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Li XL, Ezelle HJ, **Hsi TY**, Hassel BA. A central role for RNA in the induction and biological activities of type 1 IFNs. *WIRES RNA.* 2011 Jan/Feb; 2(1): 58-78

Laufer MK, van Oosterhout JJ, Thesing PC, Dzinjalama FK, **Hsi T**, Beraho L, Graham SM, Taylor TE, Plowe CV. Malaria treatment efficacy among people living with HIV: the role of host and parasite factors. *Am J Trop Med Hyg.* 2007 Oct;77(4):627-32

## ABSTRACTS

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**Lee T**, Li XL, Hassel B., Regulation of the Endoribonuclease RNase-L by the microRNA-29 family. Invited Oral Presentation at the 2<sup>nd</sup> Annual UMB - UMCP Annual Research Symposium: Crosstalk: Across Cells, Across Campuses. 2011, June 3. Baltimore, MD USA

**Hsi T**, Li XL, Hassel B., Regulation of the Endoribonuclease RNase-L by the microRNA-29 family. Poster presented at the 4th Annual RNA Stability Meeting. 2010, October 17-20. Montreal, Canada

**Hsi T**, Li XL, Hassel B., Regulation of the Endoribonuclease RNase-L by microRNAs. Poster presented at the International Society of Interferon and Cytokine Research Tri-Society Annual Conference. 2009, October 18-21. Lisbon, Portugal

**Hsi T**, Laufer M, Plowe C., Genotyping as a method for distinguishing recrudescences from reinfections in malaria drug efficacy trials. Poster presented at the 29th Annual Medical Student Research Day. 2006, September 25-26. Baltimore, MD USA

Vanderburg CR, Pfannl R, Tian D, Kiehl T-R, **Hsi T**, Hedley-Whyte ET, Frosch MP., Factors influencing post-mortem RNA integrity in human brain. Poster presented at the 29th Annual Medical Student Research Day. 2006, September 25-26. Baltimore, MD USA

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## Abstract

Title of Dissertation: Regulation of the Endoribonuclease RNase-L

by the miR-29 Family of MicroRNAs

Teresa Lee, Doctor of Philosophy, 2013

Dissertation Directed by: Bret A. Hassel, Ph.D.

Associate Professor

Department of Microbiology and Immunology

The endoribonuclease RNase-L is the terminal component of an interferon (IFN) - regulated RNA decay pathway known as the 2'-5'-oligoadenylate (2-5A) system whose established functions include antimicrobial and tumor suppressive activities. RNase-L enzymatic activity requires the binding of the small molecule 2-5A, leading to RNase-L dimerization and cleavage of single-stranded RNA. RNase-L levels are controlled post-transcriptionally by the 3'-untranslated region (3'UTR) of its messenger RNA (mRNA), which exerts a strong negative regulatory effect on RNase-L expression. MicroRNAs (miRNAs) are a class of small noncoding RNAs that repress expression of target mRNAs by binding to regions of complementarity often located in their 3'UTRs. The miR-29 family has been shown to play a tumor suppressive role in several cancers, including chronic myelogenous leukemia (CML), and has many known oncogenic targets. Here, we report that the miR-29 family represses expression of RNase-L protein across several cell types. Using a luciferase reporter, we showed that miR-29 acts via four target sites

within the *RNASEL* 3'UTR. Mutation of all sites is required for abrogation of miR-29 repression. MiR-29 repression of RNase-L expression modulates its biologic activities, such as IFN-induced protection against encephalomyocarditis virus infection. In light of the reported tumor suppressive role of miR-29 in K562 CML cells and miR-29 repression of RNase-L expression in these cells, we generated K562 cells with stable RNase-L knockdown and demonstrated that loss of RNase-L expression inhibits cell proliferation in vitro as well as tumor growth in a nude mouse xenograft model. Our findings identify a previously unknown miRNA regulator of RNase-L expression and support a novel oncogenic role for RNase-L in CML and potentially other malignancies.

Regulation of the Endoribonuclease RNase-L  
by the miR-29 Family of MicroRNAs

by  
Teresa Lee

Dissertation submitted to the faculty of the Graduate School of the  
University of Maryland, Baltimore in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2013

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## List of Abbreviations

2-5A: 2'-5'-oligoadenylate  
3'UTR (3'-untranslated region)  
5'UTR (5'-untranslated region)  
ACVR2A: activin A receptor type II a  
ADAM12: disintegrin and metalloproteinase domain-containing protein 12  
AGO: Argonaute  
ALCL: anaplastic large cell lymphomas  
ALK: anaplastic lymphoma kinase  
ALCL: anaplastic large cell lymphoma  
ALR: AIM2-like receptor  
AML: acute myeloid leukemia  
AP-1: activator protein 1  
ARD: ankyrin repeat domain  
AR: androgen receptor  
ARE: AU-rich element  
ATP: adenosine triphosphate  
ATRA: all-trans retinoic acid  
Bcl-2: B-cell lymphoma 2  
B-CLL: B-cell chronic lymphocytic leukemia  
BACE1: beta-secretase-1  
CatE: cathepsin E  
CDK6: cyclin-dependent kinase 6  
CEBPA: CCAAT/enhancer-binding protein alpha  
CFS: chronic fatigue syndrome  
ChIP: chromatin immunoprecipitation  
CHOP10: C/EBP homologous protein  
CLIP: cross-linking and immunoprecipitation  
CML: chronic myelogenous leukemia  
CTAR: C-terminus activation region  
CTGF: connective tissue growth factor  
CTNNBIP1: beta-catenin-interacting protein 1  
DC: dendritic cell  
DKK1: Dkkopf-1  
DMD: Duchenne's muscular dystrophy  
DMEM: Dulbecco's modified Eagle medium  
DNMT: DNA methyltransferase  
dsRNA: double-stranded RNA  
DUSP2: dual specificity protein phosphatase 2  
EBV: Epstein-Barr virus  
ECM: extracellular matrix  
ECyd: 1-(3-C-ethynyl- $\beta$ -D-ribo-pentofuanosyl) cytosine  
EGFR: epidermal growth factor receptor  
ELISA: enzyme-linked immunosorbent assay  
EMCV: encephalomyocarditis virus

eRF3: eukaryotic release factor 3  
FADD: Fas-associated protein with death domain  
FBS: fetal bovine serum  
FHIT: fragile histidine triad  
FIH: factor inhibiting HIF1- $\alpha$   
FRET: fluorescence resonance energy transfer  
FUSIP1: FUS-interacting protein  
FUS/TLS: fused in sarcoma/translocated in liposarcoma  
FXR: farnesoid X receptor  
FZD5: Frizzled homology 5  
GAPDH: glyceraldehyde 3-phosphate dehydrogenase  
GW182: glycine-tryptophan 182kDa  
Hbp1: HMG-box transcription factor 1  
HBV: hepatitis B virus  
HBx: hepatitis B virus X  
HCC: hepatocellular carcinoma  
HCV: hepatitis C virus  
HDAC: histone deacetylase  
HIV-1: human immunodeficiency virus-1  
HMBS: hydroxymethylbilane synthase  
HMGA1: high mobility group A1  
HPC1: Hereditary Prostate Cancer-1  
HPV: human papillomavirus  
HSC: hepatic stellate cells  
HSPC: hematopoietic stem/progenitor cells  
HuR: human antigen R  
IACUC: Institutional Animal Care and Use Committee  
ID1: DNA binding protein inhibitor 1  
IF2mt: mitochondrial initiation factor 2  
IFI16: interferon, gamma-inducible protein 16  
IFN: type I interferon  
IFN- $\gamma$ : interferon- $\gamma$  (type II interferon)  
IFN- $\lambda$ : interferon- $\lambda$  (type III interferon)  
IFNAR1: IFN- $\alpha/\beta$  receptor 1  
IFNAR2: IFN- $\alpha/\beta$  receptor 2  
IGF-I: insulin-like growth factor I  
IgV<sub>H</sub>: immunoglobulin variable-region heavy chain  
IL-1 $\beta$ : interleukin-1 $\beta$   
IL-4: interleukin-4  
IPS1: IFN- $\beta$ -promoter stimulator 1  
IRAK1: IL-1 receptor-associated kinase 1  
Ire1: inositol-requiring enzyme 1  
IRF: interferon regulatory factor  
ISG: interferon-stimulated gene  
ISG15: interferon-stimulated gene 15  
JAK1: Janus kinase 1

JNK: c-Jun N-terminal kinase  
KEN: kinase-extension-nuclease  
Kremen2: kringle containing transmembrane protein 2  
LAMP: lysosomal-associated membrane protein  
LC3: microtubule-associated protein1A/B/light chain 3  
LDH: lactate dehydrogenase  
LEF: lymphoid enhancer factor  
Lims1: LIM and senescent cell antigen-like containing domain protein  
LPL: lipoprotein lipase  
LMP1: latent membrane protein 1  
LPS: lipopolysaccharide  
Mcl-1: myeloid cell leukemia 1  
MDA5: melanoma differentiation-associated gene 5  
MEF: mouse embryonic fibroblast  
miRISC: miRNA-induced silencing complex  
miRNA: microRNA  
MMP-2: matrix-metalloproteinase-2  
MOI: multiplicity of infection  
mRNA: messenger RNA  
mtRNA: mitochondrial RNA  
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
MyD88: myeloid differentiation factor 88  
MyoD: myogenic differentiation protein  
NASP: nuclear autoantigenic sperm protein  
Narf: nuclear prelaminin A recognition factor  
NAV3: Neurone navigator 3  
ncRNA: noncoding RNA  
NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells  
NK: natural killer  
NLR: NOD-like receptor  
NOD2: nucleotide-binding oligomerization domain-containing protein 2  
NPC: nasopharyngeal carcinoma  
NPM-ALK: nucleophosmin-anaplastic lymphoma kinase  
NSCLC: non-small cell lung cancer  
OAS: oligoadenylate synthetase  
ORF: open reading frame  
oxLDL: oxidized low density lipoprotein  
P-bodies: processing bodies  
PABP: poly-A-binding protein  
PAMP: pathogen-associated molecular pattern  
PBMC: peripheral blood mononuclear cell  
PBS: phosphate buffered saline  
PDGF: platelet-derived growth factor  
PDPN: podoplanin membrane sialoglycoprotein  
PI3K $\beta$ : phosphatidylinositol 3-kinase p85 regulator subunit  
PKR: protein kinase R

PMA: phorbol-12-myristate-13-acetate  
PMP22: peripheral myelin protein 22  
Ppm1d: protein phosphatase Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent 1D  
pre-miRNA: precursor miRNA  
pri-miRNA: primary miRNA  
PRR: pattern recognition receptor  
PTEN: phosphatase and tensin binding homolog  
qRT-PCR: quantitative real-time polymerase chain reaction  
RIG-I: retinoic-inducible gene-I  
RIPA: radioimmunoprecipitation assay  
RLI: RNase-L inhibitor  
RLR: RIG-I-like receptor  
RLU: relative luciferase units  
RMS: rhabdomyosarcoma  
RNABP: RNA-binding protein  
Rpl13a: ribosomal protein L13a  
rRNA: ribosomal RNA  
Rybp: Ring and YY-1 binding protein  
SDN: small RNA degrading nuclease  
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis  
sFRP2: secreted frizzled related protein  
shRNA: short hairpin RNA  
SNP: single nucleotide polymorphism  
Sp1: specificity protein 1  
ssRNA: single-stranded RNA  
STAT: signal transducers and activators of transcription  
STING: stimulator of interferon genes  
TBS-T: Tris-buffered saline with Tween 20  
TCF: T-cell factor  
TCL1: T cell leukemia/lymphoma 1  
TDG: thymine-DNA glycosylase  
TGF- $\beta$ : transforming growth factor-beta  
TLR: Toll-like receptor  
TNF- $\alpha$ : tumor necrosis factor- $\alpha$   
TNFAIP3: tumor necrosis factor alpha-induced protein 3  
TRAIL: tumor-necrosis factor-related apoptosis-inducing ligand  
TPM1: tropomyosin alpha-1  
TRIF: TIR-domain-containing adapter-inducing interferon- $\beta$   
TRAF6: TNF receptor-associated factor 6  
TTP: tristetraprolin  
TYK2: tyrosine kinase 2  
WWOX: WW domain containing oxidoreductase  
XRN2: exoribonuclease 2  
YWHAZ: 14-3-3 zeta  
YY1: Yin-yang 1  
ZAP-70: Zeta-chain-associated protein kinase 70

# CHAPTER 1: INTRODUCTION

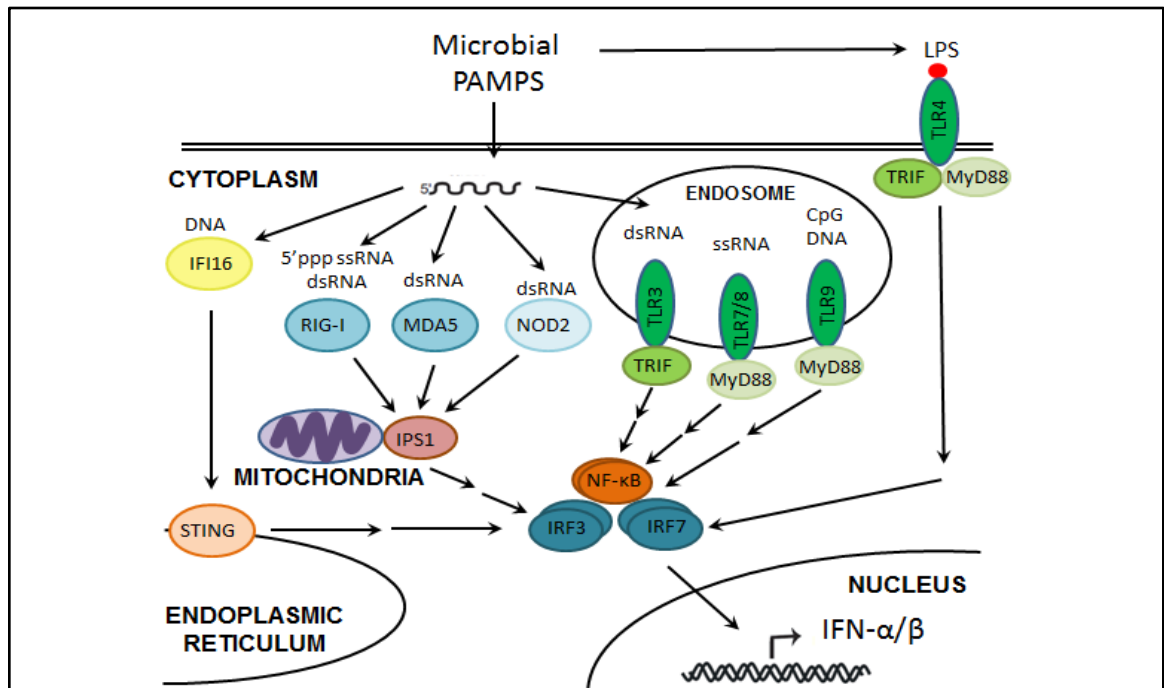
## 1.1 THE ENDORIBONUCLEASE RNASE-L

### 1.1.1 Type I Interferons

Type I interferons (IFNs) were discovered over 50 years ago as soluble factors capable of “interfering” with virus infection in cell culture (3). This family of secreted proteins was subsequently found to possess pleiotropic effects in addition to its antiviral activity, including antiproliferative/antitumor and antibacterial activities (4). IFNs are found in all jawed vertebrates (5), with humans possessing 17 subtypes to date (thirteen of IFN- $\alpha$  and one each of IFN- $\beta$ ,  $\omega$ ,  $\epsilon$ , and  $\kappa$ ) and all mammals possessing at least one IFN- $\alpha$  and one IFN- $\beta$  subtype (4). Type II and type III interferons also play important roles in antiviral immunity, but are distinct from IFNs in structure and function. The type II interferon family contains a single member, interferon- $\gamma$  (IFN- $\gamma$ ), while type III interferons are represented by three interferon- $\lambda$  (IFN- $\lambda$ ) proteins, IFN- $\lambda$ 1-3.

Production of IFN is stimulated by various viral and bacterial ligands (Fig. 1), through both membrane-bound and cytosolic pattern recognition receptors (PRR) (5). The former includes members of the Toll-like receptor (TLR) family. In particular, TLRs 3, 7/8, and 9 are alike in their endosomal location and their recognition of nucleic-acid based pathogen-associated molecular patterns (PAMPs). The surface-expressed TLR4 can also induce IFN production in response to bacterial lipopolysaccharide (LPS) and viral proteins (6). Signaling through these receptors leads to activation of interferon-regulatory factors 3 and 7 (IRF3 and IRF7) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) that initiate transcription of IFN-encoding genes. TLR-induced IFN production is particularly important in a few specialized immune cells, such

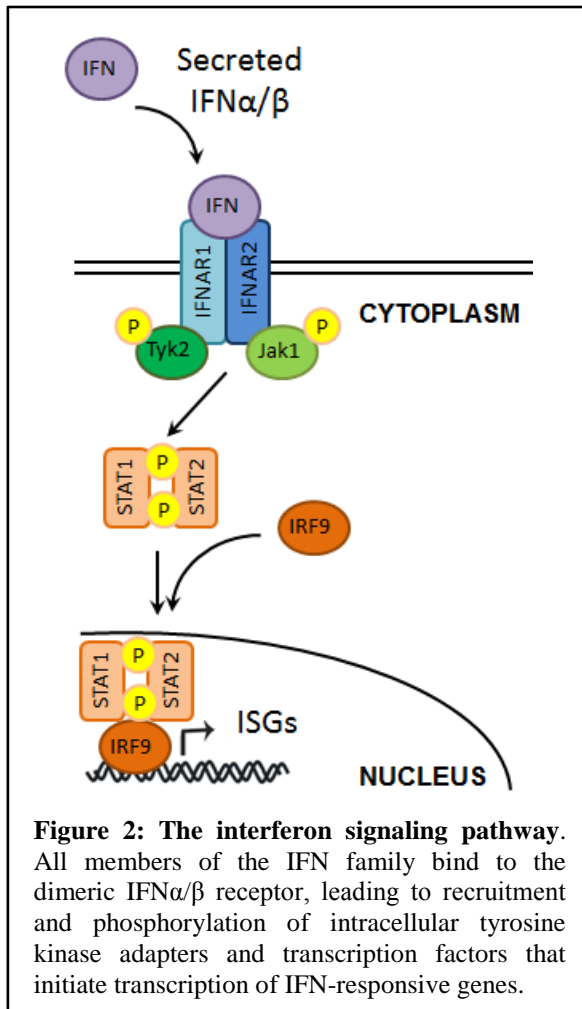
as dendritic cells (DCs), especially the plasmacytoid DCs. These cells are distinguished by high expression of TLR7, TLR9 and IRF7 and are adept at phagocytosing extracellular virus debris from virus-infected cells that can enter the endosomal pathway and trigger TLR-signaling. In contrast, almost all nucleated cells are capable of producing IFN in response to intracellular double-stranded RNA (dsRNA) via broadly expressed cytosolic receptors from the RIG-I-like receptor (RLR) family including retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-association gene 5 (MDA5) (5). More recently, PRRs from the NOD-like receptor (NLR) and AIM2-like receptor (ALR) families have also been implicated in IFN production in response to



**Figure 1: Induction of type I interferons.** IFN production is induced by various viral and bacterial PAMPs which are detected by both membrane-bound and cytosolic receptors. The former includes members of the TLR family which signal through either myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF). The latter includes members of the RLR, NLR, and ALR families. RLRs include RIG-I and MDA5. The NLR family is represented by nucleotide-binding oligomerization domain-containing protein 2 (NOD2). RLR and NLR members both signal through the mitochondrial adapter IFN- $\beta$ -promoter stimulator 1 (IPS1). The ALR family is represented by interferon, gamma-inducible protein 16 (IFI16) which signals through the endoplasmic reticulum protein stimulator of interferon genes (STING). These pathways converge on transcription factors including IRF3/7 and NF- $\kappa$ B to initiate transcription of *IFN* genes and secretion of IFN proteins. Repeated arrowheads denote intermediate signaling steps that are not shown in detail.

various intracellular microbial nucleic acids such as dsRNA and DNA (7, 8).

Secreted IFN acts in an autocrine or paracrine fashion (Fig. 2). All IFN subtypes bind and signal through a surface receptor comprised of two transmembrane proteins, IFN- $\alpha/\beta$  receptors 1 and 2 (IFNAR1 and IFNAR2) and their associated cytoplasmic kinases, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), eventually leading to recruitment and phosphorylation of two members of the signal transducers and activators of transcription (STAT) family, STAT1 and STAT2. These transcription factors dimerize and translocate to the nucleus and associate with IRF9 to activate transcription of several hundred interferon-stimulated genes (ISGs) whose products mediate the effects

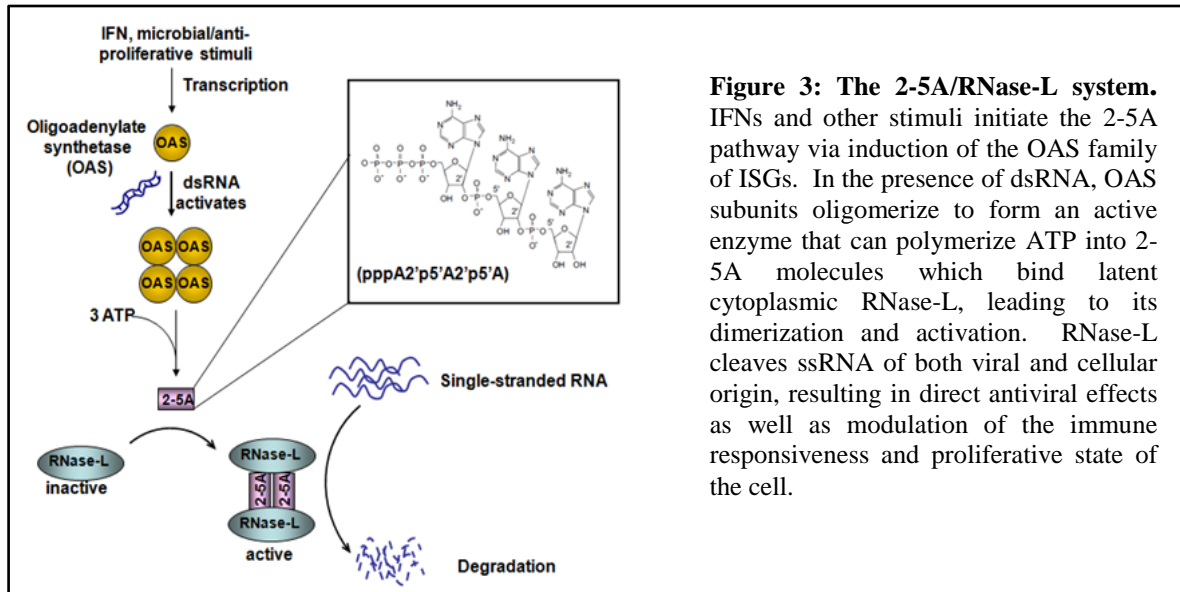


of IFN (9, 10). Though the function of many ISGs remain to be elucidated, some common features have emerged (5). For example, many ISGs have direct antiviral actions that limit viral replication or protein production. Others sensitize the cell to apoptosis (thought to be a strategy to limit viral spread if infection cannot be controlled). Finally, many PRRs are themselves ISGs, and the effect on IFN on uninfected cells is to induce an “antiviral state” where cells are primed to detect and respond to microbial stimuli. IFNs also modulate the adaptive immune system,

facilitating the cross-presentation of extracellular antigens (e.g. viral antigens within apoptotic infected parenchymal cells) by antigen-presenting cells (APCs) and enhancing the recruitment and activity of NK and cytotoxic T lymphocytes that can recognize and kill virally-infected cells.

### **1.1.2 The 2-5A/RNase-L System**

The 2'5'-oligoadenylate (2-5A)/RNase-L system is an RNA cleavage pathway that was among the first discovered mediators of IFN-induced antiviral activity (Fig. 3). The pathway is comprised of two major enzymatic components: the oligoadenylate synthetase (OAS) family of enzymes that produces the eponymous 2-5A ( $p_35'A([2'p5'A]_n, n>2)$ ) small molecules (11) as well as the endoribonuclease RNase-L which is activated by 2-5A to cleave single-stranded RNA (ssRNA) (2). The pathway is initiated by IFN or other microbial or antiproliferative stimuli which induce transcription of OAS-encoding genes. The various OAS isoforms display different subcellular localization and efficiency of 2-5A production and have been found to mediate non-redundant activities (12). OAS monomers require dsRNA for oligomerization and enzymatic activity; thus, OAS can itself function as a PRR for this class of PAMP (13). In the presence of dsRNA, such as viral replication intermediates, OAS polymerizes adenosine triphosphate (ATP) into polyadenylate molecules containing unusual 2'-5'-phosphodiester linkages. These in turn binds latent cytoplasmic RNase-L, leading to dimerization and enzymatic activation (14, 15). RNase-L selectively cleaves ssRNA targets, generating 3'-phosphorylated products and leaving substrates susceptible to further decay by cellular exonucleases. The pathway is tightly regulated by rapid



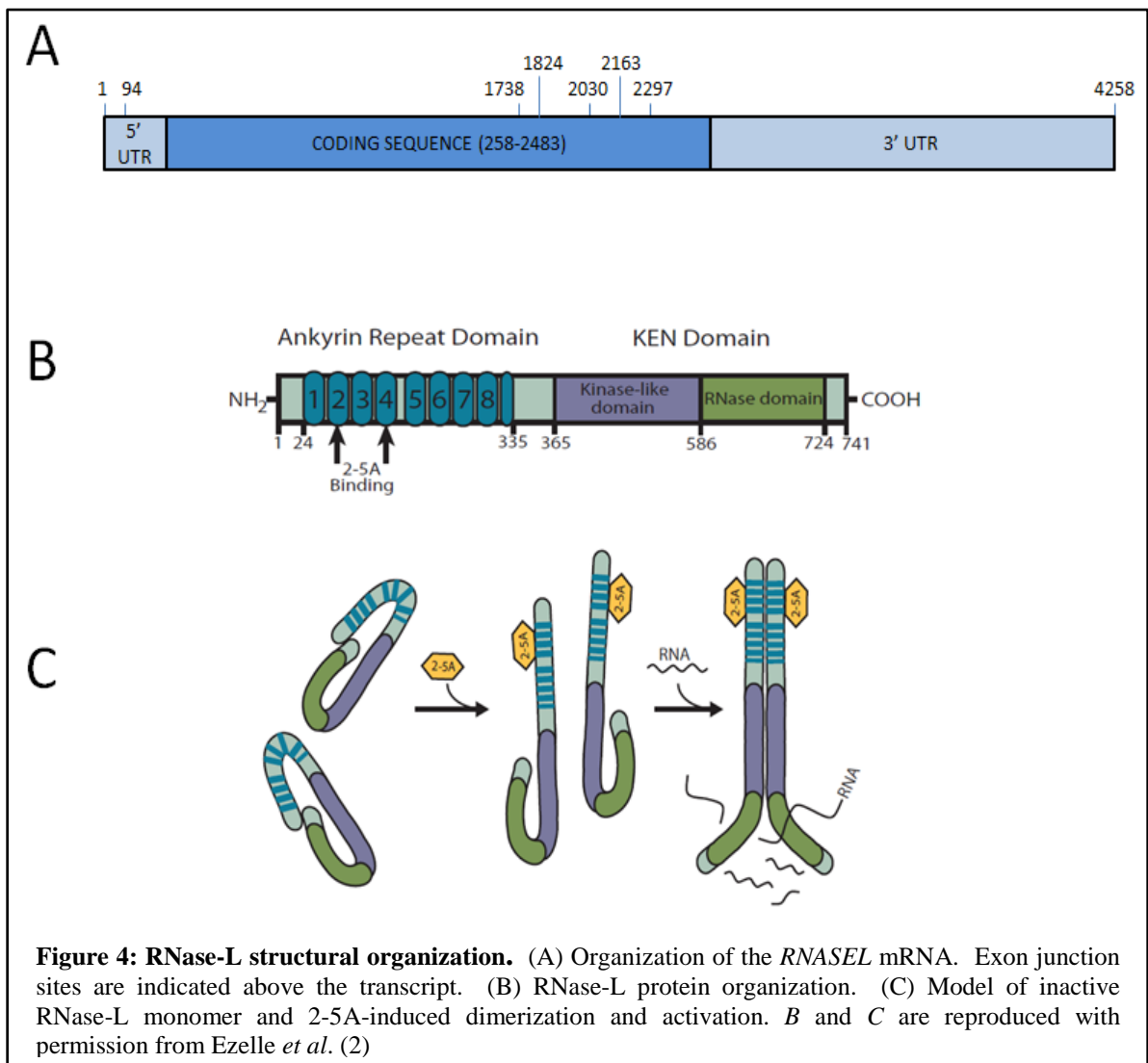
**Figure 3: The 2-5A/RNase-L system.** IFNs and other stimuli initiate the 2-5A pathway via induction of the OAS family of ISGs. In the presence of dsRNA, OAS subunits oligomerize to form an active enzyme that can polymerize ATP into 2-5A molecules which bind latent cytoplasmic RNase-L, leading to its dimerization and activation. RNase-L cleaves ssRNA of both viral and cellular origin, resulting in direct antiviral effects as well as modulation of the immune responsiveness and proliferative state of the cell.

degradation of 2-5A by nonspecific cellular phosphatases that remove the 5'-triphosphate and by a selective 2'-phosphodiesterase (16). In addition, the RNase-L inhibitor (RLI) protein is induced by certain viruses or dsRNA and directly binds to RNase-L, inhibiting its activity (17, 18).

### 1.1.3 Structure and Regulation of RNase-L Expression

The human *RNASEL* gene is located on chromosome 1q25, and is transcribed and processed into a messenger RNA (mRNA) of 4.2kb (NCBI: NM\_021133.3) containing seven exons (Fig. 4A). This mRNA has a short 5'-untranslated region (5'UTR) (257nt), a coding region of roughly 2.2kb, and a long 3'-untranslated region (3'UTR) (~1.8kb) which falls entirely within exon 7 and comprises almost half of the entire transcript. The 82 kDa RNase-L protein contains 741 amino acids, and consists of an N-terminal ankyrin repeat domain (ARD), a central kinase-like domain, and a C-terminal ribonuclease domain (Fig. 4B) (2). Collectively, the kinase-like and ribonuclease domains are referred to as the kinase-extension-nuclease (KEN) domain, which is required for RNase-L

dimerization and endonuclease activity (19-21). Both of these domains have homology with the kinase-endoribonuclease protein inositol-requiring enzyme 1 (Ire1) which functions in the endoplasmic reticulum as part of the unfolded protein response (22). However, unlike Ire1, in which both enzymatic domains are functional, there is no evidence of RNase-L kinase activity (21, 23). The RNase-L ARD contains 8 complete and 1 partial ankyrin repeats, which are 33 amino-acid motifs that typically function in protein-protein interactions and form paired antiparallel  $\alpha$ -helices connected by a  $\beta$  hairpin turn (24). Elucidation of the crystal structure of the N-terminal portion (amino



acids 1-133) of human RNase-L revealed that ankyrin repeats 2 and 4 are responsible for 2-5A-binding (25). In the absence of 2-5A, monomeric RNase-L is thought to be maintained in an inactive state through intramolecular interactions between the N-terminal ARD and the C-terminal catalytic domain (20). In this model, 2-5A binding to RNase-L induces a conformational change, dimerization, RNA binding, and ribonuclease activity (Fig. 4C) (26).

RNase-L is conserved among vertebrates, and its mRNA and protein are expressed at low levels in most human tissues (27). While significant induction of mouse RNase-L has been reported following IFN treatment (28, 29), transcription of human *RNASEL* is not markedly induced by IFN or other stimuli examined to date (27). Human *RNASEL* is, however, subject to post-transcriptional regulation, being strongly repressed by its 3'UTR in resting cells and induced in response to cell stress via binding of human antigen R (HuR) to an AU-rich element (ARE) in the 3'UTR of *RNASEL* mRNA and stabilization of the transcript (1). Post-translational regulation of RNase-L protein has also been reported, with proteasome-mediated RNase-L degradation being induced in response to phorbol-12-myristate-13-acetate (PMA) treatment in mouse fibroblasts (30). In addition, the asparaginyl hydroxylase factor inhibiting HIF1- $\alpha$  (FIH) was shown to hydroxylate RNase-L within the ankyrin repeat domain; the functional effects of this modification are unclear, but it may be responsible for regulating RNase-L protein stability in response to changes in oxygen state (31). Thus, the 2-5A/RNase-L pathway is regulated at many levels; this regulation permits both rapid activation to efficiently respond to immune and antiproliferative stimuli and rapid attenuation to prevent dysregulated RNA cleavage.

#### **1.1.4 RNase-L Cleavage Substrates and Protein Interactions**

RNase-L has been shown to selectively target specific RNAs for degradation, with the biologic effects of RNase-L endoribonuclease activity depending on the identity of its RNA substrates. No single strong consensus sequence for RNase-L recognition has been identified; however, a preference for UU and UA dinucleotides has been reported (32, 33). Microarray analyses have identified diverse RNase-L-regulated transcripts and demonstrated that RNase-L regulates a distinct set of substrates in different physiologic settings (34-36). Not all of these differentially-expressed transcripts represent direct RNase-L cleavage substrates, as modulation of some mRNAs (e.g. those encoding transcription factors) can indirectly affect many others. Indeed, RNase-L activation in prostate cancer cells has been shown to upregulate the expression of more genes than it downregulates via a c-Jun N-terminal kinase (JNK)-activated transcriptional pathway (34). Authentic cleavage substrates are expected to display not only downregulation in the presence of RNase-L but also an RNase-L-dependent decrease in RNA stability, physical association with RNase-L, and generation of detectable cleavage products. Evidence for selective RNase-L cleavage of various RNAs along with the biologic effects of this targeting is summarized in Table 1.

In addition to its endonucleolytic activity, RNase-L interaction with other proteins represents an alternative mechanism that might impact its own function as well as that of its binding partners. Table 2 lists the known protein interactors of RNase-L; however, the full functional consequences of RNase-L interaction with many of these proteins remain to be determined.

**Table 1: Candidate RNase-L Substrates**, adapted with permission from Ezelle *et al.* (2)

<b>RNASE-L-REGULATED RNA</b>	<b>EVIDENCE FOR RNASE-L CLEAVAGE</b>	<b>PROPOSED BIOLOGIC FUNCTION OF RNASE-L REGULATION</b>
Viral RNA (genomic, mRNA) (37-41)	Detection of viral RNA cleavage products following RNase-L activation	Direct antiviral effects, generation of small RNA PAMPs
Ribosomal RNA (rRNA) (42, 43)	Detection of rRNA cleavage products following RNase-L activation	Inhibition of viral protein synthesis and proliferation
mRNA encoding Protein Kinase R (PKR) (44)	Increased <i>PKR</i> mRNA stability in <i>RNASEL</i> <sup>-/-</sup> cells	Restoration of host protein synthesis following viral clearance
mRNA encoding Cathepsin E (CatE) (45)	Increased <i>CATE</i> mRNA stability in <i>RNASEL</i> <sup>-/-</sup> cells	Modulation of endolysosomal trafficking and cytokine induction
mRNAs encoding differentiation factors (46-48): a. Myogenesis differentiation protein (MyoD) b. C/EBP homologous protein (CHOP10)	Increased/decreased <i>MYOD</i> mRNA stability upon RLI/RNase-L expression  Immunoprecipitation of <i>CHOP10</i> mRNA with RNase-L and stabilization in <i>RNASEL</i> <sup>-/-</sup> cells	Inhibition of myogenesis and promotion of adipogenic differentiation
Mitochondrial RNAs (mtRNA) (49-51)	Increased mtRNA stability upon RNase-L knockdown or ectopic RLI expression	Inhibition of mitochondrial energy production and cell proliferation
mRNAs encoding ribosomal proteins (35)	Immunoprecipitation of ribosomal mRNAs with RNase-L and destabilization upon 2-5A-induced RNase-L activation	Inhibition of protein synthesis and induction of senescence
mRNA encoding Human antigen R (HuR) (36)	Increased <i>HUR</i> mRNA stability in <i>RNase-L</i> <sup>-/-</sup> cells, decreased expression of <i>HUR</i> 3'UTR reporter upon ectopic RNase-L expression	Inhibition of cell proliferation
mRNA encoding Tristetraprolin (TTP) (52)	Immunoprecipitation of <i>TTP</i> mRNA with RNase-L, destabilization upon RNase-L overexpression, and stabilization in <i>RNASEL</i> <sup>-/-</sup> cells	Inhibition of cell proliferation

**Table 2: RNase-L Protein Interactions**, adapted with permission from Ezelle *et al.* (2)

RNASE-L-BINDING PROTEIN	FUNCTION	CONSEQUENCES OF RNASE-L INTERACTION
RNase-L Inhibitor (RLI) (17)	ATP-binding cassette transporter family member, potential function in rRNA processing and/or translation	RLI forms complex with eRF3 and RNase-L, binding inhibits RNase-L interaction with 2-5A and activation
Eukaryotic release factor 3 (eRF3) (53)	Translation termination release factor, functions in ribosome recycling and nonsense-mediated decay	2-5A enhances eRF3 binding to RNase-L, RNase-L may inhibit eRF3 activity
Mitochondrial initiation factor 2 (IF2mt) (51)	Mitochondrial translation initiation factor	Active translation and RNase-L binding to IF2mt required for RNase-L degradation of mtRNAs
Androgen receptor (AR) (54)	Nuclear hormone receptor, functions in maintenance of prostate tissue and/or cancer progression	Dihydrotestosterone enhances AR binding to RNase-L, RNase-L contributes to IFN- $\gamma$ -mediated suppression of AR activity
IQ motif-containing Ras GTPase activating-like protein 1 (IQGAP1) (55)	Scaffold protein, functions in cell adhesion, migration, proliferation and differentiation	1-(3-C-ethynyl- $\beta$ -D-ribo-pentofuanosyl) cytosine (ECyd) treatment enhances IQGAP1 binding to RNase-L, binding required for ECyd-induced apoptosis

### 1.1.5 RNase-L Functions: Antimicrobial Immunity

RNase-L was originally identified as an antiviral mediator of IFN action and has been shown to have activity against many RNA and DNA viruses (56). Many viruses generate dsRNA as part of their replicative cycle, and these replicative intermediates have been shown to bind OAS and induce synthesis of 2-5A (57, 58). Inhibition or absence of RNase-L activity has been shown to compromise antiviral activity both *in vitro* and *in vivo*, with *RNASEL*<sup>-/-</sup> mice showing increased mortality in response to several

types of viral infection (18, 59-62). Conversely, many viruses have developed mechanisms for inhibiting or evading OAS or RNase-L activity. These include expression of proteins that bind dsRNA (sequestering it and preventing OAS activation) (63), induction of inhibitory OAS- and RNase-L-binding proteins (18, 64, 65), RNA secondary structures that bind RNase-L and inhibit endoribonucleolytic activity (66-68), and even mutation of the genome to reduce the frequency of RNase-L UU or UA cleavage sites (37, 38).

RNase-L has been demonstrated to directly cleave viral genomes and mRNA transcripts as a mechanism of antiviral activity (37-39). Selective regulation of host RNAs has also been shown to be important for RNase-L function. For example, inhibition of viral replication occurs through targeting of the host translational machinery, with cleavage of 18S and 28S rRNA being a hallmark of RNase-L activation (42, 43). Finally, RNase-L activation is capable of inducing cellular apoptosis (69-71), a process requiring JNK and the release of cytochrome *c* from the mitochondria (72, 73). In cases of sustained cellular infection, this mechanism is thought to be a way of limiting viral spread by elimination of infected cells (56). In addition to its direct antiviral effects, RNase-L has been increasingly shown to play an immunomodulatory role which may also contribute to its antiviral function. RNASEL<sup>-/-</sup> mice have increased numbers of thymocytes due to reduced apoptosis in general, but no overt phenotype has been associated with this condition, and generation of all other immune cell subsets appears normal in the absence of pathogens (60). Contact hypersensitivity responses were not found to be altered in these mice; however, RNase-L-deficient mice do show delayed rejection of skin allografts with class II major histocompatibility complex mismatch.

These results suggest that RNase-L may regulate CD4+ but not CD8+-mediated immune proinflammatory functions (74). RNase-L has recently been shown to contribute to the induction of IFN by generating small RNA cleavage fragments that activate the cytoplasmic PRR retinoic-inducible gene-I (RIG-I) to amplify IFN production (40, 41). Microarray studies have also shown that RNase-L regulates production of established immune mediators (e.g. chemokines, cytokines, ISGs) (34, 35, 45). While the mechanistic details of RNase-L-dependent regulation remain to be determined for most of them, the recent discovery that RNase-L binds to and regulates the mRNA encoding the RNA-binding protein (RNABP) tristetraprolin (TTP) (52) provides a novel mechanism for RNase-L regulation of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (BAH, unpublished). TNF- $\alpha$  is regulated not only by transcriptional induction, but also by control of mRNA stability via TTP binding to an ARE in its 3'UTR (75). RNase-L regulation of TTP makes it part of a network that regulates TNF- $\alpha$  and other ARE-containing mRNAs.

These proinflammatory functions are consistent with the recent finding that RNase-L plays a role in antibacterial immunity. *RNASEL*<sup>-/-</sup> mice display increased mortality following both *Escherichia coli* and *Bacillus anthracis* infection. This mortality is associated with compromised induction of proinflammatory cytokines, altered recruitment of immune cells and disrupted clearance of internalized bacteria in macrophages (45). The mRNA encoding CatE, an endolysosomal cathepsin protease that promotes pathogen targeting to the lysosome, was identified as an RNase-L substrate in macrophages. The regulation of CatE expression may be one of the mechanisms by which RNase-L enhances bacterial clearance.

### 1.1.6 RNase-L Functions: Differentiation, Senescence, and Tumorigenesis

Independent of its antiviral function, the 2-5A/RNase-L pathway has also been implicated in several of the antiproliferative and antitumor effects of IFN. Both OAS and RNase-L are elevated in conditions where cell proliferation is decreased, such as confluence-associated growth arrest (29, 76) and differentiation (77). RNase-L-deficient cells and tissues exhibit a defect in apoptosis in response to apoptotic stimuli (59, 60, 78), while overexpression or activation of RNase-L has been shown to induce apoptosis, differentiation, or senescence depending on the cell type (70, 79, 80). These processes are all thought to be important for tumor suppression *in vivo*, and indeed, exogenous RNase-L expression impairs tumor growth in a xenograft model of mouse rhabdomyosarcoma (81). Consistent with the association between cellular senescence and organismal aging, *RNASEL*<sup>-/-</sup> mice exhibit an increased lifespan (32% longer) compared to their wild-type counterparts (79).

A link between RNase-L and human cancers was established by the mapping of human *RNASEL* to the Hereditary Prostate Cancer-1 (HPC1) locus on chromosome 1q25 (82). Several germline mutations were identified in prostate cancer samples that reduce RNase-L activity and are associated with an increased risk of either hereditary or sporadic prostate cancer in some, though not all, populations examined (83-87). A later study extended the association of *RNASEL* mutations to pancreatic cancer (88). In addition, a single nucleotide polymorphism (SNP) in the *RNASEL* 5'UTR was shown to be associated with an increased risk of head and neck, cervical, and breast cancers (89); however, the effect of this SNP on RNase-L expression and function remains to be determined.

Of note, the initial reports associating RNase-L with prostate cancer were followed by studies examining a potential link between the antiviral and antitumor effects of RNase-L. A controversial paper reported the discovery a novel retrovirus known as xenotropic murine leukemia virus-related virus (XMRV) that was detected at high frequency in the tissue of prostate cancer patients homozygous for the RNase-L R462Q mutation that exhibits 3-fold reduced enzymatic activity (90). Protection against XMRV was shown to be RNase-L-dependent, and it was proposed that integration of XMRV into prostate cell genomes had the potential to disrupt the expression of cancer-associated genes and promote tumorigenesis (91). However, many subsequent reports failed to recapitulate the association between XMRV and RNase-L and suggested that XMRV may in fact be a laboratory contaminant (92, 93). Mounting evidence has suggested that XMRV actually represents a recombinant retrovirus derived from various endogenous murine retroviruses that arose during the passage of human prostate cancer cell lines as mouse xenografts, rather than a clinically significant human pathogen (94). These findings, while once again leaving unknown the mechanisms of tumor suppression by RNase-L in prostate cancer, do highlight the possibility that RNase-L protection against oncogenic viruses may be one of the mechanisms of RNase-L antitumor activity in general.

Although the majority of studies are consistent with a tumor suppressor role for RNase-L (i.e. loss of RNase-L expression increases the risk of malignancy), there have been reports of RNase-L upregulation in some cancers. For example, RNase-L is elevated in colorectal adenocarcinomas and noncancerous polyps as compared to normal mucosa in familial adenomatous polyposis patients, thus representing a potential early contributory event in colorectal tumorigenesis (95). Elevated RNase-L has also been observed in peripheral blood mononuclear cells (PBMC) from chronic myelogenous

leukemia (CML) patients (96), and we present novel evidence here that RNase-L promotes tumorigenesis in CML cells.

The oncogenic and tumor suppressor functions of RNase-L both depend on its regulation of cellular mRNA targets. Many identified targets of RNase-L are thought to contribute to its tumor suppressor function (Table 1). For example, RNase-L degradation of rRNAs, mRNAs, and mtRNAs may inhibit tumorigenesis in general by impairing protein biosynthesis and energy production, both of which are required by rapidly-proliferating cancer cells. RNase-L interaction with other proteins may also play a role in its tumor suppressor function (Table 2), such as RNase-L binding of AR and inhibition of androgen signaling in prostate cancer. However, the RNase-L targets that contribute to oncogenic function remain to be determined. Because the effects of RNase-L are likely to depend on the specific profile of targets in each cell type, additional research is necessary to determine the mechanisms by which RNase-L promotes malignancy as well as the specific cancers in which it plays an oncogenic role.

### **1.1.7 Other Functions of RNase-L**

Chronic fatigue syndrome (CFS) is a debilitating, long term disorder of unknown etiology causing extreme fatigue, short-term memory loss, musculoskeletal pain, impaired sleep, tender lymph nodes, low-grade fever, and headache in the absence of other medical conditions which may explain these symptoms (97). Immune dysregulation leading to chronic viral infection has often been proposed to be a cause of CFS, as patients frequently display signs of chronic immune activation, such as elevated CD8+ T cell counts, diminished NK cell function, and altered cytokine expression.

Significantly, PBMCs from CFS patients exhibit dysregulation of the OAS/RNase-L pathway, including upregulation of OAS and RNase-L, downregulation of RLI, and a 3-fold increase in RNA cleavage activity (98, 99). Of note, unique 30kDa and 37kDa RNase-L fragments have been observed in these cells from CFS patients compared to healthy controls (100). These truncated proteins are generated through cleavage of full-length RNase-L by cellular proteases (101), and it is believed that the absence of intermolecular interaction and repression of RNase-L activity leads to both increased RNA cleavage and possibly dysregulation of target recognition that contributes to CFS pathology (102).

Many studies have attempted to identify microbial agents responsible for CFS. Putative causative infections include human herpes virus-6, Epstein-Barr Virus (EBV), and *Mycoplasma* spp. but no clear etiologic role has been demonstrated for any of these (98, 103-105). The discovery of XMRV in prostate cancer patients with germline RNase-L mutations led to a great deal of excitement among researchers, especially when it was found that the prevalence of this virus was also significantly elevated in CFS patients compared to healthy controls (106). However, not all subsequent studies have recapitulated these results (107-109), and the eventual research showing that XMRV is likely a laboratory contaminant derived from endogenous murine leukemia viruses makes XMRV an improbable etiologic agent of CFS. At this time, the precise relationship between RNase-L and CFS remains undetermined, and further research is necessary to clarify the role of RNase-L in CFS pathogenesis.

## **1.2 THE MICRORNA-29 FAMILY**

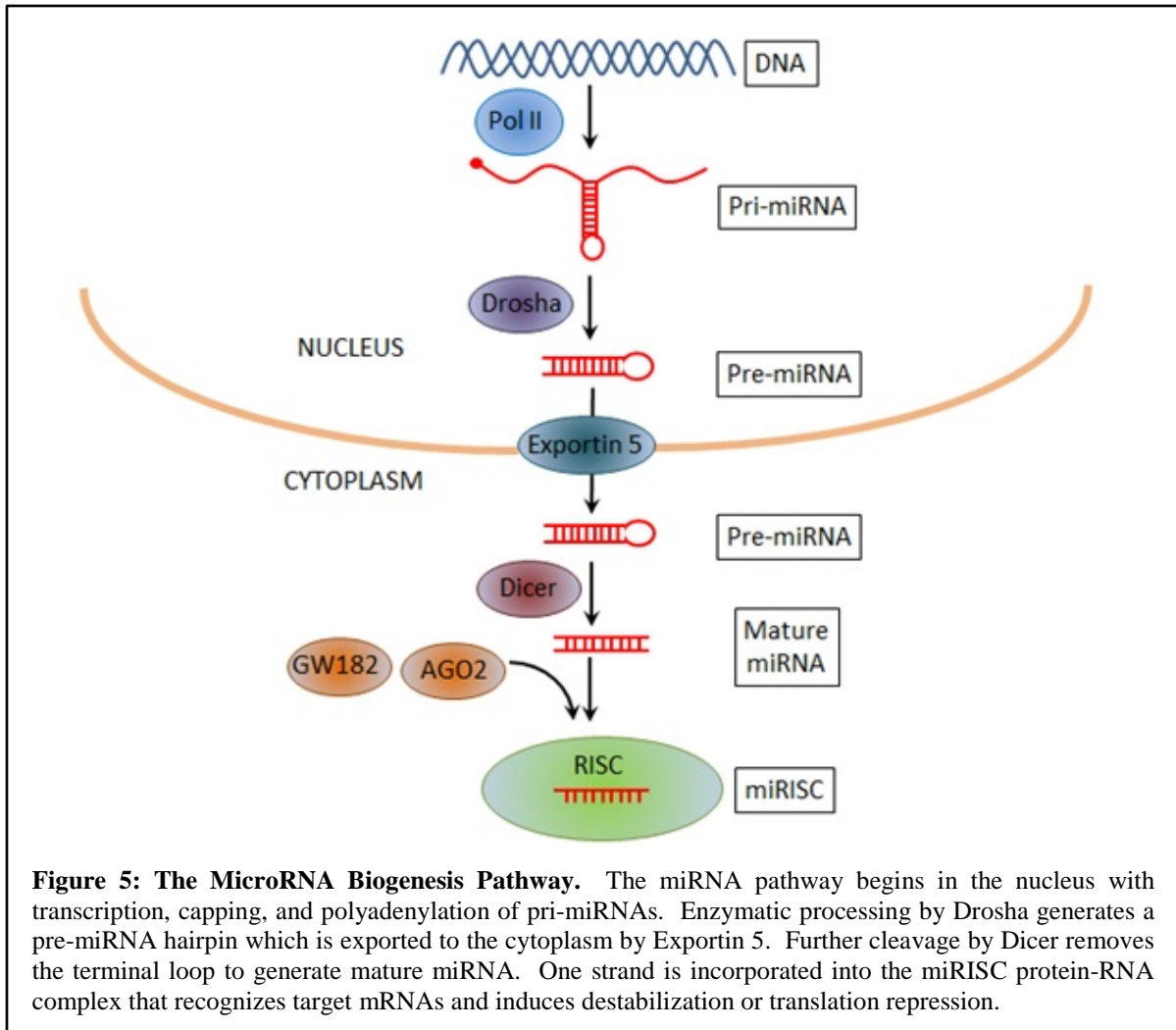
### **1.2.1 MicroRNA Biogenesis and Function**

MicroRNAs (miRNAs) are a class of small regulatory RNAs originally discovered in *C. elegans* (110, 111) which are capable of modulating protein expression based on antisense complementarity to mRNA transcripts (112-114). They have since been discovered in many eukaryotic organisms (including metazoans, plants, and green algae) as well as viruses and are estimated to regulate roughly 50% of human protein coding genes (115). As of its November 2011 release (version 18), miRBase.org reports 1,921 mature miRNAs in humans, a number that may still increase as more miRNAs are discovered (116). MiRNA regulation has been shown to be important in virtually every biologic process, from development to malignancy (117). The complementarity-based binding of miRNAs to their mRNA targets means that a single miRNA can regulate multiple targets and a single mRNA may be regulated by multiple miRNAs. This system also allows for effective fine-tuning of gene expression in distinct biologic settings. MiRNAs as a group are thought to carry out many important cellular functions, such as silencing of genes until the appropriate developmental stage, maintaining gene expression within a beneficial physiologic range, rapid reduction of gene expression after transcriptional shutoff, and cooperating with RNA-binding proteins in post-transcriptional networks that can rapidly and coordinately regulate multiple components of the same pathway (112, 118, 119).

miRNAs are part of a larger phenomenon known as RNA silencing, which includes many other noncoding RNAs (ncRNAs) of various origins. While these ncRNAs utilize many of the same processing and effector components, miRNAs are

distinguished by the unique ~70nt hairpin structure of their precursor products (112). Human miRNA biogenesis (Fig. 5) begins with RNA polymerase II transcription of the primary miRNA (pri-miRNA) in the nucleus. These can be either non-coding or protein-coding RNAs; in the latter, miRNA hairpins are usually found in introns or non-coding regions. In some cases, clusters of more than one miRNA are found on the same transcript. Precursor miRNA (pre-miRNA) hairpins of ~70nt are excised from the pri-miRNA by the RNase III family enzyme Drosha and exported to the cytoplasm. There, a second RNase III type enzyme known as Dicer removes the terminal loop, leaving a 20-22 nucleotide duplex. One strand of the duplex is preferentially loaded onto an assembly of proteins, forming what is known as the miRNA-induced silencing complex (miRISC) (112), and recruits the entire complex to complementary target mRNAs to either induce mRNA destabilization or translation repression (113).

The miRISC-mediated mRNA destabilization and translation repression pathways are not mutually exclusive. The effects of miRISC recruitment to the mRNA most likely depend on many factors, including identity of the miRNA or mRNA or varying composition of the miRISC itself. Not all of the proteins that make up the miRISC are known. However, key components that have been identified include the Argonaute (AGO) family (AGO1-4 in humans), and the glycine-tryptophan 182kDa (GW182) protein family. AGO2, in particular, is unique for its endoribonuclease activity which cleaves mRNAs that display perfect complementarity to the miRNA at nt 9-11 (112). This mRNA cleavage mechanism, while common in plants, does not appear to be the most important miRISC function in animals. Instead, miRISC recruitment more frequently appears to lead to mRNA destabilization via GW182 binding of poly-A-



**Figure 5: The MicroRNA Biogenesis Pathway.** The miRNA pathway begins in the nucleus with transcription, capping, and polyadenylation of pri-miRNAs. Enzymatic processing by Drosha generates a pre-miRNA hairpin which is exported to the cytoplasm by Exportin 5. Further cleavage by Dicer removes the terminal loop to generate mature miRNA. One strand is incorporated into the miRISC protein-RNA complex that recognizes target mRNAs and induces destabilization or translation repression.

binding protein (PABP) and mRNA deadenylases or repression of mRNA translation by all AGO isoforms through as-yet unconfirmed mechanisms (113). AGO, GW182, mature miRNAs, and repressed mRNAs are all enriched in processing bodies (P-bodies, also known as GW-bodies), cytoplasmic inclusions which are postulated to function as depots for mRNA storage and decay (113). Supporting this hypothesis is the fact that P-bodies are also enriched for mRNA processing enzymes that function in translational repression, deadenylation, decapping, and degradation. In addition, inhibition of miRNA biogenesis leads to disappearance of P-bodies. However, visible P-bodies are not necessary for

miRNA function, raising the possibility that these structures may be more a consequence rather than a cause of miRNA-mediated repression.

Regulation of miRNA processing has been demonstrated at nearly every step, starting from the transcription of the pri-miRNA. Various binding cofactors can change the efficiency of Drosha and Dicer activity, and even influence their preference for certain miRNAs (113). miRNAs themselves can be bound by cofactors that influence their processing and can even be directly edited after excision. For example, RNA editing enzymes can generate nucleotide changes in miRNAs that affect their target specificity, while 3'-polyadenylation and polyuridylation can stabilize and destabilize a miRNA, respectively (113). Modulation of miRISC components is another regulatory mechanism, with reports of AGO2 activity or localization being affected by cofactor binding as well as modifications such as hydroxylation or polyubiquitination. MiRNA incorporation into the miRISC may also be regulated. Generally, the identity of the incorporated miRNA appears to be determined by thermodynamics, with the strand possessing higher 5' stability becoming the mature miRNA and the other strand (known as the \* strand) being ejected. However, some duplexes are known to generate substantial amounts of mature miRNA from both strands or to preferentially produce mature miRNAs from different strands depending upon cellular conditions (113). The factors that regulate miRNA degradation are still under investigation, though studies in other organisms have implicated various exonucleases such as the 3'-5' small RNA degrading nucleases (SDN1-3) of *Arabidopsis thaliana*) and the 5'-3' exoribonuclease 2 (XRN2) of *C. elegans* (113). Experiments examining miRNA decay following either RNA polymerase II inhibition or depletion of miRNA processing enzymes suggest that

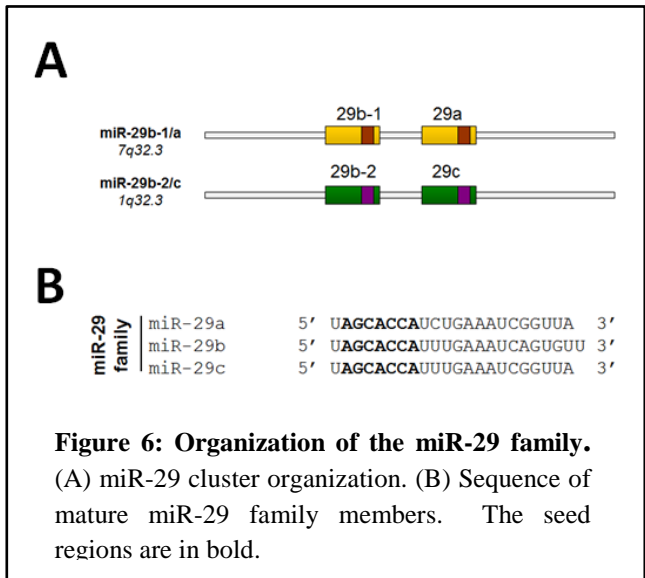
the majority of miRNAs are highly stable (with half-lives of days), but there are notable exceptions. For example, miRNAs localized to the retina and to dendritic spines undergo rapid decay (half-life of approximately 1 hour), suggesting miRNA-specific, cell-specific, or even compartment-specific factors that may also influence decay (113).

The elements that determine target recognition by miRNAs have been extensively studied, leading to the development of many target prediction tools and algorithms (112). The most important element in miRNA-mRNA binding is complementarity at the 5' end of the miRNA. This 6-8nt region of the miRNA is known as the seed region and is the basis for miRNA classification into families (113). Most algorithms use some measurement of seed-pairing efficiency as a primary criterion for miRNA binding site identification, with conservation of the site across species as another common condition (112). Though functional target sites have been described in 5'UTRs and open reading frames (ORFs), repression appears to be more frequent and effective via 3'UTR target sites. For ORFs, potential causes of decreased targeting efficiency include masking of miRISC binding sites or active displacement of miRISC by translating ribosomes (112). Additional elements, both *cis* and *trans*, can also influence miRNA target recognition. Complementarity at the miRNA 3' end can compensate for an imperfect seed match. In addition, mRNA-specific factors can influence target site efficiency. Factors such as target site distance at least 15 nucleotides from a stop codon, position away from the center of a long 3'UTR, close proximity to another miRNA binding site, and an AU-rich nucleotide context have all been reported to improve miRNA repression efficiency (112). Because of all these potential variables, experimental validation through mutational analysis of putative mRNA target sites or demonstration of direct binding (e.g. CLIP,

cross-linking and immunoprecipitation) is always required to verify predicted miRNA targeting of a given mRNA.

### 1.2.2 The MicroRNA-29 Family: Organization, Expression, and Regulation

The miR-29 family is composed of three members whose mature sequences are broadly conserved among vertebrates (115) and whose genomic organization and regulation has been thoroughly reviewed by Kriegel and colleagues (120). MiR-29 is expressed from two clusters in humans, (Fig. 6), the first on chromosome 7q32.3 containing miR-29a and one copy of miR-29b (miR-29b-1) and the second on chromosome 1q32.3 containing miR-29c and a second copy of miR-29b (miR-29b-2). According to one miRNA profiling study, miR-29a



and -29b are highly and consistently expressed across a panel of 40 normal tissue samples (representative of brain, muscle, circulatory, respiratory, lymphoid, gastrointestinal, urinary, reproductive, and endocrine systems). The authors suggest that miR-29 represents a “universal miRNA” and may therefore play an important role in metabolic functions that are common to all cells (121).

Analysis of the miR-29 promoters have demonstrated that expression of miR-29 is regulated by a variety of transcriptional elements, some of which differ between the two miR-29 clusters. For example, Wnt signaling induces transcription from the miR-

29b-1/a promoter during osteoblast differentiation. This induction occurs via transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, and mutation of two predicted TCF/LEF binding sites in the miR-29b-1/a promoter abolishes Wnt-mediated miR-29 transcription (122). The granulopoietic transcription factor CCAAT/enhancer-binding protein alpha (CEBPA) was also found to enhance transcription of the miR29b-1/a cluster; chromatin immunoprecipitation (ChIP) demonstrated CEBPA binding to the miR-29b-1/a promoter, while mutational analysis verified the involvement of two binding sites at +15 and +29 in CEBPA-induced miR-29 expression (123). ChIP was also used to demonstrate binding of the activator protein 1 (AP-1) transcription factor to the miR-29b-1/a (but not the miR29-b2/c) promoter and transcriptional activation (124). Activation of farnesoid X receptor (FXR) increased activity of the miR-29b-1/a cluster and a potential FXR-responsive element was identified though not verified in this promoter (125). Additionally, the latent membrane protein 1 (LMP1) encoded by EBV upregulates transcription of the miR-29b-1/a promoter through its C-terminus activation regions 1 and 2 (CTAR1 and CTAR2). At the miR-29b-2/c promoter, the MyoD transcription factor was confirmed by ChIP to associate with four different binding sites (-4929, -4892, -2699, -101) and induce miR-29 transcription (126, 127). Finally, although the responsible transcription factors have not been identified, both IFN- $\alpha$  treatment (128) and pre-natal and post-natal estradiol exposure (129, 130) are stimuli that have been demonstrated to induce miR-29 expression.

Repression of miR-29 expression is also controlled by many factors. Myc-binding sites in the promoters of both miR-29 clusters were identified by ChIP, and Myc

represses expression of miR-29 (131-133). A single Gli binding site at position -424 was identified in the miR-29b-1/a promoter and verified through gel-shift experiments in cholangiocarcinoma cells; induction of Gli via Hedgehog signaling represses miR-29b-1/a promoter activity (132). In the same system, gel-shift experiments identified three NF- $\kappa$ B binding sites (-561, -110, +134) in the miR-29b-1/a promoter. NF- $\kappa$ B binding to the mouse miR-29b-1/a promoter was confirmed by ChIP and mutational analyses in mouse T cells (134), and NF- $\kappa$ B activation by several different TLR ligands leads to inhibition of miR-29b-1/a promoter activity (132, 134). NF- $\kappa$ B binding to the miR-29b-1/a promoter was also reported in acute myeloid leukemia (AML) cells (135). Interestingly, this study also identified putative binding sites for specificity protein (Sp1) transcription factor in the same promoter region, and Sp1 was shown to enhance miR-29 repression in this system by forming a complex with NF- $\kappa$ B and histone deacetylases (HDACs) 1 and 3 on the miR-29b-1/a promoter. One group did report NF- $\kappa$ B-dependent upregulation of miR-29a following 8 hours of LPS stimulation in the human cholangiocyte cell line H69, but no direct binding of NF- $\kappa$ B to the miR-29b-1/a promoter was demonstrated in this system (136). In myoblasts, NF- $\kappa$ B signaling was also found to repress expression of the miR-29b-2/c cluster, although repression at this promoter occurs indirectly via upregulation of the transcription factor Yin-yang 1 (YY1). ChIP experiments demonstrated that YY1 binds the miR-29b-2/c cluster and represses expression at four binding sites (-4921, -3641, -1873, +2294) (126, 127). The transforming growth factor- $\beta$  (TGF- $\beta$ ) family of cytokines has been shown to repress miR-29 expression in numerous tissues and cell types (137-149). TGF- $\beta$  signaling activates Smad transcription factors, and Smad3 binding to the miR-29b-2/c promoter

was demonstrated by CHIP at both proximal (-4849, -2741, -692) and distal (-22kb) sites (127, 145). Finally, both platelet-derived growth factor (PDGF)-B and interleukin-4 (IL-4) signaling were shown to decrease miR-29 levels in fibroblasts (137).

In addition to the differential transcriptional regulation of the two miR-29 clusters, there is evidence that post-transcriptional mechanisms can influence the abundance of individual family members, even those expressed from the same cluster. In an analysis of miR-29 isoform expression in a panel of leukemia cell lines, the relative expression of all miR-29 family members tended to correlate across cell lines (i.e. cell lines that expressed the highest levels of one isoform also tended to express the highest levels of the other isoforms), indicating some degree of coordinate regulation. However, the absolute level of each miRNA did not reflect the genomic copy number, as might be expected if all miRNAs were processed equally following transcription. Instead, miR-29a was consistently more highly expressed than miR-29b or miR-29c (150). A similar observation was reported in HeLa cells, in which miR-29a was strongly and constitutively expressed, miR-29c was undetectable both at the primary and mature miRNA level (suggesting an absence of transcription of the miR-29b-2/c cluster), and miR-29b was highly expressed only during mitosis. The fluctuations in miR-29b levels were associated with changes in its stability, with miR-29b half-life comparable to that of miR-29a during mitosis but reduced more than 3-fold during the rest of the cell cycle (151). The cellular factors that contribute to miR-29b lability are not known, but mutational analysis in HeLa cells demonstrated that the uracil at position 10 of miR-29b itself is necessary for the accelerated decay of miR-29b during non-mitotic phases (151, 152). Significantly, miR-29c also has a uracil at position 10 as well as a shorter half-life, while

the much more stable miR-29a has a cytosine in this location. The uracils at positions 9 and 11 also enhance decay in the presence of uracil 10. Interestingly, examination of the miRNA-nome of several model species (*C. elegans*, *D. rerio*, *D. melanogaster*) revealed that miRNAs with uracils at positions 9-11 were much more likely to be downregulated during development compared to the miRNAs lacking this motif, suggesting that such uracil-mediated decay may be a common evolutionary mechanism (152).

Besides its distinctive expression profile, miR-29b is unique within the miR-29 family for its predominantly nuclear localization. One research group has reported that nuclear transport of miR-29b is stimulated by a unique hexanucleotide nuclear localization sequence at its 3' end (151). Another study of the nuclear and cytoplasmic distribution of various miRNAs in the nasopharyngeal carcinoma cell line 5-8F also found significant nuclear enrichment of miR-29b as well as miR-29c to a lesser extent. However, in this system, the previously identified miR-29b localization sequence did not appear to be necessary or sufficient for nuclear import of other miRNAs, suggesting that other features of miR-29b may also influence its intracellular distribution (153).

### **1.2.3 MicroRNA-29 Functions: Regulation of the Extracellular Matrix**

MiR-29 regulation of the extracellular matrix (ECM) exemplifies the principle that miRNAs often play a role in coordinating regulation of multiple components of a pathway or system. MiR-29 family members have been shown to directly target numerous mRNAs encoding ECM components. These include several collagen isoforms, integrins, laminins, fibrillin, elastin, matrix-metalloproteinase (MMP)-2, and disintegrin and metalloproteinase domain-containing protein 12 (ADAM12) (125, 128, 137, 139,

141, 142, 154-160). MiR-29 members also target regulators of collagen production such as the phosphatidylinositol 3-kinase p85 regulator subunit (PI3Kp85 $\alpha$ ) and the Sp1 transcription factor (157).

MiR-29 regulation of ECM components is important during physiologic processes such as exercise-induced tissue remodeling and embryonic development. For example, during aerobic exercise in rats, miR-29c induction is associated with beneficial cardiac remodeling effects such as decreased collagen expression and improvement of left ventricular compliance (161). In another study, robust miR-29 upregulation was observed during murine aortic development and was associated with direct targeting of elastin-encoding mRNA as well as downregulation of other ECM gene products (160). Dysregulation of this process in either direction can easily alter the integrity of connective tissue and lead to pathologic consequences, as evidenced by reports of both high and low miR-29 levels associated with aortic aneurysm and dissection in patient samples and mouse models (162-166). In each case, restoration of miR-29 towards normal levels either through repression or overexpression is protective against aneurysm formation and rupture.

Abnormal expression of miR-29 family members has also been strongly implicated in fibrosis. Increased expression of ECM genes is associated with downregulation of miR-29 in many fibrotic diseases and experimental models of fibrosis, including cardiac fibrosis following myocardial infarction (154), fibrosis of the trabecular meshwork of the eye (146, 147, 159), renal fibrosis (142, 145, 155, 158), pulmonary fibrosis (139, 143, 167), hepatic fibrosis (138, 140, 168-170), scleroderma (171), and systemic sclerosis (137).

TGF- $\beta$  is known to be an important pro-fibrotic factor that can directly stimulate transcription of many ECM genes (172); multiple studies show that it can also indirectly increase production of ECM components in multiple tissues and cell types by inducing Smad transcriptional repressors that downregulate miR-29 expression (137-147). Evidence from trabecular meshwork cells and lung cells shows that miR-29 in turn indirectly inhibits fibrotic stimuli such as TGF- $\beta$  and connective tissue growth factor (CTGF) in a negative feedback loop (143, 147). In hepatic stellate cells (HSCs), miR-29 has been shown to directly target mRNAs encoding profibrogenic growth factors such as PDGF-C and insulin-like growth factor-I (IGF-I) (170). As might be expected, many anti-fibrotic factors and clinical treatments have been shown to increase miR-29 levels (125, 128, 130, 137, 141, 142). Finally, direct *in vivo* miR-29 gene transfer to lung and kidney tissue inhibited the fibrotic process in animal models of bleomycin-induced pulmonary fibrosis and TGF- $\beta$ -induced renal fibrosis, respectively (143, 145).

#### **1.2.4 MicroRNA-29 Functions: Quiescence, Senescence, and Differentiation**

Quiescence is a state outside of the cell cycle (denoted by G<sub>0</sub>) in which cells are neither dividing nor preparing to divide. It can act as an intrinsic barrier to tumorigenesis, and evidence exists that it may be regulated by miR-29. In cultured HSCs, activation from the quiescent state is associated with miR-29 downregulation, while miR-29 overexpression in turn is able to inhibit HSC activation and maintain quiescence (140, 169).

Senescence is the process by which normal cells lose their ability to divide over time (173). Like quiescence, it is a mechanism that limits uncontrolled cell proliferation

and acts as a barrier to oncogenesis. MiR-29 is reported to promote both processes. Upregulation of miR-29 is reported in models of both replicative and induced senescence (174-176). In particular, miR-29 was shown to be upregulated in an Rb-dependent manner in a model of bovine papillomavirus E2 protein-induced senescence in HeLa cells. In this system, miR-29 directly targets the mRNA encoding the B-Myb oncogene, leading to inhibition of DNA synthesis (176). Interestingly, miR-29 may also play a role in senescence at the organismal level. MiR-29 expression in muscle increases significantly during normal aging and is also high in a mouse model of Hutchinson-Gilford Progeria Syndrome, a disease associated with accelerated aging (177). In this system, miR-29 expression was found to be triggered by DNA damage in a p53-dependent manner. The authors identified the mRNAs encoding nuclear prelaminin A recognition factor (Narf) and protein phosphatase Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent 1D (Ppm1d) as direct miR-29 targets. Ppm1d is a known regulator of p53, and miR-29 repression of Ppm1d expression correlates with p53 phosphorylation and stabilization, further enhancing expression of miR-29.

Finally, miR-29 has been reported to regulate differentiation in several tissue types. Like quiescence, and senescence, the development of pluripotent progenitor cells into terminally differentiated tissues serves as an obstacle to tumorigenesis. The general importance of miR-29 in promoting cell specialization was demonstrated in a model of mouse embryonic fibroblast (MEF)-derived induced pluripotent stem cells. MEFs are normally enriched in miR-29a, but depletion of this miRNA enhances reprogramming efficiency and increased pluripotency (178).

More specifically, miR-29 is required for osteoblast differentiation and is induced in response to Wnt signaling during differentiation of mesenchymal precursor cell lines as well as primary human osteoblasts (122, 179). MiR-29 functions in osteogenesis by binding and down-regulating mRNAs encoding known inhibitors of osteoblast differentiation, including HDAC4, TGF- $\beta$ 3, activin A receptor type II a (ACVR2A), beta-catenin-interacting protein 1 (CTNNBIP1), and dual specificity protein phosphatase 2 (DUSP2) (179). At the same time, miR-29 directly targets mRNAs encoding negative regulators of Wnt signaling such as Dickkopf-1 (DKK1), kringle containing transmembrane protein 2 (Kremen2), and secreted frizzle related protein (sFRP2), forming a feed-forward loop that promotes osteogenesis (122). Finally, the mRNA encoding the bone matrix protein osteonectin was found to be a direct target of miR-29; decreased expression of osteonectin and other ECM proteins was demonstrated in osteoblasts and is thought to be important to prevent premature matrix maturation and mineralization during the early stages of osteogenesis (179, 180).

MiR-29 also plays a role in muscle development. MiR-29 members are able to promote differentiation of myoblast primary cells and cell lines into myotubes (126, 181). This activity is associated with direct repression of several mRNAs encoding known inhibitors of myogenesis such as YY1, HDAC4, and Ring and YY-1 binding protein (Rybp) (126, 148, 182). Interestingly, YY1 and Rybp are responsible for binding to the miR-29b-2/c promoter and inhibiting transcription in undifferentiated myoblasts; upregulation of miR-29 during myogenic differentiation thus represents a negative feedback loop promoting myoblast differentiation (126, 182). Simultaneously, miR-29 appears to suppress differentiation towards a myofibroblast-like phenotype due to its

repression of mRNAs encoding ECM-related targets such as the LIM and senescent cell antigen-like containing domain protein (Lims1) which is involved in integrin-mediated cell adhesion (127). As in other organs, TGF- $\beta$ /Smad signaling represses miR-29 expression, inhibiting normal myogenesis and instead skewing myoblast development towards the more myofibroblast-like phenotype (127, 148, 149). Smad3 was shown to directly bind miR-29 promoter sequences, resulting in decreased binding of the MyoD transcription enhancer and increased association with the transcription inhibitor YY1 (127). Also as in other tissues, miR-29 appears to indirectly inhibit Smad3 induction in a negative feedback loop (148). Clinically, miR-29 downregulation is observed in muscle wasting that occurs in chronic kidney disease; this downregulation is due to overexpression of the miR-29 transcription inhibiting factor YY1 (181). MiR-29 is also decreased in a mouse model of Duchenne's muscular dystrophy (DMD), which is characterized by muscle wasting and replacement with fat and fibrotic tissue. In this system, direct intramuscular injection of miR-29 was reported to promote muscle regeneration, inhibit fibrinogenesis, and improve pathology (149).

MiR-29 is reported to play a role in regulating hematopoiesis, albeit a more variable one. In some cases, miR-29 is a promoter of differentiation. For example, in both CD34(+) hematopoietic stem/progenitor cells (HSPCs) and in various leukemia cell lines, miR-29 is upregulated during granulocytic differentiation induced by all-trans retinoic acid (ATRA) or monocytic differentiation induced by PMA. MiR-29 was found to enhance both programs (183). Interestingly, the pertinent targets of miR-29 differed between the two programs, with repression of the mRNA encoding cyclin T2 being necessary for monocytic differentiation and repression of the mRNA encoding cyclin-

dependent kinase 6 (CDK6) being necessary for granulocytic differentiation. In contrast, another study reported a decrease in miR-29a during development of murine HSPCs to lineage-committed progenitors (184). Stable expression of miR-29a (but not -29b or -29c) in mouse HSPCs leads to a bias towards myelopoiesis as well as a myeloproliferative disorder with features of human AML. In addition, overexpression of miR-29a in progenitor populations increases self-renewal capacity (as demonstrated by serial transplantation) as well as promotes transition from the G1 to S phase of the cell cycle. HMG-box transcription factor 1 (Hbp1) is a well-known regulator of cell-cycle progression whose mRNA was found to be directly targeted by miR-29a in this study; however, Hbp1 repression is not sufficient to recapitulate the miR-29a-induced myeloproliferative disorder. Yet another picture of miR-29 and hematopoiesis comes from a study of human CD133(+) bone marrow cells which are thought to represent HSPCs ancestral even to CD34(+) cells. This study compared CD133(+) cells to CD34(+)CD133(-) and CD34(-)CD133(-) populations, representing sequential stages of differentiation. MiR-29a is among the most differentially-expressed miRNAs between each stage, showing robust expression in CD133(+) cells, followed by a large decrease in CD34(+)CD133(-) cells and high levels again in CD34(-)CD133(-) cells. The mRNAs encoding Wnt-signaling receptor Frizzled homology 5 (FZD5) and the actin-binding protein tropomyosin (TPM1) were both identified as direct targets of miR-29a in this system (185). These various pictures of miR-29 expression during hematopoiesis suggest that it may play numerous roles at different stages of hematopoietic development.

### 1.2.5 MicroRNA-29 Functions: Tumorigenesis

The miR-29 family has been extensively studied in the context of human malignancy. Dysregulated miR-29 expression has been observed in a wide range of cancer types, with instances of both upregulation and downregulation reported. Given the evidence that miR-29 promotes quiescence, differentiation, and senescence in multiple tissues (Section 1.2.4), it is not surprising that miR-29 functions as a tumor suppressor in many malignancies. Loss of miR-29 has been reported in multiple cancers (Table 3) and experimental manipulation of miR-29 expression has provided additional support for its tumor suppressor function.

**Table 3: Cancers with Reported Loss of MicroRNA-29 Expression**

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<u>Cancer</u>	<u>References</u>
Acute myeloid leukemia	(123, 135, 183, 186-188)
ALK(+) anaplastic large cell lymphoma	(189)
B-cell chronic lymphocytic leukemia*	(190-195)
Basal cell carcinoma of the skin	(196)
Bladder cancer	(197)
Cervical carcinoma (HPV-related)	(198)
Cholangiocarcinoma	(199)
Endometrial serous adenocarcinoma	(200)
Esophageal carcinoma	(201)
Gastric cancer	(202)
Glioblastoma	(203)
Hepatocellular carcinoma	(204, 205)
Mantle cell lymphoma	(206)
Multiple myeloma	(207)
Non small-cell lung cancer	(133, 208, 209)
Nasopharyngeal carcinoma	(210)
Osteosarcoma	(211)
Ovarian serous carcinoma	(212)
Prostate cancer	(213)
Rhabdomyosarcoma	(126)

\* aggressive form only, characterized by high levels of Z-chain-associated protein kinase (ZAP-70) and unmutated immunoglobulin variable-region heavy chain (IgV<sub>H</sub>)

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The examination of miR-29 functions in different tumor types has identified many mRNA targets whose repression contributes to the tumor suppressive effects of miR-29 at varying steps of oncogenesis. Furthermore, experimental manipulation of miR-29 expression in specific malignancies has identified multiple pathways leading to miR-29 downregulation as well as diverse mechanisms of miR-29-mediated tumor suppression. For example, in rhabdomyosarcoma (RMS) cell lines and primary tumors, miR-29 is silenced by an activated NF- $\kappa$ B-YY1 pathway. MiR-29 itself can directly target and repress *YY1*, and reconstitution of miR-29 expression in RMS cell lines stimulates myogenic differentiation and inhibits tumor growth in a xenograft model (126).

Unsurprisingly, miR-29 has been demonstrated to target the mRNAs encoding many oncogenes and anti-apoptotic proteins as part of its tumor suppressor function. The oncogene T cell leukemia/lymphoma 1 (TCL1) is an Akt co-activator whose regulation is an important initiating event in the aggressive form of B-cell chronic lymphocytic leukemia (B-CLL). Aggressive B-CLL is characterized by high ZAP-70 and unmutated IgV<sub>H</sub>. *TCL1* mRNA was found to be a direct miR-29 target, and loss of miR-29 is strongly associated with aggressive (but not indolent) CLL and with the deleterious 11q deletion in particular (190-195). Decreased miR-29 expression has also been associated with other deleterious mutations in CLL, such as p53-inactivating mutations, independent of ZAP-70 or IgV<sub>H</sub> status (214). At least one study reported that loss of miR-29 was associated with overexpression of HDAC proteins and epigenetic silencing of miR-29b (215). In multiple studies, decreased miR-29 expression was predictive of clinical endpoints, including decreased treatment-free survival and overall survival of cancer patients (193, 216, 217).

The anti-apoptotic factor myeloid cell leukemia 1 (Mcl-1) is a member of the B-cell lymphoma 2 (Bcl-2) family whose mRNA was identified as another important target of miR-29 tumor suppressor activity. Mcl-1 is frequently upregulated in cholangiocarcinoma, and miR-29 overexpression in cholangiocarcinoma cell lines was able to repress Mcl-1 expression by binding to the 3'UTR of its mRNA and to sensitize cells to tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) cytotoxicity (199). Mcl-1 is also highly expressed in ALK-positive anaplastic large cell lymphomas (ALCL). Modulating expression of the oncogenic nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) fusion protein showed that ALK activation represses miR-29a expression via hypermethylation and silencing of its promoter. MiR-29 overexpression in ALK+ ALCL cell lines led to repression of Mcl-1 expression, enhanced apoptosis in response to doxorubicin treatment, and inhibition of tumor growth in a xenograft model (189). Finally, miR-29-repression of Mcl-1 was also demonstrated in multiple myeloma cell lines, with miR-29 overexpression inducing caspase-3 activation and apoptosis (207).

miR-29 targets also include several mRNAs encoding regulators of cell cycle and proliferation. MiR-29 downregulation is a poor prognostic factor in mantle cell lymphoma. In this malignancy, the mRNA encoding the cell cycle regulator CDK6 was shown to be a direct miR-29 target (206). Elevated CDK6 as well as YY1 were also associated with the miR-29 downregulation observed in human papillomavirus (HPV)-induced cervical carcinoma. Loss of miR-29 was associated with decreased apoptosis and increased accumulation of cells in the G2/M phases, and both of these targets are thought to contribute to malignant transformation in this system (198). In contrast, *CDK6* was not found to be an important miR-29 target in esophageal squamous cell

carcinoma. Instead, the tumor suppressive effects of miR-29 (induction of cell cycle G<sub>1</sub>/G<sub>0</sub> arrest and inhibition of *in vitro* proliferation and *in vivo* xenograft growth) in this malignancy were associated with direct targeting of the mRNA encoding Cyclin E (201). Another mRNA encoding the cell cycle regulator *cdc42* was identified as a direct miR-29 target in a study that screened for miR-29 targets that regulate p53 expression. In this study, all three miR-29 isoforms were shown to upregulate p53 levels and induce apoptosis in a p53-dependent manner in Hela cells. Both *cdc42* and the p85 alpha regulatory subunit of PI3 kinase were shown to be repressors of p53 whose mRNAs were in turn directly targeted by miR-29 (218), providing another mechanism of miR-29 mediated tumor suppressor function.

Some targets of miR-29 are involved in invasiveness and metastatic potential. In glioblastomas, low miR-29b was associated with increased proliferation and invasiveness. MiR-29b was found to directly target the mRNA encoding podoplanin membrane sialoglycoprotein (PDPN), which is frequently overexpressed in astrocytic tumors and which is associated with an invasive phenotype (203). In prostate cancer cells, reconstitution of miR-29 expression inhibited wound healing, invasiveness, and colonization of mouse lungs and liver after intravenous injection in an experimental model of metastasis. These effects of miR-29 were attributed to its direct targeting of the mRNA encoding Snail, a key mesenchymal marker, and to suppression of epithelial-to-mesenchymal transition (213). In nasopharyngeal carcinoma (NPC) cell lines, miR-29 was shown to repress expression of ECM components such as fibrillin, laminin  $\gamma$ 1, osteonectin, and various collagen isoforms; increased ECM expression upon loss of miR-29 is thought to increase the invasiveness and metastatic potential of NPC. Novel targets

of miR-29 were also identified in NPC. These include the mRNAs encoding FUS-interacting protein (FUSIP1), which interacts with the oncoprotein fused in sarcoma/translocated in liposarcoma (FUS/TLS) and has a role in general repression of RNA splicing (219), and thymine-DNA glycosylase (TDG), which is involved in DNA repair and is frequently dysregulated in cancer (220).

Other miR-29 targets affect tumorigenesis in a more indirect fashion. B7-H3 is a surface immunomodulatory glycoprotein that acts in a costimulatory fashion to inhibit the actions of natural killer (NK) and cytotoxic T cells. It is highly expressed in many tumors and is theorized to play an immunomodulatory role and facilitate tumor escape from immune surveillance. MiR-29 directly targets the mRNA encoding B7-H3 in neuroblastoma cell lines; thus, loss of miR-29 may upregulate B7-H3 and facilitate tumor survival (221). In non-small cell lung cancer, the mRNAs encoding the DNA methyltransferases (DNMTs) 3A and 3B were also identified as direct miR-29 targets that can mediate its tumor suppressor function. Downregulation of miR-29b is a poor prognostic factor in non-small cell lung cancer (NSCLC), with Myc reported to contribute to miR-29 repression (133). DNMT3A and DNMT3B are frequently upregulated in NSCLC and are also associated with a poor prognosis. Overexpression of miR-29 in lung cancer cell lines repressed DNMT3A and B expression, restored normal DNA methylation patterns, and induced re-expression of silenced genes encoding tumor suppressors such as fragile histidine triad (FHIT) and WW domain containing oxidoreductase (WWOX). It also inhibited *in vitro* cell proliferation as well as tumor growth in a xenograft model (222). A separate study in NSCLC cells identified phosphatase and tensin binding homolog (PTEN) as another tumor suppressor indirectly

affected by miR-29 regulation of DNMT expression. Loss of PTEN is frequently observed in NSCLC. Radiation treatment of NSCLC cell lines induces miR-29, leading to repression of DNMT3A and B, global hypomethylation and increased PTEN expression, and cellular apoptosis. The effects of radiation treatment were reversed by inhibition of miR-29, both *in vitro* and in a xenograft model (223). MiR-29 appears to have many modes of tumor suppression in NSCLC. For example, downregulation of miR-29a was found to be associated with increased invasiveness within a panel of NSCLC cell lines. Overexpression of miR-29a in these lines reduced proliferation and invasiveness *in vitro* (224). MiR-29 repression of migration and invasion was confirmed in a second study that also identified the mRNA encoding inhibitor of DNA binding/differentiation 1 (ID1) as a direct target of miR-29 (133).

AML is another malignancy in which miR-29 exerts multiple tumor suppressive mechanisms. Downregulation of miR-29 has been frequently reported in blasts taken from AML patients; this decrease has been variously attributed in different studies to deletions of chromosome 7q, loss of CEBPA transcriptional induction, and Myc-induced transcriptional repression of miR-29 (123, 135, 183, 186-188). Clinically, miR-29b levels are positively correlated with overall survival in AML patients and with improved response to cytarabine treatment (225, 226). Experimentally, restoration of miR-29 expression in AML cell lines and primary cells induced apoptosis, restored differentiation ability, and reduced tumorigenicity in a xenograft model (188, 227). MiR-29 regulation of previously-established targets such as Mcl-1 and DNMT3A and 3B was confirmed in AML and may contribute to oncogenesis (227, 228). Additionally, miR-29 was shown to directly target the mRNAs encoding the serine protease inhibitor SerpinB9, the nuclear

oncogene Ski, and the transcription factor Sp1 (135, 187, 226). Sp1 is itself a known inhibitor of miR-29 expression; thus, downregulation of miR-29 leads to increased Sp1 protein which further represses miR-29 in a feedback loop.

MiR-29 downregulation was reported in several studies of hepatocellular carcinoma (HCC) and is associated with poor prognosis and shorter disease-free survival after treatment (229-231). Reconstitution of miR-29 expression in HCC cells sensitized them to apoptotic stimuli such as serum starvation, hypoxia, and chemotherapeutic drugs and inhibited tumor growth in a xenograft model. In this system, miR-29 was found to directly repress the mRNAs encoding the previously-reported target Mcl-1 as well as its relative Bcl-2 (229). In a separate study, reconstitution of miR-29 expression in HCC was shown to repress the mRNA encoding MMP-2. Loss of miR-29-mediated repression enhanced indicators of angiogenesis such as endothelial cell capillary tube formation *in vitro* and increased microvessel density in a subcutaneous xenograft model. Metastatic ability was also increased, as measured by transwell migration *in vitro* and intrahepatic metastatic capacity in an orthotopic xenograft model (231). In a model of hepatitis B virus (HBV)-induced HCC, miR-29c overexpression inhibited cell proliferation and induced apoptosis, and it also was found to directly target the mRNA encoding tumor necrosis factor alpha-induced protein 3 (TNFAIP3), a key regulator in inflammation and immunity (230).

In contrast to the many examples of miR-29 acting as a tumor suppressor, overexpression of miR-29 has been reported in a small subset of cancers. MiR-29 may function in an oncogenic manner in these malignancies. In particular, upregulation of miR-29 has been reported in breast cancer by several groups (209, 232-234). In breast

cancer cell lines, miR-29 upregulation is associated with increased migration and a mesenchymal, metastatic phenotype. One explanation for these observations is the direct targeting of the mRNA encoding the tumor suppressor PTEN by miR-29. Repression of PTEN expression by miR-29 is associated with impaired apoptosis and increased invasion and migration of breast cancer cell lines *in vitro* (235). Another important target in this system may be the mRNA encoding TTP. TTP binds and enhances the degradation of many mRNAs encoding inflammatory cytokines, and decreased TTP expression has been reported in breast and other cancers (75, 236). MiR-29 repression of TTP in the breast is suggested to create a localized inflammatory environment that can promote tumorigenesis (237).

While the evidence supports a tumor suppressive function for miR-29 in aggressive CLL, there is evidence that miR-29 may play an oncogenic role in the more indolent forms of CLL which are characterized by low ZAP-70 expression and mutated IgV<sub>H</sub>. MiR-29 upregulation was observed in indolent CLL compared to both aggressive CLL and normal CD19<sup>+</sup> cells, and transgenic expression of the miR-29a/b-1 cluster in mouse B cells led to development of an indolent B-CLL-like disease with characteristic IgV<sub>H</sub> rearrangements (238). In human CLL samples, the mRNA encoding peroxidase was dramatically downregulated and was identified as a direct miR-29 target (238).

AML is another hematologic malignancy with varying reports of miR-29 function. While there is ample evidence that miR-29 can play a tumor suppressive role in AML, it has also been observed that ectopic expression of miR-29a in mouse HSPCs leads to increased self-renewal capacity and biased myeloid differentiation, culminating in an AML-like myeloproliferative disorder (184).

Similarly, despite many studies of miR-29 tumor suppressor activity in HCC, an oncogenic function of miR-29 in this system has also been reported. Direct repression of the mRNA encoding the tumor suppressor PTEN by miR-29 and enhanced migration was observed in HCC cell lines. MiR-29 is upregulated in a subset of HCCs. In particular, the hepatitis B virus X (HBx) protein is known to be important in development of HBV-related liver cancer, and HBV infection or HBx overexpression were shown to upregulate expression of miR-29 (239). Other solid tumors with reported elevation of miR-29 expression include pancreatic cancer, prostate cancer, and colon cancer (209, 240, 241). However, functional studies are required to elucidate the effects of miR-29 elevation in these cancers.

MiR-29 has emerged as an important player in the development of cancer, though its role is far from straightforward. Multiple studies have shown that miR-29 can either inhibit or promote tumorigenesis, depending on the cellular context. Furthermore, the experimental evidence demonstrates that miR-29 represses different targets in each situation; the specific targets in each tissue type may determine the outcome of miR-29 on tumor development.

### **1.2.6 Other Functions of MicroRNA-29**

miR-29 dysregulation has been reported in many models of diabetes and may play a role in both the development of insulin resistance as well as in the various tissue pathologies associated with uncontrolled high blood sugar (242-246). MiR-29 has also been implicated in atherosclerosis, with reports of atherogenic stimuli leading to miR-29 upregulation (124, 247, 248). Oxidized low density lipoprotein (oxLDL) stimulation of

human aortic smooth muscle cells leads to upregulation of miR-29 expression via AP-1 binding and transcriptional induction of the miR-29b-1/a promoter. oxLDL treatment also decreases HDAC1 levels and activity, and ChIP demonstrated that HDAC1 binding to the miR-29b-1/a promoter is reduced by oxLDL. Examination of the H3 histones at the mir-29b-1/a promoter by ChIP revealed modifications (i.e. reduced methylation and increased acetylation at lysine 9, increased methylation at lysine 4) that contribute to increased miR-29 expression in this setting (124). oxLDL treatment is also associated with miR-29-mediated repression of DNMT3B expression and hypomethylation/increased expression of the metalloproteinase enzymes MMP-2/9, leading to increased human aortic smooth muscle cell migration (247). In human oxLDL-treated DCs, miR-29 directly targets the mRNA encoding lipoprotein lipase (LPL), which indirectly leads to blunting of inflammatory cytokine production and increased expression of surface scavenger receptors (248).

Many direct miR-29 targets have been identified in the nervous system, and miR-29 regulation has been shown to play a role in both physiologic and pathologic processes in this organ. MiR-29b is markedly induced during neuronal maturation and inhibits neuronal apoptosis by directly targeting the mRNAs encoding several pro-apoptotic members of the Bcl-2 family (249). MiR-29 also directly targets the mRNA encoding peripheral myelin protein 22 (PMP22) in Schwann cells; dysregulated production of PMP22 can lead to hereditary neuropathy. MiR-29 and PMP22 are inversely correlated in sciatic nerves both during embryonic development as well as post-crush injury (250). Progranulin deficiency is thought to cause some forms of frontotemporal dementia, and both human and mouse progranulin-encoding mRNAs were shown to be direct targets for

miR-29b (251). Finally, miR-29 downregulation has been reported in the brains of Alzheimer's disease patients and spinocerebellar ataxia patients, and miR-29 has been shown in these studies to directly target the mRNAs encoding beta-secretase-1 (BACE1) and Neurone navigator 3 (NAV3) (252-255).

Estradiols can upregulate the expression of miR-29, most likely through the suppression of NF- $\kappa$ B signaling (129, 130). This regulation suggests that miR-29 may play some sex-specific functions, and indeed, profiling in adult rat liver and mouse embryonic gonads revealed that miR-29b is much more highly expressed in female tissues (256, 257). MiR-29 upregulation in post-natal epididymis tissue in male rats leads to direct targeting of the mRNA encoding nuclear autoantigenic sperm protein (NASP) and may be a part of the normal decrease in proliferation of this tissue after birth (258). On the other hand, exposure to environmental estradiols has been shown to induce adult germ cell apoptosis in association with an increase in miR-29 and a decrease in established targets Mcl-1, DNMT3A and DNMT3B (129). Experimentally, estradiol protects female but not male mice from carbon-tetrachloride-induced repression of miR-29 and subsequent liver fibrosis (130).

MiR-29 has been reported to repress expression of IFN- $\gamma$ , both direct targeting of IFN- $\gamma$ -encoding mRNA and as well as through targeting of the mRNAs encoding T-bet and Eomes, two transcription factors involved in IFN- $\gamma$  induction (134, 259). Under normal conditions, infectious stimuli lead to NF- $\kappa$ B repression of miR-29 and production of IFN- $\gamma$ . Mice expressing a transgenic miR-29 sponge show higher serum IFN- $\gamma$  levels and better control of *Listeria monocytogenes* infection (134).

In addition to its mammalian targets, miR-29 family members have been reported to inhibit replication of human immunodeficiency virus-1 (HIV-1) through direct binding to highly conserved portions of the *nef* gene (260). MiR-29a and -29b are expressed in human T cells, and physical association between HIV-1 mRNAs and miRISC proteins was observed in these cells. Increasing miR-29a and -29b levels in human T lymphocytes lead to decreased Nef protein expression, increased viral mRNA association with the miRISC, and reduced virus replication and infectivity, while inhibition of these miRNAs has the opposite effect (261, 262). Clinically, repression of HIV-1 replication by miR-29 members is thought to play a role in maintaining viral latency, and in fact, miR-29 expression was found to be elevated in PBMCs from HIV-1-infected long-term suppressor patients (i.e. patients who maintain long-term undetectable viral loads without antiretroviral therapy). MiR-29 expression is also able to suppress replication of both HBV (a DNA virus) and hepatitis C virus (HCV, an RNA virus); however, it is not clear whether this involves direct targeting of viral genes or genomes (140, 230)

In summary, the miR-29 family is a widely-expressed family of miRNAs with pleiotropic effects, including regulation of ECM components and tumorigenicity. Identification of new miR-29 targets will provide a better understanding of its function and suggest new systems in which it might play a regulatory role.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Plasmids and miRNA Precursors and Inhibitors

The ZC5 and ZC5+ *RNASEL* constructs were previously described (1, 15). To generate the *RNASEL* 3'UTR reporter, we made use of a reporter plasmid (a generous gift from Dr. Kara Scheibner, University of Maryland, Baltimore) in which the firefly luciferase coding sequence was cloned into the NheI and XhoI sites of the pcDNA3.1(+) plasmid. The *RNASEL* ZC5+ construct was partially digested with EcoRI and the 3'UTR fragment was gel-purified and cloned into the EcoRI site downstream of firefly luciferase. The resulting chimeric mRNA transcript contains both the luciferase coding region and the *RNASEL* 3'UTR and utilizes the pcDNA3.1-encoded polyadenylation signal. All restriction enzymes were purchased from New England Biolabs. The *Renilla* luciferase plasmid was generously provided by Dr. Myriam Gorospe (National Institute on Aging, Baltimore). MiR-29 site mutations were created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using primers which delete the 7 nucleotides predicted to bind the seed region (nt 2-8) of the miR-29 family. Primers used for mutagenesis are as follows: site A (forward 5'-GCACTTTATAAATTTATGATTGGTACCTCTCATTTGGGC-3', reverse 5'-GCCCAAATGAGAGGTACCAATCATAAATTTATAAAGTGC-3'), site B (forward 5'-CCAGACAAAATATCAAGAGGTTGAGAAAACCTGAC-3', reverse 5'-GTCAGGTTTTCTCAACCTCTTGATATTTTTGTCTGG-3'), site C (forward 5'-CTGTCTTACGTTTTTCTTATAATGTATACATTACATCTGAG-3', reverse 5'-CTCAGATGTAATGTATACATTATAAGAAAACGTAAGACAG-3'), site D (forward 5'-CTTGATTTGAACAAATTTTCAAGTCTGATGTTCTTCCATG-3', reverse 5'-CATGGAAAGAACATCAGACTTGAAAATTTGTTCAAAT

CAAG-3'). Constructs were verified by sequencing (Biopolymer/Genomics Core Facility, University of Maryland, Baltimore). pGIPZ-encoded nonspecific, *DICER*-specific, and *RNASEL*-specific short hairpin RNAs (shRNAs) were purchased from Open Biosystems. The pcDNA3-miR-146a plasmid was obtained from Addgene.org (deposited by Dr. David Baltimore, California Institute of Technology, Pasadena). pcDNA3.1/V5-His-TOPO-mir17-92 was a generous gift from Dr. Joshua Mendell (UT Southwestern Medical Center, Dallas). MiR-29 Pre-miR™ miRNA precursors and mirVana™ miRNA inhibitors were purchased from Invitrogen.

## **2.2 Isolation of RNase-L Wild-type and Knockout Macrophages**

*RNASEL*<sup>-/-</sup> mice (60) and wild-type C57BL/6 mice were housed at the University of Maryland, Baltimore animal facility according to Institutional Animal Care and Use Committee (IACUC)-approved protocols. For induction of peritoneal macrophages, mice were injected intraperitoneally with 3mL of 3% thioglycollate (Rennel). 3-4 days after injection, macrophages were harvested by peritoneal lavage with phosphate buffered saline (PBS, Cellgro). Peritoneal cells were centrifuged, washed, and incubated with red blood cell lysis buffer (Sigma) according to the manufacturer's instructions before being counted and plated.

## **2.3 Cell culture, Transfection, and Transduction**

293T, HeLa, and MDA-MB-231 cells were passaged in Dulbecco's Modified Eagle Medium (DMEM, Cellgro) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), antibiotic/antimycotic (Invitrogen) and 2.5µg/mL

Plasmocin (Invivogen). 293T-ZC5+ cell lines stably expressing RNase-L were grown in complete DMEM with geneticin (200 $\mu$ g/mL, Invitrogen). HeLa cells with stable knockdown of a nonspecific sequence (shNS) or RNase-L (shRNL) were grown in complete DMEM with 1 $\mu$ g/mL puromycin (Sigma). K562 cells and mouse peritoneal macrophages were maintained in RPMI 1640 (Cellgro) with L-Glutamine supplemented with 10% FBS, antibiotic/antimycotic and Plasmocin. All cell lines were maintained in a humidified incubator at 37°C and 95% balanced air plus 5% CO<sub>2</sub>. 293T cells were transfected using Lipofectamine 2000 (Invitrogen), and all other cell types were reverse-transfected using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's recommendations. K562 cells stably expressing shRNA against a nonspecific sequence or *RNASEL* were generated by lentiviral transduction. Virus- containing pGIPZ-encoded shRNA constructs were generated using the Trans-Lentiviral shRNA packaging system from Open Biosystems according to the manufacturer's instructions. 48 hours after transduction, cells were moved into complete RPMI supplemented with 4 $\mu$ g/mL puromycin. 72 hours after transduction, cells were counted and seeded into 96 well plates at varying serial dilutions. Plates in which fewer than 10% of wells gave rise to colonies were considered likely to represent single cell clones and individual wells were expanded for testing of RNase-L expression by protein immunoblot. Pools and selected clones were subsequently passaged in complete RPMI with 1 $\mu$ g/mL puromycin.

## **2.4 Antibodies and Western blotting**

Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Millipore) plus protease inhibitor cocktail (Sigma). Protein concentrations were

determined using the Bradford protein assay (Bio-Rad) and equal amounts of protein per sample were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad). Proteins were then electrotransferred to Immobilon-P membranes (Millipore). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline with Tween 20 (TBS-T: 10mM Tris, pH 8.0, 150mM NaCl, 0.1% Tween 20) plus either 10% FBS (RNase-L antibody only) or 5% nonfat milk (all other antibodies). Anti-human RNase-L mouse monoclonal antibody (clone 2E9, Alexis Biochemicals) was used at a 1:1000 dilution in TBS-T plus 5% bovine serum albumin. Anti-human MCL-1 mouse monoclonal antibody (clone 22, BD Biosciences) was used at 2 $\mu$ g/mL. Anti-human DNMT3A rabbit polyclonal antibody (Cell Signaling) was used at a 1:1000 dilution. Anti-human Dicer mouse monoclonal antibody (clone 13D6, Abcam) was used at a 1:1000 dilution. Anti-human  $\beta$ -actin (clone AC15) and  $\alpha$ -tubulin (clone B-5-1-2) mouse monoclonal antibodies (Sigma) were used at 1:10,000 dilutions. Goat anti-human interleukin-1 beta (IL-1 $\beta$ ) IgG polyclonal antibody (clone M-20, Santa Cruz) was used at a 1:200 dilution. Rabbit anti-human caspase-1 p10 polyclonal antibody (clone M-20, Santa Cruz) was used at a 1:200 dilution. Rabbit anti-human microtubule-associated protein1A/B/light chain 3 (LC3) polyclonal antibody (Abgent) was used at a 1:500 dilution. Membranes were incubated with primary antibody at 4°C overnight and then with species-appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) at a 1:10,000 dilution in TBS-T plus 5% nonfat milk for 1 hour at room temperature. Membranes were washed again and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and Hyblot CL autoradiography film (Denville Scientific).

## 2.5 Quantitative Real-time Polymerase Chain Reaction

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Concentration and 260/280 ratios were determined on a Tecan Infinite 200 Pro multimode reader. mRNA quantitative real-time polymerase chain reaction (qRT-PCR) analysis was carried out on a CFX96 Touch Real-Time PCR Detection System using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad), using 0.5 $\mu$ g total RNA per triplicate reaction. Primers for human Dicer were as follows: forward 5'-AGTGCCAGTCCTGCAGTAGTTGAT-3', reverse 5'-ATGTGATTCACCAACATGCCAGCC-3'. Primers for human ribosomal protein L13a (rpl13a) were as follows: forward 5'-CTCAAGGTCGTGCGTCTG-3', reverse 5'-TGGCTTTCTCTTTCC TCTTCTC-3'. Primers for human RNase-L were as follows: forward 5'-CAGGATCTGCAACCACAAAA-3', reverse 5'-CCCCTTGATGCTCTTATCAAA-3'. Primers for the internal controls human hydroxymethylbilane synthase (HMBS) and 14-3-3 zeta (YWHAZ) were designed or adapted from Vandesompele and colleagues (263). Primers for HMBS were: forward 5'-GGCAATGCGGCTGCAA-3', reverse 5'-GGGTACCCACGCGAATCAC-3'. Primers for YWHAZ were: forward 5'-ACTTTTGGTACATTGTG GCTTCAA-3', reverse 5'-GAACCAATGATCCCTTTTATTCC-3'. Primers for human IFN- $\beta$  were kindly shared with us by the Vogel Laboratory (University of Maryland, Baltimore) and were: forward 5'-CACTTGAAGAGCTATTACTGGAGGG-3', reverse 5'-CTCGGACCACCATCCAGG-3'. Primers for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: forward 5'-TGTGATGGGTGTGAACCACGAGAA-3', reverse 5'-GAGCCCTTCCACAATGCCAAAGTT-3'. Primers for human IL-1 $\beta$  were:

forward 5'-TGGAGAGTGTGGATCCCAAGCAAT-3', reverse 5'-TGTCCTGACCAC TGTTGTTTCCCA-3'. Cycling conditions were as follows: 50°C for 5 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds and 58°C for 30 seconds. qRT-PCR detection of miRNAs and control U6 small RNAs was carried out using Taqman miRNA Assays (Applied Biosystems) according to the manufacturer's instructions.

## **2.6 Luciferase Assays**

293T cells were seeded in 12-well plates at 350,000 cells per well and allowed to adhere overnight. Cells were then transfected with a combination of firefly luciferase reporter (0.5µg per well) and *Renilla* internal control (5ng per well) plasmids, along with miRNA mimetics at 25nM or RNase-L plasmids at the indicated concentrations. Cells were harvested 24 hours later and analyzed for luciferase activity on a PerkinElmer Victor X3 Multilabel Plate Reader using the Dual Luciferase Reporter Assay (Promega) according to the manufacturer's instructions. Within each sample, firefly was normalized to *Renilla* luciferase, and for each reporter construct, the firefly/*Renilla* ratio of the non-specific control-transfected sample was set to 1.

## **2.7 Encephalomyocarditis Protection Assays**

For encephalomyocarditis (EMCV) protection studies, Hela cells were co-transfected with 50nM miRNA mimetics and any indicated plasmids using Lipofectamine 2000. 24 hours post-transfection, cells were trypsinized, counted, and seeded in triplicate into 96-well plates with or without human IFN- $\alpha$  (1000 U/mL, PBL Interferon Source). 16 hours after IFN- $\alpha$  treatment, cells were washed with PBS and EMCV at a multiplicity

of infection (MOI) of 1 was added in 20 $\mu$ L/well of infection media (DMEM plus 2% FBS without antibiotics) and incubated at 37°C with occasional shaking. After 1 hour, virus was removed, cells were washed with PBS again and given 100 $\mu$ L/well complete DMEM. Cells were monitored for cytopathic effects and survival measured using the Promega Cell Titer 96 Non-Radioactive Cell Proliferation Assay according to the manufacturer's instructions. Approximately 8 hours after infection, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye was added to each well. STOP solution was added after 4 hours and absorbance measured at 570nm. Percent protection was calculated according to the following formula: (IFN- $\alpha$  treated plus EMCV-infected – EMCV-infected)/(uninfected – EMCV-infected). For K562 cells, reverse transfection was performed using Lipofectamine RNAiMax, and IFN- $\alpha$  treatment was performed in 40 $\mu$ L of infection media. After 16 hours, EMCV was added directly to wells without washing, followed by addition of complete media to a final volume of 100 $\mu$ L after 1 hour of infection.

## **2.8 Interferon- $\beta$ Induction**

1.5 million HeLa cells were co-transfected with 50nM miRNA mimetics and 3 $\mu$ g of the indicated plasmids in a 10cm dish using Lipofectamine 2000. After 24 hours, cells were trypsinized, counted, and seeded at 50% density in 6-well plates. At 48 hours, cells were transfected with 0.5 $\mu$ g polyI:C (Sigma) per well using Lipofectamine 2000. Cells were harvested at the indicated time points using TRIzol Reagent and extracted RNA was analyzed by qRT-PCR.

## **2.9 Proliferation and Chemotherapeutic Agent Studies**

K562 cell proliferation was measured by the MTT assay. For proliferation studies, K562 cells were counted and 1000 cells/well were seeded in triplicate in 100 $\mu$ L complete RPMI in 96 well plates. MTT dye was added to a new plate and absorbance measured at 570nm daily over 4 days. For plates measured on later days, cells were centrifuged and 25 $\mu$ L of fresh media replaced daily. Adriamycin, camptothecin, staurosporine, cytarabine, imatinib mesylate, and diethyl maleate were purchased from Sigma. For drug treatment, all cells were seeded in media containing the indicated drug concentrations or an equal amount of the appropriate vehicle (e.g. dimethyl sulfoxide) and survival assessed by MTT assay at 24 hours, except for imatinib and cytarabine, which were assessed at 72 hours. No media changes were performed in the latter.

## **2.10 Xenograft Studies**

All mouse experiments were carried out by the University of Maryland Translational Core Laboratory (Baltimore, MD) in accordance with protocols approved by the IACUC at the University of Maryland. For xenograft studies, 6-week old female nude (*nu/nu*) mice were subcutaneously injected in the flank with K562 cells. Cells were washed and reconstituted in PBS, and 100 $\mu$ L of PBS containing 10 million cells was mixed with an equal amount of Matrigel (BD Biosciences) before injection. Each mouse was injected with a matched pair of K562 cell lines, the shNS cells in the left flank and the shRNL cells in the right flank. Each pair of cells was tested in 10 mice (n=10). Mice were monitored every two to three days for weight loss, and tumor size. Tumor length and width measurements were taken with calipers and tumor volume was calculated

according to the following formula: (volume = width<sup>2</sup> x length/2). Mice were euthanized and tumors frozen for protein analysis when tumor volume on either side exceeded 1500mm<sup>3</sup>.

### **2.11 Pyroptosis and release of Lactate Dehydrogenase and Interleukin-1 $\beta$**

Peritoneal macrophages were treated with LPS (1 $\mu$ g/mL, O111:B4, Sigma) for 2 hours followed by ATP (5mM, Sigma) for the indicated times to induce pyroptosis, before cells were harvested for protein immunoblot. Supernatants were saved and analyzed for LDH release using the Cytotoxicity LDH Detection Kit (Clontech) according to the manufacturer's instructions, or concentrated using Amicon Ultra-15 Centrifugal Filters (Millipore) and analyzed for mouse IL-1 $\beta$  by enzyme-linked immunosorbent assay (ELISA) (University of Maryland, Baltimore, Cytokine Core).

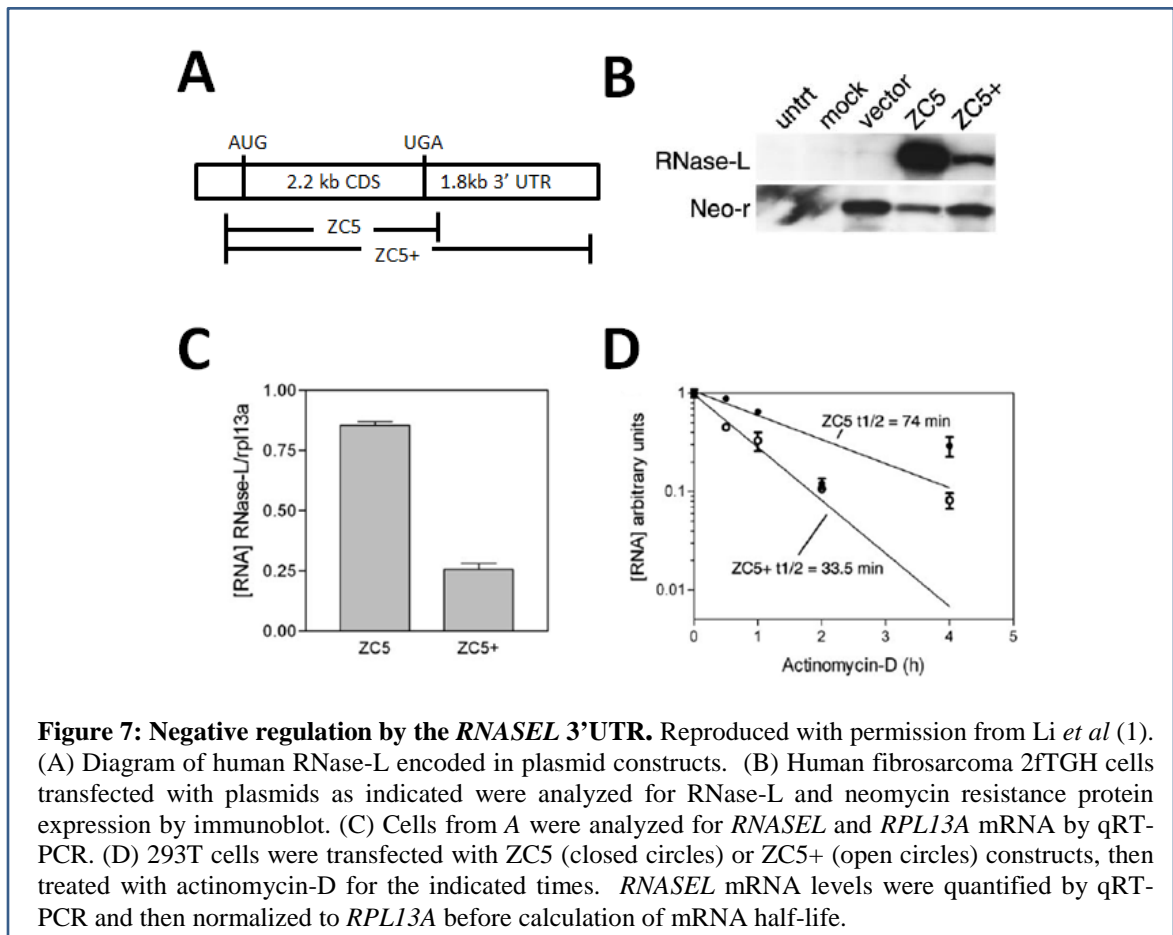
### **2.12 Induction of Autophagy**

For EMCV induction of autophagy, peritoneal macrophages were infected with EMCV at MOI of 1 as described for HeLa cells (Section 2.7) and harvested at indicated times for protein immunoblot. Alternatively, macrophages were treated with LPS (1 $\mu$ g/mL, O111:B4, Sigma), rapamycin (5mM, Sigma), and/or chloroquine (3mM, Sigma) for the indicated times before harvest for protein immunoblot.

# CHAPTER 3: REPRESSION OF RNASE-L EXPRESSION BY THE MICRORNA-29 FAMILY

## 3.1 INTRODUCTION

Our laboratory previously demonstrated that RNase-L expression is robustly regulated by the 3'UTR of the *RNASEL* mRNA (1). This regulation is inhibitory in the basal state, with the presence of the *RNASEL* 3'UTR repressing RNase-L protein expression as well as destabilizing the *RNASEL* mRNA (Fig. 7). In the same study, sequential deletion of the 3'UTR identified a positive regulatory region that was regulated by the RNABP HuR (nt 3755-4258). Ectopic expression of HuR or induction



of endogenous HuR by cellular stress leads to increased *RNASEL* mRNA stability as well as RNase-L protein expression and activity (1). The deletion analysis also uncovered two negative regulatory regions between nt 2872-3001 and nt 3193-3753. In particular, a 27 nucleotide stretch from nt 2891-2917 was shown to contribute to *RNASEL* repression. However, the cellular elements that mediated repression were not identified. We hypothesized that this regulation may be mediated by miRNAs. To that end, we used a combination of *in silico* and *in vitro* approaches to identify miRNAs that regulate RNase-L expression.

## **3.2 RESULTS**

### **3.2.1 MicroRNA-dependent Regulation of RNase-L Expression**

As a preliminary assessment of the potential contribution of miRNAs to RNase-L regulation, we inhibited the essential miRNA-processing enzyme, Dicer, in 293T cells using transiently-transfected shRNA (Fig. 8A). We observed a reduction in *DICER1* mRNA of roughly 70% (Fig. 8B) by qRT-PCR which resulted in a substantial increase in RNase-L protein (Fig. 8C), suggesting that production of mature miRNAs is necessary for repression of RNase-L expression.

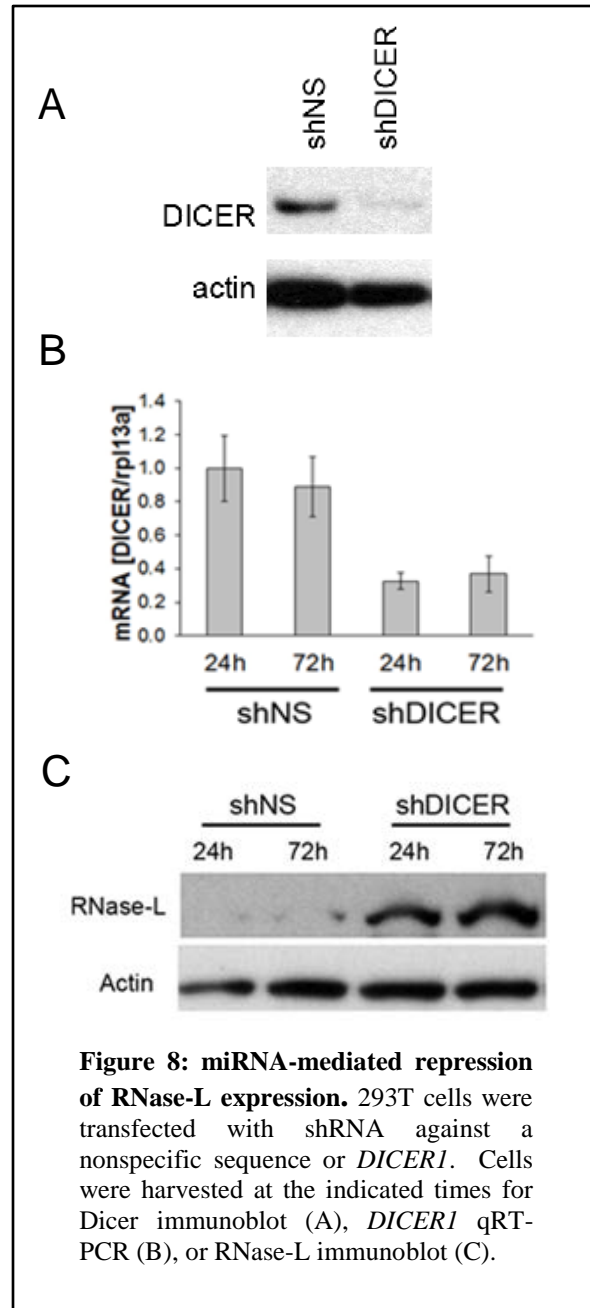
#### **3.2.1 *In silico* Analysis and Selection of Candidate MicroRNAs**

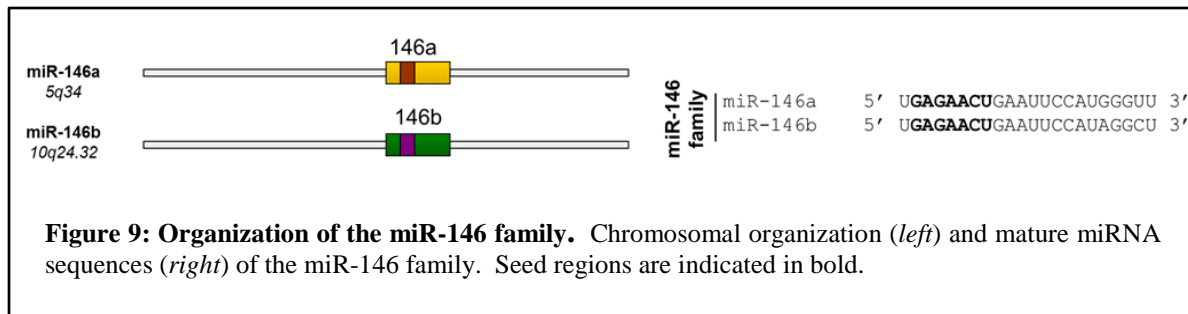
Dicer-dependent regulation of RNase-L does not rule out the possibility of indirect regulation (i.e. miRNAs targeting a regulator of RNase-L); therefore, we used miRNA-target prediction algorithms to identify miRNAs that may directly interact with the *RNASEL* mRNA. Analysis of the *RNASEL* 3'UTR (NM\_021133) using TargetScan

(version 5.1) (264) and microRNA.org (September 2008 release) (265) identified 114 predicted *RNASEL*-targeting miRNAs that were common to both algorithms; we focused our investigation on these, selecting the miR-146 family, the miR-17-92 cluster, and the miR-29 family for further analysis. All of these miRNAs are considered highly conserved (i.e. across vertebrates) by TargetScan.org (115).

The miR-146 family has two members, miR-146a and miR-146b, expressed on human chromosomes 5 and 10, respectively (Fig. 9). The predicted binding site for miR-146 was the only *RNASEL* target site that was broadly conserved according to TargetScan; thus we included it in our subsequent analyses.

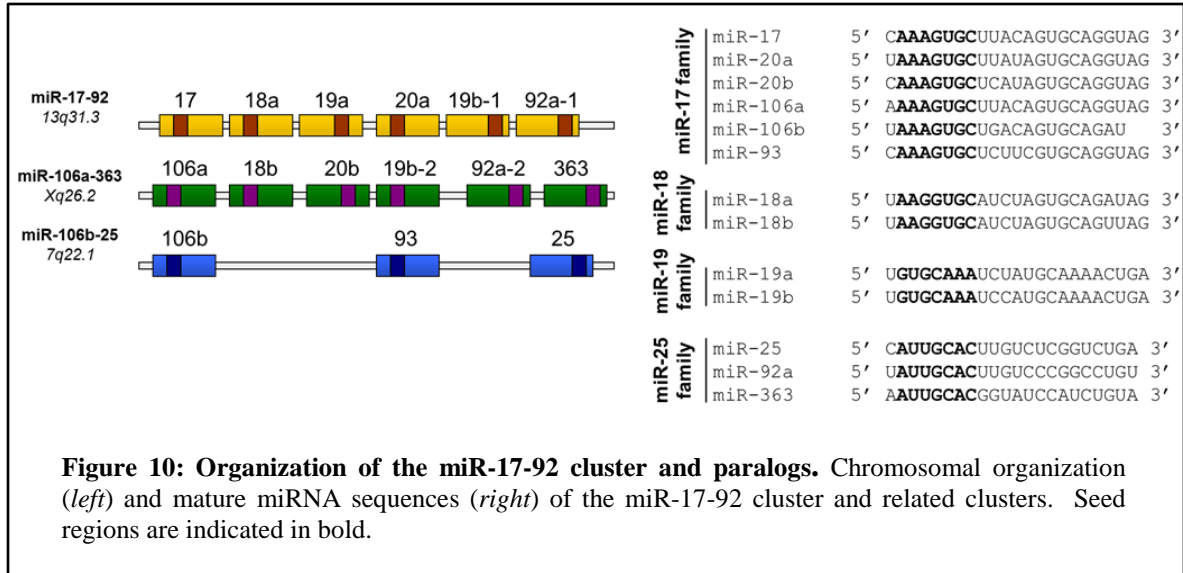
Moreover, miR-146 family members are of interest because of their involvement in several RNase-L-regulated activities, such as inflammation, differentiation, and tumorigenesis (266). MiR-146a in particular is induced by LPS-stimulated NF- $\kappa$ B signaling and feeds back to directly inhibit components of NF- $\kappa$ B pathway components such as TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1





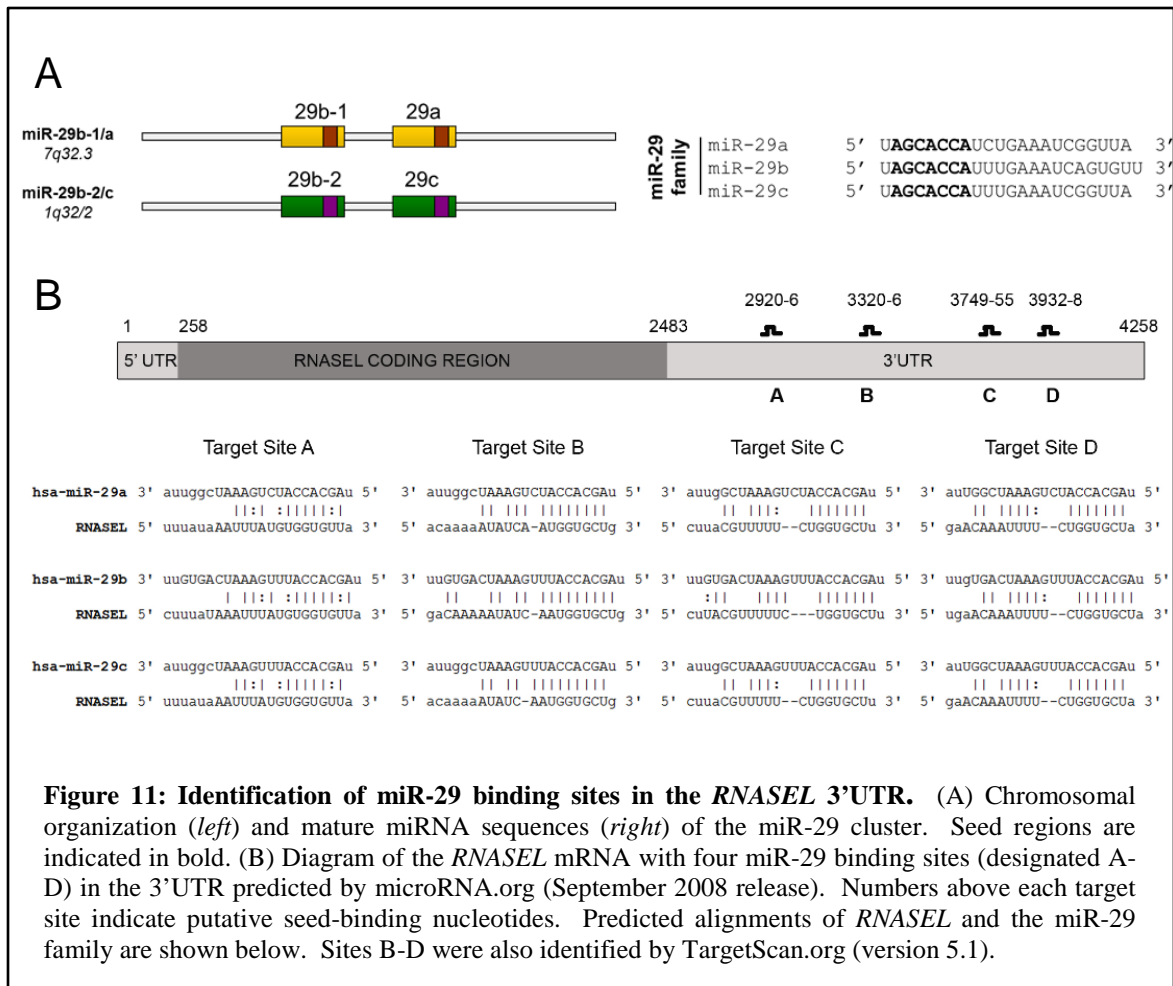
(IRAK1) (267). MiR-146 also appears to have roles in adaptive immunity, promoting survival in memory T cells by targeting Fas-associated protein with death domain (FADD) and promoting regulatory T cell survival and function. MiR-146a is also important in regulating hematopoiesis; it is induced by the PU.1 transcription factor, and absence of miR-146 leads to myeloid neoplasia. MiR-146 has several targets that can contribute to tumorigenesis, including NOTCH1 and the epidermal growth factor receptor (EGFR). Consequently, miR-146 dysregulation has been reported in several cancers, including papillary thyroid cancer, pancreatic cancer, gastric cancer, breast cancer, androgen-resistant prostate cancer, gliomas, cervical cancer, and AML (266). In particular, SNPs within the miR-146a hairpin have been associated with both papillary thyroid cancer and glioma development.

The miR-17-92 cluster is one of three paralogous clusters that encodes miRNAs representing four different miRNA families (Fig. 10). All but one of the four families are predicted to target *RNASEL*, with a different binding site predicted for the miR-17, miR-18, and miR-19 families, respectively. The binding sites for the miR-17 and miR-18 families are located in the nt 2782-3001 negative regulatory region, with the miR-17 site falling directly within the repressive 27 nucleotide-element identified during deletion analysis (1); therefore, we selected the cluster for analysis. Functionally, the miR-17-92 cluster was also of interest due to its reputation as an oncogene (268). Overexpression of



the miR-17-92 cluster has been reported in a variety of cancers, including diffuse large B-cell lymphoma, small cell lung cancer, colon cancer, neuroblastoma, medulloblastoma, retinoblastoma, and gastric cancer. In some cases, upregulation of miR-17-92 is due to genomic amplification. In addition, the miR-17-92 cluster is transcriptionally activated by c-Myc as well as the E2F family of transcription factors. MiR-17 and miR-20 directly inhibit translation of E2F1, E2F2, and E2F3, thus forming an autoregulatory network. In many experimental models of cancer, miR-17-92 overexpression appears to collaborate with Myc to promote tumorigenesis. Unlike many other miRNAs which do not appear to be essential for development, deletion of the miR-17-92 cluster in mice has profound phenotypic effects. While deletion of miR-106-363 or miR-106b-25 does not lead to obvious developmental abnormalities, homozygous deletion of the miR-17-92 cluster alone leads to perinatal lethality due to lung hypoplasia and cardiac defects (269). It also causes a block in B-cell development during the pro-B to pre-B cell transition. Hemizygous deletions of the miR-17-92 cluster in patients have recently been shown to be associated with the rare autosomal dominant condition known as Feingold syndrome, which presents with a wide array of skeletal defects (270).

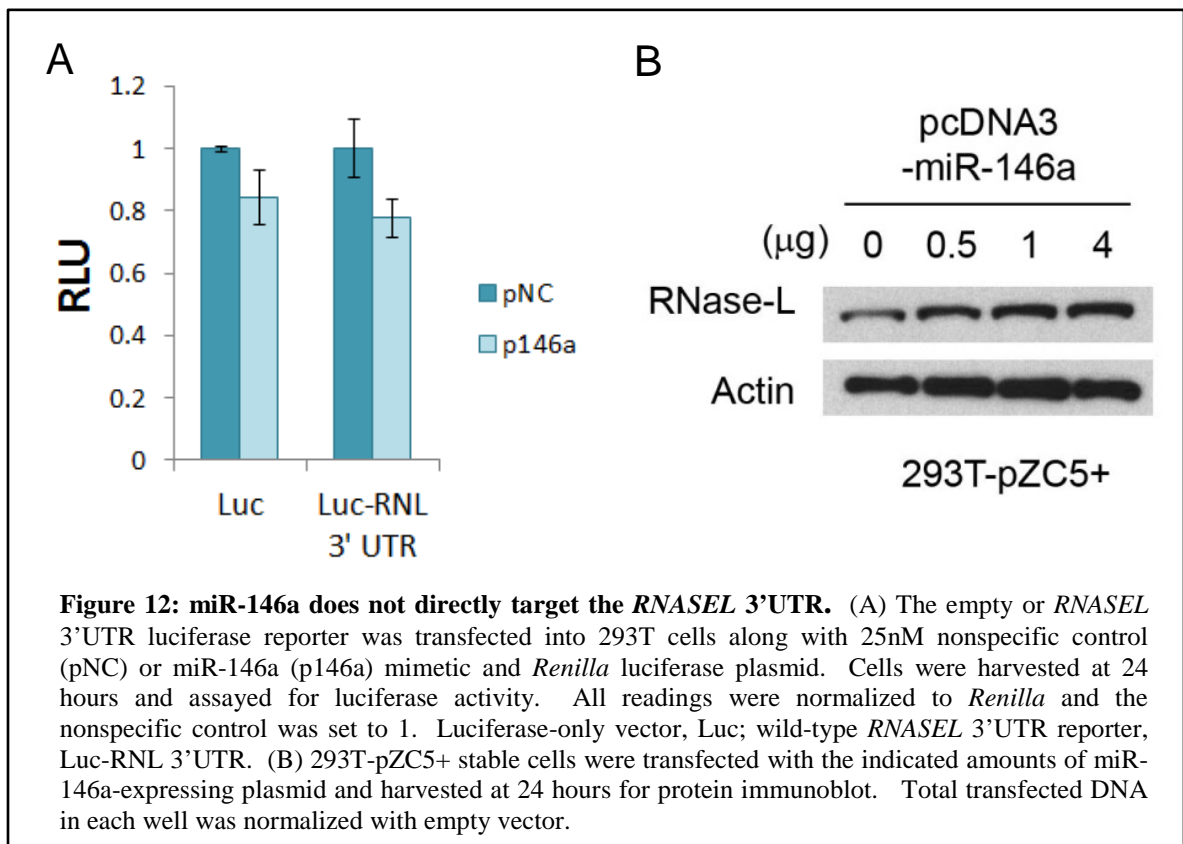
As discussed previously, the miR-29 family (Fig. 11A) is involved in many of the same processes as RNase-L, including immunity, differentiation, senescence, and tumorigenesis (Section 1.2). The miR-29 family is also notable among predicted *RNASEL*-targeting miRNAs for having the highest number of putative binding sites in the *RNASEL* 3'UTR among the conserved miRNA families (Fig. 11B). For this reason, we included it in the analysis of RNase-L-regulating miRNAs.



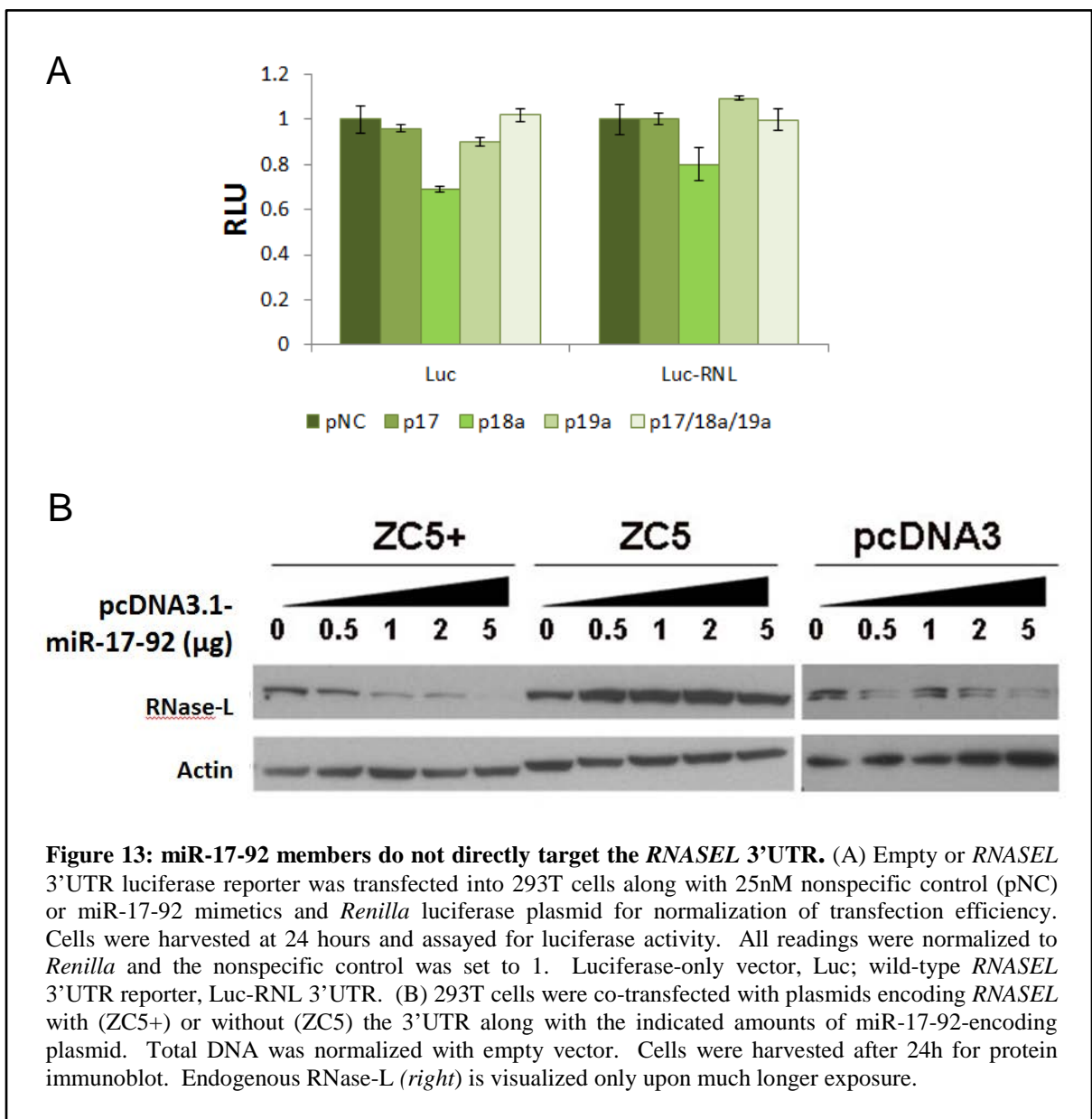
### 3.2.2 Verification of Predicted *RNASEL*-targeting MicroRNAs

To verify direct miRNA repression of RNase-L expression, we generated a luciferase reporter in which the human *RNASEL* 3'UTR was cloned downstream of the

firefly luciferase reporter gene. The reporter construct was then co-transfected into 293T cells with the indicated synthetic miRNA mimetics or a nonspecific control, as well as a *Renilla* luciferase plasmid for normalization of transfection. First, we examined miR-146a as a representative of the miR-146 family (Fig. 12). Upon co-transfection of the miRNA mimetic, no repression of the *RNASEL* 3'UTR reporter was observed beyond that of the empty luciferase vector, indicating that any changes observed were not based on miR-146a binding to the *RNASEL* 3'UTR. Similarly, in 293T cells with constitutive overexpression of a 3'UTR-containing *RNASEL* construct (ZC5+), transfection of a miR-146a-encoding plasmid did not repress RNase-L protein expression, suggesting that RNase-L is not regulated by the miR-146 family.

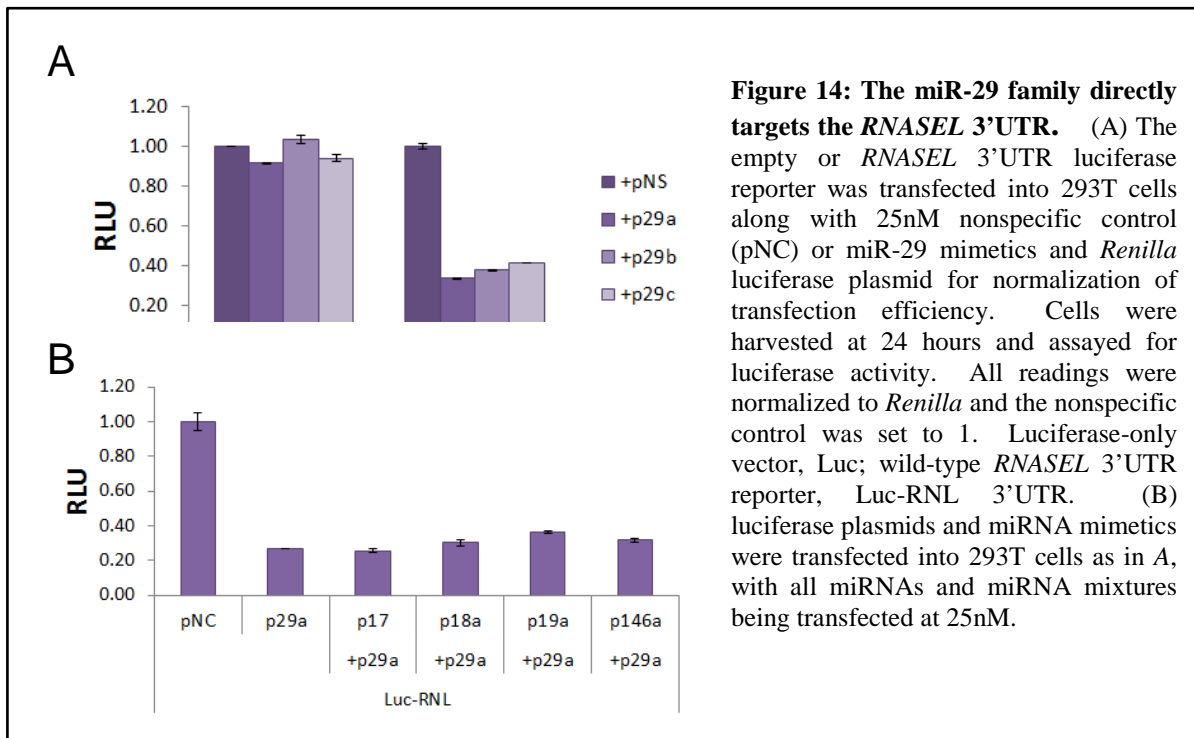


Next, we looked at *RNASEL* regulation by the miR-17-92 cluster of miRNAs. We selected miR-17, miR-18a, and miR-19a to represent the three families that are predicted to regulate RNase-L expression. None of these miRNAs specifically repressed expression of the *RNASEL* 3'UTR reporter beyond what was observed in the empty vector (Fig. 13A). There have been reports of cooperative miRNA action which demonstrate that some miRNAs must work in concert with each other to repress their

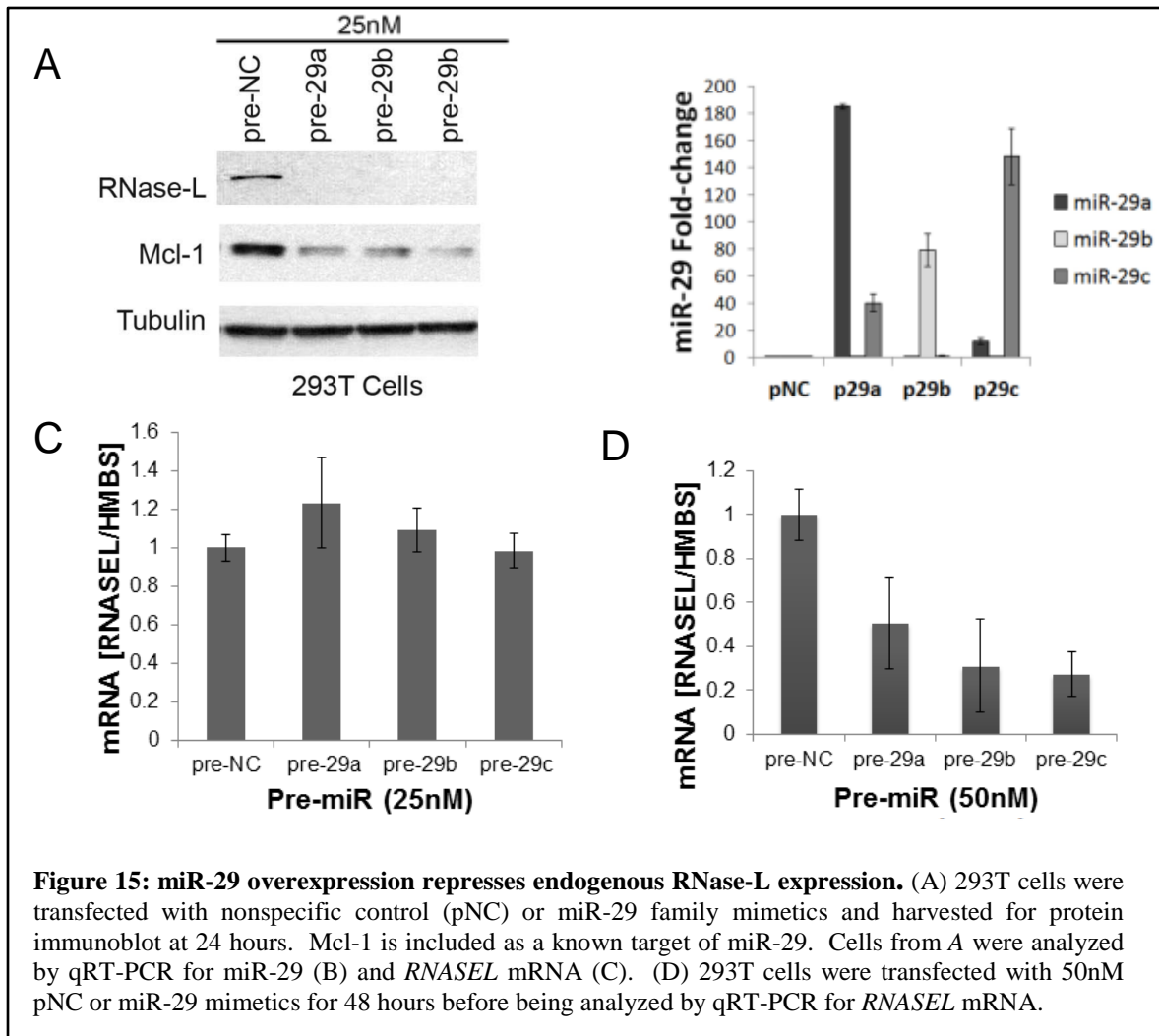


mutual targets (271). To assess the possibility of cooperative action between the miRNA families, we also evaluated a mixture of the three miRNAs; however, we still did not observe any *RNASEL* 3'UTR-specific repression (Fig. 10A). Surprisingly, endogenous RNase-L protein expression was repressed by overexpression of a plasmid encoding the entire miR-17-92 cluster. Moreover, this repression was 3'UTR-dependent, as a cotransfected *RNASEL* construct with the 3'UTR (ZC5+) was repressed while one lacking the 3'UTR (ZC5) was not (Fig. 13B). Overall, these data suggest that the miR-17-92 cluster and its paralogs may regulate RNase-L expression in a more complex manner than can be detected by our assay. For example, regulation may only occur only in the presence of the both coding region and the 3'UTR through a combination of regulatory sites. Alternatively, expression of the entire cluster may have effects that are not observable upon examining individual family members independently.

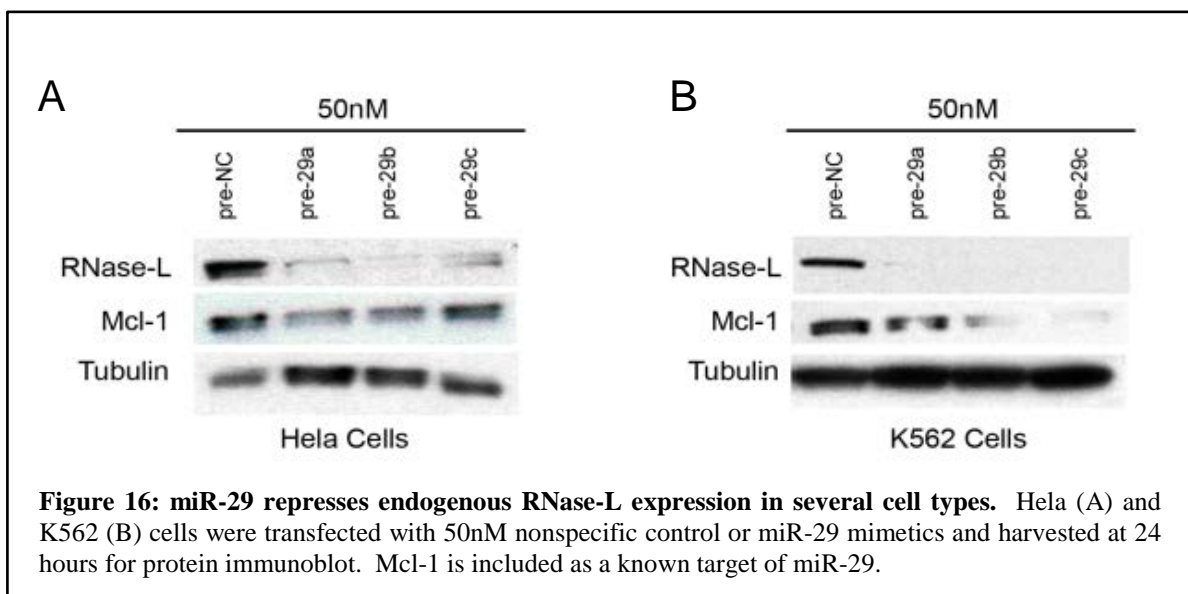
Finally, we analyzed the three members of the miR-29 family. Co-transfection of



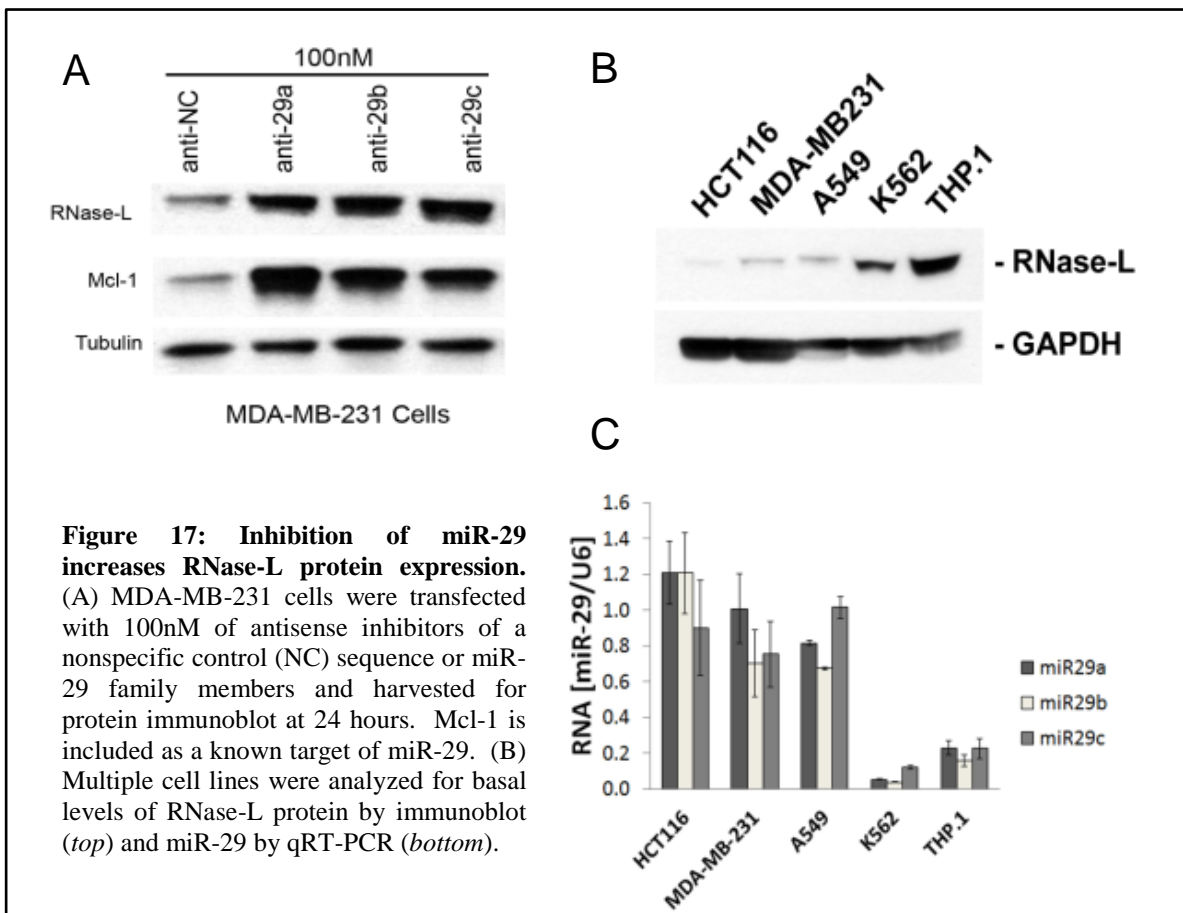
the reporter construct and miR-29 family mimetics into 293T cells resulted in a significant inhibition of luciferase activity that was not observed in the absence of the 3'UTR; thus the *RNASEL* 3'UTR conferred miR-29-dependent regulation by all three family members (Fig. 14A). Despite the lack of repression of the *RNASEL* 3'UTR-luciferase reporter by miR-146a and miR-17-92 cluster members individually, we speculated that they might still coordinate with miR-29 to enhance *RNASEL* repression. Co-expression of these other miRNAs with miR-29 did not increase repression of the reporter, suggesting that these miRNAs do not enhance RNase-L repression in the presence of miR-29 (Fig. 14B).



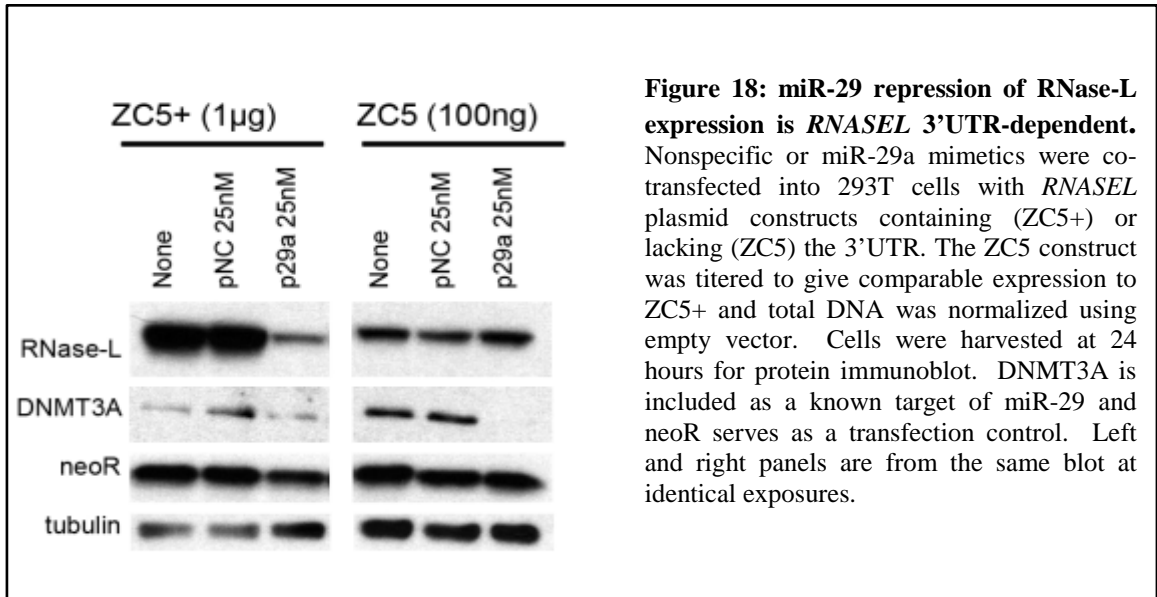
We then assessed the ability of miR-29 family members to repress endogenous RNase-L expression by transfecting miR-29 mimetics into 293T cells. All three miR-29 family members potently repressed expression of RNase-L protein as well as the previously-identified miR-29 target, Mcl-1 (199), while no repression was observed following transfection with a non-specific miRNA control (Fig. 15A). Expression of the mature miR-29 family members was confirmed by qRT-PCR, with transfected miRNAs increasing endogenous levels between 80- and 180-fold (Fig. 15B). The decrease in RNase-L protein in 293T cells occurred without a change in steady-state *RNASEL* mRNA (Fig. 15C), suggesting that miR-29 inhibits RNase-L translation. However, a reduction in *RNASEL* mRNA was observed in some experiments at higher miR-29 concentrations and later time points (Fig. 15D). This finding suggests that miR-29 is capable of influencing both *RNASEL* mRNA stability and translation, though the two processes may occur with different kinetics and efficiencies depending on miRNA concentration. MiR-29-mediated repression of RNase-L expression was also observed in HeLa and K562 cells (Fig. 16).



Consistent with the repression of RNase-L expression by ectopically expressed miR-29, knockdown of endogenous miR-29 using antagonomirs in MDA-MB-231 breast cancer cells (which express high levels of miR-29) resulted in an upregulation of RNase-L protein (Fig. 17A). The inverse relationship between miR-29 and RNase-L observed following miR-29 overexpression or knockdown suggests that RNase-L is a direct miR-29 target. In agreement with this conclusion, expression of miR-29 and RNase-L was inversely correlated in several cell lines examined (Fig. 17B).



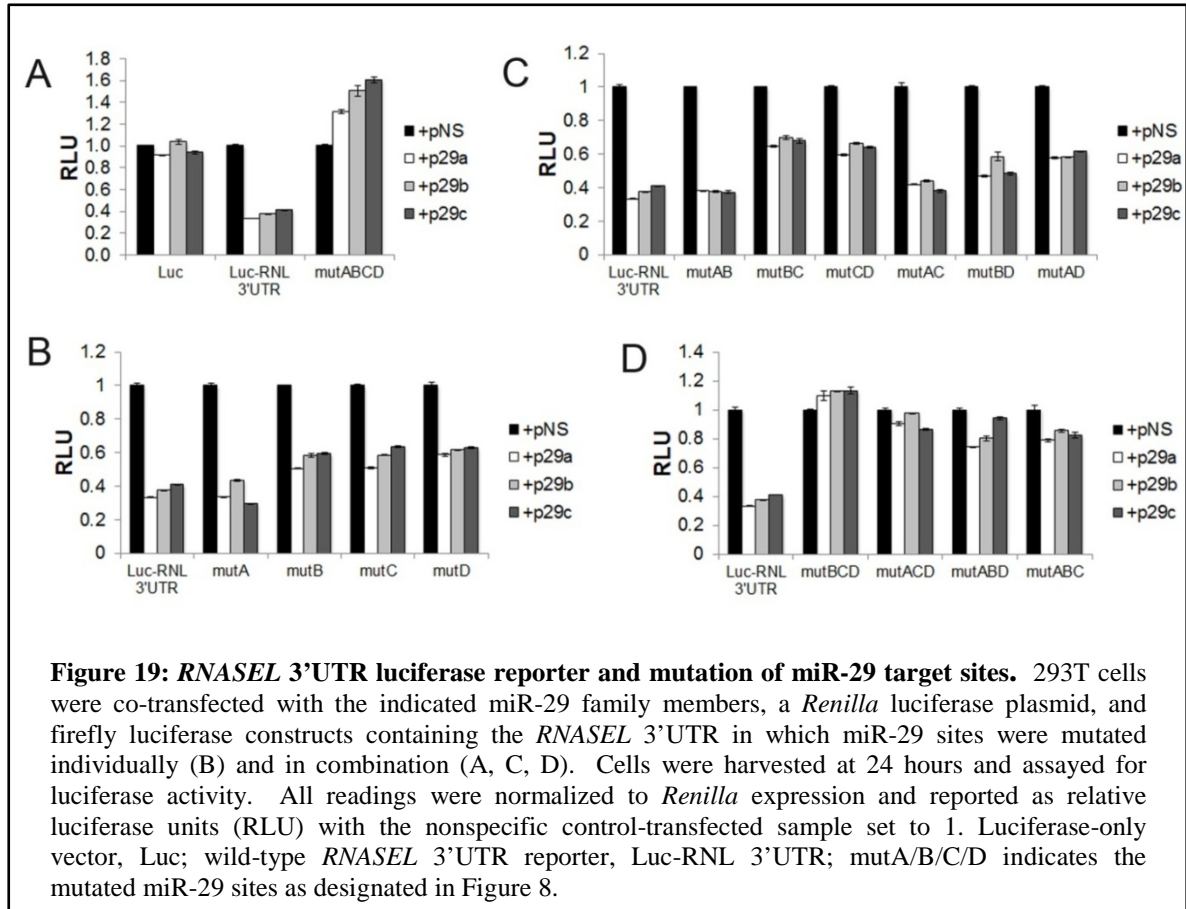
We expected that deletion of the *RNASEL* 3'UTR would abrogate miR-29-mediated regulation. Accordingly, a miR-29a mimetic was co-transfected with an *RNASEL* expression construct that contained (ZC5+) or lacked (ZC5-) the 3'UTR. As



would be expected from the presence of miR-29 binding sites in the *RNASEL* 3'UTR, miR-29a potentially repressed RNase-L expression from the ZC5+ construct but did not markedly alter expression from ZC5 (Fig. 18). Similar results were observed for miR-29b and miR-29c (data not shown). Together, these findings identify miR-29-mediated repression as a novel mechanism regulating RNase-L expression.

### 3.2.3 Analysis of MicroRNA-29 Target Sites in the *RNASEL* 3'UTR

Mutation of miR-29 target sites that are critical for repression of RNase-L expression is predicted to rescue expression and luciferase activity. Therefore, to evaluate the contributions of individual target sites to miR-29-mediated repression, we generated mutations in which the seed regions (nt 2-8) of predicted miR-29 target sites (designated A-D) were deleted. Mutation of all four target sites (*mutABCD*) resulted in the complete loss of miR-29-mediated repression and full rescue of luciferase activity (Fig. 19A) while deletion of between one and three target sites led to partial repression that was proportional to the number of intact target sites (Fig 16B-D). Target site A was



the weakest, as it was neither necessary for complete repression (*mutA*), nor capable of mediating repression on its own (*mutBCD*). Nonetheless, site A exhibited some functionality, as its presence enhanced repression by other more robust target sites (e.g. *mutABD* vs. *mutBD*).

### 3.3 DISCUSSION

RNase-L is generally maintained at fairly low levels in most tissues (27). Given that overexpression of RNase-L has been shown experimentally to induce cellular apoptosis (70), the mechanisms that control RNase-L expression are of significant research interest. In this study we demonstrate that RNase-L expression is post-

transcriptionally regulated by the miR-29 family of miRNAs via four target sites in the 3'UTR of the *RNASEL* mRNA. Repression of an *RNASEL* 3'UTR-luciferase reporter occurred via the additive effects of multiple miR-29 sites, and deletion of at least three sites was necessary to rescue expression (Fig. 19D). We found target site A to be the least effective of the four sites in its individual action. Notably, this site was also the least supported by *in silico* evidence. While it was predicted by microRNA.org (September 2008 release) to be a miR-29 site at the time of our analysis, it was not identified as a site by Targetscan.org (version 5.1) or by later releases of microRNA.org. At the same time, we saw evidence that target site A might nonetheless play a supporting function in miR-29 targeting (*mutABD* vs. *mutBD* or *mutACD* vs. *mutCD*), enhancing repression when stronger target sites were present. Our findings are also consistent with the published deletion analysis of the *RNASEL* 3'UTR (1), with site A falling within the negative regulatory region at nt 2871-3001 and sites B and C falling within another at nt 3193-3753. In this work, removal of a large segment of the 3'UTR that contains all four miR-29 sites gave comparable RNase-L protein expression to the coding sequence construct alone. Finally, while the coding-sequence (ZC5) *RNASEL* construct was significantly more resistant to miR-29-mediated repression than the 3'UTR-containing construct (ZC5+, Fig. 18), we did observe a small amount of miR-29-mediated repression of the ZC5 construct in some experiments (data not shown). This suggests that miR-29 may also directly target one or more as-yet unidentified sequences within the *RNASEL* coding sequence, a region that is not analyzed by most available miRNA target prediction algorithms. RNA22 is a tool that analyzes interactions between any two RNA sequences, allowing for examination of the coding region of *RNASEL* (272). A potential binding site

for miR-29 members is predicted ~200bp downstream of the translation start site by this algorithm, although the contribution of this putative site to miR-29 mediated repression of RNase-L expression remains to be experimentally verified.

While our reporter assays show that target site D is a direct miR-29 binding site that contributes to repression of RNase-L (Fig. 19), previously published deletion analysis places it within a positive regulatory region that enhances basal RNase-L expression (nt 3755-4258). This effect is mediated at least in part through HuR binding and stabilization of *RNASEL* mRNA (1). We predict that binding of miR-29 to the *RNASEL* 3'UTR may impact the activities of additional 3'UTR regulatory elements and their cognate binding factors, such as HuR. Indeed, evidence is emerging that miRNAs and RNABPs can either compete or coordinate to regulate mRNA stability and translation (118, 273). Interestingly, target site D and the predicted HuR binding site lie only ~60bp apart. Binding of either miR-29 or HuR could theoretically alter accessibility to the other regulatory element through changes in local mRNA secondary structure or recruitment of protein partners. The data so far imply an antagonistic relationship between miR-29 and HuR actions; however, further experiments are needed to determine exactly how miR-29 works together with HuR and other potential RNABP regulators of RNase-L to determine expression and whether the outcome of regulation differs between normal and stressed conditions.

We confirmed an inverse relationship between miR-29 and RNase-L protein levels in several different cell types (Fig. 15-17). MiR-29 repression of RNase-L protein expression was observed in the absence of changes in steady state *RNASEL* mRNA, suggesting inhibition of translation. However, we did detect decreases in *RNASEL*

mRNA in experiments using higher amounts of miR-29 and longer transfection times (Fig. 15C-D). It is not clear whether this represents a true mRNA-destabilizing effect of miR-29 that occurs with a slower onset than translational inhibition, an indirect effect mediated by other RNase-L-regulating factors affected by miR-29 overexpression, or an artifact of supra-physiologic miRNA concentrations. The previously-published data are consistent with multiple mechanisms of *RNASEL* 3'UTR-mediated repression (1), as the repression of RNase-L protein expression mediated by the 3'UTR is much stronger than the repression of *RNASEL* mRNA (Fig. 7). Overall, we conclude that miR-29 is capable of repressing RNase-L expression by inhibiting its translation (i.e. without changing its mRNA level), with potential additional repression occurring through destabilization of *RNASEL* mRNA.

In addition to the miR-29 family, we evaluated several other miRNAs predicted to regulate RNase-L expression. MiR-146a did not appear to target *RNASEL* in either luciferase reporter or protein experiments (Fig. 12). This finding is somewhat unexpected given that the miR-146a target site is the only one that shows evolutionary conservation, often presumed to be a predictor of functionality. However, it does not correlate with previously-published data, as the predicted miR-146a site does not fall in any of the regulatory regions identified during deletion analysis. Similarly, members of the miR-17-92 cluster failed to repress expression from the *RNASEL* 3'UTR luciferase reporter (Fig. 13), despite the location of the miR-17a and -18a sites within a confirmed repressive element. Nonetheless, 3'UTR-dependent repression of RNase-L protein expression was demonstrated by the entire miR-17-92 cluster, suggesting that this group of miRNAs may still play a role in regulating RNase-L levels *in vivo*. This regulation

may occur in ways that cannot be detected by a 3'UTR reporter assay. For example, repression may require the combined presence of regulatory elements in both the *RNASEL* coding region and the 3'UTR. Non-3'UTR-restricted RNA binding algorithms like RNA22 could identify potential miR-17-92 binding sites in the coding region for further evaluation. Additionally, the observed repression may be a result of combined expression of the entire cluster. While co-expression of representatives from the three miRNA families predicted to target RNase-L was not-sufficient to affect expression from the luciferase reporter, the presence of the entire plasmid may be required for biologic effect. This may be due to the presence of the remaining cluster members that are not predicted to target *RNASEL* by conventional algorithms. While uncommon, non-seed-based miRNA target recognition has been reported (274-276), and it is possible that these additional miRNAs do in fact target the *RNASEL* mRNA. They also could potentially affect RNase-L expression indirectly by regulating other targets such as RNABP that then act on *RNASEL*, for example.

Our data show that miR-29 plays an important role in modifying post-transcriptional RNase-L expression. However, other miRNA and RNABP regulatory factors that contribute to RNase-L regulation remain to be identified. For example, the *trans* factors that act on the inhibitory 27nt *cis* element at nt 2891-2917 are still unknown. Though we could not evaluate them all, several other miRNAs are predicted to bind within this region as well as within the other negative regulatory regions in the *RNASEL* 3'UTR. Combined with the multiple miR-29 target sites verified here, these candidate miRNA/RNABP binding sites form a potential platform for complex regulatory interactions that mediate rapid and tightly controlled changes in RNase-L expression.

Identification of the specific *cis*-elements and *trans*-acting regulators that control RNase-L expression in distinct biologic settings may reveal strategies to modulate its expression for therapeutic applications.

## **CHAPTER 4: BIOLOGICAL IMPLICATIONS OF REGULATION OF RNASE-L EXPRESSION BY MICRORNA-29**

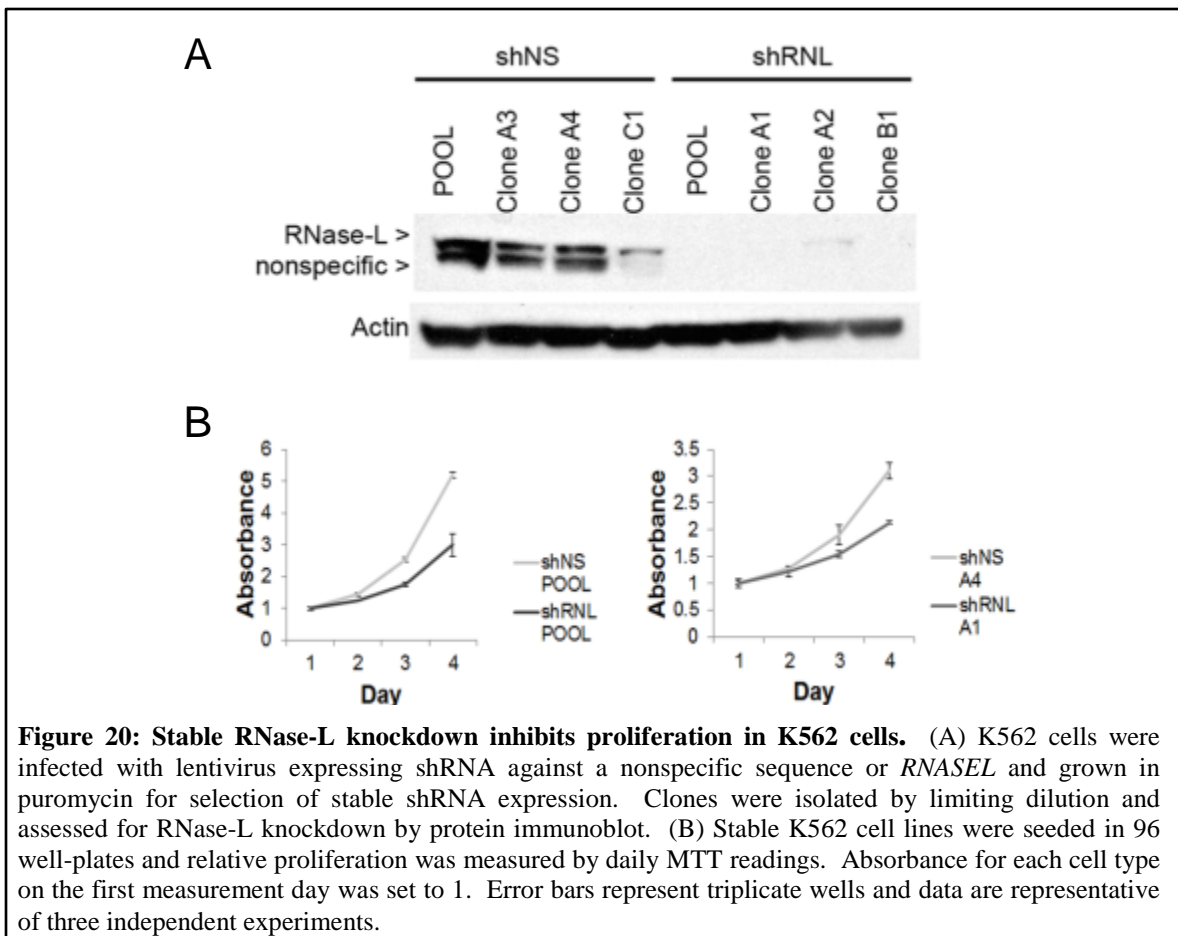
### **4.1 INTRODUCTION**

Our data demonstrating robust miR-29-dependent regulation of RNase-L in cell culture systems suggest that RNase-L is an important miR-29 target and contributes to its biologic functions in physiologic and pathologic settings. Consistent with this prediction, a study of miR-29 tumor suppressor activity in myelogenous leukemias identified *RNASEL* as the most highly repressed mRNA transcript following miR-29 transfection of K562 CML cells (227). We demonstrated that miR-29 transfection also downregulated RNase-L protein expression in K562 cells, validating their inverse relationship (Fig. 16B). The role of RNase-L in antiproliferative and tumor suppressor activities is well established (59, 70, 79), so its identification as a target of miR-29 repression and tumor suppressor activity is surprising and raises the possibility that RNase-L may serve a novel oncogenic function in this setting. Alternatively, if RNase-L is not playing a direct tumorigenic role in this system, loss of miR-29 expression and the resulting elevation in RNase-L might leave cells vulnerable to therapeutic interventions that trigger RNase-L-dependent apoptosis. MiR-29 is known to regulate the expression of many transcripts which may contribute to its tumor suppressor activity (e.g. Mcl-1, Tcl1, CDK6, DNMT3A and 3B) (192, 199, 206, 222); therefore, we took the approach of stably inhibiting RNase-L expression in K562 cells to examine what role RNase-L plays in tumorigenesis in this system independently of other miR-29 targets. In addition, we examined the effects of miR-29 repression on established RNase-L immune functions.

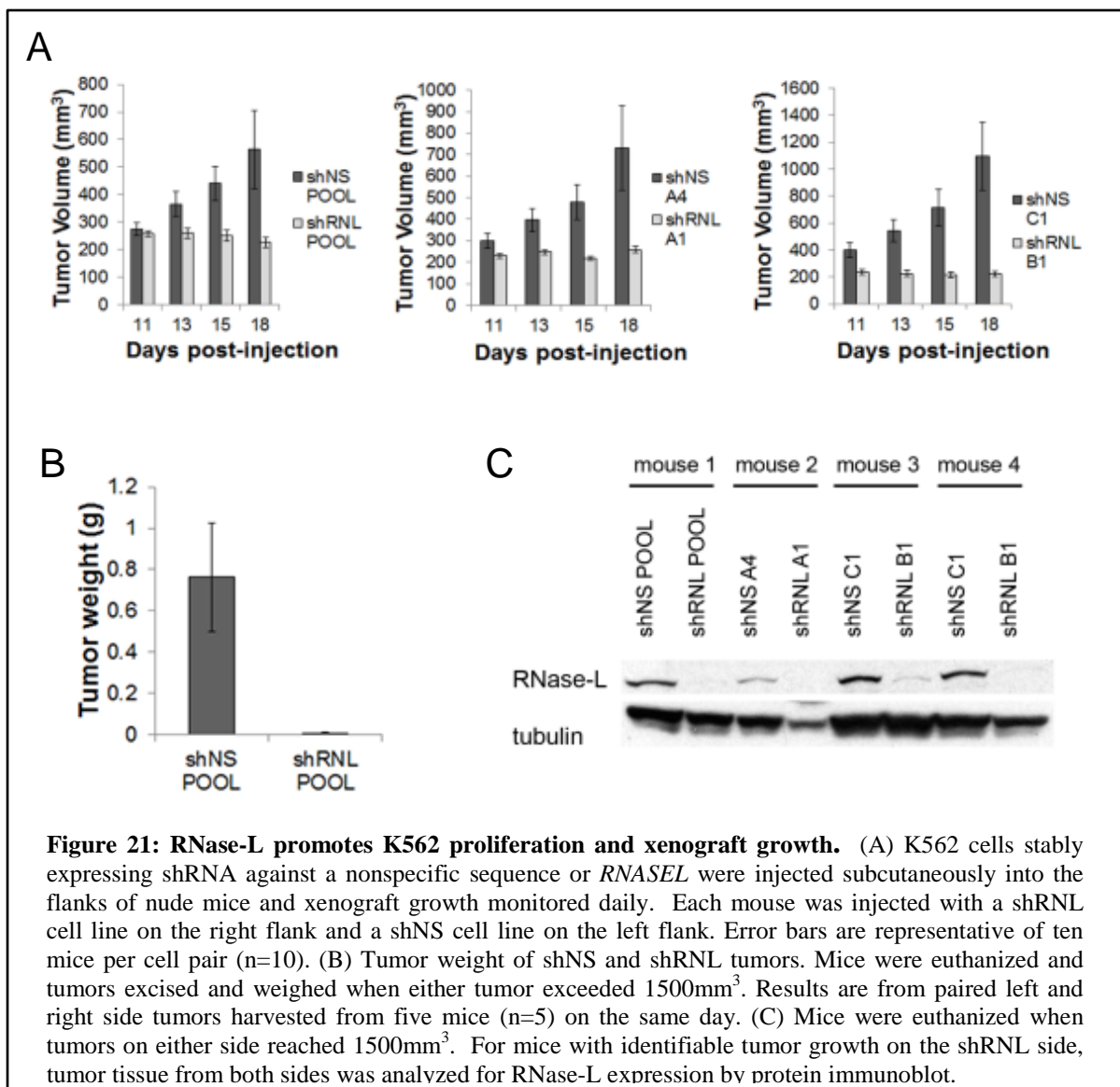
## 4.2 RESULTS

### 4.2.1 MicroRNA-29 Regulation of RNase-L Expression Reveals a Novel Role for RNase-L in Tumorigenesis

We used lentivirus-expressed shRNA to inhibit RNase-L expression in K562 cells, selecting clones that showed stable knockdown of RNase-L (Fig. 20A). We observed that RNase-L knockdown resulted in a reduction in the proliferation of cultured cells (42% in the pools and 31% in the clones at day 4, Fig. 20B), suggesting that RNase-L functions to stimulate proliferation in K562 cells. The same trend was observed in all clones tested (data not shown). More strikingly, RNase-L knockdown dramatically inhibited tumorigenesis in nude mouse xenografts, whereas nonspecific shRNA-



transfected control cells exhibited robust tumor growth (Fig. 21A-B). All control shRNA K562 cells formed detectable tumors by 11 days post-injection whereas the majority of mice injected with RNase-L knockdown cells failed to produce tumors even at 26 days post-injection. Furthermore, RNase-L knockdown cells failed to produce tumors in several mice that were monitored through day 46 post-injection (data not shown). In the few small tumors generated from RNase-L knockdown cells, functional knockdown of RNase-L expression was maintained at the time of tumor harvest (Fig. 21C). Thus,



RNase-L knockdown phenocopied the tumor suppressive activity of miR-29 in K562 xenografts, revealing a novel tumorigenic role for RNase-L in this setting.

Unlike miR-29 overexpression, which sensitizes cells to apoptosis induced by various chemotherapeutic agents (199, 227), RNase-L knockdown did not enhance cell death in response to a panel of cytotoxic compounds (Table 4), suggesting that other miR-29 targets are responsible for this aspect of miR-29 tumor suppressive activity. More research is needed to determine the mechanisms by which RNase-L promotes tumorigenesis in CML cells.

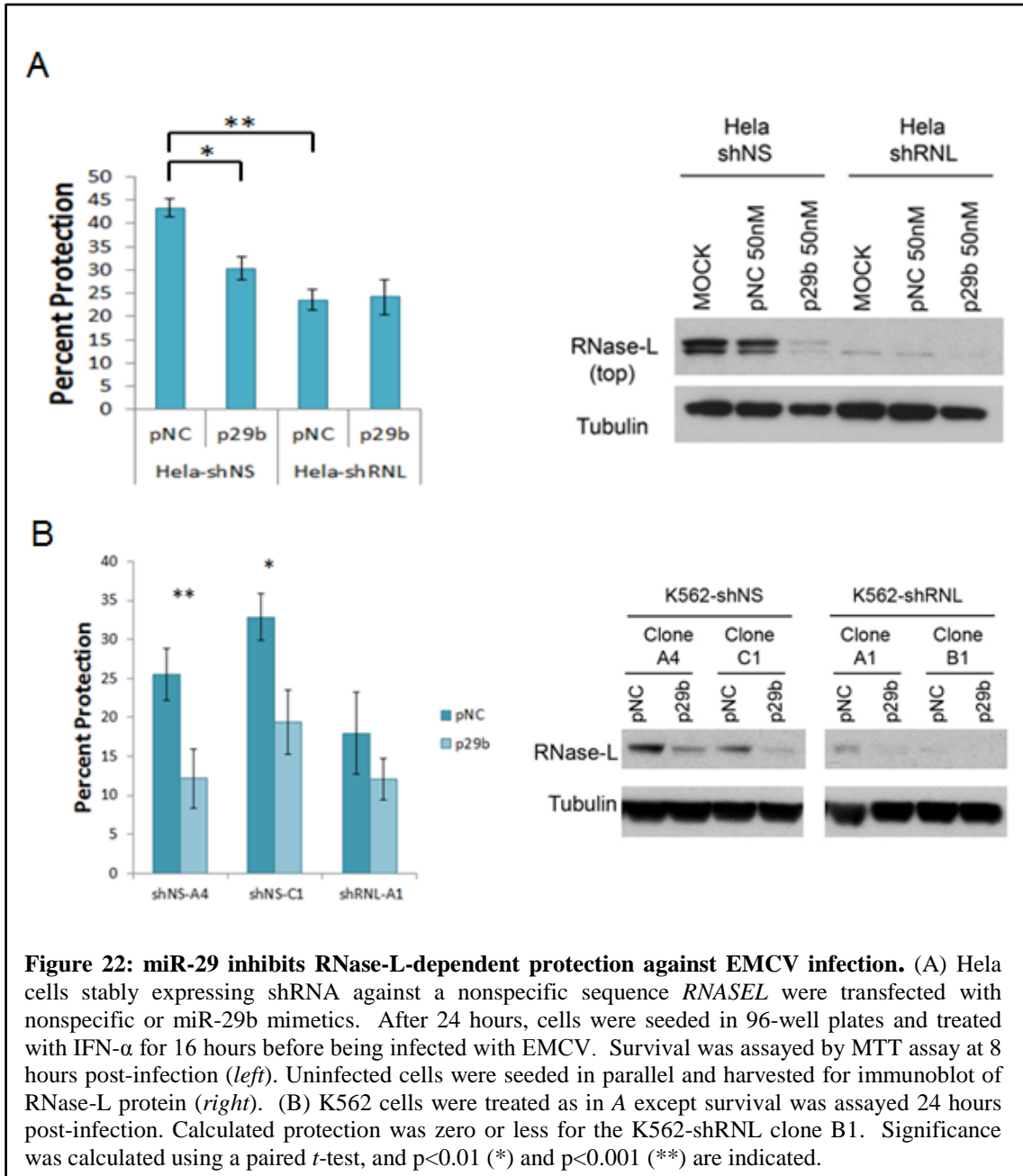
DRUG	SURVIVING CELLS (%) $\pm$ S.D.		p value
	shNS clones	shRNL clones	
Adriamycin (5 $\mu$ M)	68.8 $\pm$ 13.4	55.8 $\pm$ 15.9	0.3398
Camptothecin (5 $\mu$ M)	66.3 $\pm$ 12.6	53.3 $\pm$ 10.9	0.2482
Staurosporine (500nM)	64.4 $\pm$ 9.2	73.4 $\pm$ 16.0	0.4445
Diethyl Maleate (1mM)	39.6 $\pm$ 5.5	42.4 $\pm$ 4.7	0.5412
Cytarabine (50nM)	45.9 $\pm$ 2.9	62.1 $\pm$ 10.3	0.0579
Imatinib (5uM)	48.8 $\pm$ 4.09	54.2 $\pm$ 24.4	0.7438

**Table 4: RNase-L Knockdown does not Affect Response to Cytotoxic Agents.** K562 cells stably expressing shRNA against a nonspecific sequence or *RNASEL* were seeded in 96-well plates and exposed to drugs at the indicated concentrations for 24-72 hours. Surviving cells were measured by MTT assay. shNS clones and shRNL clones were compared using Student's t-test.

#### 4.2.2 MicroRNA-29 Regulation of RNase-L Expression Affects RNase-L

##### Antiviral Function

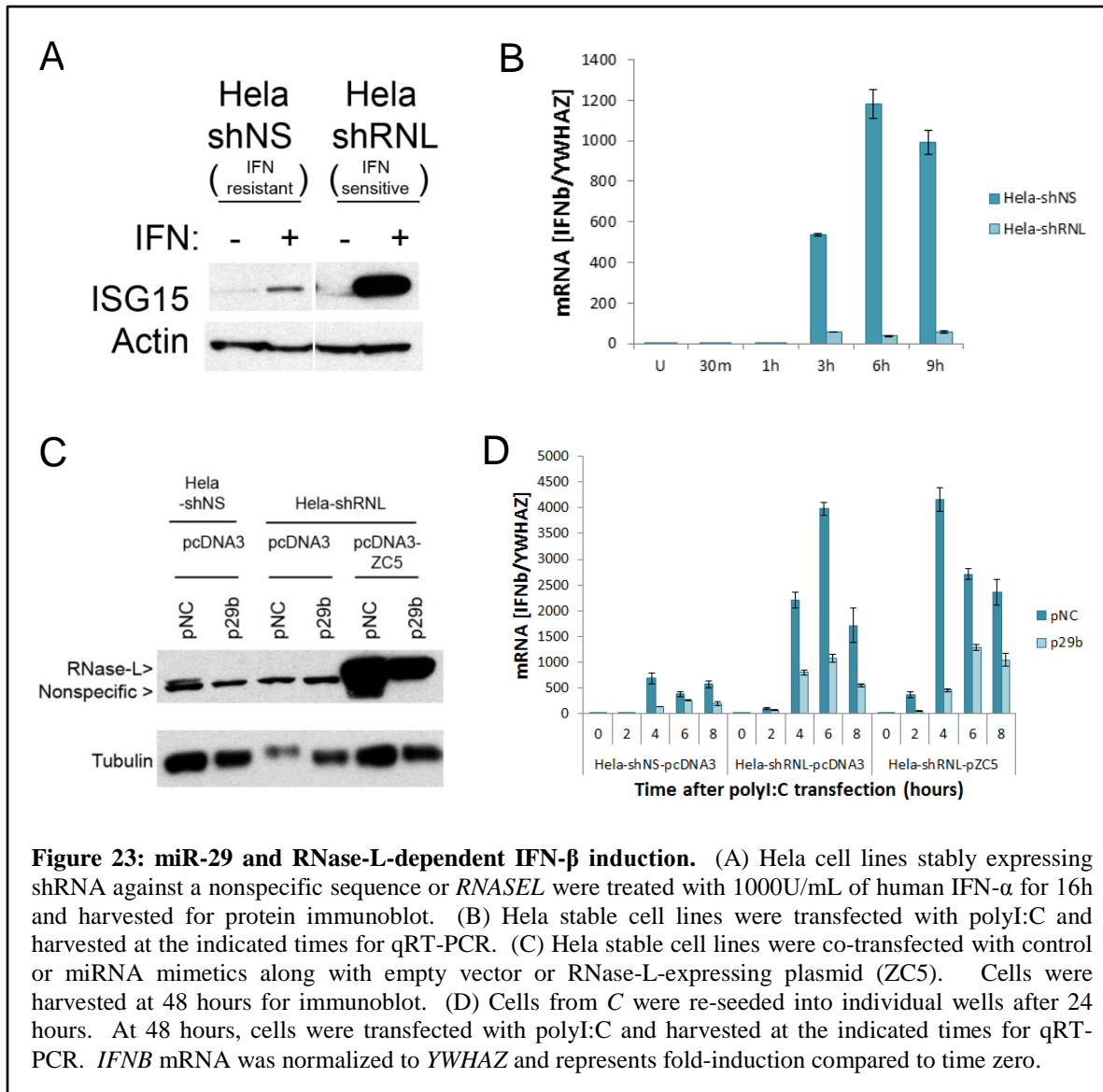
We hypothesized that miR-29 repression of RNase-L expression would affect its biologic function. Therefore, we also examined the effect of miR-29 repression on a



known RNase-L activity, namely protection from viral infection. RNase-L is necessary for IFN-induced protection against EMCV infection (59), as we confirmed in pooled HeLa cells stably expressing a nonspecific or RNase-L-specific shRNA (Fig. 22A). IFN treatment led to more than 40 percent protection from EMCV infection in shNS cells and about half that amount in shRNL cells. When transfected into HeLa cells, miR-29b was

able to inhibit both RNase-L protein expression and IFN-induced protection from EMCV to levels similar to that observed in the shRNL cells. This effect was RNase-L dependent, as miR-29b transfection into shRNL cells did not cause further inhibition of protection. MiR-29 repression of RNase-L protein expression and antiviral activity was also observed in our K562 cells with stable RNase-L knockdown (Fig. 22B). We attempted to rescue miR-29-mediated repression of RNase-L expression and antiviral activity by co-transfection of an RNase-L plasmid construct (ZC5), but were unable to complete the experiment in our HeLa stable cells due to loss of IFN-responsiveness that developed in our cell lines that abrogated the protective effects of IFN. Measurement of the interferon-stimulated gene 15 (ISG15) protein was used as a measure of a functional IFN signaling pathway activity, and ISG15 induction was markedly diminished in IFN-resistant as compared to IFN-sensitive HeLa cells (Fig. 23A). We also attempted rescue of miR-29 inhibition of RNase-L antiviral activity in K562 cells, but were not able to successfully co-transfect both the RNase-L construct and miR-29 mimetics (data not shown).

In an attempt to bypass the requirement for IFN signaling, we turned instead to another RNase-L immune function, namely, the enhancement of primary IFN production in IFN-naïve cells through the cleavage of viral RNA or RNA mimetics (e.g. polyI:C) into PAMPs that can activate the cytosolic dsRNA sensor RIG-I (40, 41). We confirmed that RNase-L is required for maximal IFN- $\beta$  induction following transfection of polyI:C (Fig. 23B), and we were able to demonstrate that transfection of miR-29b alone or with the ZC5 *RNASEL* coding sequence construct repressed and rescued RNase-L expression, respectively (Fig. 23C). However, following co-transfection of miRNA mimetics and



plasmid-encoded RNase-L, polyI:C-induced IFN-β production was also observed in RNase-L knockdown cells (Fig. 23D). This effect did not appear to be RNase-L-dependent, as IFN-β production was similar in knockdown cells rescued with ectopic RNase-L expression. In contrast, the IFN-resistant HeLa-shNS cells did not show an enhanced polyI:C-induced IFN-β response following DNA/miRNA transfection as compared to naïve cells (Fig. 23B), suggesting that our manipulations themselves may have induced IFN production and autocrine signaling that sensitized the IFN-responsive

cells to respond more strongly to polyI:C transfection. Interestingly, miR-29 expression inhibited IFN- $\beta$  production in all conditions, suggesting that besides RNase-L, it may regulate other innate immune components involved in sensing and responding to dsRNA.

### 4.3 DISCUSSION

The strong miR-29-dependent regulation of RNase-L involving multiple mRNA target sites suggests that it is an important target in miR-29-mediated biologic functions. Indeed, miR-29 family members and RNase-L are implicated in an overlapping set of physiologic and pathologic activities including antiviral immunity (56, 140, 261), tumorigenesis (81, 83, 89, 126, 192, 199, 209, 210, 222) and myogenesis (47, 126, 148). In this study, we focused on the regulation of RNase-L in the context of miR-29 tumor suppression of myelogenous leukemias, as *RNASEL* was identified as a candidate miR-29 target in this system (227). MiR-29 transfection of K562 CML cells was reported to reduce proliferation, enhance basal and drug-induced apoptosis, and inhibit tumorigenesis. Therefore, our validation of *RNASEL* as a miR-29-repressed transcript in K562 cells suggested that RNase-L serves a novel oncogenic function in this setting. Consistent with this prediction, stable knockdown of RNase-L, independent of other miR-29 targets, decreased K562 proliferation and inhibited tumorigenesis in a xenograft model (Fig. 21). Surprisingly, RNase-L knockdown did not phenocopy the enhanced sensitivity to proapoptotic chemotherapeutic agents observed following ectopic expression of miR-29 (199, 227); these phenotypes may be mediated by other miR-29 targets (e.g. *Mcl-1*). Further research is necessary to determine the effect of RNase-L on

basal apoptosis in CML, along with other processes that naturally limit tumorigenesis such as senescence or differentiation.

The defect in K562-shRNL xenograft growth manifested early, with most injections of shRNL cells not establishing detectable tumors. The generation of K562 cells with inducible knockdown would allow for the examination of RNase-L function in tumor progression rather than initiation, by turning off RNase-L expression in tumors that are already established. K562-shRNL tumors did not provide enough tissue for microarray analysis; an inducible shRNA system would also allow us to determine which mRNAs are regulated by RNase-L in CML. Importantly, RNase-L endoribonuclease activity is intact in K562 cells (69), therefore its cleavage of RNA targets is expected to play a role in its oncogenic function. The identity of RNase-L targets in this setting would provide insight into the mechanisms by which RNase-L might promote tumor growth in CML and other malignancies.

At this point, the mechanisms of RNase-L oncogenic function are still unclear. However, its role in innate immunity and particularly its regulation of inflammatory mediators suggest several productive directions for future inquiry. The link between inflammation and cancer has been well-studied. On one hand, inflammation can contribute to tumor suppression by recruiting and activating the immune system to kill tumor cells. However, sustained inflammation can also promote malignancy by causing tissue damage, chronic proliferative repair, and tumor invasion (277, 278). RNase-L activation is known to regulate production of several inflammatory cytokines including interleukins-1, -8, and -15 (IL-1, IL-8, IL-15) and TNF- $\alpha$  (34, 45). Dysregulated secretion of such factors could recruit and activate immune cells that promote

inflammation and tissue damage. Assessment of inflammatory cytokine production from our stable K562 cell lines and histological examination of xenografts (including tumor architecture and immune cell infiltration) will shed light on the role of inflammation in RNase-L-induced oncogenesis.

In addition to uncovering a novel oncogenic function for RNase-L, we demonstrated that miR-29 regulation affects the established antiviral activity of RNase-L. Overexpression of miR-29 repressed RNase-L protein expression as well as RNase-L-dependent protection against EMCV infection (Fig. 22). We also examined the effect of miR-29 on the RNase-L-dependent induction of IFN- $\beta$  in naïve cells, but encountered difficulties when our transfection procedures apparently sensitized cells to produce IFN- $\beta$  in a non-RNase-L-dependent manner. The fact that the enhancement occurred only in IFN-responsive cells suggested to us that it was itself IFN-mediated. This was supported by the upregulation of ISG15 in parental Hela cells we subjected to the same transfection protocol (data not shown); therefore, we tried several approaches to minimize the effects of transfection. We ensured that our plasmid stocks were free of endotoxin that might induce IFN- $\beta$ , and we extended the post-transfection rest period in the hopes that cells would return to an unstimulated state. We also attempted to carry out our transfection in the presence of IFN- $\beta$  neutralizing antibody to counter any potential IFN- $\beta$  secretion. However, none of these approaches eliminated the priming effect. Generation of cell lines with stable miR-29 expression or knockdown might overcome this barrier for future experiments and allow for examination of miR-29 regulation of this particular RNase-L function.

Finally, our data also suggest that miR-29 might inhibit the expression other innate immune effectors involved in IFN- $\beta$  production besides RNase-L. If true, this would demonstrate an important immune function for miR-29 that, like the coordinated regulation of the ECM (139, 145), involves targeting of multiple steps of the same process. Future research is also needed to determine how miR-29 regulation affects other RNase-L functions such as antibacterial immunity and differentiation (45, 46) and whether RNase-L plays any role in known miR-29-regulated processes, such as the TGF- $\beta$ -induced production of ECM components and resulting fibrosis.

## CHAPTER 5: SUMMARY AND PERSPECTIVES

### 5.1 INTRODUCTION

RNase-L is an IFN-regulated endoribonuclease whose activation can have potent antimicrobial effects as well as profound consequences for cell survival and proliferation (2, 279, 280). Activation of this enzyme is controlled at multiple levels, from transcription and activation of upstream OAS enzymes to formation and degradation of its small molecule ligand, 2-5A. However, until recently, less was known about the control of RNase-L expression itself. The *RNASEL* 3'UTR was discovered to mediate robust post-transcriptional regulation of RNase-L protein expression (1), and we presented evidence in this work that miRNAs, specifically the miR-29, family are involved in RNase-L repression in many cell types, including CML cells (Chapter 3).

We observed that miR-29 repression can modulate RNase-L antiviral function. In addition, the known tumor suppressor role of miR-29 in cancer in general and in CML in particular raised questions about the role of RNase-L in this malignancy. Our findings provide the first evidence of a causative role for RNase-L in tumorigenesis (Chapter 4) and are supported by previous reports of cancer-associated increases in RNase-L activity and expression in CML and colon cancer (95, 96). Furthermore, microarray analyses have revealed that RNase-L is upregulated in diverse types of leukemias, being among the top 10% of upregulated genes in certain studies (281, 282). RNase-L, however, was not altered in a focused analysis of AML (227), suggesting that it contributes to the oncogenic phenotype only in a subset of malignancies. Additional studies are required to determine the specific settings in which RNase-L mediates tumorigenic functions and the molecular mechanisms involved in these activities.

In the following sections, we discuss some of the major implications of our findings, both in terms of how they fit into our current understanding of RNase-L function, as well as prospective future experiments that could expand our knowledge of the mechanisms and biologic consequences of miR-29 regulation of RNase-L.

## **5.2 DUAL FUNCTIONS FOR RNASE-L IN CANCER**

Our data supporting a tumorigenic role for RNase-L contrasts with the established antiproliferative and tumor suppressor activities of RNase-L (59, 70, 79) and suggest that it mediates context-specific tumor suppressor versus oncogenic activities. In fact, many proteins and regulatory RNAs that play contrasting roles in tumorigenesis have been reported, with the Myc transcription factor family being a notable example (283). On one hand, Myc is a prototypical oncogene which is frequently overexpressed in cancers. However, Myc also has many effects that inhibit tumorigenesis, such as Myc-induced sensitization to apoptotic stimuli and induction of senescence. These effects are barriers to tumor development that must be overcome for the oncogenic functions of Myc to predominate; hence, additional cooperative mutations that inactivate such pathways are often necessary for Myc-induced tumorigenesis. Interestingly, miR-29 itself is another regulator of gene expression that has been reported to have dual functions in malignancy (284) (Section 1.2.3). These opposing functions may reflect distinct mRNA target profiles in different cells or environmental stimuli that tip the balance towards repression of tumor suppressor or oncogenic gene products, respectively. Indeed, some verified miR-29 targets are repressed in one cell type but not another (199, 201, 206, 238). In addition, miR-29 repression of different targets within the same system has been shown

to lead to divergent functional outcomes (e.g. granulocytic vs. monocytic differentiation in various leukemia cell lines) (183).

These observations also have relevance for RNase-L and tumorigenesis. Like Myc, RNase-L has been well-established as an inducer of apoptosis and senescence (69, 79), while in this work it has instead been shown to promote tumor growth. It is therefore possible that, as with Myc, mutations that suppress the tumor suppressor pathways downstream of RNase-L may be necessary for its oncogenic functions to prevail. Additionally, the recognition of RNase-L-dependent regulation of specific targets in different conditions (35, 36, 285) suggests that, like miR-29, its functions may also depend on the identity of its substrates in individual malignancies.

### **5.3 POTENTIAL MECHANISMS OF RNASE-L SELECTIVE TARGETING AND RNASE-L INVOLVEMENT IN A POST-TRANSCRIPTIONAL REGULATORY NETWORK**

The specific mechanisms by which RNase-L impacts the global RNA expression profile to exert tumorigenic activity remain to be determined. Despite evidence of selective targeting of specific RNAs, a strict consensus sequence for RNase-L binding and cleavage has not been identified. One hypothesis is that secondary structures in specific RNAs (e.g. folded stretches of dsRNA) could activate OAS enzymes to produce 2-5A and activate RNase-L in a localized manner, providing a mechanism of target selectivity. This model has been supported in experiments using synthetic RNAs, but it remains to be tested with endogenous substrates (286).

In addition to substrate-specific characteristics, we also hypothesize that RNase-L target selectivity may depend on its interaction with various RNABPs; in this model, the RNABPs expressed in different cell types or cancers bind and recruit RNase-L to cleave specific RNAs and thus dictate its function as an oncogene or tumor suppressor. To date, RNase-L has been reported to interact with three RNABPs: the translation termination factor eRF3 (53), the mitochondrial translation initiation factor IF2mt (51), and the ARE-binding protein TTP (BAH unpublished). Of these, eRF3 and IF2mt are general factors involved in the translation of mRNAs and mtRNAs, respectively; it is therefore unclear whether or how they would be capable of mediating selective RNase-L cleavage of specific mRNAs or mtRNAs. In contrast, we observed a protein-protein interaction between RNase-L and the selective ARE-binding protein TTP as well as RNase-L-dependent regulation of a subset of TTP targets. Whether this effect represents direct RNase-L cleavage of these mRNAs remains to be determined, but we hypothesize that the binding of RNase-L to TTP may play a role in TTP-dependent regulation of inflammatory mediators such as TNF- $\alpha$  that could promote cancer (75).

The link between RNase-L and TTP is complicated by several other factors. One is the fact that RNase-L regulates *TTP* mRNA in addition to binding TTP protein (52) (BAH, unpublished). A recent study reported that RNase-L destabilization of *TTP* mRNA led to de-repression of the TTP target *p21<sup>Cip1/WAF1</sup>*, whose protein product is an inducer of cell cycle arrest. The authors hypothesized that this pathway represents another mechanism of RNase-L tumor suppressor activity (52). This dichotomy highlights the importance of target identity in determining functions of RNA regulatory proteins and RNAs. Finally, an additional level of complexity is provided by the fact that

both *TTP* and *RNASEL* have been shown to be miR-29 targets (237) (Chapter 3). Further research is required to understand the complicated relationships between miR-29, TTP, and RNase-L.

On a larger scale, a picture is emerging of an intricate post-transcriptional regulatory network that involves many RNABPs and regulatory RNAs. The interplay between RNase-L, TTP, and miR-29 represent only one portion of this network. The regulation of *RNASEL* mRNA by the ARE-binding protein HuR forms another known element of the network. HuR has been reported to stabilize *RNASEL* mRNA and increase RNase-L protein expression under conditions of cell stress (1). RNase-L, in turn, destabilizes the mRNA encoding HuR (36), forming a negative feedback loop. In addition, both HuR and TTP are reported to regulate their own expression, binding to their own mRNAs and stabilizing or destabilizing them, respectively (287, 288). MiRNAs are also an important part of this network, functioning both to regulate expression of RNABPs (237, 289) as well as to act either cooperatively or competitively with RNABPs in the regulation of their targets (273). An interesting as-yet unanswered question is whether RNase-L activity affects expression of miRNAs, either directly through RNA cleavage or indirectly through regulation of miRNA processing components. Our laboratory is currently performing microarray analyses to determine which miRNAs might be subject to RNase-L regulation. Identification of such miRNAs would expand the repertoire of known RNase-L-targeted RNAs and open up new questions regarding its physiologic functions. In summary, the effect of RNase-L in cancer appears to involve significant reprogramming of a large post-transcriptional

network that cooperates to affect the gene expression profile to promote or inhibit tumorigenesis.

#### **5.4 RATIONALE FOR A PROTEIN-CENTRIC APPROACH TOWARDS RNASE-L RESEARCH**

The post-transcriptional regulation of RNase-L has an important implication for cancer research methodology. Given that at least some of the effects of miR-29 on RNase-L expression occur via translation inhibition (Fig. 15), we predict that genomic mutation-based or microarray-based studies may not accurately reflect the true level of RNase-L expression and activity. Indeed, RNase-L germline mutations have been discovered to have a range of effects on the mature protein, from completely or partially repressing expression to reducing enzymatic activity (83, 84). One analysis of RNase-L mutations and prostate cancer risk found that a missense mutation leading to reduced RNase-L dimerization and catalytic activity was associated with increased prostate cancer risk, while another mutation which completely abolished RNase-L protein expression was not (86). The authors suggested that some mutations might actually create new RNase-L protein products that exhibit dysregulated or novel activities, therefore representing gain-of-function rather than loss-of-function mutations. Alternatively, post-translational mechanisms can lead to altered RNase-L protein activity independent of genomic mutation. For example, dysregulation of RNase-L in the PBMCs of CFS patients is associated with the generation of unique 30kDa and 37kDa RNase-L protein fragments which display increased RNA cleavage activity (98, 99). These fragments have been hypothesized to differ from intact RNase-L in their regulation, activity, and

even target specificity. In light of our hypothesis that the protein binding partners of RNase-L may play a role in determining target specificity (Section 5.3), it is plausible that RNase-L post-translational modifications can lead to altered interaction with RNABPs and dysregulated recognition of mRNA targets. Ultimately, research that examines actual levels of the various RNase-L protein products, RNase-L enzymatic activity, and even RNase-L protein-protein interactions in specific malignancies may be instrumental in identifying cancers in which RNase-L plays a functional role. Conventional RNase-L cleavage assays based on degradation of radioactively-labeled RNA species or ribosomal RNA (43, 290) are cumbersome or relatively insensitive, respectively. However, recently-developed assays for detection of 2-5A or RNase-L activity that are based on fluorescence resonance energy transfer (FRET) (291) may facilitate a more functional approach to RNase-L research.

## **5.5 ADDITIONAL STUDIES OF RNASE-L FUNCTION**

Besides the miR-29/RNase-L story, our laboratory has pursued several lines of experimentation involving other regulatory mechanisms and functions of RNase-L, and our inquiries have yielded interesting preliminary data that also suggest avenues for future research (Appendix). The self-regulatory nature of some RNABP led us to investigate the possibility that RNase-L regulates its own expression by binding to the *RNASEL* 3'UTR (Fig. 24). We found no evidence that RNase-L is able to regulate expression from a *RNASEL* 3'UTR luciferase reporter in this manner. It is possible that RNase-L could recognize the *RNASEL* mRNA through motifs not located in the 3'UTR; in this case, studies using differentially-tagged RNase-L plasmid constructs could provide

definitive evidence for or against RNase-L regulation of its own expression. Next, the link between RNase-L and induction of inflammatory cytokines led us to examine the specialized pathway of inflammatory cytokine production known as pyroptosis (Fig. 25). *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> macrophages clearly displayed dissimilar responses to pyroptotic stimuli, with differences in detectable intracellular pro-IL-1 $\beta$  and cell survival. The evidence suggests that RNase-L may facilitate IL-1 $\beta$  release without associated cell death, but further research is needed to definitively demonstrate this and determine what the consequences of this protection are *in vivo*. Finally, we explored the possibility that RNase-L is involved in regulation of the cytoplasmic recycling pathway known as autophagy (Fig. 26). We observed consistent differences in the level of autophagosome membrane marker proteins between *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> cells in response to a number of autophagic stimuli; however, our data does not allow us to state at this point whether this represents an enhancement or a blockage of autophagy in RNase-L deficient cells. Functional studies of autophagic activity (e.g. recycling of a labeled cytosolic target or autophagic degradation of virus) are necessary to determine what role RNase-L plays in this process.

## 5.6 CONCLUSIONS

The 2-5A/RNase-L system is a tightly-regulated IFN-responsive RNA decay pathway that is involved in both antimicrobial activity and regulation of cell-proliferation. Control of this pathway involves the rapid regulation of the molecular ligand 2-5A; in this work, we also demonstrate that RNase-L expression itself can be repressed within hours by the miR-29 family of microRNAs, leading to detectable

inhibition in RNase-L antiviral activity. This regulation occurs via four different miR-29 binding sites, allowing for both additive repression and for combinatorial regulatory interplay with other RNABP and miRNAs that may act on the *RNASEL* 3'UTR. MiR-29 regulation of RNase-L expression in CML led us to examine the function of RNase-L in this system. We observed a novel effect whereby RNase-L promotes CML cell proliferation and xenograft growth in a nude mouse model. This oncogenic role for RNase-L conflicts with established reports of antiproliferative and tumor suppressive effects of RNase-L and opens up the question of how and in what settings does RNase-L promote proliferation and cell growth. Ultimately, it is our hope that an increasing understanding of the cancer-specific functions of RNase-L combined with the development of RNase-L-targeted therapeutics (292) will lead to the advent of clinical applications that modulate this pathway.

## **APPENDIX**

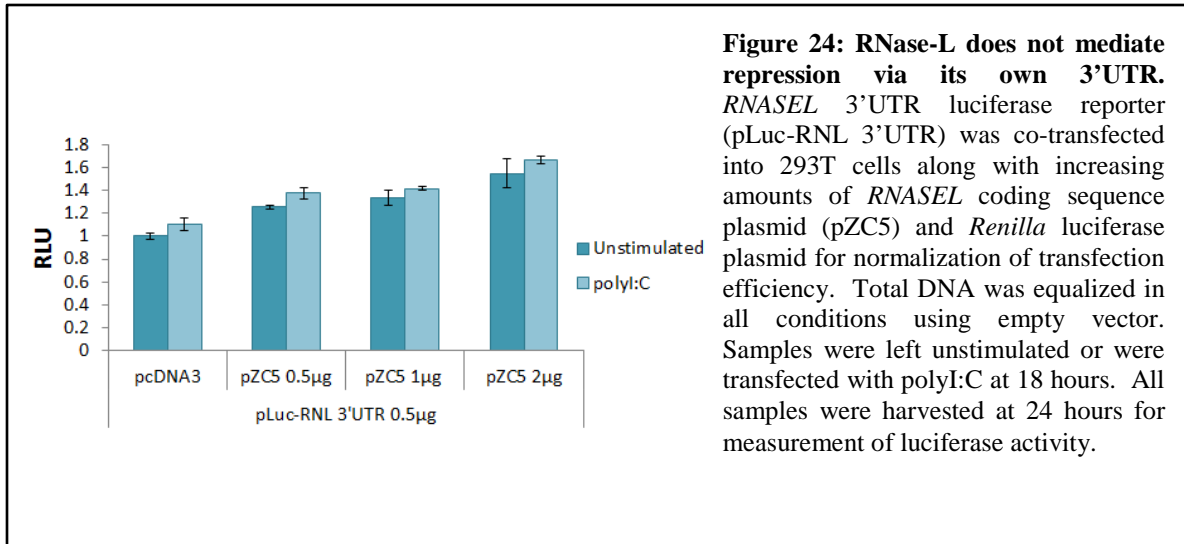
### **6.1 RNASE-L PROTEIN DOES NOT ACT DIRECTLY ON THE *RNASEL* 3'UTR TO REGULATE EXPRESSION**

#### **6.1.1 Introduction**

The *RNASEL* 3'UTR exerts a strong regulatory effect on its expression. This work describes the role of the miR-29 family in mediating repression of RNase-L expression via the *RNASEL* 3'UTR, while previous reports have identified a HuR-binding region in the *RNASEL* 3'UTR that mediates increased RNase-L expression under conditions that induce HuR cytoplasmic translocation (1). We predict that other RNABPs and miRNAs may also play a role in RNase-L regulation. HuR and another RNABP, TTP, are both ARE-binding proteins whose mRNAs are regulated by RNase-L (36, 52). In addition, they both regulate their own expression, binding to AREs in the 3'UTR sequences of their own mRNAs to stabilize and destabilize the transcripts, respectively (287, 288). We hypothesized that RNase-L might also modulate its own expression in a similar fashion. Therefore, we used an *RNASEL* 3'UTR luciferase reporter to evaluate RNase-L-dependent regulation via its own 3'UTR.

#### **6.1.2 Results and Discussion**

We co-transfected the *RNASEL* 3'UTR luciferase reporter into 293T cells with increasing amounts of ZC5 *RNASEL* plasmid lacking the 3'UTR. Ectopic RNase-L protein expression did not repress luciferase production from the *RNASEL* 3'UTR reporter, even when polyI:C was transfected to ensure RNase-L activation (Fig. 24).



The long 3'UTR of *RNASEL* (1.8kb) contains both known and suspected *cis* elements that mediate post-transcriptional regulation. We asked whether RNase-L can regulate its own expression via the 3'UTR of its mRNA, a mechanism that is observed among other RNABPs. Our preliminary data do not support a role for *RNASEL* 3'UTR-dependent autoregulation. However, the possibility that RNase-L acts on regions of its mRNA other than the 3'UTR (i.e. the coding region or 5'UTR) cannot be excluded; this hypothesis could be addressed in future experiments.

## 6.2 RNASE-L REGULATES THE PRODUCTION OF INFLAMMATORY CYTOKINES VIA PYROPTOSIS

### 6.2.1 Introduction

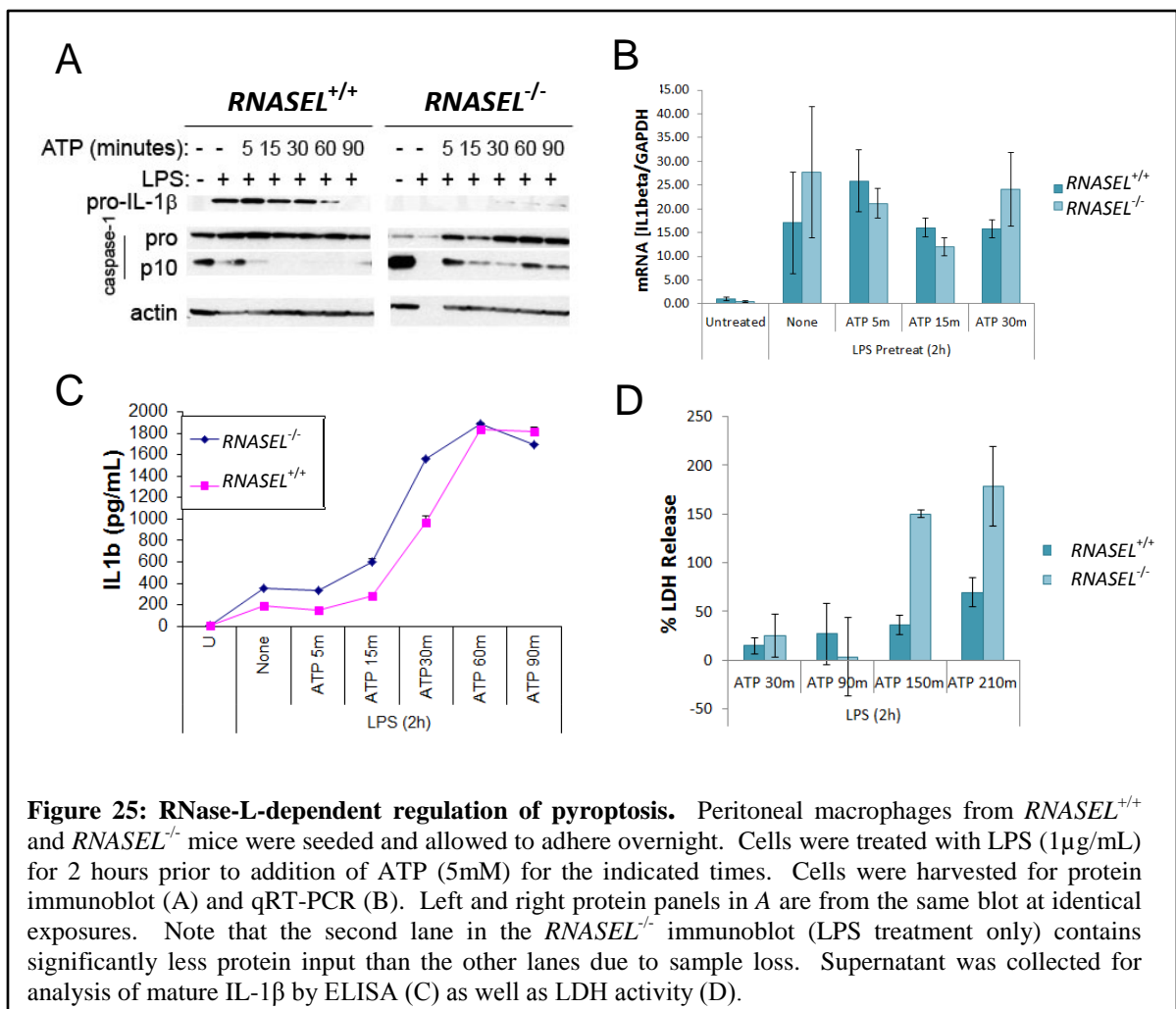
RNase-L has been reported to promote production of pro-inflammatory cytokines during both Gram-positive and Gram-negative infection *in vivo* (45). In particular, macrophages from *RNASEL*<sup>-/-</sup> mice show decreased production of IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\beta$  in response to *E. coli* infection. This defect was associated with delayed activation

of *RNASEL*<sup>-/-</sup> macrophages and impaired bacterial clearance. Production of IL-1 $\beta$  requires processing of the inactive precursor, pro-IL-1 $\beta$ , into its mature form by caspase-1. Caspase-1 is also required for a unique form of inflammatory cell death known as pyroptosis (293). In contrast to the immunologically silent apoptosis pathway, pyroptosis results in cell rupture and release of pro-inflammatory cytokines including IL-1 $\beta$ , IL-18, and others (294). However, cytokine production can occur independently of cell death (295). Caspase-1 itself is normally inactive until cleaved; various pathogen-associated and host-associated stimuli (e.g. intracellular bacteria, uric acid crystals, K<sup>+</sup>-depletion triggered by extracellular ATP, others) can signal through cytosolic receptors including members of the cytoplasmic NLR family to induce an assembly of proteins known as the inflammasome which activates caspase-1 (296). A commonly used experimental model induces pyroptosis using LPS pretreatment as a “first signal” to induce transcription and translation of pro-IL1- $\beta$ . ATP is then added as a “second signal” to induce inflammasome formation and caspase-1 autoproteolysis and activation (297, 298). IL-1 $\beta$  release occurs rapidly within minutes of ATP treatment. The observation that RNase-L promotes IL-1 $\beta$  production prompted us to examine whether it may play a role in pyroptosis.

### **6.2.2 Results and Discussion**

We examined peritoneal macrophages from *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> mice, comparing their ability to undergo caspase-1 activation, IL-1 $\beta$  production, and cell death. LPS treatment led to induction of *IL1B* mRNA and pro-IL1 $\beta$  protein in *RNASEL*<sup>+/+</sup> macrophages (Fig. 25A-B). *RNASEL*<sup>-/-</sup> macrophages showed comparable IL-1 $\beta$  mRNA

induction to the wild-type cells, but much lower levels of pro-IL-1 $\beta$  protein. We did not detect any intracellular mature IL-1 $\beta$  for either cell type, most likely due to its rapid release following formation. Surprisingly, measurement of mature IL-1 $\beta$  in the supernatant by ELISA showed comparable induction of mature IL-1 $\beta$  in both cell types, with slightly faster kinetics in the *RNASEL*<sup>-/-</sup> cells (Fig. 25C). In general, pro-caspase-1 levels did not vary significantly with RNase-L status; however, RNase-L<sup>-/-</sup> macrophages displayed higher levels of the active p10 subunit of caspase-1, even in the untreated cells (Fig. 25A). Cell death was measured by LDH release into the supernatant (Fig. 25D). Though no differences were detected at early time points, LDH release was higher in



*RNASEL*<sup>-/-</sup> cells starting at 150 minutes after ATP treatment.

The regulation of pyroptosis is clearly altered in RNase-L-deficient macrophages, though the exact nature of this dysregulation is not yet clear. Both the published data on RNase-L-dependent production of inflammatory cytokines (45) and our pro-IL-1 $\beta$  protein data (Fig. 25A) would suggest that RNase-L promotes IL-1 $\beta$  production. However, release of mature IL-1 $\beta$  was just as efficient if not slightly more so in the *RNASEL*<sup>-/-</sup> macrophages (Fig. 25C). Further research is necessary to determine the significance of these conflicting results. One possible interpretation is that while RNase-L-deficient cells are able to produce IL-1 $\beta$ , their increased susceptibility to cell death ultimately leads to depletion of the macrophage population and attenuation of the *in vivo* inflammatory response. This hypothesis is supported by the basal elevation of active caspase-1 in *RNASEL*<sup>-/-</sup> macrophages as well as the increased cell death following ATP treatment (Fig. 25A and D). The lower levels of pro-IL-1 $\beta$  in these cells would thus represent rapid cleavage and secretion following translation rather than a failure of induction. Experimentally, treatment of cells with caspase-1 inhibitors prior to stimulation of pyroptosis could reduce the effects of differential IL-1 $\beta$  cleavage/release and allow for comparison of basal pro-IL-1 $\beta$  protein production. Additionally, while it was previously observed that *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> mice injected intraperitoneally with *E. coli* had similar percentages of macrophages in their peritoneal fluid at 72 hours by light microscopy (45), examination at later time points post-infection or the use of functional endpoints (e.g. resistance to secondary infection) might clarify the role of RNase-L in pyroptosis-related inflammation.

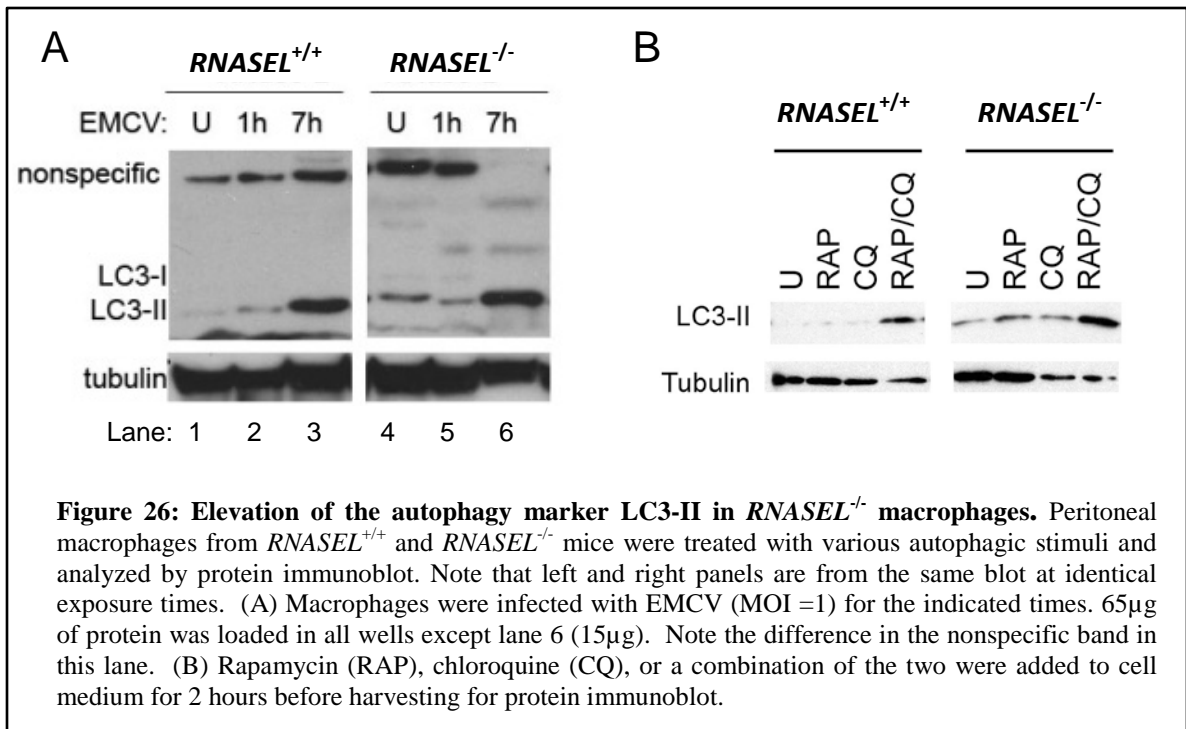
## 6.3 RNASE-L REGULATION OF THE AUTOPHAGIC PATHWAY

### 6.3.1 Introduction

Autophagy is a mechanism by which intracellular material can be targeted to the endolysosomal pathway for degradation (299). During autophagy, cytoplasmic components, even structure as large as whole organelles, become surrounded by a unique autophagosomal double-membrane that then fuses with lysosomes, leading to degradation of the contents. Autophagosome membranes are distinguished by the presence of the small protein marker known as LC3. During membrane formation, LC3 becomes modified post-translationally and attaches to the autophagosome membrane (300). The 18kDa unmodified and 16kDa modified forms are referred to as LC3-I and LC3-II, respectively, and the conversion from LC-I to LC3-II is frequently used as an indicator of autophagosome formation (301, 302). This pathway is important for recycling of cellular components and is important during cell stress (303). Additionally, it serves an immune function by allowing for degradation of intracellular pathogens and exposure of their antigens to the immune system via cross-presentation (304). Microarray analysis of *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> macrophages identified the mRNA encoding the aspartic protease CatE as a substrate of RNase-L (45). One of the functions of CatE is the degradation of the lysosomal-associated membrane proteins-1 and -2 (LAMP-1 and -2) which are necessary for fusion of various vesicular bodies with lysosomes (305). Indeed, *RNASEL*<sup>-/-</sup> mouse macrophages showed an increase in *CATE* mRNA stability and elevated CatE protein, as well as a decrease in LAMP-1/2 protein (45). We hypothesized that a defect in RNase-L could lead to dysregulation of LAMP-1/2 expression and impairment of processes involving lysosomal fusion such as autophagy.

### 6.3.2 Results and Discussion

We measured LC3 proteins in *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> peritoneal macrophages exposed to a variety of autophagic stimuli. LC3-I protein was not always detectable, but we consistently observed higher levels of LC3-II in RNase-L-deficient cells even at basal level (Fig. 26). LC3-II levels increased with autophagic stimuli such as EMCV infection, rapamycin treatment, or chloroquine treatment and were also generally higher in the *RNASEL*<sup>-/-</sup> macrophages.



Our preliminary observation that LC3-II levels differ consistently in *RNASEL*<sup>+/+</sup> and *RNASEL*<sup>-/-</sup> cells supports the idea that RNase-L is involved in regulating autophagy. However, the nature of this regulation is not yet clear. The direct use of LC3-II level as an indicator of autophagy or even the monitoring of LC3-positive autophagosomes by microscopy has been questioned in recent years (306, 307). LC3-II may reflect an increase in autophagosome production, but could also indicate blockage of lysosomal

fusion and degradation. Accurate differentiation between the two requires either the use of known inhibitors of lysosomal fusion (which are expected to increase autophagosome accumulation except in the presence of an existing block in fusion) or measurement of degradation rates of known autophagy substrates such as the autofluorescent drug monodansylcadaverine (308, 309). Such techniques could clarify the nature of RNase-L regulation of the autophagic pathway.

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