), passed through a needle and syringe to fragment DNA, clarified by centrifugation at 18,800 g for 10 min, and incubated overnight with antibody (10ug anti-Ago21 or 1ug anti- HA at 4°C. Immunoprecipitates were collected with protein G magnetic beads (Dynabeads, Invitrogen) for one hour at 4°C, washed and analyzed by western blotting.

Mouse ES cells. Mouse embryonic stem cell protein and cDNA samples were generously provided by R. Krishnakumar, R. Blelloch lab, UCSF. ES cells were V6.5 mouse embryonic stem cells grown in 15% FBS and supplemented with Lif and 2i. cDNA was prepared using random hexamer primers.

Results

We previously showed that helper T cell differentiation is associated with major changes in miRNA expression (Monticelli et al., 2005). To determine the kinetics with which these changes occur, we measured miRNA expression during a time course of T cell activation. Spleen and lymph node $CD4^+$ cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 3 days and then rested in media containing IL-2 until day 7. Equal quantities of RNA from cells harvested at various time points were subjected to Northern blot analysis. As expected, T cell activation induced an increase in the total RNA yield per cell, and the relative expression of rRNA remained stable throughout the time course (Fig. 1A). Although many "housekeeping" mRNA transcripts, including miRNA targets, are induced upon T cell activation, rRNA makes up a constant proportion of cellular RNA across many cell types and experimental conditions (Bas et al., 2004).

The 5.8S, 18S, and 28S rRNAs are co-transcribed and processed from a single precursor and maintained at a constant stoichiometric ratio with each other and the 5S rRNA. Transfer RNA (tRNA) abundance also tracked closely with 5.8S rRNA in activated T cells (Fig. 1A). Therefore, we used rRNAs as a standard for normalization throughout this study, with attention to matching the size of the standard and analytes in each assay. Consistent with previous reports, miR-155 was upregulated in activated T cells, while miR-150 and miR-146 were downregulated (Banerjee et al., 2010; Monticelli et al., 2005; Rodriguez et al., 2007; Thai et al., 2007). Surprisingly, several other miRNAs were also downregulated (Fig. 1A and data not shown), including miRNAs that are highly expressed throughout the T cell lineage (e.g. miR-142), and several miRNAs that are expressed broadly in mouse tissues (e.g. miR-16). MicroRNA expression decreased within 4 hours of activation, and the largest reduction was observed by 44 hours (Fig. 1A and data not shown).

We measured the expression of all miRNAs across a time course of T cell activation using miRNA microarrays. Microarray data are typically normalized using quantile normalization or other algorithms that adjust the hybridization intensity of each probe relative to the intensity of all other probes on the array. This approach yields information about the expression of each miRNA relative to the other miRNAs in a sample, but it fails to capture global changes in the expression of miRNAs as a class. Therefore, we customized our arrays to include probes for several control noncoding RNAs, including tRNAs. Compared with tRNA, global miRNA expression decreased measurably within 12 hours of T cell activation, long before any cell division has occurred. Within 2 days, a large majority of all expressed miRNAs were downregulated,

with an average reduction to less than 25% of that seen in naïve T cells (Fig. 1B). Very similar results were obtained using naïve $CD4^+$ T cells isolated from human peripheral blood (Fig. 1C).

We confirmed the array data by qPCR measurement of a panel of miRNAs with a range of expression in T cells (Fig. 1D-F). As shown in the array, the abundance of most miRNAs decreased relative to tRNA in T cells activated for 42 hours (Fig. 1E). Since qPCR does not suffer from the data compression inherent in hybridization-based array analyses, it provides a more accurate measure of the magnitude of miRNA downregulation (Shi et al., 2006). The most highly downregulated miRNAs, miR-150 and miR-181a, decreased to less than 2% of their abundance in naïve T cells (Fig. 1F).

To directly monitor miRNA activity during T cell activation, we constructed slicer-dependent miRNA sensors with perfectly complementary miRNA binding sites cloned downstream of *Renilla* luciferase (Fig. 1G). T cells were transfected with sensors for miR-150 (most highly downregulated), miR-191 (moderately downregulated), miR-19b (upregulated), or a control vector without miRNA binding sites. Primary T cells were transfected at various times following stimulation, and luciferase activity was measured 18 hours later. These experiments revealed the expected reciprocal relationship between sensor activity and miRNA expression. As miR-150 and miR-191 levels decreased, their luciferase sensors exhibited increased luciferase activity. Conversely, as miR-19b expression increased, there was a decrease in luciferase activity from this sensor. The vector control exhibited no significant change in luciferase activity over the time course of activation.

Finally we asked if resting T cells would exhibit similar miRNA downregulation upon restimulation. T cells were rested until day 6 and restimulated on plate-bound anti-CD3 and CD28 for 24 hours. Microarray profiling revealed that restimulated cells display a similar pattern of miRNA expression changes as that which was seen in naïve T cells stimulated for the first time (Fig. 1H). Thus, restimulation of resting in vitro derived effector T cells recapitulates the activation-induced downregulation of most miRNAs.

Activation-induced downregulation of Ago proteins

To explore the possibility that activation-induced miRNA downregulation could be the result of reduced expression of key proteins in the miRNA biogenesis pathway, we measured Drosha, Dicer, and TRBP during T cell activation. The mRNA of the genes encoding all three proteins were induced to varying degrees within 14 hours, then returned to baseline in resting T cells (Fig. 2A). The abundance of Drosha and Dicer protein followed the same pattern with a slight delay. Both exhibited strong induction followed by a return to baseline levels (Fig. 2B). In contrast, increased TRBP protein expression persisted even when T cells were removed from stimulus and allowed to rest in IL-2-containing media for >3 days. The induction of these miRNA biogenesis proteins in activated T cells is interesting, but it cannot account for the concurrent reduction in mature miRNA expression.

Argonaute (Ago) protein abundance has emerged as a common limiting factor that determines miRNA levels, and Ago stability and miRNA-binding activity is subject

to regulation by post-translational modification (Adams et al., 2009; Diederichs and Haber, 2007; Lund et al., 2011; O'Carroll et al., 2007; Qi et al., 2008a; Rybak et al., 2009). Like Drosha, Dicer, and TRBP, Ago1, Ago2, and Ago3 were moderately induced at the transcriptional level by 14 hours (Fig. 2C). Ago4 mRNA could not be detected in these cells. Ago1 and Ago2 protein expression did not follow the same pattern, instead resembling the kinetics of miRNA downregulation in activated T cells. Ago1 and Ago2 decreased significantly within 24 hours, reached a minimum at 48 to 72 hours of activation and remained very low for the remainder of the time course (Fig. 2D). Probing with a new monoclonal antibody that recognizes all 4 mouse Ago proteins (Fig. 3) revealed a similar pattern for total Ago protein expression (Fig. 2E).

To determine whether Ago protein abundance is a limiting factor for global miRNA homeostasis in T cells, we measured miRNA levels in Ago2-deficient T cells. Spleen and lymph nodes of *Ago2*$^{fl/fl}$*; CD4-cre* mice and their *Ago2*$^{+/+}$*; CD4-cre* (WT) littermates contained similar numbers of $CD4^+$ and $CD8^+$ T cells, and similar proportions of naïve ($CD62L^{hi}CD44^{lo}$) and memory ($CD62L^{lo}CD44^{hi}$) cells (Fig. 4A). Western blot analysis confirmed the absence of Ago2 protein in bead-sorted T cells from *Ago2*$^{fl/fl}$*; CD4-cre* mice (Fig. 4B). Importantly, miRNA expression was also significantly lower in these cells (Fig. 4C). Twenty-three out of 24 members of a panel of T cell-expressed miRNAs were decreased by an average of 60% in *Ago2*$^{fl/fl}$*; CD4-cre* T cells, and 30% in heterozygous *Ago2*$^{+/fl}$*; CD4-cre* T cells relative to 5.8S rRNA. Other noncoding small RNAs snoRNA-202 and U7 snRNA were unaffected. The remaining miRNAs may be associated with other Argonaute family proteins. To further test if Argonaute proteins are limiting for T cell miRNA expression, we re-expressed Ago2 in differentiating Ago2-

deficient T cells. Cells were transduced with Ago2 or control retrovirus at 80-90% efficiency as measured by expression of the GFP marker gene (data not shown). Ago2 protein was detected only in Ago2-transduced cells (Fig. 4D). MicroRNA expression increased in these cells by an average of 145% (Fig. 4E). These results demonstrate that miRNA expression in T cells is highly sensitive to changes in Ago protein expression, and indicate that activation-induced downregulation of Ago1 and Ago2 could be the proximate cause of the observed decrease in mature miRNA expression in activated T cells.

MicroRNAs restrain helper T cell differentiation and cytokine production

Dicer-, *Drosha*-, or *Dgcr8*-deficient T cells that lack all miRNAs undergo abnormally rapid and robust differentiation into cytokine-producing effector cells upon activation (Chong et al., 2008; Liston et al., 2008; Muljo et al., 2005; Steiner et al., 2011a). However, these cells also expand poorly due to reduced proliferation and increased apoptosis. In contrast, activated Ago2-deficient and heterozygous T cells divided at a similar rate as control cells, and expanded to similar cell numbers in in vitro cultures (Fig 5A and data not shown). This indicates that T cell proliferation does not require the "slicer" activity of Ago2 that mediates RNA interference, and allowed us to test for proliferation and survival-independent effects of reduced miRNA expression on T cell differentiation and cytokine production. $CD4^+$ T cells were activated for 3 days in conditions that drive Th1 (10 ng/ml IL-12, anti-IL-4) or Th2 (500 units/mL IL-4, anti-IFN-γ) differentiation, in limiting concentrations of IL-4 (10 units/ml) or IL-12 (10 pg/ml), and in nonpolarizing conditions ('ThN'; no cytokines or blocking antibodies

added), allowed to rest for 3 days in the presence of IL-2, and then restimulated to induce cytokine production. The percentage of cytokine-producing cells increased in all conditions tested (Fig. 5B). In non-polarizing conditions, a 70% increase in the proportion of IFN-γ producing cells was coupled with a significant increase in the Th2 cytokines IL-4 and IL-13. Very similar results were obtained with limiting IL-12, and Th2 cytokines were increased even in strong Th1 and Th2 polarizing conditions. In limiting IL-4 conditions, there was a significant increase in the production of all 3 cytokines. Thus, Ago2-deficient T cells produced more lineage-specific effector cytokines without a particular bias toward Th1 or Th2 differentiation. These data indicate that miRNAs are required in naïve T cells to restrain their differentiation. When miRNAs are depleted, as in the case of Ago2-deficiency, cells are more apt to differentiate into cytokine-producing effectors.

The pathways and specific mRNAs targeted by miRNAs to restrain T cell differentiation and cytokine production are mostly unknown, and likely complex. However, as an example of a relevant established miRNA target, we also measured the expression of T-bet in differentiating Ago2-deficient T cells (Fig. 5C). T-bet is a key transcription factor required for Th1 differentiation recently shown to be a direct target of miR-29 in T cells (Steiner et al., 2011a) T-bet expression increased in Ago2-deficient cells in non-polarizing, limiting IL-4 and limiting IL-12 conditions. No differences were evident in Th1 conditions, which presumably have maximal T-bet expression, or in Th2 conditions in which both T-bet and IFN-γ expression is very low. Thus, the increased T-bet in Ago2-deficient cells correlates with increased IFN-γ in those cells (compare Fig. 5B and C), and may directly contribute to this effect.

Specific transcriptional changes and global post-transcriptional downregulation reset the miRNA repertoire in activated T cells

Downregulation of Ago proteins could plausibly account for the widespread reduction in miRNA abundance in activated T cells. This mechanism would be unlikely to act in a miRNA-specific fashion. However, we observed significant heterogeneity in the expression of individual miRNAs in response to T cell activation, indicating that one or more additional layers of regulation remodel the miRNA repertoire during T cell differentiation (Fig. 1B). Specifically, we identified three groups of miRNAs for further investigation: *(i)* miRNAs like miR-150 that are heavily downregulated, decreasing to 1-2% of their starting levels during T cell activation (Fig. 6A, top), *(ii)* miRNAs like miR-106a that remain at relatively constant abundance or are slightly upregulated (Fig. 6A, middle), and *(iii)* the largest group of miRNAs that are moderately downregulated to the global average of about 25% of their starting abundance (Fig. 6A, bottom).

To determine whether miRNA gene-specific transcriptional regulation may explain these different expression patterns, we measured the corresponding pri-miRNAs. Primary miRNAs whose corresponding mature miRNA exhibited the greatest downregulation also fell precipitously during T cell activation, reaching 3-10% of their starting levels within 24 hours (Fig. 6B, top). These data suggest that miRNAs in this class are regulated by activation-induced repression of pri-miRNA transcription. However, a post-transcriptional mechanism also appears to contribute, since the mature miRNAs were downregulated further and faster than can be accounted for by transcriptional repression alone. MicroRNAs whose levels increased or remained

unchanged during T cell activation exhibited robust upregulation of at least one of their corresponding pri-miRNAs (Fig. 6B, middle). For these miRNAs, the magnitude of pri-miRNA induction exceeded miRNA upregulation 4 to 20-fold, suggesting that these miRNAs are also subject to post-transcriptional downregulation even as they are transcriptionally induced. In addition, pri-miRNAs corresponding to miRNAs that were moderately downregulated during activation did not exhibit decreased expression, but instead exhibited an early transient increase followed by a return to baseline levels for the remainder of the time course (Fig. 6B, bottom). Taken together, these findings indicate that miRNA-specific transcriptional regulation is layered over a global post-transcriptional mechanism of downregulation. Together, these mechanisms allow rapid changes in the miRNA expression patterns of activated T cells that are entering a phase of growth and differentiation.

In addition to changes in miRISC stability and regulation of miRNA gene transcription, we investigated the possibility that activated T cells may exhibit a block in the enzymatic steps in miRNA biogenesis. To this end, we assayed pre-miRNA abundance during an activation time-course. Since pre-miRNAs share their entire sequence with the corresponding pri-miRNA, we devised a method to distinguish them in qPCR assays by separating cellular RNA into small (<200nt) and large fractions. Two primers that bind to the stem loop sequence were then used to measure pre-miRNAs in the small RNA fraction (Fig. 7A, right). Primary miRNAs can be unambiguously detected using PCR primers spanning the Drosha cleavage site in the miRNA-containing stem-loop structure (Fig. 7A, left). Primary miRNA-specific primers amplified their product from the large fraction, but no product was detected in the small RNA fraction,

indicating that pri-miRNAs were effectively removed (Fig. 7B, left). Pre-miRNA primers amplified a product in both fractions (Fig. 7B, right). Both pri-miRNA and contaminating pre-miRNAs may contribute to the products amplified from the large fraction, but pre-miRNA abundance could be specifically measured in the small fraction. We routinely confirmed the absence of the corresponding pri-miRNAs in the small fraction when measuring pre-miRNAs.

If pri-miRNA processing to pre-miRNA were blocked in activated T cells, pre-miRNA expression would be expected to fall rapidly during an activation time course. If pre-miRNA to miRNA expression were blocked, some pre-miRNAs would be expected to accumulate, as has been shown for pre-miR-21 and pre-miR-150 in Dicer-deficient $CD4^+$ T cells (Muljo et al., 2005). However, pre-miR-21 expression consistently tracked closely behind pri-miR-21 expression across the activation time-course. Pri-miR-21 was transiently upregulated during the first 6 hours of activation, and a corresponding transient increase in pre-miR-21 was observed at 12 hours (Fig. 7C). In contrast, mature miR-21 was clearly downregulated within 24 hours of T cell activation. Even during the early burst of pri-miR-21 transcription and processing, mature miR-21 remained at or below its concentration in naïve T cell. We confirmed these data by Northern blot using a miR-21-specific probe that recognizes both mature and pre-miR-21 (Fig. 7D). Pre-miR-150 was detected only in naïve T cells, consistent with transcriptional silencing (data not shown). We conclude that neither Drosha nor Dicer processing is blocked in activated T cells, though the processing efficiency of specific miRNAs could be subject to regulation.

In vivo activated T cells exhibit global miRNA downregulation

We also investigated whether similar miRNA regulation occurs in T cells activated in vivo. Ovalbumin (OVA)-specific TCR-transgenic naïve $CD4^+$ T cells were adoptively transferred into wildtype congenic hosts, some of which were then immunized with OVA-pulsed dendritic cells. At 5 days post transfer, $CD44^{hi}$ effector $CD4^+$ T cells were purified from immunized mice, and naïve cells were recovered from unimmunized mice (Fig. 8). Quantitative PCR showed that miRNAs were also downregulated in T cells activated in vivo (Fig. 9A and data not shown). Microarray analysis showed close correlation between the miRNA expression changes induced in effector T cells in vivo and those induced by activation in vitro for 65 hours (Fig. 9B). In vivo activated T cells also downregulated Ago2 protein to a similar extent as was observed in vitro, becoming almost undetectable (Fig. 9C). Taken together, these data demonstrate that Ago protein and global miRNA downregulation occurs in physiologically relevant T cell responses.

Ago2 is ubiquitinated and degraded by the proteasome

Finally, we investigated the mechanism of activation-induced Ago2 downregulation. To make biochemical analyses more feasible, we performed our studies in in vitro cultured T cells, which downregulated miRNAs upon restimulation with anti-CD3 and anti-CD28 (Fig. 1H). Ago2 was also downregulated in restimulated cells, reaching near undetectable levels within 24 hours (Fig. 10A). Since previous studies have indicated that Ago2 can be ubiquitinated and degraded by the proteasome, we wondered whether this mechanism occurs in activated T cells (Adams et al., 2009; Rybak et al., 2009). Treatment with MG-132 during the final 2 hours of restimulation largely restored Ago2 protein abundance to the quantity found in unstimulated cells. This finding shows a

critical role for the proteasome in Ago2 downregulation, and indicates that Ago2 undergoes a high rate of turnover in activated T cells.

Next we tested whether Ago2 itself becomes ubiquitinated during T cell activation. T cells were restimulated or left at rest in media supplemented with MG-132 for the final 4 hours to stabilize ubiquitinated proteins, and lysates were prepared under stringent conditions that disrupt non-covalent protein interactions. Anti-Ago2 immunoprecipitates from activated T cells contained an accumulation of high molecular weight forms of immunoprecipitated Ago2 (>90kD) that were recognized by ubiquitin immunoblotting (Fig. 10B). Ubiquitinated Ago2 was far less abundant in resting T cells, and was not detected in control immunoprecipitations with non-specific antibody. Importantly, the high molecular weight proteins that we identified as ubiquitinated Ago2 were detected in restimulated wildtype T cells, but not in Ago2-deficient T cells (Fig. 10C). To confirm these results with an independent antibody for immunoprecipitation, we transduced wildtype T cells with HA-tagged Ago2. Like the endogenous protein, retrovirally encoded HA-Ago2 was downregulated upon T cell activation in an MG132-sensitive manner (Fig. 10D). In addition, anti-HA specifically immunoprecipitated high molecular weight ubiquitinated proteins only in restimulated cells expressing HA-Ago2 (Fig. 10E). Ago2 ubiquitination could be detected as early as 12 hours following stimulation (Fig. 10F). These data strongly support a model in which T cell activation induces ubiquitination and proteasomal degradation of Ago2, and probably other Ago proteins as well.

We asked which signals downstream of TCR engagement are required for Ago2 downregulation. Inhibition of proximal TCR signaling with the Src-family kinase

inhibitor PP2 partially rescued Ago2 protein expression (Fig. 11A). A robust increase in Ago2 was observed in cells activated in the presence of the Phosphatidylinositol 3-Kinase (PI3K) inhibitor LY294002 (Fig. 11A) or the mTOR inhibitor rapamycin (Fig. 11B). LY294002 is also a potent inhibitor of mTOR (Brunn et al., 1996). In contrast, calcineurin and MAPK activity were dispensable, as blocking these pathways with Cyclosporin A or U0126, respectively, had no effect on Ago2 downregulation (Fig. 11B). Continuous mTOR signaling was required to maintain low Ago2 expression, since rapamycin treatment restored it to unstimulated T cell levels within 2 hours (Fig. 11C).

Treatment with Actinomycin D or cycloheximide also rapidly rescued Ago2 expression (Fig. 11D), indicating that continued transcription and translation are required for Ago2 degradation. These results suggest that an induced effector protein may need to be continuously generated to maintain low Ago2 protein expression in activated T cells. The ubiquitin ligase Trim71 (a.k.a. mLin41) has been implicated in the regulation of Ago2 stability (Rybak et al., 2009), though two recent studies countered this claim (Chang et al., 2012; Chen et al., 2012). Trim71 mRNA was almost undetectable in resting and restimulated T cells, in comparison to robust expression in embryonic stem (ES) cell positive control samples (Fig. 12A). Trim71 protein was also undetectable in T cells, in contrast to ES cell lysate (Fig. 12B).

Discussion

Naïve T cells that encounter their cognate antigen dramatically remodel their gene expression program as they proliferate and acquire effector functions. The data presented here demonstrate that these cells also actively reset their miRNA repertoire (Fig. 13).

Activation-induced global post-transcriptional downregulation of mature miRNAs allows concurrent changes in pri-miRNA gene transcription to rapidly establish a new pattern of miRNA expression in effector cells. Ago1 and Ago2, which bind miRNAs in the miRISC and mediate target mRNA repression, are also downregulated during T cell activation in a process of ubiquitination and proteasomal degradation. This degradation depends on intact PI3k/mTOR signaling and continuous transcription and translation. Ago2 is a limiting factor for miRNA expression in T cells, with the abundance of a large panel of miRNAs reduced by 30% for each disrupted allele of *Ago2*. Taken together, our data support a model in which TCR engagement leads to downregulation of Ago proteins, resulting in decreased miRNA expression. Alternatively, inducible miRNA degradation may destabilize Ago proteins in activated T cells.

These findings have important implications for miRNA regulation of immune responses. Naïve $Ago2^{-/-}$ and $Ago2^{+/-}$ T cells appeared phenotypically normal at baseline, but were more prone than wildtype cells to differentiate into cytokine-producing effector cells when activated. These data are reminiscent of previous observations of *Dicer*-, *Dgcr8*-, and *Drosha*-deficient cells which lack essentially all miRNAs and display even more aberrantly unrestrained differentiation, especially into IFN-γ-producing Th1 cells (Chong et al., 2008; Cobb et al., 2006; Liston et al., 2008; Muljo et al., 2005; Steiner et al., 2011a; Zhou et al., 2008). Also in accordance with data from *Dicer*- deficient T cells, *Ago2*-deficient T cells have a large percentage of cells producing cytokines that are associated with more than one helper T cell lineage, suggesting a dysfunction in lineage commitment (Muljo et al., 2005). Thus, global miRNA abundance correlates with propensity to differentiate into effector cells, and activation-induced miRISC

downregulation may be important to relax miRNA-mediated gene repression, allowing activated T cells to change their gene expression program and differentiate into effector cells. For example, consider miR-29a, which was among the most highly downregulated miRNAs in activated T cells. In this context and in Ago2-deficient T cells, decreased miR-29a correlated well with increased expression of its target, T-bet, which is a major determinant of Th1 differentiation and IFN-γ production (Steiner et al., 2011a; Szabo et al., 2003). We predict that other miRNAs that are downregulated during T cell activation play similar roles in restraining Th2 and Th17 gene expression programs. MicroRNAs may also target common determinants of helper T cell differentiation rather than lineage specific genes.

In this model, miRNAs expressed in naïve T cells act as a "brake" against changes in gene expression, and they are actively eliminated to promote differentiation. However, miRNA-deficient T cells also display survival and proliferation defects, indicating that some miRNAs need to be able to overcome global miRNA downregulation to support clonal expansion (Chong et al., 2008; Cobb et al., 2006; Liston et al., 2008; Muljo et al., 2005; Steiner et al., 2011a; Zhou et al., 2008). Indeed, our data indicate that miRNAs of the miR-17~92 and miR-106a~25 clusters, which can support T cell proliferation and survival in the absence of other miRNAs and are also implicated in lymphoma, are maintained or even upregulated during T cell activation (He et al., 2005; Steiner et al., 2011a; Xiao et al., 2008). Comparison of matched pri-miRNA and mature miRNA measurements indicated that a disproportionately large increase in transcription of these particular miRNA genes offsets the general reduction in miRNA.

MicroRNAs that cannot compensate with increased transcription during T cell activation are downregulated relative to their targets (mRNAs) and the translational machinery (rRNA) through a combination of lower Argonaute protein abundance and dilution with newly transcribed cellular RNAs. To support clonal expansion, activated T cells increase their cellular metabolism, and the total per cell RNA content increases approximately 10-fold in the first 24-48h post-activation (Frauwirth and Thompson, 2004). Thus, there is major dilution of the existing miRNAs. This effect allows pri-miRNA transcriptional changes to be rapidly translated into changes in mature miRNA levels. This is true not only for upregulated miRNAs, but for those that are transcriptionally repressed. The case of miR-150 illustrates the layered regulation of miRNA expression in activated T cells, and permits an estimation of the rate of miRISC turnover in activated T cells. Primary miR-150 transcription is rapidly silenced in activated T cells, and this combined with Ago downregulation, dilution with other cellular RNAs, and cell division reduces the abundance of mature miR-150 as a proportion of total RNA to 1-2% of its original level within 2 days. The effect of dilution can be eliminated by considering the amount of miR-150 on a per cell basis, leaving only transcription and mature miRNA elimination as factors in the expression of mature miR-150 over time. Making the conservative assumption that pri-miRNA transcription ceases completely and immediately upon T cell activation, we calculated a half-life between 11 and 18 hours for pre-existing miR-150 detected by qPCR or Northern blot in several independent experiments (Fig. 1A, 2C, and data not shown). This is considerably shorter than the 12-day half-life of mir-208 in propylthiouracil-treated cardiomyocytes and other estimates that have gained general acceptance in the miRNA research community (van

Rooij et al., 2007). The rapid recovery of Ago2 levels upon inhibition of mTOR signaling or proteasome activity suggests that the half-life of miRISC complexes may be as short as 1-2 hours at the height of T cell activation.

Our data reveal a crucial and regulated role for Ago proteins in maintaining mature miRNA homeostasis in T cells. Since siRNAs also depend on Ago proteins for their activity, this finding may also have implications for therapeutic siRNA delivery to immune cells. Indeed, short hairpin RNA transgenes produce relatively inefficient target gene knockdown in lymphoid organs where T cells and other lymphocytes predominate (Oberdoerffer et al., 2005). Several previous studies used Ago2 deficiency, knockdown, or overexpression to implicate Ago2 as a limiting factor for miRNA expression (Wang et al., 2012). Ago2 is required for normal hematopoiesis, and Ago2-deficient erythroblasts display a global reduction in miRNAs (O'Carroll et al., 2007). MicroRNA expression is also reduced in Ago2-deficient mouse embryonic fibroblasts and in Ago-deficient *Xenopus* embryos during early development (Diederichs and Haber, 2007; Lund et al., 2011). Conversely, overexpression of Ago2, but not other proteins in the miRNA biogenesis pathway, increased miRNA expression in HEK293 cells (Diederichs and Haber, 2007). It will be interesting to determine what role Ago2's slicer activity plays in T cells, and whether depletion of other Ago proteins will lead to a similar T cell phenotype.

The ubiquitin ligase Trim71 (mLin41) can ubiquitinate Ago2 in overexpression systems, and siRNA experiments indicated that it may regulate Ago2 abundance and ubiquitination in mouse embryonic stem cells (Rybak et al., 2009). However, neither overexpression nor genetic deficiency for Trim71 altered Ago2 expression in neural

progenitors (Chen et al., 2012). Moreover, a recent study corroborated these findings in mouse embryonic stem cells, and identified a novel function for Trim71 in enhancing miRNA activity (Chang et al., 2012). Our own analyses of Trim71 expression indicate that it is not expressed in T cells. A broader search will be necessary to identify the proteins that mediate Ago2 ubiquitination and degradation in activated T cells.

It is also important to consider whether and how other known posttranslational modifications of Ago proteins may regulate ubiquitination and protein stability in T cells (Adams et al., 2009; Qi et al., 2008a; Rybak et al., 2009). Our work has identified an essential role for mTOR signaling in Ago2 downregulation. This suggests that the mTOR pathway regulates protein synthesis at multiple nodes, both by stimulating ribosome assembly and function, and through inhibiting miRNA activity (Wang and Proud, 2006). Rapamycin, which inhibits mTOR activity, is a potent immunosuppressant that is used to prevent organ rejection (Weichhart and Saemann, 2009). Our work has shown that miRNAs also exhibit immunosuppressive properties, as Ago2-deficient T cells more readily differentiate into cytokine-producing effectors. Thus activation of the miRNA pathway may represent another mode by which rapamycin prevents immune activation. Because the signaling pathways induced by TCR engagement are common to many cell types, it will also be useful to investigate how general this mechanism may be and to determine if other cell types downregulate Ago proteins and miRNAs upon activation. We note that miRNA turnover is also accelerated in neurons in an activity-dependent manner (Krol et al., 2010). Reduced miRNA abundance is a common feature of many transformed cells, suggesting that Ago protein regulation may also occur in some cancer

settings, and contribute to tumorigenesis and metastatic potential (Hwang et al., 2009; Ventura and Jacks, 2009).

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Footnotes

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Figures

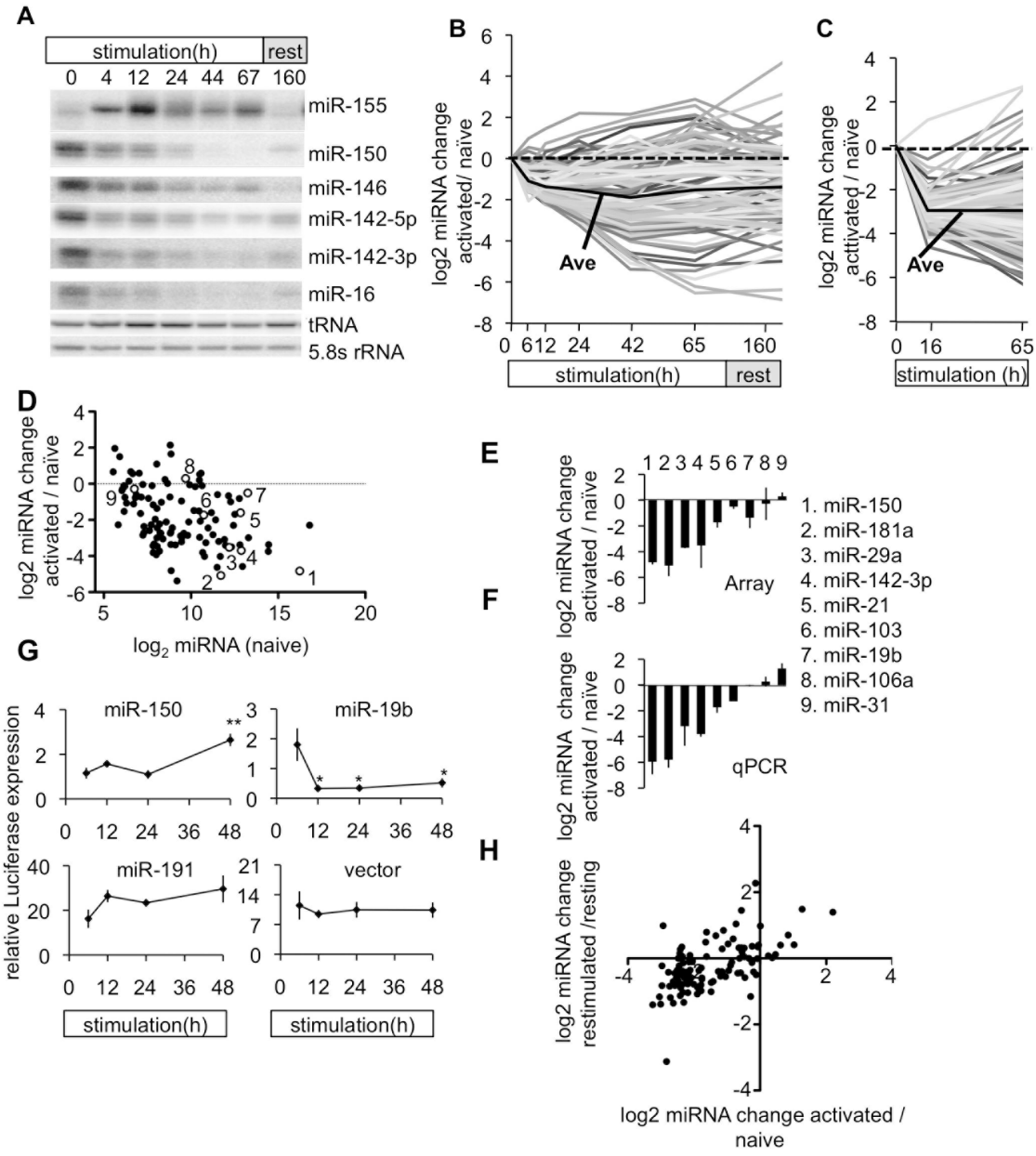


Figure 1. T cell activation leads to global miRNA depletion. (A) Northern blot analysis of indicated miRNAs in naïve CD4$^+$ T cells, and CD4$^+$ T cells stimulated for the indicated amounts of time with anti-CD3 and anti-CD28. tRNA and 5.8S rRNA are used as loading controls. (B) Array analysis of 114 miRNAs in naïve CD4$^+$ T cells, and CD4$^+$

T cells stimulated for the indicated amounts of time with anti-CD3 and anti-CD28. Data are relative to naïve, normalized to tRNA. (C) Array analysis of 174 miRNAs in naïve human peripheral blood $CD4^+$ T cells and $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28. Data are relative to naïve, normalized to tRNA. (D) MicroRNA expression in naive and 48h in vitro activated T cells. X-axis denotes raw intensity of all probes in the naive group while Y-axis denotes the fold difference between activated and naive groups, normalized to tRNA (Log2) Open circles with numbers correspond to chart in (E). (E) Bar graph denotes expression of selected miRNAs in the activated group as measured by array normalized to tRNA. Each bar corresponds to the numbered points on the plot above. (F) Bar graph denotes expression of selected miRNAs in the activated group as measured by qPCR normalized to 5.8S rRNA. (G) Relative Luciferase activity from sensors for miR-150, 19b, 191 and vector control at indicated times. Data are an average of at least 5 replicates and 4 independent experiments. $*p<0.05$, $**p<0.01$; Two-way ANOVA. (H) Log2 fold difference between 24 h activated and naïve T cells (X-axis) and log2 fold difference between 24h restimulated and resting T cells (Y-axis). Data are representative of at least 2 independent experiments.

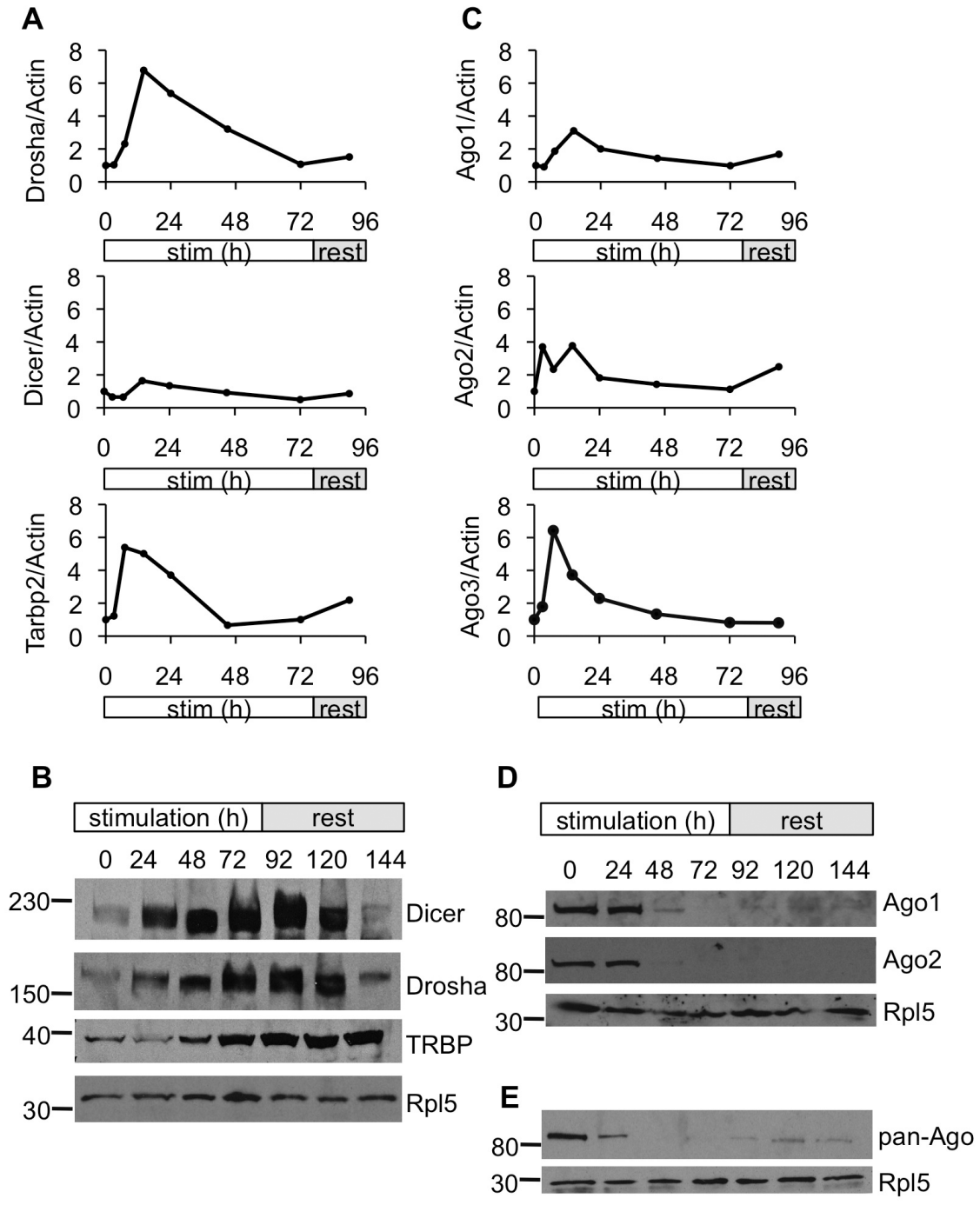


Figure 2. Argonaute proteins are post-transcriptionally downregulated upon T cell activation. (A) Quantitative PCR analysis of indicated mRNAs in naïve and stimulated

$CD4^+$ T cells. Data is normalized to β-actin. (B) Immunoblot analysis of indicated proteins in naïve and stimulated $CD4^+$ T cells. Rpl5 serves as a loading control. (C) qPCR analysis of Ago1, Ago2, and Ago3 in naïve and stimulated $CD4^+$ T cells. Data is normalized to β-actin. (D) Immunoblot analysis of Ago1 and Ago2 in naïve and stimulated $CD4^+$ T cells. Rpl5 serves as a loading control. (E) Immunoblot analysis of pan-Ago in naïve and stimulated $CD4^+$ T cells as in D. PCR data are an average of two duplicate reactions. Data are representative of at least 3 experiments.

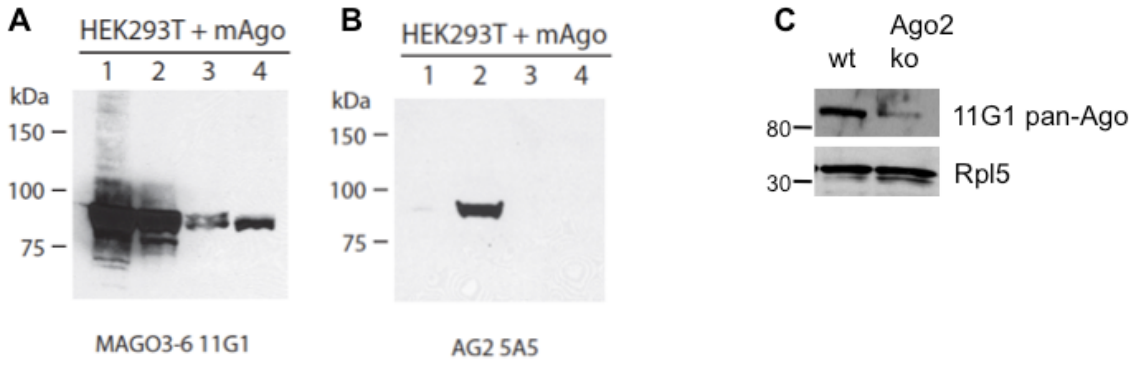


Figure 3. Verification of 11G1 pan-Ago antibody. (A) Immunoblot analysis of HEK293T cells overexpressing Ago 1, 2, 3, or 4. Blot is probed with 11G1 pan-Ago antibody (B) Immunoblot with Ago2-specific control antibody of same samples as above. (C) Immunoblot analysis of $CD4^+$ T cells from wt (left) or Ago2-deficient (right) mice. Blot is probed with 11G1 pan-Ago and Rpl5 serves as a loading control.

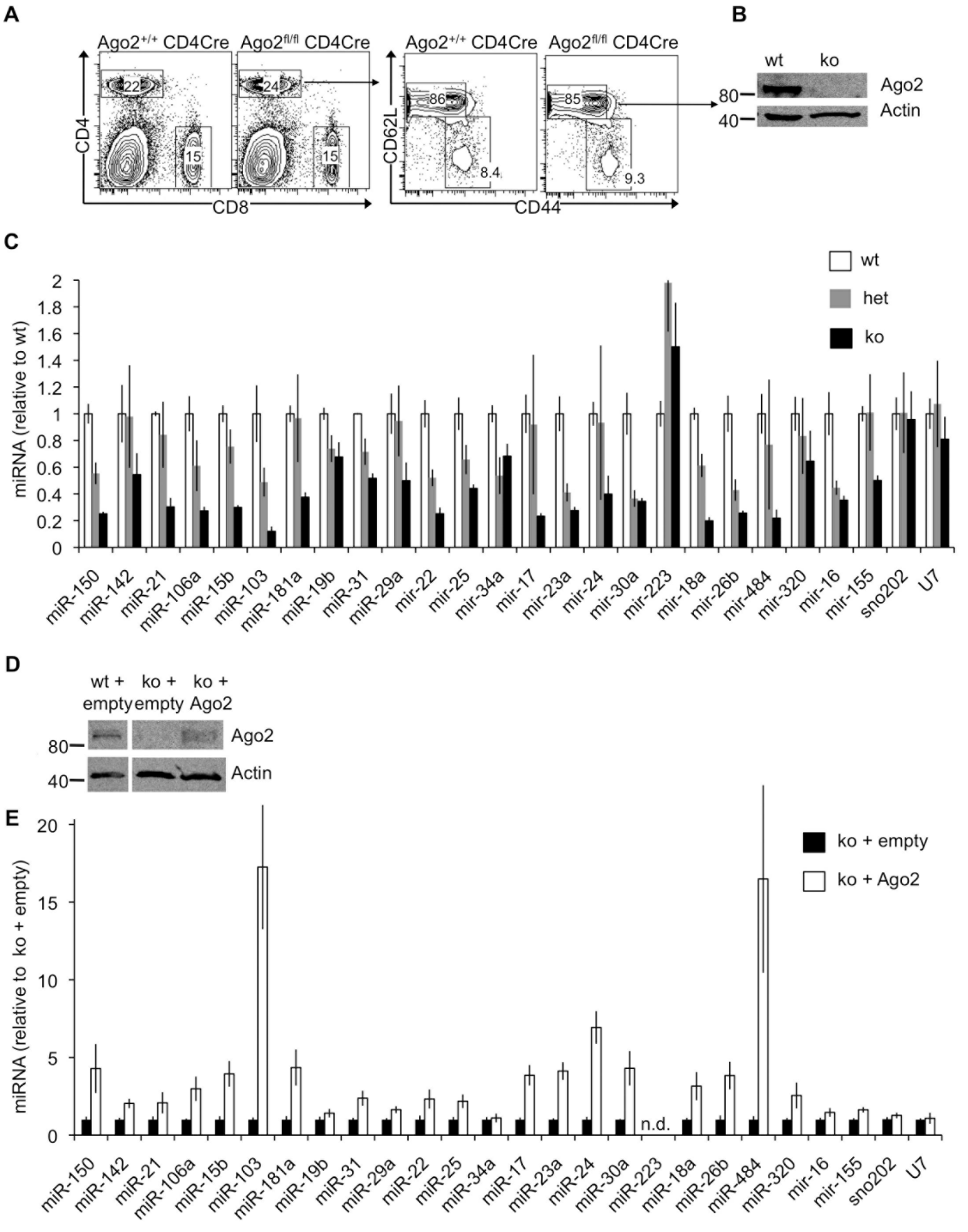
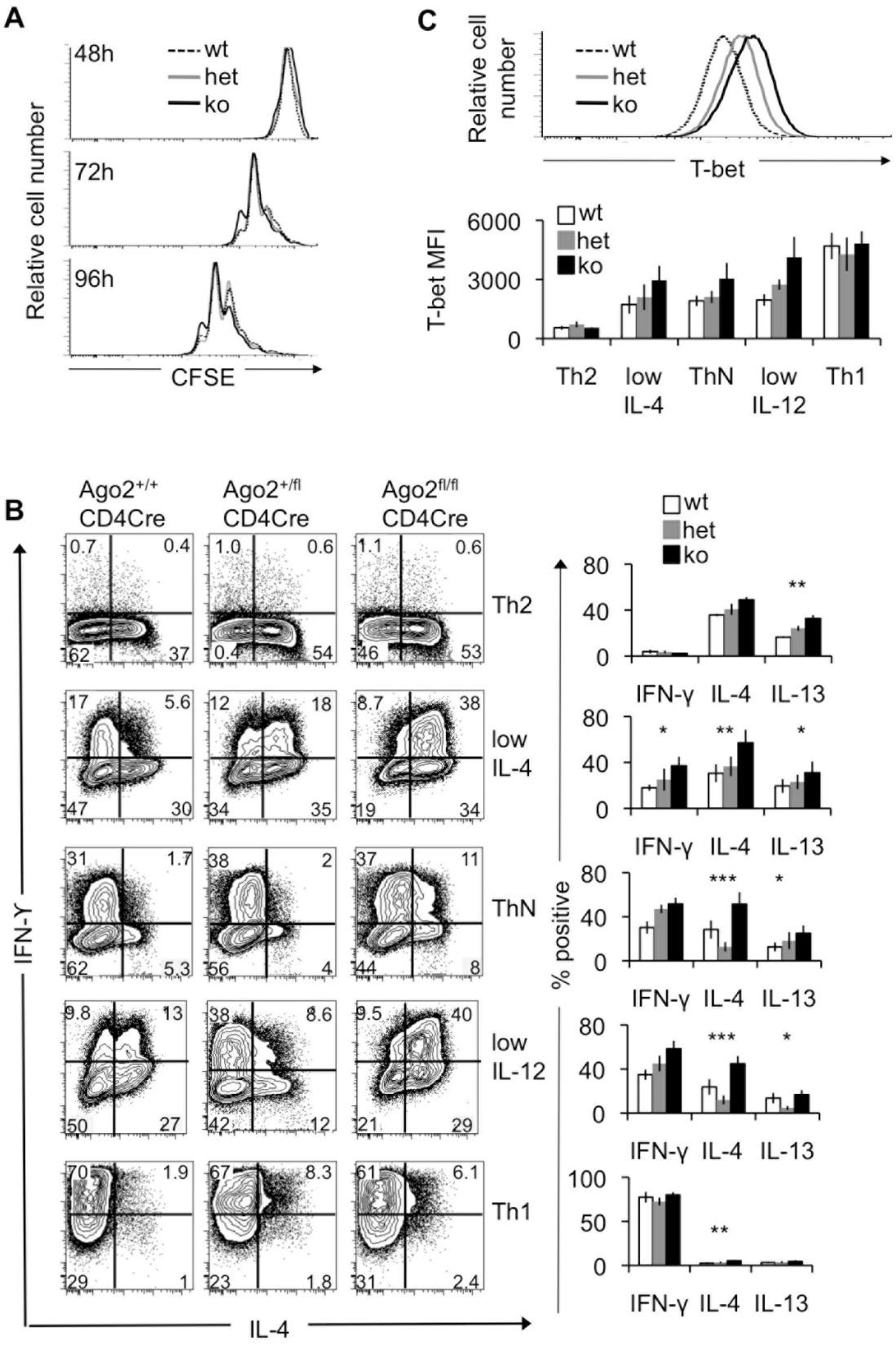


Figure 4. Ago2 is limiting for T cell miRNA expression. (A) Flow cytometry analysis of staining for CD4 and CD8 (left) in mixed spleen and lymph nodes from 4-6 week old *Ago2*$^{+/+}$; *CD4-cre* and *Ago2*$^{fl/fl}$; *CD4-cre* mice. Flow cytometry analysis of staining for

CD62L and CD44 (right) in mixed spleen and lymph nodes. (B) Immunoblot analysis of Ago2 in naïve $CD4^+$ T cells from *Ago2*$^{+/+}$, and *Ago2*$^{fl/fl}$; *CD4-cre* mice. β-actin serves as a loading control. (C) qPCR analysis of indicated miRNAs in naïve $CD4^+$ T cells from *Ago2*$^{+/+}$, *Ago2*$^{+/fl}$ and *Ago2*$^{fl/fl}$; *CD4-cre* mice. Data is normalized to 5.8S, and is represented relative to wt. (D) Immunoblot analysis of Ago2 in 6 day cultured *Ago2*$^{+/+}$ and *Ago2*$^{fl/fl}$; *CD4-cre* $CD4^+$ T cells transduced on day 2 with Ago2 retrovirus or empty retrovirus control. (E) qPCR analysis of indicated miRNAs in 6 day cultured *Ago2*$^{fl/fl}$; *CD4-cre* $CD4^+$ T cells transduced on day 2 with Ago2 retrovirus or empty retrovirus control. Data are representative of 3 experiments and error bars represent standard error of the mean. n.d., not detected.


A
48h
72h
96h
wt
het
ko
Relative cell number
CFSE
C
Relative cell number
T-bet
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6000
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ThN
low IL-12
Th1
B
Ago2+/+ CD4Cre
Ago2+/fl CD4Cre
Ago2fl/fl CD4Cre
IFN-γ
IL-4
Th2
low IL-4
ThN
low IL-12
Th1
% positive
IFN-γ
IL-4
IL-13

Figure 5. Increased effector cytokine production in Ago2-deficient T cells. (A) Flow cytometry analysis of staining for CFSE in $CD4^+$ T cells cultured for the indicated times in non-polarizing conditions. (B) Flow cytometry analysis (left) of intracellular staining for IFN-ϒ and IL-4 production by restimulated T cells isolated from *CD4-cre* $Ago2^{fl/fl}$ (ko), $Ago2^{+/fl}$ (het) or $Ago2^{+/+}$ (wt) mice differentiated for one week under Th2, low IL-4, ThN, low IL-12, and Th1 conditions. Numbers indicate percentage of cells in each quadrant. Bar graphs (right) show percentage of $CD4^+$ cells producing IFN-ϒ, IL-4 and IL-13 in the conditions on the left. **p*<0.05, **p<0.01, ***p<.001; Two-way ANOVA. (C) Flow cytometry analysis (top) of intracellular staining for T-bet in T cells differentiated for one week in low IL-12 conditions. Bar graphs (bottom) show T-bet mean fluorescence intensity (MFI) in $CD4^+$ cells under Th2, low IL-4, ThN, low IL-12, and Th1 conditions. Data are representative of 3 experiments and error bars represent standard error of the mean.

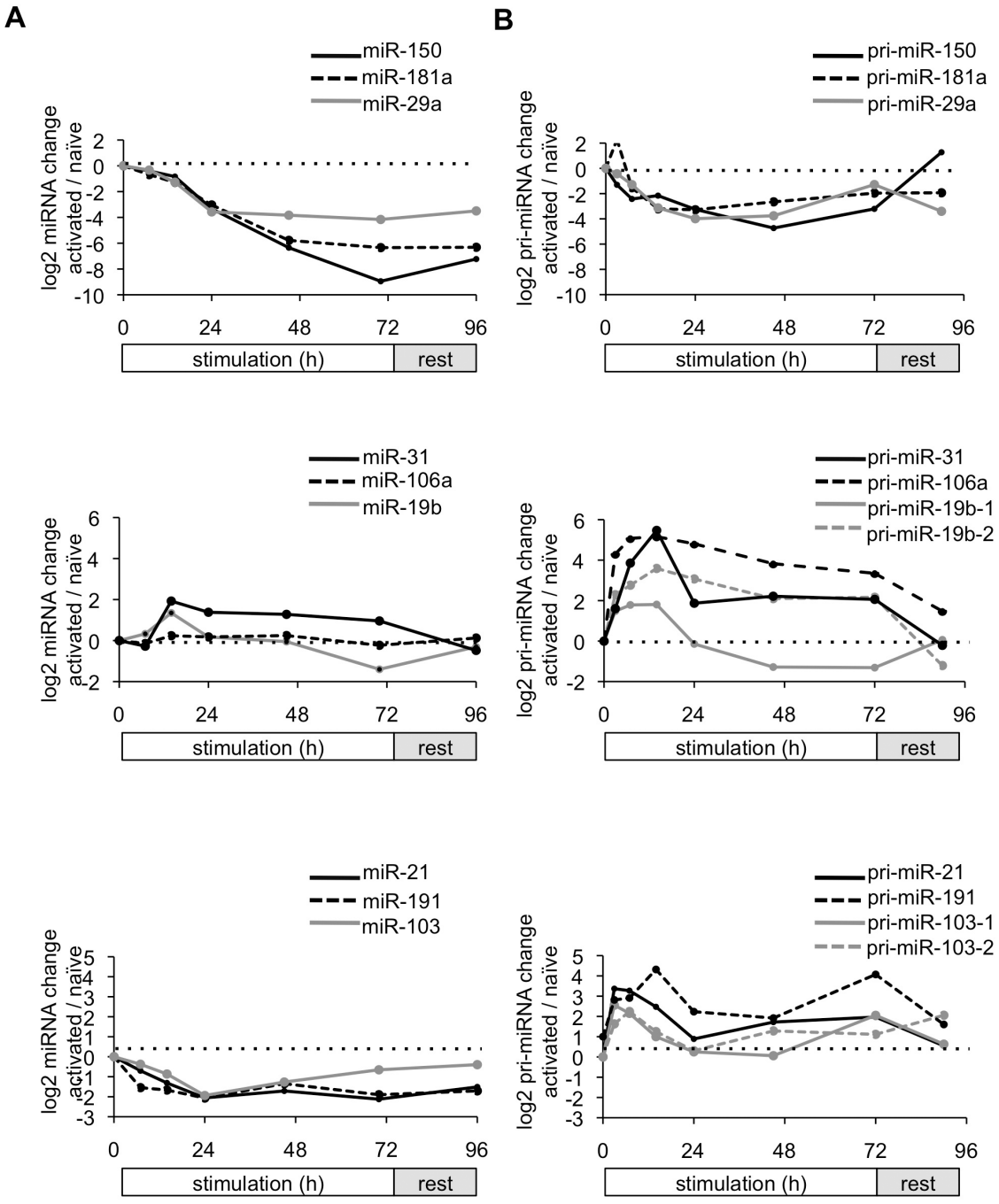

Figure 6. Activation-induced transcriptional regulation leads to differential expression of mature miRNAs. (A) Quantitative PCR analysis of indicated miRNAs in naïve and stimulated $CD4^+$ T cells. Data is relative to naïve, normalized to 5.8S rRNA.

(B) PCR analysis of indicated pri-miRNAs in naïve and stimulated $CD4^+$ T cells. Data are normalized to 28S rRNA. qPCR data are representative of 10 experiments.

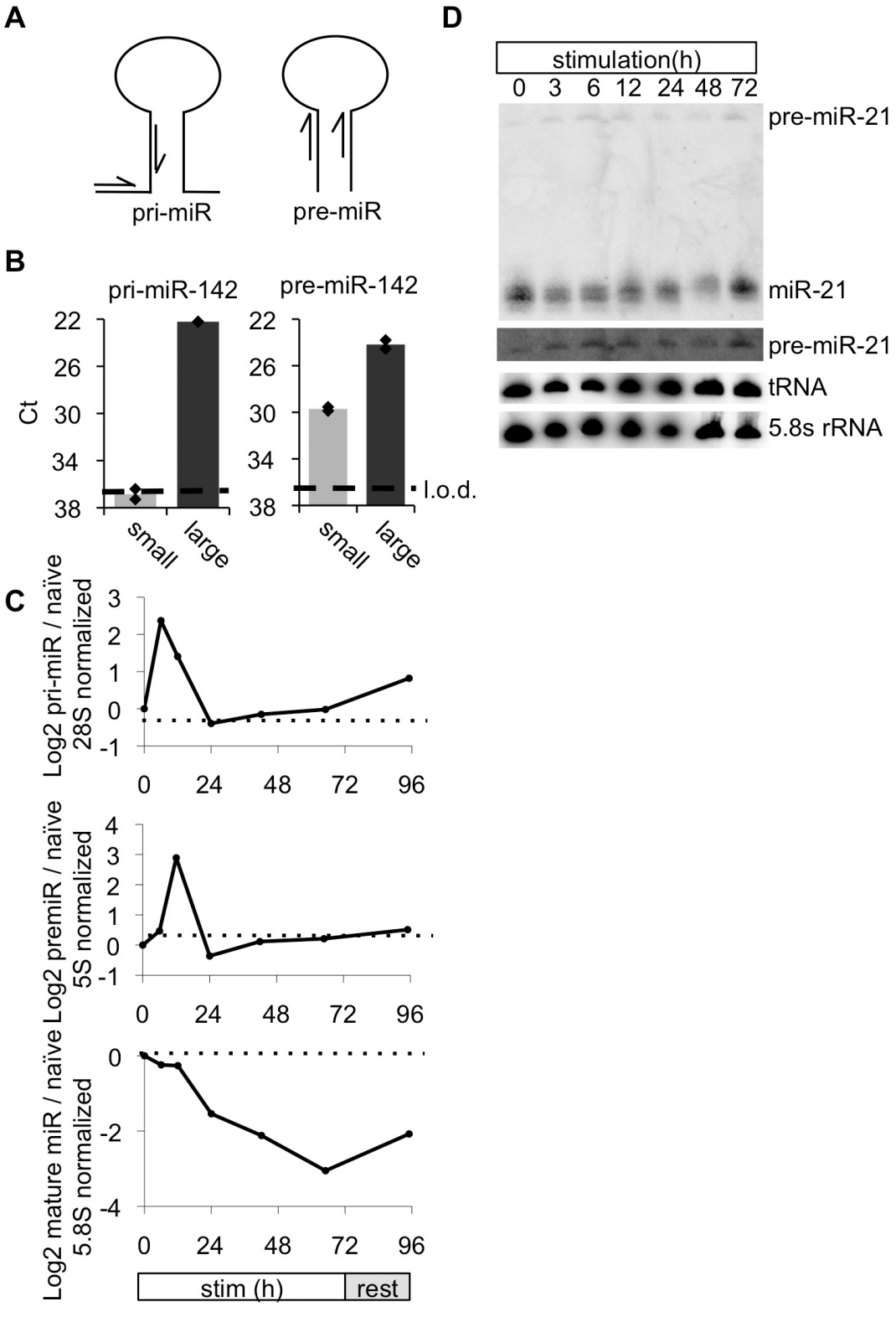
A
pri-miR
pre-miR
B
pri-miR-142
pre-miR-142
Ct
22
26
30
34
38
small
large
l.o.d.
C
Log2 pri-miR / naïve 28S normalized
Log2 premiR / naïve 5S normalized
Log2 mature miR / naïve 5.8S normalized
0
24
48
72
96
stim (h)
rest
D
stimulation(h)
0 3 6 12 24 48 72
pre-miR-21
miR-21
pre-miR-21
tRNA
5.8s rRNA

Figure 7. Efficient miRNA processing occurs in activated T cells. (A) Graphical representation of pri-miRNA and pre-miRNA primers shows those spanning one arm of the stem loop and the flanking sequence (pri-miR), and those within the stem loop (pre-miR). (B) Validation of size fractionation technique shows thermal cycle (Ct) values for qPCR performed with pri-miR-142 and pre-miR-142 primers in the small and large RNA fractions. Dotted line represents limit of detection. (C) qPCR analysis of pri-, pre- and mature miR-21 in naïve and stimulated $CD4^+$ T cells. Data is relative to naïve, normalized to 28S rRNA (pri-miRNA), 5S rRNA (pre-miRNA) and 5.8S rRNA (mature miRNA). Data in A-C are representative of 5 experiments. (D) A Northern blot analysis of pre- and mature miR-21, tRNA, and 5.8S rRNA in naïve $CD4^+$ T cells and $CD4^+$ T cells stimulated for the indicated amounts of time with anti-CD3 and anti-CD28. Lower pre-miR-21 panel is a higher exposure.

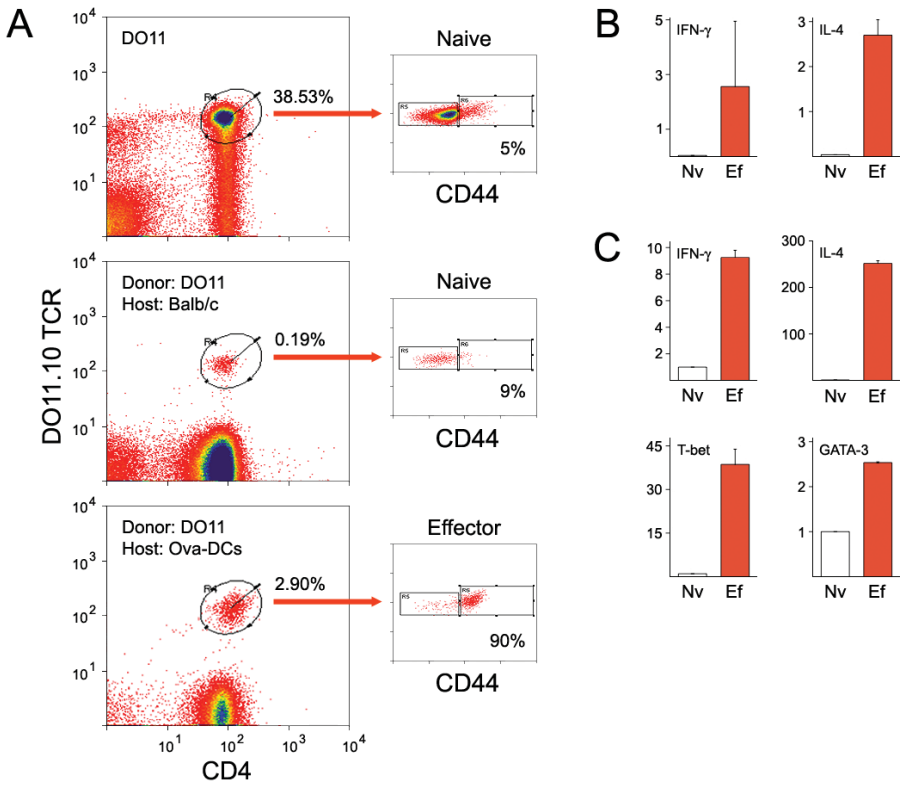

Figure 8. Functional analysis of in vivo generated effector T cells. (A) OVA-reactive T cells were transferred into congenic hosts (naive) or congenic hosts which were then immunized with OVA-pulsed DCs (effector). At day 5 post-transfer (P.T.), $CD4^+$ cells were purified from LNs and spleens of recipient mice and the percentage of total or activated ($CD44^{hi}$) donor T cells was measured by flow cytometry. (B) Adoptive transfers were performed as in (A). $CD44^{hi}$ donor T cells were then purified from the immunized group, re-stimulated ex vivo and cytokine production measured by ELISA. (C) mRNA expression was measured by PCR in purified naive and effector T cells. Data are presented as the fold change relative to naive controls (naive=1). Data are representative of 5 individual experiments.

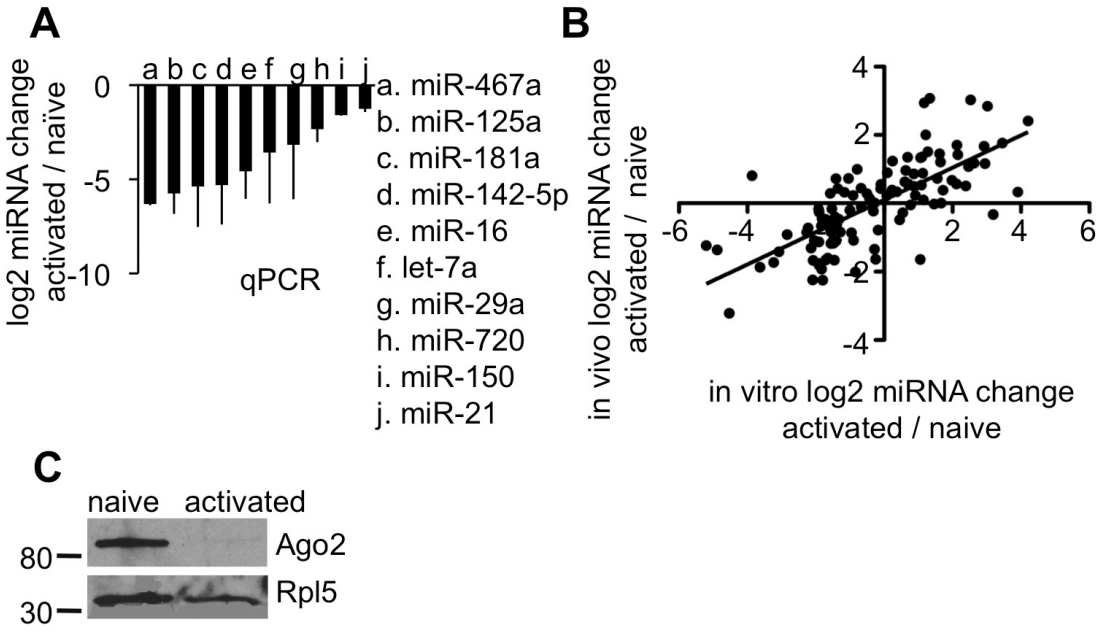

Figure 9. MicroRNA depletion and Ago2 downregulation occurs during in vivo T cell activation. (A) MicroRNA expression in in vivo activated OVA-specific T cells as

measured by qPCR normalized to 18S rRNA. Data are representative of at least 5 experiments and error bars indicate the standard deviation between replicate measurements. (B) Log2 fold difference between in vivo activated and naïve T cells (X-axis) and log2 fold difference between 65h in vitro activated and naïve T cells (Y-axis). (C) Immunoblot analysis of Ago2 and RPL5 protein in naïve and in vivo activated OVA-specific T cells. Data are representative of 2 independent experiments.

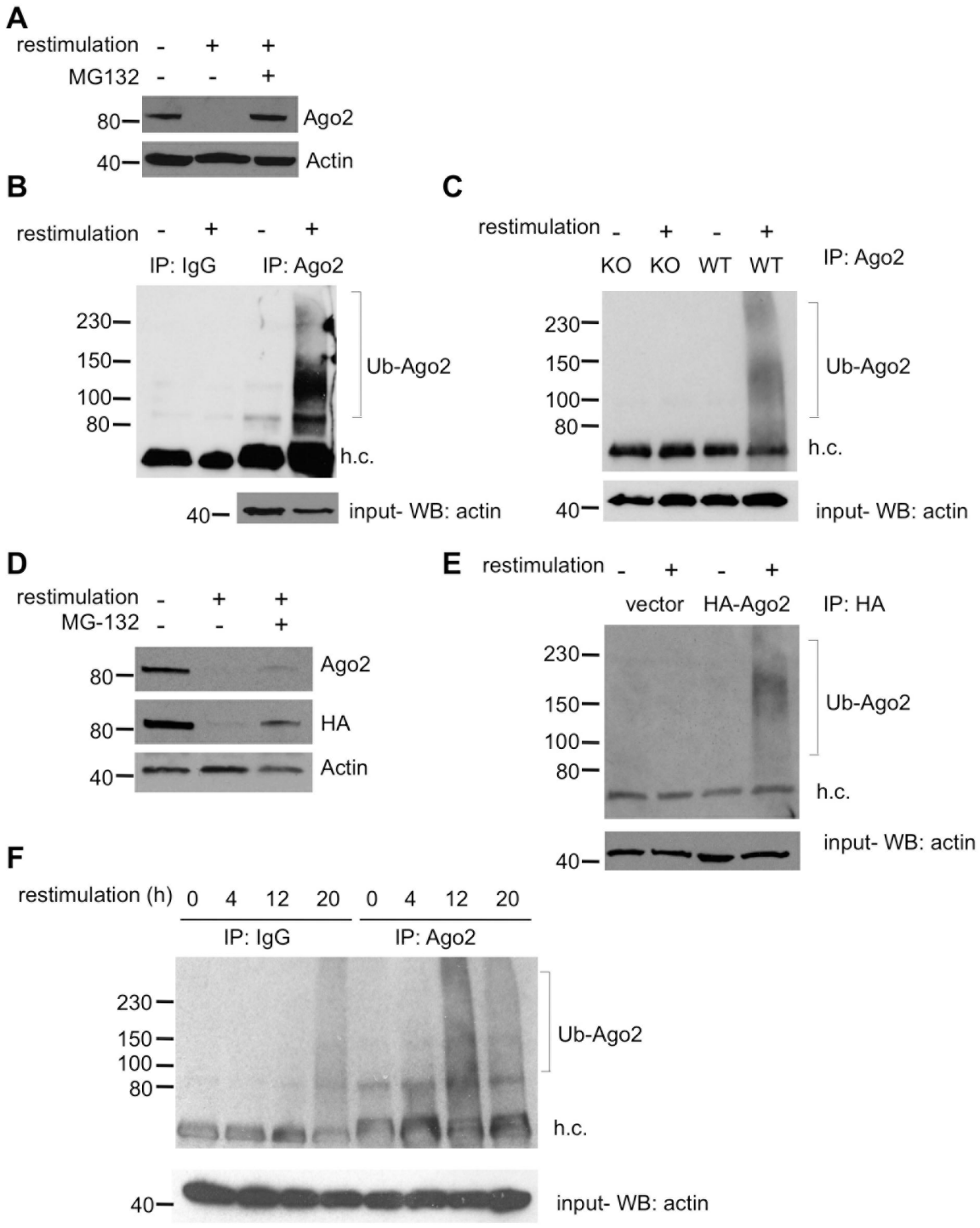

Figure 10. Ago2 is ubiquitinated and degraded by the proteasome (A) Immunoblot analysis of Ago2 and RPL5 protein in resting, 24 h restimulated T cells, and 24 h restimulated cells with MG-132 added in the last 2 h of culture. (B) IgG (left) or Ago2

(right) immunoprecipitation from resting and 24 h restimulated T cells. All cultures were treated with MG-132 in the last 4 h of culture. Immunoprecipitates were blotted with anti-ubiquitin and 4% input from resting and restimulated cells was blotted with anti-β-actin. (C) Resting and 24 h restimulated Ago2-deficient (left) or wildtype (right) T cells. Data in A-B are representative of at least 5 independent experiments. Immunoprecipitation was performed with anti-Ago2 antibody and blotted with anti-ubiquitin antibody. 4% input from resting and restimulated cells was blotted with anti-β-actin. (D) Immunoblot of Ago2, HA, and β-actin from cells transduced with HA-Ago2 retrovirus. Cells were rested until day 5 and restimulated for 24 h with MG-132 or DMSO in the last 4 h of culture. (E) Empty vector (left) and HA-Ago2 retrovirus-transduced (right) T cells that were either rested or restimulated for 24 h. Immunoprecipitation was performed with anti-HA antibody and were blotted with anti-ubiquitin antibody. 4% input from resting and restimulated cells were blotted with anti-β-actin. (F) IgG (left) or Ago2 (right) immunoprecipitation from resting, 4 h, 12 h, and 20 h restimulated T cells. All cultures were treated with MG-132 in the last 4 h of culture. Immunoprecipitates were blotted with anti-ubiquitin and 4% input from resting and restimulated cells was blotted with anti-β-actin. Data in C-F are representative of 2 independent experiments. h.c., heavy chain. Ub-Ago2, ubiquitinated Ago2.

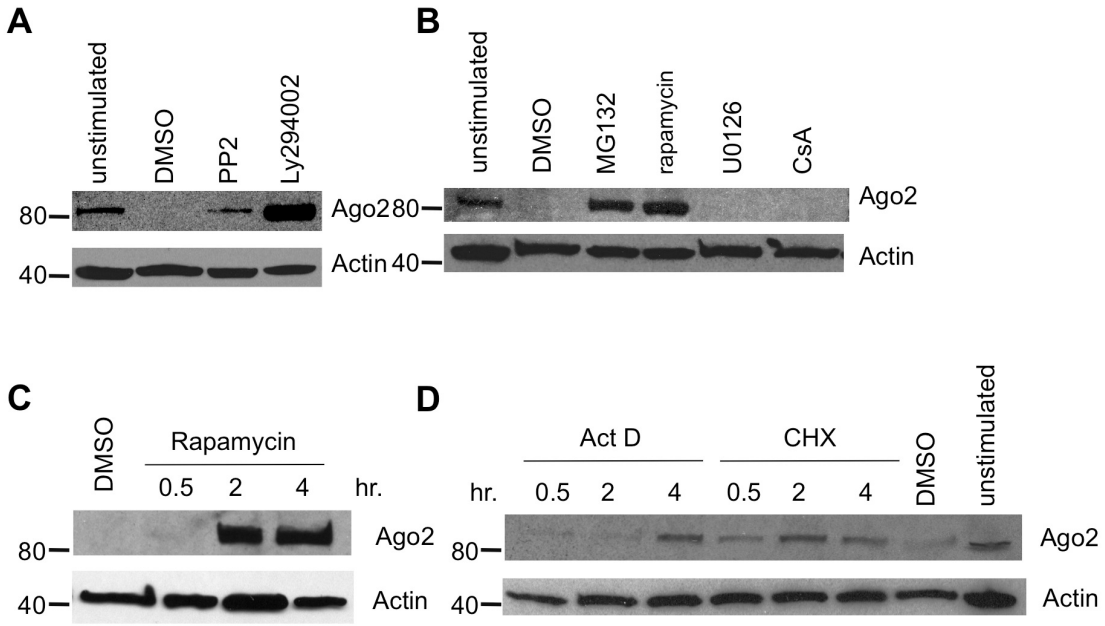

Figure 11. Continuous mTOR signaling is required for Ago2 degradation. (A) Immunoblot analysis of Ago2 and β-actin from resting T cells and T cells restimulated for 16 h plus 4 h treatment with indicated inhibitor. (B) Immunoblot analysis of Ago2 and β-actin from resting T cells and T cells restimulated for 16 h plus 4 h treatment with indicated inhibitor. (C) Immunoblot analysis of Ago2 and β-actin from 16 h restimulated T cells. Cells were then treated with 4 h DMSO (left) or Rapamycin for indicated times (right). (D) Immunoblot analysis of Ago2 and β-actin from resting and 16 h restimulated T cells. Cells were then treated with 4 h DMSO (right), or Actinomycin (left) or Cycloheximide (right) for indicated times. Data are representative of at least 3 independent experiments.

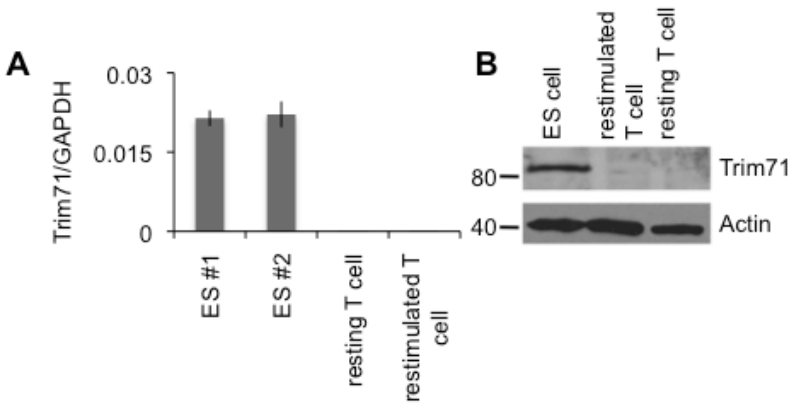

Figure 12. Trim71 expression in activated T cells. (A) qPCR analysis of Trim71 in ES cells and resting and restimulated T cells. Data are normalized to GAPDH. (B) Immunoblot analysis of Trim71 protein in ES cells and resting and restimulated T cells. β-actin serves as a loading control.

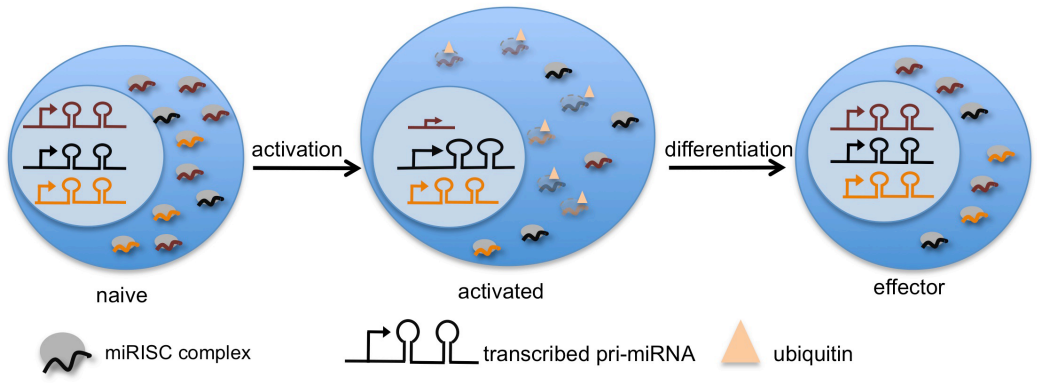

Figure 13. Activated T cells reset their miRNA repertoire. Upon T cell activation, cells increase cellular metabolism to support cell growth, proliferation and effector functions. Activated T cells also downregulate Argonaute proteins, the core component of the miRISC complex (grey ovals), through a process of ubiquitination (orange triangles) and proteasomal degradation. This leads to global miRNA depletion. Layered

atop this post-transcriptional regulation is programmed modulation of miRNA gene transcription (arrows). Together, these mechanisms rapidly remodel, the miRNA repertoire of activated T cells as they differentiate into effector cells.

CHAPTER IV. CONCLUSIONS AND FUTURE DIRECTIONS

Summary

Our work focused on investigating the mechanisms underlying the global changes in miRNA expression that occur following $CD4^+$ T cell activation. We used Northern blot, microarray, qPCR, and luciferase sensor analysis to determine that activated T cells exhibit downregulation of virtually all miRNAs within 12-48h following in vitro stimulation. We also observed a concurrent post-transcriptional downregulation of Ago proteins, wth near undetectable levels observed by 48-72h. These same phenomena were observed in T cells activated in vivo. Using T cell-specific deletion of Ago2, we also determined that Ago2 is a limiting factor for miRNA homeostasis in T cells. Further, Ago2-deficient T cells exhibit increased production of effector cytokines, such as IL-4 and IFN-γ. We also observed significant heterogenity in the expression of individual miRNAs in response to T cell activation, and identified a mechanism of miRNA-specific transcriptional regulation. Finally we investigated the mechanism underlying activation-induced Ago downregulation, and determined that Ago2 is ubiquitinated and degraded by

the proteasome. This degradation is dependent on continuous signaling through the mTOR pathway.

Mechanism of Argonaute degradation

Our work has determined that Ago2 is ubiquitinated within 12-24h following T cell activation. Further, Ago2 stability is sensitive to the proteasome inhibitor, MG-132, indicating that the protein is degraded via the proteasome. By treating cells with inhibitors of several signaling molecules, we determined that calcineurin and MAPK activity is dispensible for Ago2 degradation, as blocking these pathways with Cyclosporin A or U0126 had no effect on activation-induced Ago2 downregulation. In contrast, inhibiting PI3K or mTOR activity by treating with LY294002 or rapamycin, completely rescued Ago2 levels following T cell activation. Using inhibitors of transcription and translation, Actinomycin D and cyclohexamide, we further determined that continued transcription and translation are required for Ago2 degradation. Together, these results suggest that an induced protein is required to maintain low Ago2 expression in activated T cells.

Several questions remain regarding the regulation of Ago2 degradation in activated T cells. The late onset of Ago2 downregulation following T cell stimulation (visible by western blot at 24h) suggests that it could be mediated, at least in part, through cytokine signaling. Because the PI3K-Akt and mTOR pathways are activated downstream of the IL-2 receptor, IL-2 signaling could enhance Ago2 degradation (Malek and Castro, 2010). Our preliminary experiments have indicated that, in the absence of restimulation, increasing IL-2 concentrations in resting T cells leads to decreased Ago2

protein. It would also be interesting to assay the effect of short-term IL-2 blockade with and without T cell stimulation. Furthermore, the JAK nonreceptor tyrosine kinase inhibitor CP-690,550 blocks both the STAT5 and Akt signaling pathways downstream of the IL-2 receptor (Ghoreschi et al., 2011). This compound, in conjunction with an Akt inhibitor, could therefore be used to determine if IL-2 signaling is required for activation-induced Ago2 degradation.

It will also be important to determine the sites of Ago2 ubiquitination and the E3 ubiquitin ligase that targets Ago2. Ubiquitination of proteins occurs by a cascade of enzymatic reactions involving ubiquitin-activation (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes (Deshaies and Joazeiro, 2009). Ubiquitination occurs when an E3 ligase binds to an E2 conjugated to ubiquitin, allowing for the transfer of the ubiquitin from the E2 to a lysine residue in the substrate protein. Specificity in ubiquitination is determined by E3s pairing E2s with substrates. Identification of the E3 ligase that targets a particular protein is challenging, due to the ability of several ligases to redundantly target a single protein. Thus a combination of experimental approaches is required to elucidate the mechanism of Ago2 degradation. Using our optimized Ago2 immunoprecipitation protocol, Ago2 can be purified from restimulated T cells and analyzed by mass spectrometry to determine the lysine residues that are ubiquitinated (Kaiser and Wohlschlegel, 2005). Expression of HA-tagged Ago2 can also be used to increase specificity of immunoprecipitation. Identified lysine residues can be mutated to arginines and reexpressed in T cells to assess the importance of these sites for Ago2 stability. In order to identify the relevant E3 ligase, an siRNA screen against E3 ligases can be performed, though our current Western Blot readout is probably too low

throughput to be practical. Instead it will be useful to generate a flow cytometry based assay, perhaps using a GFP-tagged Ago2, and a cell line system, in order to make the screen more tractable.

Characterization of Ago-deficient T cells

Our data identified an important role for Ago2 in restricting helper T cell differentiation. Specifically, Ago2-deficient T cells, which have an average 60% reduction in miRNA levels, are phenotypically normal at baseline, but are more prone than wildtype cells to differentiate into cytokine-producing effector cells when activated. These data are similar to observations in Dicer- and DGCR8-deficient cells, which lack virtually all miRNAs, and display even more aberrant differentiation, especially into IFN-γ-producing Th1 cells (Chong et al., 2008; Cobb et al., 2006; Liston et al., 2008; Muljo et al., 2005; Steiner et al., 2011b; Zhou et al., 2008). Thus global miRNA abundance correlates with a helper T cell's propensity to differentiate into effector cells, and activation-induced miRISC downregulation may be important to relax miRNA-mediated gene repression, and allow activated T cells to change their gene expression program and differentiate into effectors. It will be interesting to determine if this phenomenom holds true in vivo. To this end, in vivo infection and/or autoimmunity models should be performed in the T cell-specific Ago2-deleted mice.

In contrast to the cell survival and proliferation defect observed in complete miRNA-deficient T cells, Ago2-deficient cells proliferate similarly to wild type cells in vitro. Presumably this discrepancy results from the remaining 40% of miRNAs found in Ago2-deficient cells, and that a complete Ago knockout T cell would more closely

resemble Dicer- and DGCR8-deficient T cells. Furthermore, Ago2 is unique amongst Ago proteins in its slicer activity. Thus it will be useful to compare the phenotypes of Ago2-, Ago1-, and Ago3-deficient T cells. To that end we obtained mice carrying an Ago1 floxed allele, which was crossed to CD4-Cre to obtain T cell-specific deletion of Ago1 (Skarnes et al., 2011). Preliminary analyses of these mice, and those intercrossed to the Ago2 floxed allele have revealed that Ago1-deficient T cells have a slight but inconsistent decrease in miRNA levels, while double-deficient T cells exhibit very low miRNA expression (Fig. 1). Upon in vitro polarization and cytokine analsysis, Ago1-deficient cells exhibit no differentiation defect, while significantly more double-deficient cells, as compared to wildtype cells, produce IFN-γ. This phenotype is reminiscent of Dicer or DGCR8-deficient T cells, and in fact, Ago1,Ago2-double-deficient T cells also exhibit reduced proliferation in vitro. Further work is required to determine how invidual Agos and miRNAs, and the balanced expression of these miRNAs, affect helper T cell differentiation.

Relevance of Ago in cancer

Global miRNA downregulation has been observed in numerous human tumors, as compared to normal tissue (Gaur et al., 2007; Lu et al., 2005). Further, poorly differentiated tumors have lower global levels of miRNA expression compared with more differentiated samples. Global repression of miRNA maturation promotes transformation and tumorigenesis, and Dicer1 can function as a haploinsufficient tumor suppressor (Kumar et al., 2007; Kumar et al., 2009). Data from our work and others has determined that Ago proteins are limiting factors in miRNA abundance, such that decreased Ago is

associated with decreased miRNA levels (Diederichs and Haber, 2007; Lund et al., 2011; O'Carroll et al., 2007; Wang et al., 2012). Therefore it will be interesting to determine if Agos can also function as tumor suppressors.

Several studies have addressed the potential association between Ago expression and tumorigenesis. Parisi et al. showed that overexpressing Ago1 in neuroblastoma cells led to decreased proliferation and cellular motility and a stronger apoptotic response upon UV irradiation (Parisi et al., 2011). Zhang et al. further exhibited that Ago2 overexpression in 293T and H1299 cells inhibited cell proliferation and migration in vitro and in a xenograft tumor model in nude mice (Zhang et al., 2012). They further showed that Ago2 expression is lower in human lung adenocarcinomas than in the paired, non-cancerous tissues. These data are supported by several microarray studies of differentiating and tumorigenic cells that reveal an inverse correlation between Ago1 expression and cellular division rates (Barrett et al., 2011; Talantov et al., 2005). Additionally, region 1p34-35 of chromosome 1, which includes the Ago1 gene, is often deleted in Wilm's tumors and has been associated with neuro-ectodermal tumors (Dome and Coppes, 2002; Koesters et al., 1999). Genome wide association studies (GWAS) of single nucleotide polymorphisms (SNPs) associated with human cancers have provided further support for this model. Kim et al. determined that a SNP in Ago1 was significantly associated with a decreased risk of lung cancer compared with those with the standard allele (Kim et al., 2010a). Further, Ago2 mutations were found in gastric cancers with high microsatellite instability, and loss of Ago2 protein was observed in 40% of analyzed gastric cancers and 35% of colorectal cancers (Kim et al., 2010b). In contrast, another studied found that Ago proteins are actually overexpressed in colon

cancer relative to adjacent non-cancer tissue and that expression is increased in advanced tumors with distant metastasis, arguing that further research is required (Li et al., 2010).

To date, no associations between Ago expression and hematopoietic malignancies have been reported. Our data in Ago2-deficient T cells suggests that loss of Ago could predispose to tumorigenesis, either through increased inflammation or through in vivo proliferation effects (Grivennikov et al., 2010). One method to address a role for Ago2 in hematopoietic transformation is to use Ago2-deficient cells in a model of Notch-induced T-lineage acute lymphoblastic leukemia (T-ALL). Most cases of T-ALL exhibit mutations or translocations that result in increased expression or constitutive *Notch1* activity (Weng et al., 2004). To model this disease, hematopoietic progenitor cells (HPCs) from fetal livers or bone marrows are tranduced with a vector expressing Notch-ICN (intracellular cleaved Notch1) (Mavrakis et al., 2010; Pear et al., 1996). Transduced HPCs are transplanted into irradiated recipients, who then develop leukemia within 60-100 days following transplant. A similar experiment could be performed using HPCs from $Ago2^{fl/fl}$; CD4-Cre mice to determine if loss of Ago2 predisposes recipients to faster or more aggressive leukemogenesis. It will also be interesting to investigate the expression of Ago protein in additional human cancer settings. Given our results describing a key role for mTOR signaling in regulating Ago2 stability, it will be especially informative to assay Ago protein in settings of anti-cancer rapamycin treatment (Powell et al., 2012).

FIGURES

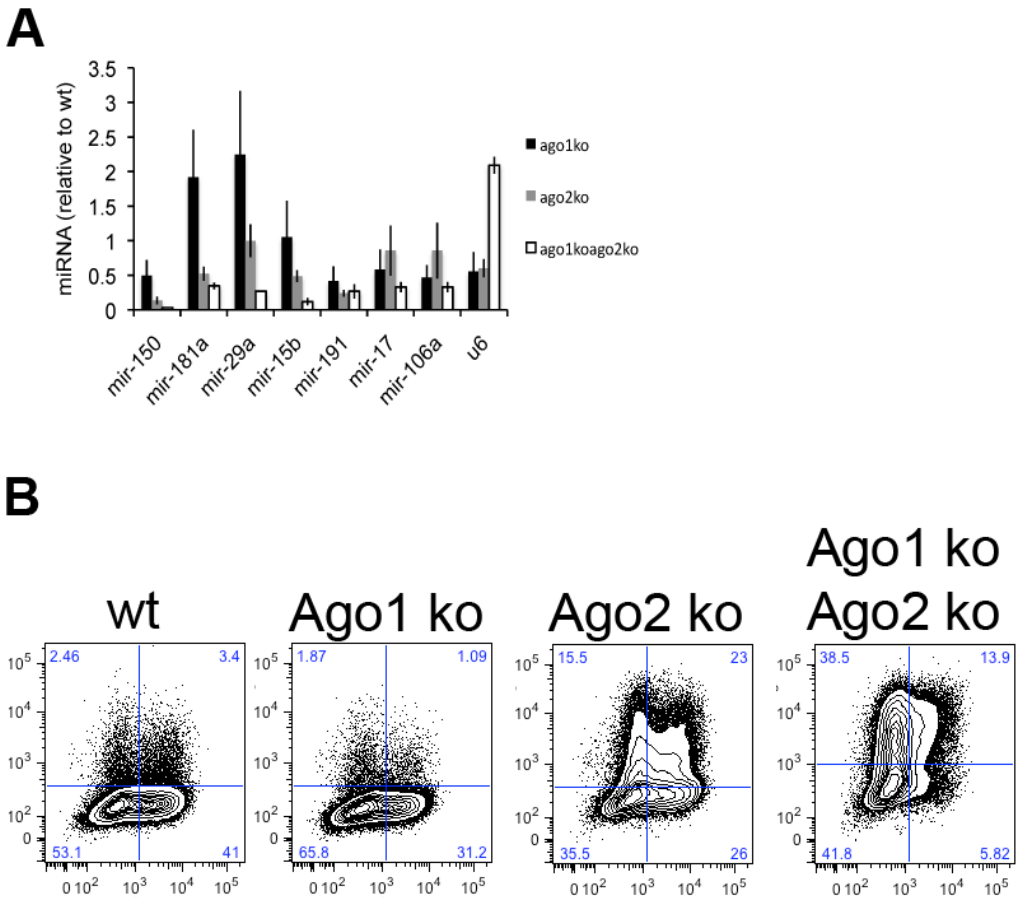

Figure 1. MicroRNA expression and cytokine expression in Ago-deficient T cells. (A) qPCR analysis of indicated miRNAs in naïve CD4$^+$ T cells from *Ago1*$^{+/+}$Ago2$^{+/+}$, *Ago1*$^{fl/fl}$, *Ago2*$^{fl/fl}$, and *Ago1*$^{fl/fl}$*Ago2*$^{fl/fl}$; *CD4-cre* mice. Data is normalized to 5.8S, and is represented relative to wt. (B) Flow cytometry analysis intracellular staining for IFN-ϒ and IL-4 production by restimulated T cells isolated from *CD4-cre Ago1*$^{+/+}$Ago2$^{+/+}$ (wt), *Ago1*$^{fl/fl}$ (Ago1 ko), *Ago2*$^{fl/fl}$ (Ago2 ko), and *Ago1*$^{fl/fl}$*Ago2*$^{fl/fl}$ (Ago1 ko Ago2 ko) mice differentiated for one week under ThN conditions. Numbers indicate percentage of cells in each quadrant.

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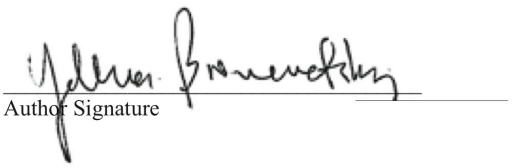

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