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Small RNA Regulation of the Innate Immune Response: A Role for Dicer in the Control of Viral Production and Sensing of Nucleic Acids: A Dissertation

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SMALL RNA REGULATION OF THE INNATE IMMUNE RESPONSE: A ROLE FOR DICER IN THE CONTROL OF VIRAL PRODUCTION AND SENSING OF NUCLEIC ACIDS

A Dissertation Presented By

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“I was gratified to be able to answer promptly and I did. I said I didn’t know.”

-Mark Twain, Life on the Mississippi

“Do not take life too seriously. You’ll never get out of it alive.”

-Elbert Hubbard

“There are already surgeons in France and in Germany, who introduce into the abdominal cavity or under the skin of their patients either warmed blood serum or ***nucleic acid*** *or other substance, with the object of bringing to the scene a protective army of phagocytes to ward the microbes off. The results achieved are so encouraging that it is possible to predict new progress in the approach to the dressing of wounds. “*

-Ilya Mechnikov, excerpt from his Nobel lecture, 1908.
Emphasis added.

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Thank you, thank you, thank you.

ABSTRACT

All organisms exist in some sort of symbiosis with their environment. The food we eat, air we breathe, and things we touch all have their own microbiota and we interact with these microbiota on a daily basis. As such, we employ a method of compartmentalization in order to keep foreign entities outside of the protected internal environments of the body. However, as other organisms seek to replicate themselves, they may invade our sterile compartments in order to do so. To protect ourselves from unfettered replication of pathogens or from cellular damage, we have developed a series of receptors and signaling pathways that detect foreign bodies as well as abnormal signals from our own perturbed cells. The downstream effector molecules that these signaling pathways initiate can be toxic and damaging to both pathogen and host, so special care is given to the regulation of these systems. One method of regulation is the production of endogenous small ribonucleic acids that can regulate the expression of various receptors and adaptors in the immune signaling pathways. In this dissertation, I present work that establishes an important protein in small ribonucleic acid regulation, Dicer, as an essential protein for regulating the innate immune response to immuno-stimulatory nucleic acids as well as regulating the productive infection of encephalomyocarditis virus. Depleting Dicer from murine embryonic fibroblasts renders a disparate type I interferon response where nucleic acid stimulation in the Dicer null cells fails to produce an appreciable interferon response while infection with the paramyxovirus, Sendai, induces a

more robust interferon response than the wild-type control. Additionally, I show that Dicer plays a vital role in controlling infection by the picornavirus, encephalomyocarditis virus. Encephalomyocarditis virus fails to grow efficiently in Dicer null cells due to the inability for the virus to bind to the outside of the cell, suggesting that Dicer has a role in modulating viral infection by affecting host cellular protein levels. Together, this work identifies Dicer as a key protein in viral innate immunology by regulating both the growth of virus and also the immune response generated by exposure to pathogen associated molecular patterns. Understanding this regulation will be vital for future development of small molecule therapeutics that can either modulate the innate immune response or directly affect viral growth.

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18S	refers to the ribosomal subunit
5’ pp	5 prime diphosphate
5’ ppp	5 prime triphosphate
AP-1	activator protein 1
APOBEC3	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BHK-21	baby hamster kidney 21 cell line
BLV	bovine leukemia virus
BMDMØ	bone marrow derived macrophage
CARD	caspase activation and recruitment domain
cDNA	coding DNA
cGAS	cyclic GMP-AMP synthase
CRE	causes recombination
CTD	C-terminal domain
DAI	DNA-dependent activator of interferon
DAMP	damage-associated molecular pattern
DCL	Dicer-like
DExD/H box	aspartate-glutamate-any amino acid-aspartate/histidine-box
DI	defective interfering
$Dicer^{O}$	Dicer oocyte
$Dicer^{S}$	Dicer somatic
DNA	deoxyribonucleic acid
DNA-PK	deoxyribonucleic acid-dependent protein kinase
ds	indicates “double-stranded” when preceding DNA or RNA
EIF3C	eukaryotic initiation factor 3C
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
ESC	embryonic stem cell
ESR1	estrogen receptor 1
flox	flanking of loxP sites
GFP	green fluorescent protein
GMP	guanosine monophosphate
GusB	glucuronidase beta
HA	hemagglutination
HEK293	human embryonic kidney 293 cell line

HIV	human immunodeficiency virus
HPRT	hypoxanthine-guanine phosphoribosyltransferase
hs	refers to “homo sapiens” when in front of a gene name
HSV	herpes simplex virus
HSV60mer	herpes simplex virus derived 60mer oligo
IFI16	interferon-gamma-inducible protein 16
IFI204	interferon activated gene 204
IFIT	interferon-induced protein with tetratricopeptide repeats
IFITM	interferon induced trans-membrane
IFN	interferon
IFNAR	interferon alpha/beta receptor
IPS-1	interferon beta promoter stimulator 1
IRAK	interleukin 1 receptor-associated kinase
IRES	internal ribosome entry site
IRF	interferon regulatory factor
IKK	I kappa B kinase
ISG	interferon stimulated gene
JAK	Janus kinase
kb	refers to “kilobase” when discussing nucleic acids
kDa	refers to “kiloDalton” when discussing protein size
KO	refers to “knockout” when discussing genotypes
LENDIS	length distribution
LGP2	laboratory of genetics and physiology 2
LINE	long interspersed nuclear elements
loxP	locus of crossing over P1
LPS	lipopolysaccharide
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling
MDA5	melanoma differentiation-associated gene 5
MEF	murine embryonic fibroblast cell
MOI	multiplicity of infection
MRE11	meiotic recombination 11
mRNA	messenger ribonucleic acid
miRNA	micro ribonucleic acid
MT-C	mouse transposon C
MX1	myxovirus resistance 1
NA	nucleic acid
NCoR	nuclear receptor co-repressor
NF-κB	nuclear factor kappa B
NLRP3	NOD-like receptor family, porin domain containing 3
NS	refers to “non-silencing” when discussing siRNAs
nt	refers to “nucleotide” when discussion nucleic acids

-OH	hydroxyl group
PACT	protein kinase R activating protein
PAMP	pathogen-associated molecular pattern
PAZ	Piwi-Argonaute-Zwille
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque-forming units
piRNA	Piwi ribonucleic acid
Piwi	P-element induced wimpy testis
PKR	protein kinase R
poly dA:dT	polydeoxyadenylic:polydeoxythymidylic acid
poly I:C	polyriboinosinic:polyribocytidilic acid
PRR	pattern recognition receptor
PYHIN	Pyrin and hematopoetic expression, interferon-inducible nature, and nuclear localization
qPCR	quantitative polymerase chain reaction
Ran-GTPase	Ras-related nuclear guanosine triphosphatase
RANTES	regulated on activation, normal T cell expressed and secreted
rasiRNAs	repeat-associated short interfering RNAs
RdRP	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene I
RISC	RNA induced silencing complex
RLH	RIG-I like helices
RLR	RIF-I like receptor
RNA	ribonucleic acid
RNAa	ribonucleic acid activation
RNAi	ribonucleic acid interference
RNase	ribonuclease
RNA Pol II	ribonucleic acid polymerase II
RNA Pol III	ribonucleic acid polymerase III
RSAD2	radical s-adenosyl methionine domain-containing protein 2
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
SARS	severe acute respiratory syndrome
SeV	Sendai virus
SINE	short interspersed nuclear elements
siRNA	short interfering ribonucleic acid
SMRT	silencing mediator of retinoid acid and thyroid hormone receptors
SOCS1	suppressor of cytokine signaling 1
ss	indicates “single-stranded” when preceding DNA or RNA

STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TAK1	transforming growth factor beta-activated kinase 1
TBK1	Tank-binding kinase 1
TLR	Toll-like receptor
TRAF6	TNF receptor-associated factor 6
TRBP	trans-activating response RNA binding protein
UTR	untranslated region
VACV70mer	vaccinia virus derived 70mer oligo
VCAM	vascular cell adhesion molecule
VISA	virus-induced signaling adaptor
VLDLR	very low-density lipoprotein receptor
vsRNAs	virus-derived small RNAs
vsiRNAs	virus-derived short interfering RNAs
VSV	vesicular stomatitis virus
WT	refers to “wild-type” when discussing genotypes
ZBP-1	Z-DNA binding protein 1

CHAPTER I – Introduction

ANTI-VIRAL IMMUNOLOGY

OVERVIEW

For most organisms, there is a constant battle between the host and a number of invading pathogens. These pathogens often use host resources for replication, nourishment and transmission. Thus, in order to protect oneself, an organism must constantly evolve ways to defend itself against invasion from foreign pathogens. This evolutionary arms race has resulted in systems that are potent and can be deadly to both pathogen and host. Dysregulation of the immune response can cause everything from minor perturbations, such as allergy or hypersensitivity response, up to death if the commensurate response to infection is too severe or too weak. Thus a tight, yet agile regulation of these systems must be maintained at all times in order to protect the host from collateral damage as well as to provide for the rapid and correct response of the immune system to combat invading pathogens. Additionally, there must be specificity to distinguish between foreign and self-materials as well as breadth in order to defend against pathogens that vary greatly in size, shape, function and location. Distinguishing between foreign and self-antigens allows the host to avoid induction of auto-immunity while providing a comprehensive defense against infection. In order to develop new strategies and treatments of disease, it is vital not only to understand the many forms of recognition but also the mechanisms of regulation present in the immune response.

The mammalian immune response can be broken down into two distinct but connected systems: the innate immune response and the adaptive immune response. Charles Janeway first proposed the idea that the two systems work in a coordinated response in order to provide general and specific immunity against a variety of pathogens.[1] Briefly, the innate immune system relies on recognizing a limited number of foreign motifs called pathogen-associated molecular patterns (PAMPs) or danger motifs called DAMPs damage-associated molecular patterns (DAMPs). In both cases, pattern recognition receptors (PRR) have evolved to detect evolutionarily-conserved motifs, rather than species-specific antigens.[2] For example, the first PRR whose function was clearly described in mammals, Toll-like receptor 4, recognizes the common Gram-negative bacterial cell wall component LPS, without needing to make a number of distinctions for the multitude of species of Gram negative bacteria.[3,4] The recognition of PAMPs or DAMPs then starts a variety of signaling cascades that produce different outcomes depending on the type of receptor and adaptor proteins that are engaged. Many of the components arising from a bacterial, parasitic, or fungal infections can give rise to an inflammatory response, where a series of cytokines, chemokines and effector molecules are released from infected cells or surveying innate immune cells and cause chemotaxis of other immune cells, edema, and pyrexia. Recognition of replicating virus during infection more commonly give rise to a type-I interferon based response, which can have a mild or non-existent inflammatory component. The activation of the inflammatory or interferon

pathways depends on the type of pathogen detected, the location and availability of host innate immune receptors as well as the role of immunity counter-acting components produced by the pathogen. Regardless of the response, infection and activation of the innate immune response has two primary functions: first, the immediate control of pathogen replication and spread, and second, the priming of the adaptive immune response.[2] The adaptive immune response, in the most simplistic terminology, consists of circulating cells in the bloodstream (T and B cell lymphocytes) that have the ability to tailor their receptors to specific pathogens and thus can generate a targeted response to each individual pathogen. Whereas innate immunity receptors are partially defined by their static detection of evolutionarily conserved molecules, the adaptive immune cells are partially defined by their ability to edit their T and B cell receptors to target species-specific antigens and create long term memory so that if a pathogen is seen again, the response can be generated in a much more timely manner. While both arms of the immune system are essential for effective immunity, for this dissertation, most of the discussion will be focused on elements more commonly associated with the anti-viral innate immune response.

INNATE ANTIVIRAL IMMUNITY

The innate immune response can be activated by all manners of pathogens, including viruses, bacteria, parasites, and others. Depending on the

type of pathogen, the innate immune response that is activated is specific to limiting the replication and spread of that particular class of pathogen. For viruses, the most common and important anti-viral molecules are the interferons (IFN).[5]

INTERFERONS

IFNs are glycoproteins that are part of the larger family of cytokines. They are divided into three subgroups based on their function and expression.[6] In general, IFNs are involved in anti-viral signaling by upregulating a series of interferon sensitive genes (ISGs) that lead to the establishment of the anti-viral state: a series of cellular modifications that make the cells inhospitable to viral replication.[5] There are potentially hundreds of physiologically relevant ISGs.[7,8] Some of the most well-studied ISGs include RNA-activated protein kinase R, RNase L, myxoma resistance protein 1, oligoadenylate synthase, APOBEC3, and RSAD2. These ISGs collectively serve as a series of restriction factors that control viruses by restricting infection, targeting viral and host RNA for degradation, inhibiting protein synthesis, and disrupting lipid rafts, amongst others. (Reviewed in[9]) In addition to ISG upregulation, interferons also function as signals to the adaptive immune response that a pathogen is present and lead to the induction of the adaptive immune response along with other cytokines. While all three classes of IFN are important for protection against infection, for the purposes of this thesis, only type I IFN is explored in detail. For a detailed

exploration of the type II Interferons, which are primarily produced by leukocytes and lymphocytes, please refer to an excellent review by Schroder et. al.[10] The type III interferons were discovered in 2003 and thus are the newest class of IFN to be described. While their functions are similar to the type I IFNs, type III IFNS are largely restricted to the mucosal membranes and protect the mucosa from the array of viruses that attempt to enter through this exposed route. For a comprehensive, up-to-date review, please see Wach et. al.[6]

TYPE I INTERFERON

Type I IFNs consist of two well-characterized types: IFNα, IFNβ and several poorly understood subtypes: IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ. In mice and humans, IFNβ is a single gene, predominately expressed in fibroblasts, but capable of being synthesized in almost any differentiated cell type. In contrast, IFNα exists as a series of individual genes[a] that are primarily expressed in leukocytes. They share roughly 70-80% protein homology while maintaining ~35% homology to IFNβ.[5,11] Engagement of various PRRs induces downstream signaling that leads to the activation and dimerization of Interferon Regulatory Factor 3 (IRF3). IRF3 translocates into the nucleus of the cell and binds to the IFNβ promoter and initializes the transcription of IFNβ.[12,13] IFNβ, upon secretion from the cell, acts in an autocrine and paracrine loop, engaging the IFN α/β receptor (IFNAR) on neighboring cells. IFNAR is a hetero-dimeric receptor that,

[a] In humans, there are 13 IFNα genes. In mice there are 14 IFNα genes.

when bound by IFN, initiates a signaling cascade that ultimately leads to the activation of a series of transcription factors that upregulate the ISGs responsible for the aforementioned anti-viral state.[5]

NUCLEIC ACID SENSING INNATE IMMUNITY RECEPTORS

Innate immune receptors are categorically different from adaptive immune receptors in that they recognize broadly conserved molecular patterns rather than pathogen specific sequences. These molecular patterns can be a variety of molecules, while the majority recognized are conserved protein, nucleic acid, carbohydrate, or danger signal motifs. The detection of foreign or self nucleic acids (NA) depends on a variety of factors, including sequence or motif-dependent recognition, as well as spatial and temporal recognition. Some receptors, such as the NA sensing Toll-like receptors (TLRs) are able to recognize NAs based on their motifs as well as their location within the endosomal compartment, which suggests the uptake of NA from an exogenous source. Four TLRs have been described that bind NA. TLR3 binds dsRNA[14], TLR7 and 8 can both bind ssRNA[15,16], and TLR9 recognizes both ssDNA and dsDNA.[17] While there are a wide variety of receptors that recognize many different PAMPs and all play a vital role in the innate immune response against pathogens, for the purpose of this dissertation I will focus on the intracellular RNA and DNA sensors.

RNA SENSORS

RIG-I and MDA5

Retinoic Acid Inducible Gene -1 (RIG-I) was the first intracellular RNA sensor to be described and is the eponym for the family of receptors that followed: the RIG-I like receptors (RLR[b]).[18] Structurally, RIG-I shares homology with another RLR, melanoma differentiation-associated gene-5 (MDA5). Both proteins contain a helicase domain with a conserved aspartate-glutamate-x-aspartate/histidine box motif (DExD/H)[c], which allows for the binding of RNA. They also both contain two Caspase Activation and Recruitment Domains or CARDS.[19] These CARD domains were found to be essential for RIG-I and MDA5 to interact with an adaptor protein located on the outer mitochondrial membrane called MAVS.[d][20-23] **(Fig. 1.1)** Eliminating either the helicase or CARDs results in the abrogation of signaling and downstream production of type I IFN.[19] Like other superfamily class 2 helicases, the RLR's require ATP hydrolysis for the proper function of their helicase and, in the case of RIG-I and MDA5, downstream activation of the IFN response.[24] One major structural difference between RIG-I and MDA5 is that in its steady-state inactive form, RIG-I exists in an auto-inhibited configuration where salt bridges and hydrophobic interactions between the CARD domains and the Hel2i domain cause the N-

[b] RLR's are sometimes referred to as RLH, or RIG-I Like Helicases.
[c] DExD/H helicases are part of the conserved Helicase Superfamily 2 (SF2). The "x" denotes any amino acid.
[d] MAVS is also known as Cardif, VISA, and IPS-1.

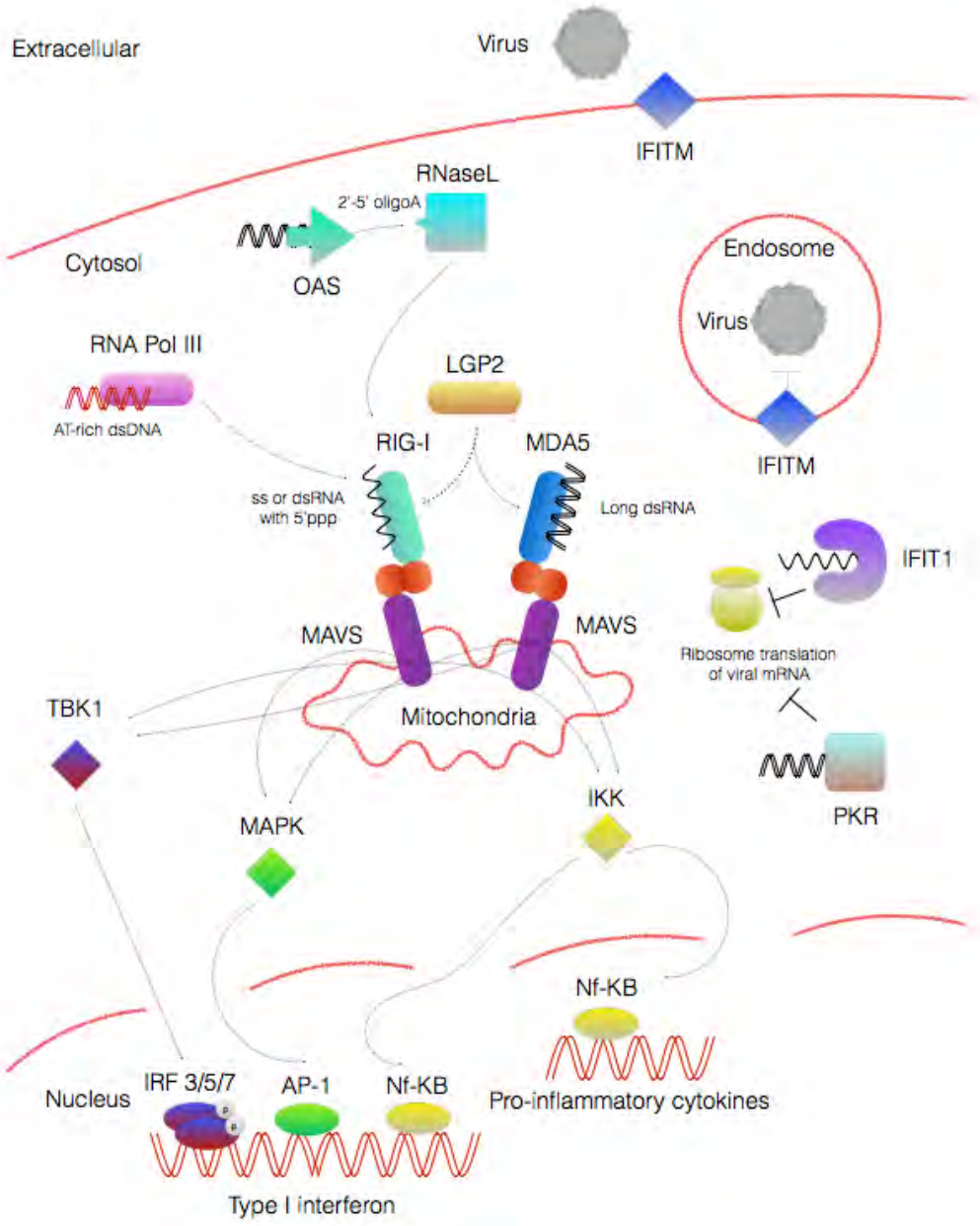

Fig. 1.1 Simplified model of cytosolic RNA-induced IFN expression
ss and dsRNA are recognized by a variety of receptors in the cell. Recognition of foreign RNA leads to the production of IFN by signaling to transcription factors in the nucleus. In addition, IFITM can restrict viral entry and IFIT1 and PKR can inhibit translation of viral RNA by blocking ribosome formation.
Solid lines indicate well-established pathways. Dashed lines indicate interactions that are undefined. DNA is in red, while RNA is in black.

terminal domain of RIG-I to fold back on itself.[25-27] Thus, expression or over-expression of RIG-I is not sufficient to drive IFN induction on its own. After the C-terminal domain (CTD) engages an appropriate ligand, ATP hydrolysis allows for the de-repression of the CARD domains in a manner that is not fully understood and signaling can continue.[19] Alternatively, MDA5 has no such auto-inhibition, and over-expression of MDA5 can drive IFN induction on its own.[19] Further work has identified that RIG-I and MDA5 do not share common ligands, with RIG-I being shown to predominately detect short (<300 nucleotide) blunt-ended and base-paired dsRNA that contains either a 5' triphosphate or diphosphate. (5'ppp or 5'pp RNA)[28-31] MDA5, however, preferentially binds long double stranded RNA longer than 2kb, or branched RNA structures, such as those found in the synthetic MDA5 ligand polyinosinic:polycytidylic acid (polyI:C). [32-35] Many viruses, such as encephalomyocarditis virus (EMCV) and nodamura virus are detected by MDA5 but not by RIG-I, in part because they mask the 5' ends of their genome. [36-38] Regardless of the ligand, engagement of the CTD of RIG-I and MDA5 causes aggregation of the receptors on the RNA strand, with RIG-I favoring a 5' end directed filamentation while MDA5 favors internal dsRNA binding and the creation of a monofilament in either direction along the dsRNA. The creation of the monofilament of receptors along the substrate is vital for downstream signaling. [39-43] The accumulation of repeated CARD domains along

the RNA strand leads to translocation of the RLR-RNA complex to the mitochondrial membrane and activation of MAVS. The CARD domains on MAVS interact with the CARD domains of RIG-I or MDA5 and cause the formation of large MAVS polymer units that are essential for further downstream activation.[44,45]

LGP2

There is a third member of the RLRs, Laboratory of Genetics and Physiology 2 (LGP2), that shares the same DExD/H box helicase domain as RIG-I and MDA5 but completely lacks the CARDs.[19] Without CARDs, LGP2 has no ability to directly influence downstream signaling, therefore the function of LGP2 has been difficult to describe. It was initially reported to be an inhibitor of RIG-I and activator of MDA5, but subsequent experimentation has shown that it is can have negative or positive regulatory effects for IFN production depending on the type of virus used for infection.[46], [47], [48] **(Fig. 1.1)** Differing LGP2 knockout mouse models have been inconsistent when the mice are infected with viruses detected by RIG-I, such as VSV, but they have all shown that LGP2 is vital for IFN signaling following infection with picornaviruses such as EMCV that signal through MDA5.[47,48] An elegant study by Bruns and Horvath et.al. revealed that LGP2 acts as a primer for MDA5-RNA monofilament formation.[49] **(Fig. 1.2)** In the absence of LGP2, MDA5 bound dsRNA slowly, dissociated quickly, and formed few monofilament loci per RNA. In the presence of LGP2 and ATP,

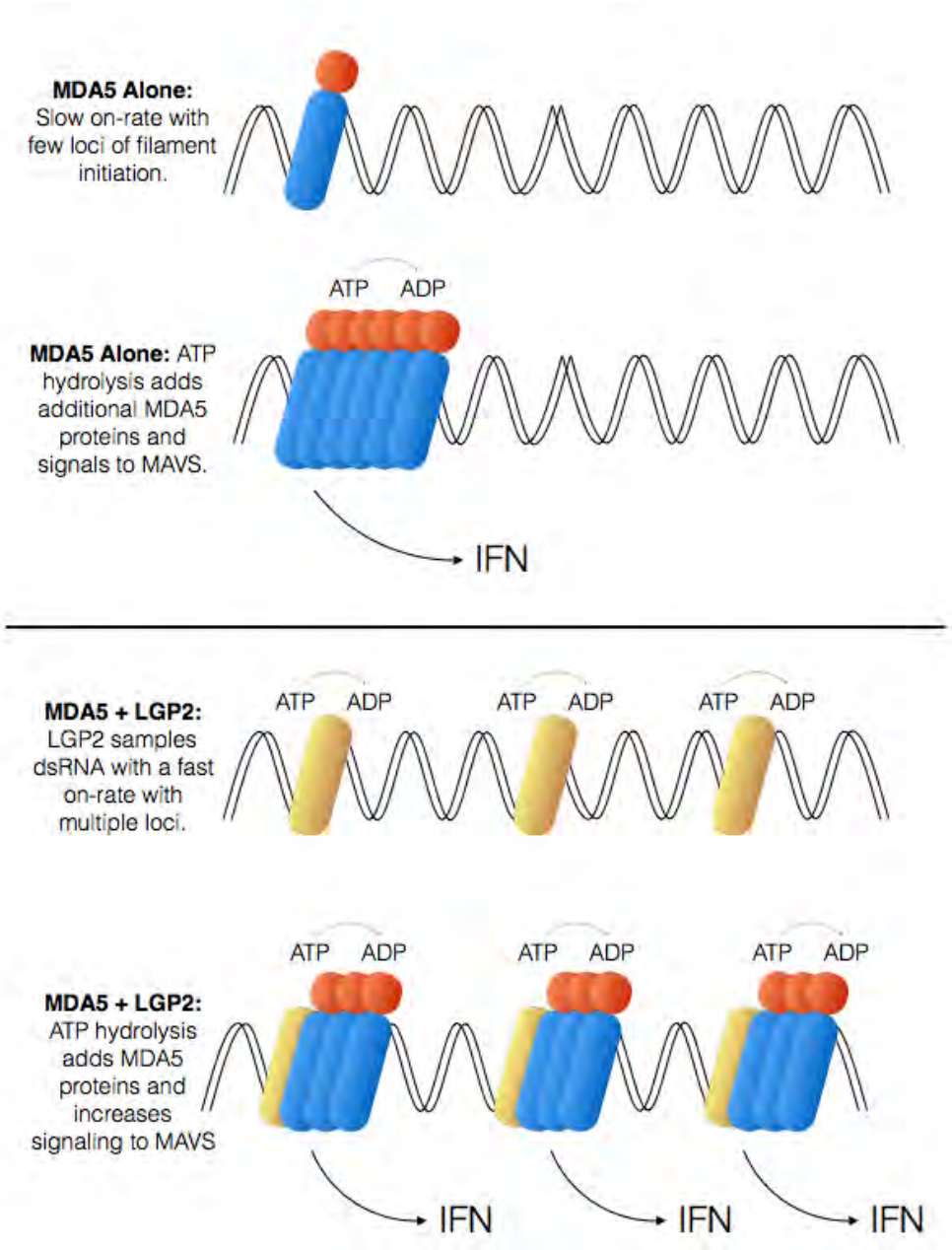

Fig. 1.2 Model of cooperative enhancement of MDA5 signaling by LGP2
Top panel: MDA5 binds dsRNA slowly and multiple MDA5 proteins are added in an ATP hydrolysis-dependent manner. When the filament reaches a suitable length, CARD clustering initiates signaling to MAVS and downstream expression of IFN.

LGP2 facilitated a faster and more stable interaction between MDA5 and the RNA and allowed for a greater quantity of shorter monofilaments. LGP2 was unable to generate monofilaments on its own.[49] Thus, LGP2 acts as a necessary, but not sufficient component of the MDA5 signaling pathway.

IFIT and IFITM

Interferon-induced protein with tetratricopeptide repeats (shortened to IFIT) are a family of genes conserved across many mammalian species. Humans have four IFIT proteins while mice have three. Under basal conditions, IFIT proteins are expressed at low or non-existent levels, but are upregulated rapidly upon viral infection.[50] They can also be upregulated by direct addition of IFN or by the engagement of various PRRs.[51,52] IFIT proteins, through their tetratricopeptide repeats, are able to inhibit the translation of viral mRNAs by binding to the eukaryotic initiation factor 3C (EIF3C) and inhibiting the assembly of the 43s-mRNA translation complex.[53] **(Fig. 1.1)** More recently, the IFIT1 protein was shown to recognize 5'ppp RNA and sequester the RNA, preventing it from being used for replication or protein translation.[54,55] Since the IFIT proteins have no signaling domains or enzymatic activity, it appears IFIT proteins cannot signal to induce more IFN, unlike the other 5'ppp RNA binding protein RIG-I.

The IFN induced trans-membrane (IFITM) genes are a distinct anti-viral family with different subcellular localization, expression, and function. Unlike other proteins described in this section, they do not appear to recognize nucleic

acids. Most cells express the IFITM proteins at basal levels.[e] While all IFITM proteins can be upregulated by IFN[56,57], IFITM3 is particularly strongly induced.[58] The function and mechanism of antiviral activity by the IFITM proteins are not entirely understood, however several studies have implicated the IFITM proteins as important restriction factors in enveloped viral fusion.[59-61] **(Fig. 1.1)** IFITM proteins are normally expressed in the endosome and lysosome compartments, where fusion events of many enveloped viruses take place. [62,63] Huang et. al. performed an intriguing experiment where they forced SARS-CoV to fuse with the plasma membrane of the cell and bypass endosomal fusion. They saw that IFITM's ability to restrict SARS was abolished, suggesting that the route of entry is key for IFITM restriction.[63] The sub-cellular localization and high levels of basal IFITM expression may be key to its function. Since it restricts viral infection at such an early stage, it must be present at high enough quantities in the correct compartment to be effective at preventing infection.

DNA SENSORS

Considering that the immune response relies on distinguishing between self and foreign motifs to identify signs of infection, it was little surprise that the cytosolic RNA sensors detect nucleic acids that are not traditionally seen in the cell. Long dsRNA and 5' triphosphorylated RNA are uncommon in the normal cellular environment since most cellular RNAs are single-stranded and capped.

[e] Humans have four IFITM proteins while mice have six.

However, since many pathogens use DNA as their genomic material instead of RNA, the host cell has developed an ability to detect foreign DNA while dealing with the presence of copious amounts of self DNA in every cell. Here, I will describe a few of the pertinent DNA receptors that have been implicated in viral detection.

DAI

DNA-dependent Activator of Interferon-regulatory factors (DAI)[f] was the first described intracellular receptor for DNA. Takaoka et. al. found that DAI was upregulated in L929 cells following the addition of poly dA:dT or other synthetic DNA and that knocking down DAI via siRNA blunted the cells' ability to activate IRF3 and lead to IFN production.[64] Surprisingly, when the DAI knockout mouse was generated, no phenotype could be observed in relation to DNA sensing. Work using different cell lines has shown that DAI has a moderate to inexistent effect depending on the type of cell used; thus, there must be a redundancy in DNA sensing. Important for this dissertation is that DAI has been shown to have no effect in MEFs[65-67]

IFI16

Interferon gamma-inducible protein 16 (IFI16) was first identified using a vaccinia derived nucleic acid motif (VACV70mer) to affinity-purify proteins from the cytosol of monocytes that were capable of binding DNA.[68] Unterholzner et.

[f] DAI is also known as DLM-1 or ZBP1, Z-DNA binding protein 1.

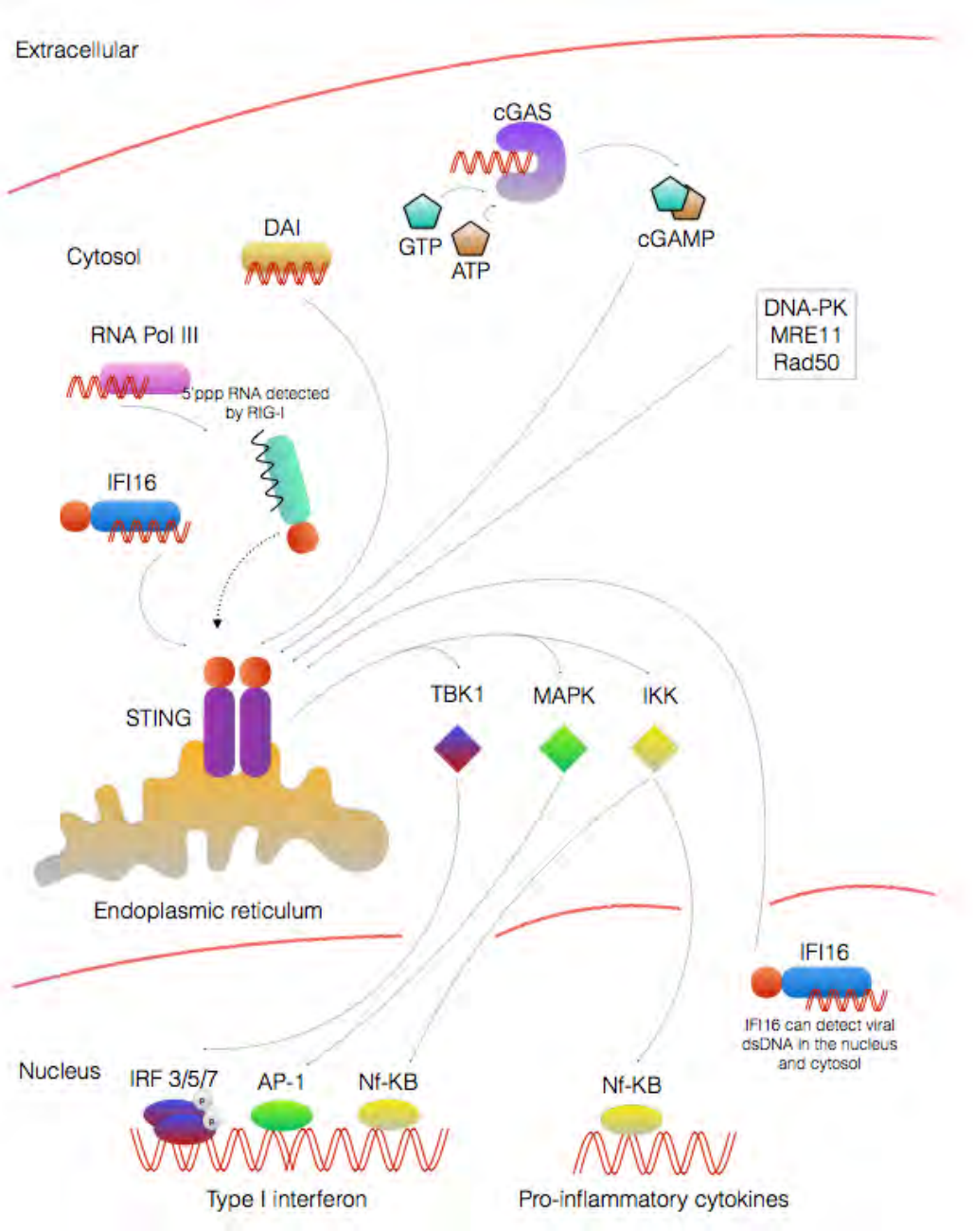

Fig. 1.3 Simplified model of cytosolic DNA-induced IFN expression
dsDNA can be recognized by a variety of sensors in the cell. Many receptors must engage STING before the signaling cascade can continue and activate interferon or inflammation via translocation of transcription factors. STING can also act as the direct receptor of cGAMP created after cGAS detects foreign DNA.
Solid lines indicate well-established pathways. Dashed lines indicate interactions that are undefined. DNA is in red, while RNA is in black.

al. showed that VACV70mer stimulated IFNβ induction only in its dsDNA form and not in a ssDNA form and that VACV70mer associated with IFI16 in the cytosol.[68] Most importantly, siRNA experiments silencing IFI16 revealed a deficiency in IFNβ signaling when IFI16, or its mouse ortholog IFI204, was depleted. IFI16 has also been observed in the nucleus of infected cells, suggesting it is able to detect DNA viruses regardless of what cellular compartment they replicate in.[69] **(Fig. 1.3)** IFI16's role in DNA sensing has been complicated by reports that IFI16 can play a role as a transcriptional activator and drive the expression of ISG's regardless of the ligand used to initialize signaling. Thus it has a role in the proper expression of ISG's post DNA and RNA stimulation.[296] It has been shown to activate transcription of IFN downstream of STING and binds directly to the IFNα promoter, suggesting a role beyond that of being just a sensor.[296] Most of what is known about IFI16 was discovered in human cell lines, and while its mouse ortholog IFI204 is thought to behave in a similar manner, the role of IF204 as a transcriptional activator has not been verified in mouse cells.

RNA Polymerase III and RIG-I

An interesting observation was made by two groups that reported that AT-rich DNA could be bound and transcribed by RNA Polymerase III (RNA Pol III) in the cytosol of transfected cells or cells infected with certain DNA viruses. This

RNA, since it was transcribed by RNA Pol III, does not contain the canonical 5' methyl cap and instead has a 5' ppp at the end of the RNA. This RNA is then recognized by the RNA sensor RIG-I and induces IFNβ.[70,71] Knocking down RNA Pol III ablated this pathway. However, the importance of RNA Pol III and RIG-I in sensing DNA is still controversial, since this pathway appears to be restricted to cell types that lack other receptors. Additionally, non-AT rich DNA can still activate IFNβ despite RNA Pol III's inability to transcribe non-AT rich DNA.[72] Therefore it is unlikely this is a key player in IFN induction.

STING

Stimulator of Interferon Genes or STING[g] was first described in 2008 as an important adaptor involved in innate immune signaling after exposure to a variety of pathogens. It was identified as an endoplasmic reticulum bound protein that appeared to regulate responses to DNA and RIG-I based stimulation.[73] **(Fig. 1.3)** Work from various labs since the discovery of STING has helped reveal STING's role as a primary crossroad of signaling in DNA cytosolic sensing. IFI16 signals to STING, as do several other DNA damage sensing proteins such as DNA-PK, Rad50 and MRE11, which detect blunt ended DNA in the cytosol (typically from replicating DNA viruses) and signal to STING.[68,74-76] STING can also be activated by cyclic di-GMP, which suggested that another protein must exist and be responsible for generating the cyclic di-

[g] STING is also known as transmembrane protein 173

GMP from dsDNA.[77,78] Following engagement with the various DNA sensors or cyclic di-GMP, STING activates IFNβ induction through recruitment of TBK-1 to the ER membrane.[79]

cGAS

Since the modified cyclic di-GMP that stimulated STING had to come from a processed DNA source, it was reasonable to hypothesize that a different receptor was detecting the cytosolic DNA and processing it to a form that could be detected by STING. Wu and Chen *et. al.* performed an experiment where they stimulated cells with nucleic acids and then extracted the cytosolic fragments and subjected them to a series of treatments including heat denaturation, protease treatment, and benzonase treatment, which degrades DNA and RNA. They found that the fractions still stimulated STING and IRF3 dimerization when transfected into unstimulated cells. Mass spectrometry analysis revealed 2'3'-cGAMP, which is a cyclic di-nucleotide with a noncanonical 2'-OH on the GMP linked to the 5' phosphate of AMP, combined with a canonical 3'-OH on the AMP linked to the 5' phosphate of GMP.[80] Further work revealed that cyclic GMP-AMP synthase (cGAS) is a DNA specific sensor that binds DNA and synthesizes cGAMP which can drive STING-mediated IFN induction in the autologous cell as well as neighboring cells, since cGAS can diffuse from cell to cell.[81,82] **(Fig. 1.3)**

SMALL RNA PATHWAYS

OVERVIEW

Since DNA and RNA are common molecules to both host and pathogen, the importance of carefully delineating what constitutes pathogenic genomic material from host genomic material is key to using nucleic acids as an innate immunity stimulant. Several common long-standing hypotheses revolved around detecting types of nucleic acid that were not thought to have host counterparts, such as dsRNA. Common cellular RNA is 5' methyl capped ssRNA or 5' monophosphate ssRNA. Thus, dsRNA was thought to primarily arise during the replicative cycle of a variety of RNA and DNA viruses since dsRNA would arise during the replication of either a positive or negative sense virus or during bi-directional RNA synthesis from a double stranded genome.[83] However, this theory had to be revisited with the discovery of the noncoding dsRNA regulatory pathways consisting of RNA interference and microRNAs. Intriguingly, the history of small RNAs can be traced back to the desire to create a more vibrant color of petunia. By overexpressing a transgene encoding chalcone synthase, Napoli and Jorgensen hoped to determine if chalcone synthase was the rate limiting step in developing violet petunia petals. Instead, the plants they produced came out solid white. They hypothesized that the strong expression of chalcone synthase somehow had co-suppressed the endogenous gene.[84] At the time, a popular way of suppressing the translation of a protein was to use

antisense RNA against an mRNA to theoretically bind the mRNA and prevent its translation. However, Guo and Kemphues showed that both antisense *and* sense RNA suppressed an mRNA signal in *C. elegans*.[85] In 1998, a seminal paper by Fire and Mello revealed the actual cause of this strong suppression: dsRNA. They termed this phenomenon “RNA interference” (RNAi) and showed that adding dsRNA had a potent and sequence-specific capacity to suppress the mRNA signal.[86] A similar effect had been seen in the earlier studies due to small amounts of dsRNA contamination or the generation of dsRNA *in vivo* by adding the antisense RNA into the organism, where the cellular machinery took over and started the RNAi process. Here, I will describe the two main small RNA pathways in general before getting into more detail on the specific proteins involved. Since there are considerable differences between the small RNA pathways of different species, unless otherwise specified I will be referring to the mammalian systems.

RNAi

In simple terms, RNA interference describes a system where primarily exogenous dsRNA enters a cell (such as that arising from a viral infection) and is processed into short dsRNA oligonucleotides (oligos) that are loaded into a complex that uses the RNA as a guide to seek out a homologous RNA sequence and cleave it, thus suppressing the ability of that RNA to be replicated or translated. **(Fig. 1.4, bottom right)** Evidence suggests that RNAi acts as an

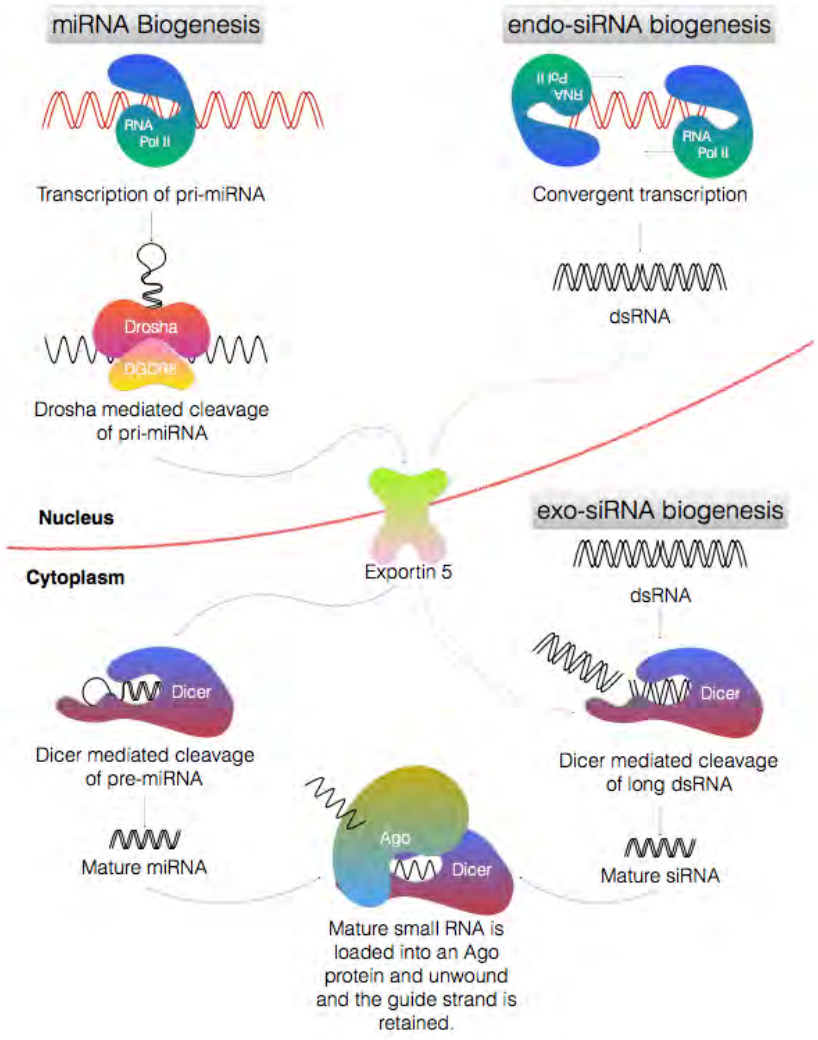

Fig. 1.4 Simplified model of canonical small RNA pathways
Left: miRNA biogenesis begins with RNA Pol II transcription of endogenous miRNA genes. Drosha and DGCR8 coordinate in processing the stem-loop from the primary transcript. The pre-miRNA stem-loop is exported through Exportin5 and further processed by Dicer in the cytosol. The mature 22t miRNA is loaded into Ago and the RNA induced silencing complex (RISC), where the RNA is unwound and a single strand is retained to act as the guide.
Top right: Convergent transcription from promoters on both strands results in ssRNAs that pair as dsRNA and are exported to the cytosol. ~22nt siRNAs are cleaved and mature siRNA is loaded into Ago and RISC where the dsRNA is unwound and the guide strand retained.
Bottom right: dsRNA from an exogenous source is processed in the cytosol by Dicer into ~22nt siRNAs. The mature siRNA is loaded into Ago and RISC where the dsRNA is unwound and the guide strand retained.

ancient defense mechanism against infection by cleaving the pathogen's own dsRNA and using it to further silence the pathogen's genome or mRNAs.[87,88] However, while exogenous RNA remains a major source for the initialization of RNAi, there have been multiple reports describing a role for endogenous "short interfering RNAs" (siRNAs) as well. These endo-siRNAs in many ways act more like microRNAs (miRNAs; defined below), as they are genome encoded and are not always amplified (though this varies from species to species). These endogenous siRNAs appear to be functionally associated with the piwi-RNAs (further discussed below), in that they largely provide for genome-defense against transposons and retrotransposons.[89-91] **(Fig. 1.4, top right)** While there is a significant amount of variation from species to species, at its core, for an organism to truly have RNAi it needs two or three proteins:

1) A type III RNase, such as Dicer. While Dicer is the most well-known and studied of the type III RNases involved in RNAi, other potential orthologs, such as the Dicer-like RNase in *Giardia intestinalis,* have significant enough diversions from the structure of Dicer that they may have evolved from a different RNase. The Type III RNase is responsible for cleaving the long dsRNA into short dsRNA fragments (commonly referred to as short interfering RNA (siRNA)) that vary between 19-23 nucleotides depending on the species.
2) RNA-dependent RNA polymerase (RdRP). While RdRP is not required for RNAi to function, it does greatly enhance its efficacy. By

binding the cleaved dsRNA and replicating it, a small amount of initial dsRNA can generate large amount of effector siRNAs that can be used in the cell that they originated in, or can be disseminated throughout the organism. In that way, a single intruding dsRNA can be made into multiple identical siRNAs that are loaded into Argonaute (Ago) proteins (discussed below) and can be used to seek out and silence homologous targets. Worms and plants both employ RdRP to greatly enhance their RNAi responses and provide an effective immunity against pathogens that generate dsRNA.[92,93] The lack of an effective RdRP in mammals[h] may help explain why RNAi does not constitute a major role in host defense from pathogens; nevertheless, flies such as *Drosophilia melanogaster* use RNAi as a potent antiviral defense without the added benefit of an RdRP.[94]

3) An Argonaute-like protein. Argonaute, or Ago, is the core protein involved in binding both mature siRNAs and miRNAs in the single stranded format. The dsRNA is loaded and unwound in the Ago protein and acts as the guide RNA, which is the basis by which homology to the target RNA is determined.[95-99] Ago does not act alone and is joined by several other proteins to form the actual effector complex referred to as the RNA-induced silencing complex or RISC.[100]

[h] The only known RdRP in humans is hTERT, which is responsible for maintaining the ends of telomeres.

Once the siRNA is loaded into Ago and the RISC has assembled, the siRNA is used as a template strand for the siRISC to bind to other ssRNAs and looks for perfect base-pair homology between the siRNA and target RNA.[99] Perfect or near-perfect homology results in the catalytic slicing activity of Argonaute 2[i] to activate and cleave the target RNA between residues 10 and 11.[99] The cleavage does not result in the degradation of the siRNA, so a single siRNA can be used catalytically to process multiple target RNAs.[95]

RNA interference has been observed in the majority of eukaryotes with some noticeable exceptions: *Saccharomyces cerevisiae*, *Leishmania major, Trypanosoma cruzi*, *Plasmodium falciparum*, and *Cyanidioschyzon merolae*.[101] Surely, more will be described as whole-genomics based techniques are applied to more species. The fact that RNAi appears to have been lost independently from these different lineages supports the hypothesis that RNAi may actually be dispensable for some eukaryotic organisms and that it primarily perseveres because it shares two of the three required proteins with the miRNA pathway.

miRNAs

The microRNA pathway is a primarily endogenous small RNA pathway, compared to the primarily exogenous RNAi pathway. miRNAs are genome

[i] In humans there are four Argonaute proteins, however only Argonaute 2 has “slicer” activity and can cut target RNA.

encoded and are expressed as a primary transcript, as part of the 3' UTR of another gene, as part of the intron of another mRNA, or as a cluster of related miRNAs.[102] **(Fig. 1.4, left)** miRNAs largely act as regulators of gene expression. Initially, miRNAs were thought to only repress translation of mRNAs, but further work has shown that miRNAs can activate gene expression as well. The first miRNA was discovered prior to knowing that dsRNA was the trigger for processing and generating mature miRNAs. The Ambros and Ruvkun labs, working with *C. elegans*, discovered that *lin*-4 was not a protein-coding RNA, but was instead a noncoding RNA that could base pair to *lin*-14, which is a protein-encoding mRNA, and that this binding resulted in suppression of LIN-14 protein production.[103,104] The primary miRNA (pri-miRNA), *lin*-4, is transcribed by RNA Pol II and exists as a single stranded RNA with a canonical stem loop, where the ssRNA folds back and pairs to itself to create secondary structure.[105-107] The stem-loop structure is bound in the nucleus by the Type III RNase Drosha and its RNA binding partner DGCR8[j], both of which are required to process the pri-miRNA. The pri-miRNA is cleaved at the base of the stem, leaving an approximately 60-70 nucleotide dsRNA stem-loop structure referred to as the pre-miRNA.[108-111] Due to the nature of the Drosha cleavage, the dsRNA contains a canonical 2nt overhang. The pre-miRNA binds to the nuclear pore protein Exportin-5 and is transferred through the nuclear membrane in a Ran-GTPase dependent manner.[112,113] Once in the cytosol, the pre-miRNA is processed by a

[j] DGCR8 is short for DiGeorge syndrome chromosome 8

second endonuclease, Dicer, in concert with its RNA binding partners TRBP[k] and PACT[l].[114-116] Dicer cleaves the loop structure off the stem and the resulting ~22nt dsRNA is known as a mature miRNA.[117,118]

The mature miRNA is loaded into an Ago protein and the two strands dissociate by one of two methods: If the miRNA has complete homology at nucleotides 9-11 and the miRNA was loaded into Ago2, then the strand referred to as the guide strand can be used to drive cleavage of the other strand, referred to as the star (or *) strand.[119,120] If the miRNA is not base-paired at residues 9-11, or if the miRNA was loaded into Ago 1,3,or 4, the miRNA must be unwound by one of the RISC-associated helicases.[121,122] After being unwound, the star strand is generally degraded; however, this is not universally true and as more miRNA sequencing data is obtained, there have been increasing reports that the star strand can be loaded into Ago to act as a miRNA as well.[m] Once the miRNA is loaded into Ago and the RISC has assembled, the miRNA guide is used to find homologous RNA targets. Unlike siRNAs, which require near-perfect homology with their target, miRNAs have much more flexibility in base-pairing their target. Still, miRNAs generally require perfect homology in what is called the “seed sequence” of the mature miRNA. The seed sequence consists of nucleotides 2-7. These nucleotides, in a manner that is still not completely understood, form the basis of miRNA recognition of their target.[123,124] Additional recognition comes

[k] TRBP is short for TAR RNA binding protein. TAR stands for trans-activation response element.
[l] PACT is short for PKR activating protein. PKR stands for protein kinase R
[m] The nomenclature for miRNAs is currently disorganized. However, a push to label the miRNAs by the relative position on the stem (5p arm versus 3p arm) should help improve the situation.

from the central region (nucleotides 9-11) and the 3' end of the miRNA.[125,126] miRNAs typically do not initiate the cleavage of their targets, although cleavage can occur if the central region has perfect homology.[119,120] miRNAs can repress their targets by a variety of mechanisms, and the importance of the different methods is still debated. miRNAs and the RISC can directly cleave the mRNA, prevent translation through steric hindrance of the ribosome, and deadenylate the 3' polyA tail and increase mRNA degradation and turnover.[127-129]

Piwi-RNAs

P-element inducing wimpy testis (Piwi) RNAs (piRNAs) are a class of Dicer –independent small RNAs[130-132] that are larger than siRNA or miRNAs, at ~27 nt.[n132] The Piwi protein involved in the piRNA pathway was discovered well before the piRNAs during a screen for factors impacting germline stem cell maintenance in *Drosophilia melanogaster*.[134] Orthologs of piwi were soon discovered in humans and *C. elegans*.[135] It was almost 10 years later that several groups simultaneously published the identification of the class of RNAs that were bound by the Piwi-class proteins. [136-140] Their main function appears to be the silencing of retrotransposable elements in the germline.[141,142] However, since piRNAs are Dicer-independent, they are not explored in detail in this body of work.

[n] Piwi RNAs in *C. elegans* are 21 nt and not ~27 nt.[133]

NON-CANONICAL SMALL RNA PATHWAYS

The field of small RNA pathways is rapidly changing; new pathways are continuously being described, and previously discovered pathways are still being elucidated. Here, I will briefly describe a few of the non-canonical[o] pathways related to mammalian Dicer.

1. Endo-siRNAs. Briefly mentioned earlier, endo-siRNAs arise in the nucleus of mammalian cells by several pathways. Convergent transcription occurs when two promoters face each other and an RNA polymerase transcribes ssRNAs overlapping on complementary strands, which base-pair and feed into the RNAi pathway. **(Fig. 1.4, top right)** Transcription of repetitive loci can also cause ssRNA to double back on itself to generate dsRNA fragments with loops.[89,90] Long interspersed nuclear elements (LINE) and short interspersed nuclear elements (SINE) are both retrotransposon-associated elements that are transcribed as non-coding RNAs in mammals. These can be processed into repeat-associated siRNAs (rasiRNAs). Deleting Dicer leads to an accumulation of LINE transcription since the rasiRNAS are not silencing the output of the LINE transcription.[91,143] SINEs are regulated in a similar manner by Dicer as the LINEs. Dicer depletion results in an increase of SINE transcripts such as ALU, the

[o] I understand describing these pathways as “non-canonical” may be controversial, however I use the term to denote the difference between pathways where the mechanism is largely understood versus pathways where the basics are still being investigated.

accumulation of which triggers apoptosis in mouse macular tissue, suggesting that Dicer may help clean up dsRNA fragments that could potentially trigger a cytotoxic response in normal cells.[144,145]

2. Dicer-independent miRNA. Typical miRNA biogenesis requires both Drosha and Dicer for the miRNA to be processed into a mature form. However three independent groups have confirmed the existence of a Dicer-independent miRNA: miR-451.[146-148] While the primary miR-451 transcript is expressed and processed by Drosha in the typical manner, the resulting stem-loop is much shorter than traditional miRNAs, at approximately 42nt instead of the normal 60-70nt. The pre-miRNA therefore bypasses Dicer entirely and is loaded into Ago2, which uses its catalytic activity to cleave the loop off and become a mature miRNA.
3. RNA activation (RNAa). Contrary to the common understanding that both siRNAs and miRNAs suppress translation of mRNAs, a newer pathway has been described where small dsRNA can transcriptionally activate genes by changing the histone profile at the promoter or by targeting antisense transcripts that target the activated RNA.[149-152] While the mechanism and biological significance is not fully understood, RNAa adds a layer of complexity to the already complex small RNA pathways.

DICER

While there are many proteins that are involved in both the RNAi and miRNA pathways, one in particular is vital for both: Dicer. Since the data chapters of this dissertation revolve around the role of Dicer in viral immunity, I'd like to provide a little more detail on Dicer.

Dicer appears to have evolved from prokaryotic type III RNases and can be found in all eukaryotic organisms that have functional small RNA pathways.[153] Because Dicer evolved early in the evolutionary timeframe, it has had time to evolve different functions in different families, through gene duplication and loss-of-function. Plants have the most diverse Dicer family with 4 distinct Dicer genes: DCL1-4.[154] Each Dicer serves a different function, with DCL-1 functioning primarily in miRNA pathways, while DCL-4 provides the core antiviral processing for RNAi.[155,156] Arthropods have two Dicers, *Dicer-1* and *Dicer-2.* *Dicer-1* is involved in miRNA maturation, while *Dicer-2* is essential for antiviral activity.[157] *C. elegans* and other nematodes have only one Dicer that is essential for both miRNA and siRNA processing.[158] Mammals also have only one Dicer, which can process a variety of substrates including the stem-loops of pre-miRNAs, dsRNA, dsRNA-DNA hybrids, and dsDNA. [159-161]

MOUSE DICER

Humans and mice each have a single Dicer protein.[p] For this review of Dicer, I will be referring to mouse Dicer unless otherwise specified, as there are several mouse-specific functions of Dicer. Dicer1 is a cytosolic protein of approximately 220 kDa that consists of the following domains, from N terminal to C terminal:

1. DExD/H box helicase. The helicase contains a Walker A ATP binding motif, but ATP studies have found that ATP is largely dispensable for long dsRNA processing ability of Dicer.[162] Mutation studies have actually revealed that removing the helicase domain increases the processivity of Dicer on longer dsRNA dramatically.[163] Currently, the model for Dicer suggests that the helicase domain is important for distinguishing pre-miRNA from linear dsRNA by engaging the loop structure on the pre-miRNA.[164]
2. DUF283. Domain of unknown function 283 largely remains a domain of unknown function, despite initial reports suggesting that it has a role in processing pre-miRNA.[163]
3. PAZ. The Piwi-Argonaute-Zwille domain is important for binding to the terminus of a dsRNA. It has two unique binding pockets within this domain, one for binding the 3' end of one strand of

[p] Human and mouse dicer share ~97% homology at the amino acid level.

RNA while the other binds the 5’ end of the second strand.[165-168] The 5’ pocket is especially conserved amongst Dicer proteins that preferentially bind pre-miRNAs and appears to favor 5’ ends that are less thermodynamically stable, which is a characteristic of miRNAs since they do not typically have perfect base-pair complementarity and thus have less stable Watson-Crick base pairing.

4. RNase III. Dicer has two RNase III domains and together they form a single catalytic center for dsRNA cleavage. The two domains, RNase IIIa and IIIb are offset so that the aspartic acid residues that are essential for the RNase function cleave the two phosphodiester bonds of the RNA backbone with a 2 nt differential, thus also generating a 2 nt overhang. The cuts are made approximately 20-21 nt away from the terminus of the dsRNA that is bound up in the PAZ binding pockets.[167] This was originally thought to act as a molecular ruler and that the length between the PAZ domain and RNase domains contributed to the establishment of the ~21 nt cleavage length. However, a recent report by Ma et. al. has shown that Dicer reconstituted from individual fragments that are not physically connected can still produce the ~21 nt dsRNA fragments.[169]

DICER1 is encoded on the chromosome 12[q] and disruption of the gene is embryonic lethal. It exists as two primary isoforms: Dicer^{S} and Dicer^{O}.[r] Dicer^{S} is 1,916bp and is considered the canonical form of Dicer. It uses exons 1-27 to form the mRNA transcript and is found in almost every cell type. Processing studies have shown that this canonical form of Dicer primarily processes dsRNA that contain a stem-loop structure, similar to the structure of a pre-miRNA.[160] The second isoform is 1,678bp and is found predominately in murine embryonic stem cells and oocytes.[170] It uses an alternative mouse transposon C (MT-C) driven element in the sixth intron of Dicer to initialize the short Dicer^{O} version that includes the MT-C leading directly into exons 7-27.[170] This alternate transcript largely removes the helicase domain from the protein and, as mentioned before, the removal of the helicase increases processivity of longer dsRNA. Thus, in normal somatic cells, the longer isoform of Dicer favors processing of miRNAs while in oocytes and murine ESC's, the transposon driven short form Dicer^{O} favors the processing of endo-siRNAs arising from transposable elements and contributes to genome defense while largely ignoring miRNA processing.[171,172]

[q] Chromosome 12 in mice, chromosome 14 in humans.
[r] Dicer^{S} is for somatic while Dicer^{O} is for oocyte.

SMALL RNA PATHWAYS IN VIRAL INNATE IMMUNOLOGY

There are several factors that led to a burst of research into small RNA pathways in mammalian and, in particular, human immunology:

1. RNAi had an established role as a major anti-viral system in plants, worms, and flies. (Reviewed in[173,174])
2. miRNAs and their targets were being discovered at an intense rate and it became clear they have a major role in regulating the activation and maturation of a variety of immune functions. (Reviewed in[175-177])
3. Viral infection produces dsRNA, the substrate by which RNAi is initiated.
4. Dicer shares a conserved DExD/H helicase that places it in part of the Helicase Superfamily 2 that also contains RNA helicases involved in mammalian anti-viral immunity: RIG-I, MDA5 and LGP2.
5. Small RNA pathway cofactors, such as TRBP and PACT are essential for binding to Dicer, viral factors (HIV TAR, in the case of TRBP) and the innate immune response (RIG-I, in the case of PACT), suggesting a common link between the systems. (Reviewed in[178,179])

Despite efforts by a number of labs to show that RNAi functioned as a vital anti-viral pathway in mammals, research by a variety of groups revealed little to no direct RNAi activity in mammalian cells. This led several prominent researchers to claim, perhaps prematurely, that RNAi played no role in

mammalian anti-viral immunity.[180-182] There have now been a plethora of papers describing the role for multiple miRNAs in the regulation of immune responses to a variety of pathogens and for the general maturation of immune cells. However, for the sake of this dissertation, I will limit this review to the papers most pertinent to the roles of miRNAs in regulating viral infections and IFN signaling pathways.

miRNAS REGULATING TYPE I IFN

miRNAs regulate many cellular functions and the signaling pathways that lead to Type I IFN expression are no exception. There are actually very few reports of miRNAs directly targeting IFN mRNA. Witwer et. al. and Li et. al. have reported miRNAs that target and regulate IFNβ and a variety of IFNα mRNAs.[183,184] The physiological relevance of these targeting miRNAs is currently unknown since several of them are expressed at very low levels in innate immune cells. Interestingly though, these miRNAs can be upregulated by IFN, thus suggesting a potential negative feedback mechanism.[183] Despite few miRNAs directly targeting IFN, miRNAs can regulate IFN by targeting components of the IFN signaling pathways. Upregulation of miR-146a has been shown to diminish IFN signaling by targeting the IFN induction pathway proteins TRAF6, IRAK1, and IRAK2.[185,186] Another miRNA, miR-155, which was first described to play a major role in the development of macrophages and activated B and T lymphocytes[187-189], has been shown to regulate IFN responses by targeting the 3' UTR's of SOCS1 and TAK1 binding protein.[190,191] Similarly, miR-

221 was able to enhance the anti-viral effects of IFN on Hepatitis C virus infection by repressing SOCS1 and SOCS3, which are both known inhibitors of JAK/STAT signaling pathways.[192]

CELLULAR ANTIVIRAL miRNAS

Several reports have focused on miRNAs that share seed sequence homology with viral sequences, whereby there is the potential that conserved miRNA sequences could repress viral genomic or messenger RNA. One of the first reports used a Dicer deficient cell line to identify miR-24 and miR-93 as cellular microRNAs that shared sequence with Vesicular Stomatitis virus (VSV).[193] VSV replication was increased in Dicer deficient mice and by adding miR-24 and miR-93 back to Dicer deficient macrophages, they were able to suppress VSV replication. A series of studies then identified miR-122 is a hepatic miRNA that can be upregulated by IFN and targets Hep B and Hep C viruses in the liver and inhibits their replication.[194-196] Similarly, a miRNA found abundantly in T lymphocytes, miR-29a, was shown to suppress HIV replication following its overexpression.[197] Conversely, depleting T cells of miR-29a led to an increase in HIV virion production. Despite these reports, the true question of the physiological relevance of these miRNAs in control of viral infection remains to be seen. Further complicating matters is that miRNAs, through their ability to theoretically target any sequence that matches their seed sequence, can have potential targets in the tens to hundreds to thousands of mRNAs. As an

example, TargetScan miRNA prediction software has 2,109 potential targets listed for miR-155-5p, whereas there are currently 118 confirmed, published targets of miR-155.[198] Thus, determining what true role a miRNA has in suppressing a target is difficult when reports are typically only testing a few miRNAs or miRNA targets at a time. In addition, considering that viruses are in an evolutionary arms race with their hosts, encoding a potentially potent anti-viral agent as a static miRNA would have limited effect on rapidly evolving viruses, unless the miRNA targeted a highly conserved region of the viral genome. Therefore, it is likely that miRNA regulation and targeting is focused on cellular processes, and the targeting of a viral RNA by a cellular miRNA is due to the promiscuity associated with miRNA:target RNA homology.

VIRAL miRNAS

As part of the evolutionary arms race, viruses are known to adapt cellular factors or machinery to enhance their own replication. The very fact that they are obligate intracellular parasites requires them to commandeer cellular machinery to survive. Thus it is not surprising that viruses have also adopted the cellular miRNA pathway for their own purposes. The first viruses shown to encode miRNAs are also in the virus family with the most miRNAs: the herpesviruses. Alpha, beta, and gamma herpesviruses all encode viral miRNAs that enter the canonical miRNA processing pathway. These miRNAs have functions that range from evading the host immune response to the establishment of latency.

(Reviewed in[199]). The majority of viruses that encode miRNAs are DNA viruses. As DNA viruses, they are able to transcribe their miRNAs from their DNA genomes without compromising the integrity of their genome by offering it up for Drosha and Dicer cleavage. RNA viruses are largely encoded as a single piece of RNA and would risk losing their whole genome to miRNA processing.[s] There is, however, one RNA virus confirmed to encode a miRNA: bovine leukemia virus (BLV)[t]. As a retrovirus, BLV has an RNA genome, but uses a DNA intermediate to integrate itself into the host genome as well as replicate. However, unlike most cellular miRNAs, the miRNA encoded by BLV is transcribed by RNA Pol III instead of RNA Pol II. The pri-miRNA encoded in BLV is short, and thus is incapable of being cleaved by Drosha, but is still cleaved by Dicer. Thus, it appears that by using Pol III as the transcription enzyme, BLV is able to express its miRNA independently from the replication of its genome, so that the same sequence embedded in its genome won't be processed.[200]

DIRECT CLEAVAGE OF VIRAL dsRNA BY DICER OR DROSHA

While the process of virus-initiated RNAi in a mammalian system has remained elusive, one step that has been reported multiple times is the cleavage of viral RNA by Dicer. In an extensive report, Parameswaran et. al. showed that very small quantities of virus derived small RNAs (vsRNAs) could be detected

[s] There are, of course, segmented RNA viruses and dsRNA viruses.
[t] BLV, as a retrovirus, is in Group VI of the Baltimore classification of viruses, some consider the retroviruses to be RNA viruses, while others consider them to be DNA viruses.

following infection of mouse and human cells with VSV, West Nile virus, poliovirus, dengue virus, and hepatitis C virus. While Dicer depletion had a modest effect (approximately 2.1 fold decrease in vsRNAs), vsRNAs still remained, suggesting that these vsRNAs may not be entirely Dicer dependent, and may in fact be degradation products.[180] It wasn't until a series of reports looking at vsRNA formation in undifferentiated cells were published that a true Dicer-dependent cleavage of viral RNA was revealed. Li et. al. and Maillard et. al. published back to back articles reporting that viral infection with nodamura virus or encephalomyocarditis virus generated a pool of ~21 nt Dicer dependent vsRNAs in undifferentiated cells like oocytes and murine embryonic stem cells (mESCs) as well as newborn suckling mice.[201,202] Of particular interest in these papers is that the generation of the vsRNAs went away as the cell differentiated. Since oocytes and mESCs do not express a functional IFN system, this suggests that the Type I IFN pathways supersedes RNAi as the cell differentiates.[203] Additionally, it had previously been published that these cell types express the alternate short form of Dicer ($Dicer^{O}$, discussed earlier) and that this isoform of Dicer preferentially processes long dsRNA instead of hairpins. Therefore, it may be that RNAi functions in these cells specifically because they have no IFN and express a Dicer that can actually process the viral dsRNA. Whether this holds any relevance in actual disease remains to be seen.

In addition to the reports showing Dicer cleavage of virus, a report from the TenOever lab identified Drosha as an interferon-independent antiviral

factor.[204] Upon infection with Sindbis virus, VSV, ΔNS-influenza virus, or upon poly I:C transfection, they observed translocation of Drosha from its usual location in the nucleus into the cytoplasm. Following Sindbis infection of wild-type (WT), Dicer knockout (KO) or Drosha KO MEFs, they observed an increase in Sindbis replication in the Drosha KO cells only, suggesting a Dicer independent processing of viral RNA. Deep sequencing of infected cells revealed an accumulation of Drosha-dependent small RNAs mapping to the Sindbis virus genome.[204] Intriguingly, one reason that Sindbis was chosen as the model virus was a previous report that Sindbis produced a miRNA-like RNA after infection of mammalian cells.[205] While there was an overall increase in Drosha-dependent processing of the Sindbis genome, no accumulation of ~21 nt vsiRNAs was observed, suggesting that Drosha cleavage does not feed into the canonical RNAi pathway. Instead, the presence of stem-loops in the secondary structure of Sindbis RNA appear to be the targets, and the reduction of viral titer in Drosha-competent cells may be due to direct cleavage of Sindbis genomic RNA by Drosha.

THESIS RATIONALE AND OBJECTIVES

The role of small RNA pathways in antiviral immunity and regulation of the immune response is an intense field of research. However, the field is complicated by seemingly conflicting results depending on the cell types, viruses,

and immune stimulants used in individual studies. The potential dual-role of the small RNA pathways as immunity-relevant processors of viral RNA and as gene-regulatory processors in the miRNA pathways make interpretation of results complex. Often when a system is first described, it is explored in broad strokes, when the reality is that as our understanding matures, the nuances of each pathway come to the foreground.

Considering both the depth and breadth of small RNA pathways in governing antiviral immunity, we sought to explore how eliminating one gene in these pathways, Dicer, could impact Type I IFN induction. This work explores the role of Dicer in the Type I IFN response to several model RNA viruses as well as in the response to several non-viral nucleic acid ligands. Chapter II is a brief presentation of the steady-state status of the system used for our work: Dicer WT or KO murine embryonic fibroblasts (MEFs). Chapter III presents data involving the use of model viruses to explore the Type I IFN response in the presence and absence of Dicer and how the loss of Dicer can impact the replication of one of the model viruses, EMCV. Chapter IV presents data on type I IFN production following stimulation with several non-viral nucleic acid ligands. Chapter V discusses how the data presented in this thesis fits into the panorama of currently available research and provides future directions that could be built upon the data in this thesis.

This work contributes to our understanding of how Dicer can impact the expression of innate immunology sensors as well as impact the replication of

viral pathogens. Research in this field will add insight into human disease models as well as potential targets for therapeutic interventions.

CHAPTER II – Dicer null MEFs: A model

ABSTRACT

Studying any complex system can result in difficulty when trying to carefully control experiments: thus, many researchers make use of models to ask their questions and then extrapolate their results. Any good model needs to be defined, however, to ensure that the phenotypes that are observed are due to the changes the researcher has made and not an artifact of the system. In this chapter, I define the Dicer null murine embryonic fibroblast (MEF) model and describe long term perturbations of the innate immune pathways. Additionally, I show that reconstituting Dicer in the Dicer null MEFs results in the rescue of miRNA maturation, but does not completely rescue the expression of various innate immune factors.

INTRODUCTION

The knockout mouse has been a potent tool for understanding gene function since it was first described. (Discussed in [206]). However it has limitations when the gene being knocked out is embryonic lethal. The initial attempts to create a Dicer knockout mouse were unsuccessful due to the lethality of Dicer deletion. [207] Dicer knockout embryos die by day 7.5. Thus, conditional knockout models were created where the targeting cassette for Dicer was flanked by loxP.[208] Upon addition of Cre recombinase from an internal source (Cre driven by a cell specific promoter), or from an external source (such as a retrovirus expressing Cre), the loxP sites recombine and Dicer is excised. However even this proved to be difficult as cells lacking Dicer quickly underwent senescence due to the loss of miRNA regulation of the $p16^{INK4A}$/$p19^{ARF}$ locus. Knocking out the $p16^{INK4A}$/$p19^{ARF}$ locus or p53 relieved Dicer knockout cells from senescence and allowed conditional Dicer knockout lines to undergo Cre recombination and survive.[208] Several lines deficient in Dicer have been created, ranging from embryonic stem cells, to MEFs, to a variety of conditionally knocked out Dicer in mature lineages.[209-213] Considering that Dicer can behave differently in different cell lineages, it is important to characterize the system that will be used for future experimentation. In this chapter I will define the Dicer deficient MEFs used in our experiments.

RESULTS

Dicer1 KO cells are unable to process precursor miRNAs into mature miRNAs.

In order to ascertain what role Dicer may play in regulating antiviral immunity, we used cells from previously described *Dicer1* conditional knockout mice (*Dicer1*$^{c/c}$ p53-/-).[208] A single line of *Dicer1*$^{c/c}$ cells were infected with an GFP or Cre expressing adenovirus and the cells were serially diluted to form single cell colonies. The colonies were verified by PCR to be WT, heterozygous or homozygous deleted. To verify that DICER1 function had been ablated or reduced in the *Dicer1* null and heterozygous MEFs, I analyzed the ability of each of the three genotypes to generate mature miRNAs. MEFs that maintained two functional alleles of *Dicer1* were able to convert endogenous pre-Let7 to its mature 22 nt form **(Fig. 2.1a)** MEFs that were heterozygous for *Dicer1* showed a marked decrease in the amount of mature Let-7 generated and also showed an accumulation of pre-Let7, suggesting that one allele of *Dicer1* is not sufficient to maintain miRNA homeostasis in rapidly dividing cells **(Fig. 2.1a)**. *Dicer1* null MEFs showed no mature Let-7 and an accumulation of the precursor, indicating that Cre recombination had produced a true *Dicer1* null cell with no functional DICER1 protein. **(Figure 2.1a)** Small RNAs from the *Dicer1* WT and KO cells were sequenced using the Solexa deep sequencing platform. Size selected RNAs were mapped to the mouse genome (mm9). [214] Far fewer sequences

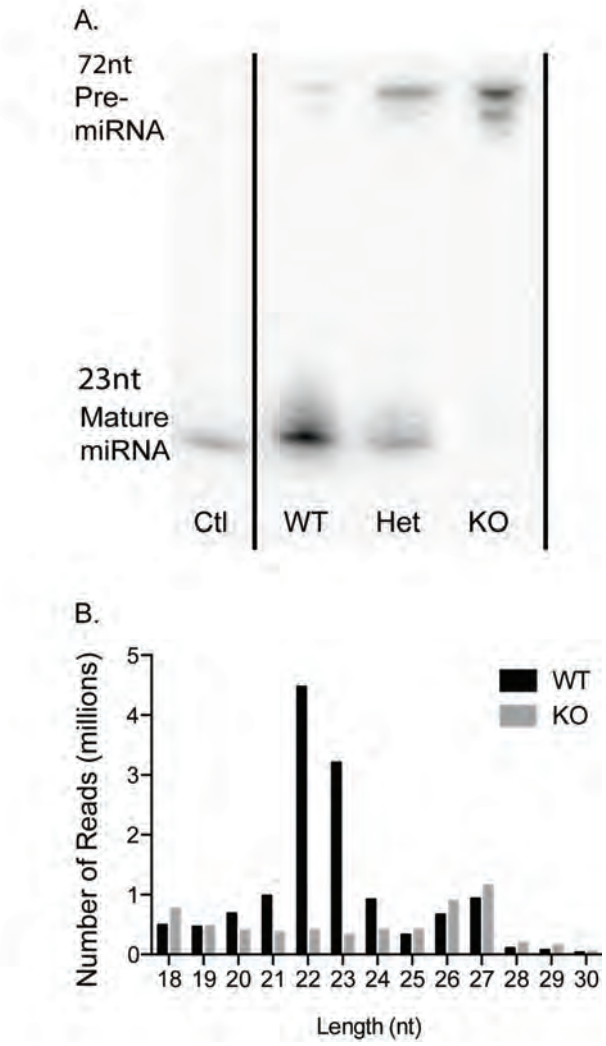

Figure 2.1 *Dicer1* KO cells do not produce mature miRNAs
(A.) Northern blot showing the accumulation of mature and precursor Let-7 miRNA in *Dicer1* WT, Heterozygous and KO mice. 4ug of small RNA (<200nt) were isolated from each genotype and synthetic Drosophila Let-7 was used as a positive control. The RNA was loaded onto a 15% PAGE urea gel. The RNA was transferred to a positively charged Nylon membrane and blocked with UltraHyb Oligo hybridization buffer. p32 α-ATP labeled Let-7 Starfire probe hybridized overnight and the gel was sequentially washed with 20X SSC and .5X SSC. **(B.)** Length distribution of small RNAs sequenced from *Dicer1* WT and *Dicer1* KO Total RNA from the two genotypes was isolated and size selected on a 15% PAGE gel for RNAs between 18-30nt. Following small RNA library preparation (see Materials and Methods), the RNA was mapped to the mouse genome (mm9).

were read in the *Dicer1* KO cells and the peak of canonical miRNAs at 21-23nt was absent, whereas piRNAs at 27nt were unaffected. **(Fig 2.1b)** When the reads from Dicer WT, Dicer heterozygous, and Dicer KO were analyzed, a marked decrease in miRNAs was observed, however over 600,000 reads still matched to putative miRNAs. **(Table 2.1)** Upon further investigation, the majority of the miRNAs that were still observed were found to be artificial. That is, computational analysis had identified them as potential miRNAs, but when they were tested experimentally, they were found to not be true miRNAs. [215] Still, the majority of miRNAs followed the expected outcome and were seen expressed at 10 to 100 fold more in the WT than the KO. **(Fig. 2.2)**

***Dicer1* WT and KO cells generate limited vsRNAs from Sendai virus.**

While previous reports have shown that virus-derived small RNA (vsRNA) formation in mammalian cells is limited at best, we wanted to see if our cells generated any vsRNAs when infected with an RNA virus. Sendai virus is a paramyxovirus with a negative sense ssRNA genome. [216] Sendai virus is also known for having a RNA-dependent RNA polymerase (RdRp) that is error-prone during replication initiation. It often makes runs along the genome before falling off, thus generating small 5'ppp RNAs that are too short to support any protein translation.[217] These RNAs are known as copyback defective interfering (DI) particles, due to their ability to promote detection by Rig-I and lead to IFN production that interferes with a successful viral infection.[217,218] Various strains of

Genotype	Total Inserts	Genome Mapping Inserts	Non-coding RNAs	Genome mapped Excluding ncRNAs	piRNAs	miRNAs	Other RNAs
Dicer WT	19695829	13365465	2739459	10626006	532343	9541266	552397
Dicer KO	10595727	6009340	3861734	2147606	673078	615132	859396

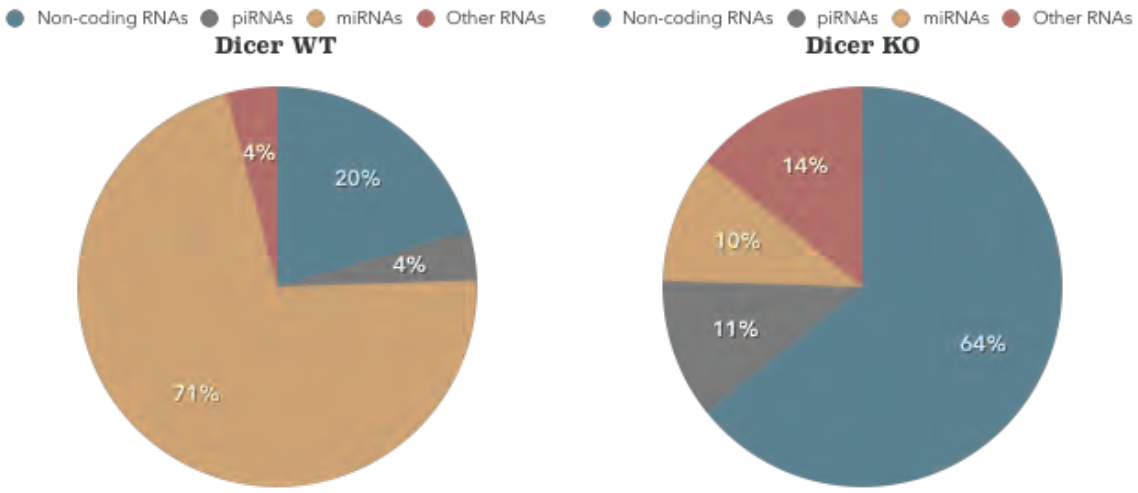

Table 2.1 RNA Deep sequencing of Dicer WT and KO cells.
RNA was isolated from Dicer WT and KO cells and size-selected (18-30nt) deep sequencing libraries were prepared. Sequencing reads are presented as raw numerical count of the different matched RNAs. Dicer KO cells generated roughly half the total reads of WT. Non-coding RNAs are RNAs that matched the genome, but are not part of any known transcribed region. piRNAs mapped equally in both cell types to annotated piRNAs. miRNAs are mapped according to putative miRNAs determined both experimentally and computationally. Other RNAs refer to transcribed RNAs that matched other sequences, some of which are not normally expected to be found in an 18-30nt sample, such as ribosomal RNA fragments. Pie charts represent the percentage of each type of RNA matched to the genome in Dicer WT and KO cells.

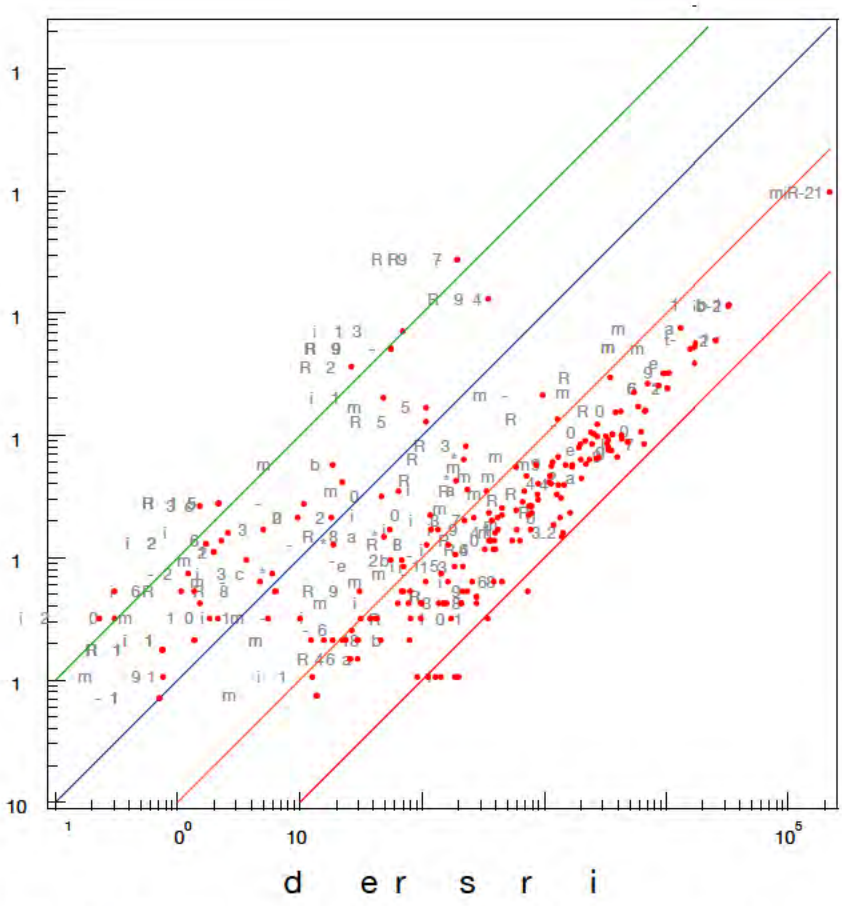

Fig 2.2 Dicer KO cells only express computationally defined miRNAs
RNA sequencing reads were matched to putative mouse miRNA sequences and quantified. As expected, most miRNAs were expressed at 10-100 fold higher levels in WT than KO cells. The miRNAs that were detected in Dicer KO cells were found to be computational defined as potential miRNAs but had been largely discredited as real miRNAs by the Bartel group. Lines: Green - 10 fold increase in Dicer KO / WT. Blue - 1:1 ratio in Dicer WT / KO. Orange - 10 fold increase in Dicer WT / KO. Red - 100 fold increase in Dicer WT / KO.

Sendai virus have differing degrees in which they generate DI particles and thus activate the interferon response. The Cantell strain of Sendai virus is known to generate large quantities of DI particles, to the point where the DI particles interfere with viral replication by activation of the immune response as well as acting as ribosomal decoys, where no productive protein production is generated from them. Of note though is that these copy-back particles in Sendai Cantell can be large, on the order of 2kb of dsRNA. [217,218] Thus, these large dsRNAs would be a prime candidate for processing by Dicer. Deep sequencing analysis of Dicer WT and KO MEFs infected with Sendai virus revealed that both WT and KO produced vsRNAs, primarily located at the 5' and 3' end of the viral genome. **(Fig. 2.3a)** However, more reads were mapped to Sendai in the Dicer KO samples than in the WT samples, suggesting a Dicer independent phenotype. Length distribution (LENDIS) of the mapped reads showed most reads were at the lower cutoff of 18nt, also suggesting a Dicer independent phenotype. **(Fig. 2.3b)**

***Dicer1* KO cells have altered levels of innate immune receptors and adaptors.**

In order to provide a robust picture of the state of the immune system in a Dicer-depleted cell, I assessed the levels of some of the nucleic acid receptors at steady-state levels in the WT and KO cells. Nanostring analysis of steady-state mRNA revealed that while some receptors and adaptors, such as Rig-I, MDA5,

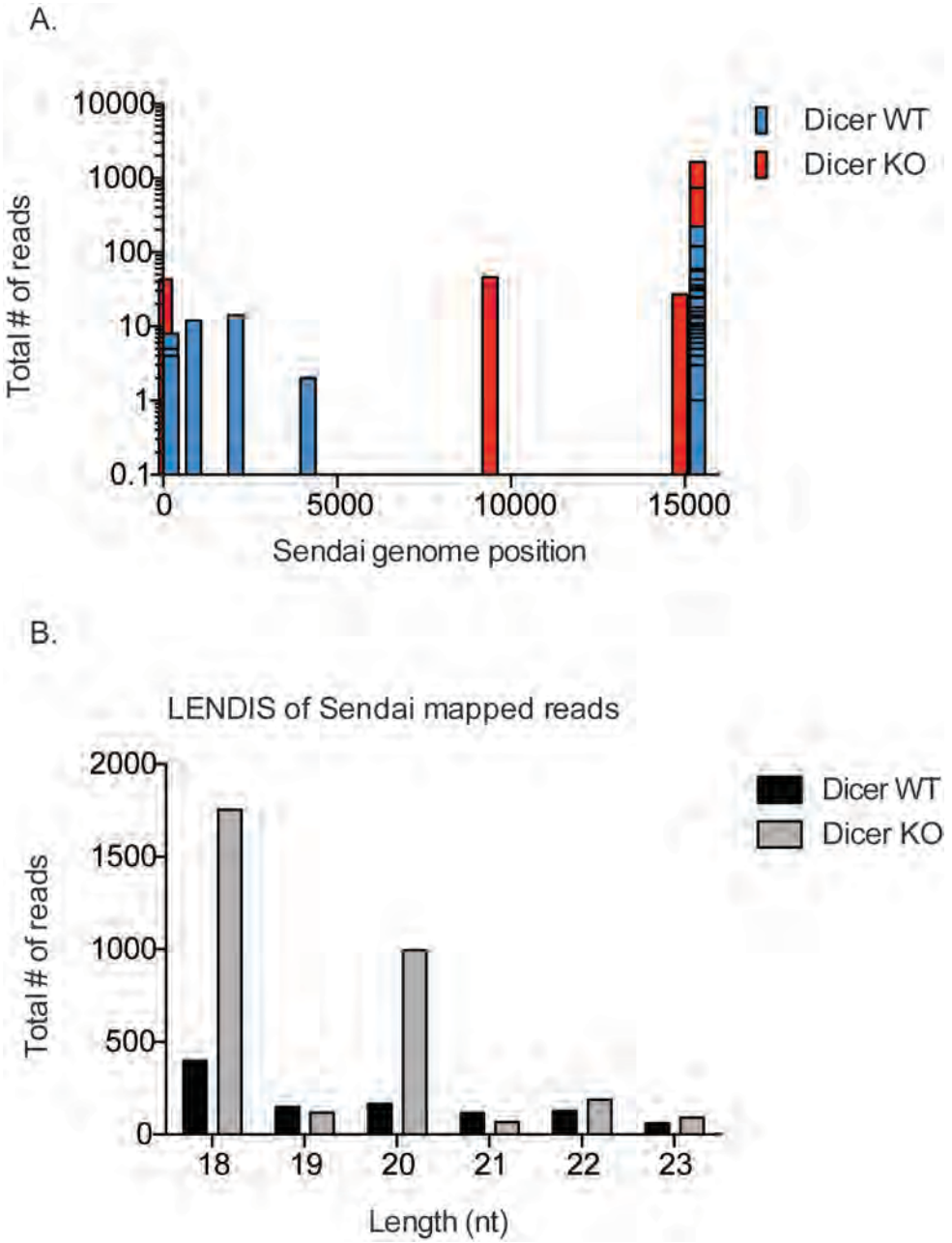

Fig. 2.3 Sendai virus mapped RNA deep sequencing of Dicer WT and KO cells.

Dicer WT and KO were infected with [80HA/mL] of Sendai virus and RNA was isolated from Dicer WT and KO cells after 8 hours. Size-selected (18-30 nt) deep sequencing libraries were prepared. (A.) Results are presented as matched small RNAs to Sendai virus genome position. The majority of reads matched to the 5' or 3' end. (B.) LENDIS distribution revealed no increase of small RNAs in Dicer WT cells and no uniformity to the size of the RNA, suggesting Dicer independent formation of the Sendai mapped small RNAs.

IFIT1,and MAVS showed equivalent levels between WT and KO cells, the general trend was that KO cells had reduced levels of receptors when compared to their WT counterparts. **(Fig. 2.4a)** The DExD/H RNA helicase LGP2 showed a marked decrease in expression, as well as the PYHIN containing DNA helicases IFI205 and IFI204, and the DNA sensor STING. The GTPase MX1, which in mice has broad anti-viral activity against nuclear-replicating viruses and in particular against Influenza, is down-regulated as well. IFIT3 and IFITM2 and M3 proteins, which are known to restrict the replication of enveloped viruses as well as sequester 5'ppp RNA, are all significantly reduced compared to WT. To test whether these genes are permanently down-regulated, we added exogenous Type I IFN to the supernatants of the cells for 2 hours and then looked at the upregulation of IFN stimulated genes (ISGs). In all cases, adding exogenous IFN upregulated ISGs in both the Dicer WT and KO cells, and in the case of MDA5, actually produced an increase in the levels of MDA5 transcript. **(Fig. 2.4b-e)** This suggests that both the WT and KO cells have an intact IFN receptor and signaling pathways and that if properly stimulated, the ISGs can be upregulated. However in the unstimulated basal-state, the cells have reduced levels of some nucleic acid detectors.

Reconstitution of Dicer rescues mature miRNA production.

Dicer is involved in regulating many genes through its function as a miRNA processor. Given that a prolonged loss of miRNAs may have unforeseen

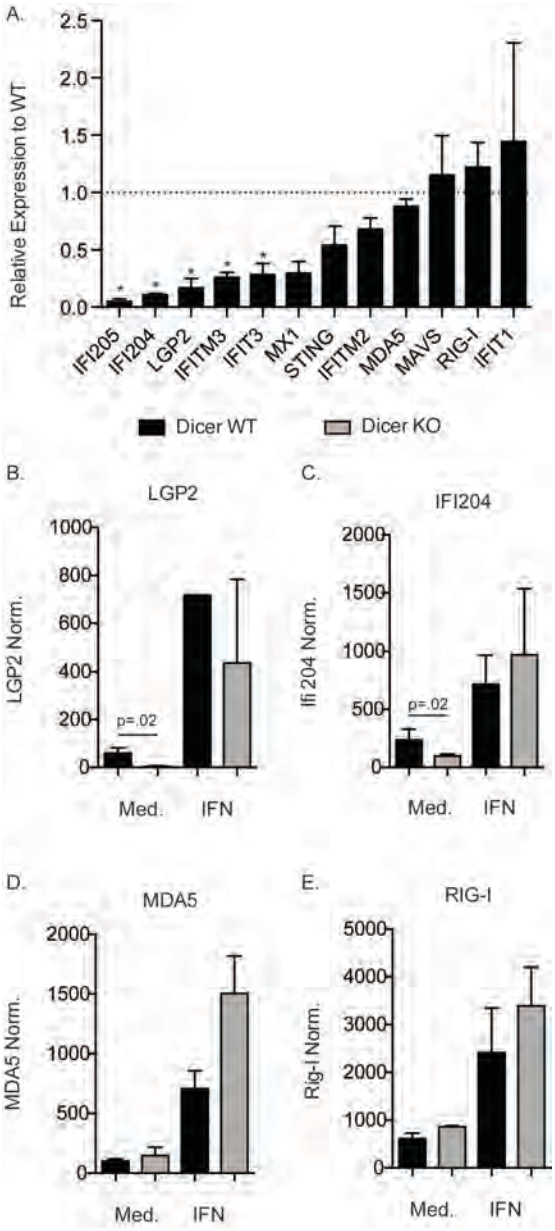

Fig. 2.4 Nucleic acid receptors and ISGs have variable expression in Dicer KO cells.

(A.) Dicer WT and KO MEF RNA at steady-state levels. RNA was counted on a Nanostring and results are shown normalized to the geometric mean of *GusB* and *HPRT* expression. Results are average of three biological replicates. **(B.-E.)** WT and KO cells were stimulated with medium or exogenous IFN (100U/mL) for 1 hour and RNA was isolated and counted on Nanostring. Results are shown normalized to the geometric mean of *GusB* and *HPRT*. Results are the average of two biological replicates.

effects on the overall fitness of the cell, we reconstituted expression of Dicer in the KO cells using a codon-optimized version of human *DICER1*. Initial reconstitutions all failed due to random recombination events of Dicer during the cloning process. Only when the sequence of Dicer was codon-optimized for maximal expression in mammalian cells and minimal expression in bacterial cells were we able to generate the lentivirus constructs necessary for reconstitution. I transduced *Dicer1* WT and KO cells with a lentivirus based vector containing GFP, full length human *DICER1* (hsDicer), or a truncated *DICER1* missing the helicase domain (ΔHelicase). I then assessed expression of hsDicer by both Western blot **(Fig. 2.5a)** and hsDicer mRNA levels **(Fig. 2.5b)** and the rescue of the common miRNA Let-7 **(Fig. 2.5c)**. Let-7 expression was normalized to the Dicer-independent small RNA Sno202 and expression in both the hsDicer and ΔHelicase reconstituted cells showed comparable levels to the *Dicer1* WT cells. Interestingly, the ΔHelicase mutant showed increased levels protein expression, mRNA expression and Let-7 cleavage. I attempted to modulate the expression of the ΔHelicase mutant, however I was unable to normalize the expression profile in relation to hsDicer. The shorter sequence may have increased the rate at which the ΔHelicase mutant could be expressed, leading to higher levels, while the increased Let-7 cleavage could be due to increased protein levels or may be due to the auto-inhibitory nature of the Dicer helicase domain.[163]

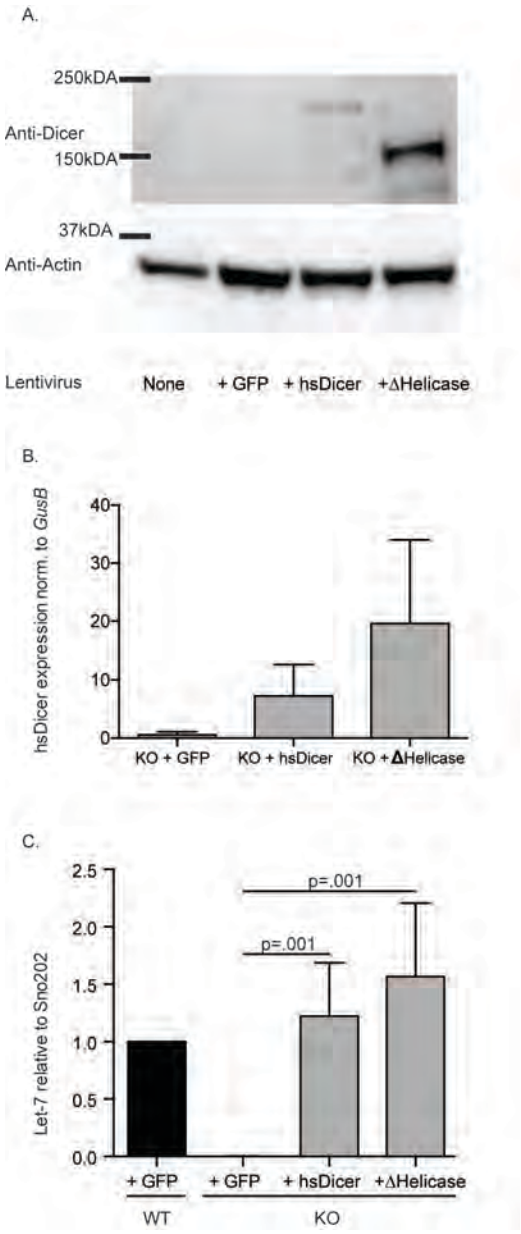

Fig. 2.5 miRNA maturation can be rescued after reconstitution with WT or ΔHelicase Dicer.

(A.) *Dicer1* WT and KO cells were transduced with a lentivirus that expressed GFP, human *DICER1* (hsDicer) or *DICER1* missing the helicase domain (ΔHelicase). Following 24 hours, the cells were selected using puromycin and allowed to recover for 3 days. Protein lysates showed the presence of full length human DICER1 and a smaller helicase-deleted variant. **(B.)** RNA from rescued cells was isolated and Taqman assays for Let-7 and Sno202 were run according to the manufacturer's specifications. Let-7 expression is shown normalized to Sno202 . Data is shown as the mean of four biological replicates.

Dicer reconstitution fails to rescue all innate immune receptors and adaptors.

While the reconstitution showed a complete rescue of miRNA maturation as assessed by Let-7 maturation, the reconstitution failed to rescue all of the receptors and adaptors that had been downregulated at the basal state in Dicer KO cells. While STING, IFI204, and MX1 all showed some level of rescue, LGP2, IFI205, and IFIT3 all failed to return to WT levels following the reconstitution of Dicer. **(Fig 2.6a-c)** Since Dicer can only process miRNAs that exist in pre-miRNA form, it is possible that some alteration of the transcription profile may have occurred in the KO cells; thus, without the proper stimuli, the reconstituted KO cell may never see the proper regulatory pre-miRNA. In this case, the reconstituted Dicer would have no effect on the rescue of any gene downstream of an unexpressed pre-miRNA.

DISCUSSION

Since any model system is only that, a model, special care must be made not to draw too broad of a conclusion from the data generated. What is important is that careful consideration be given to the strengths and weaknesses at hand. Since Dicer is required for embryogenesis, there is no way to study the lack of Dicer on a whole organism. Therefore we developed a system that could be used to explore the questions asked later in this thesis. That is, my model had to

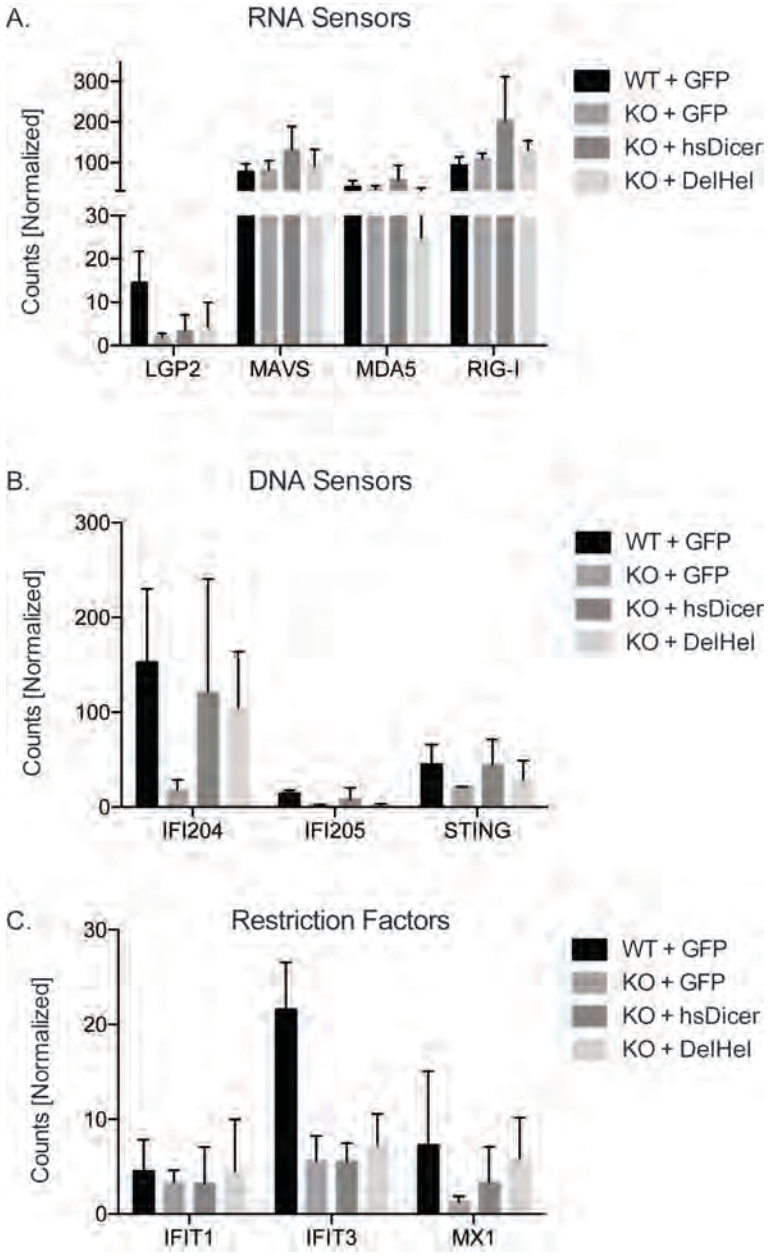

Fig. 2.6 Dicer reconstitution fails to rescue expression of LGP2 and IFIT3
RNA was extracted from WT, KO, KO +Dicer and KO + ΔHelicase MEFs at steady state. The RNA was hybridized to a custom nanostring codeset overnight and counted. Results are presented as total counts normalized to the geometric mean of *GusB* and *HPRT*. Results are the average of three biological replicates. The panels are divided into **(A.)** RLRs, **(B.)** DNA Sensors and **(C)** restriction factors.

be able to produce IFN, support viral infection and replication, be able to be reconstituted with Dicer or mutant Dicer, and be easy enough to maintain that experiments could be run in replicates. There are many other Dicer deficient models that I could have used, and in fact I did use some of them but found them unsuitable for my studies. Since Dicer depletion causes an immediate shift to senescence, I found that by the time we were able to deplete Dicer with either drugs or a Cre delivering virus, select the cells, and let them recover, the Dicer depleted cells were already unsuitable for studying the IFN response. Thus I decided to continue using our p53-/- cells as I could study the effects of Dicer knockout without the worry of senescence. Of course, introducing a mutation such as this, depleting p53, will have a major impact on other pathways in the cell. p53 has a major role in sensing DNA damage and regulating the cell cycle to prevent replication of a cell with damaged DNA.[297] In order to provide some assurance that deleting p53 would not fundamentally alter the results we obtained from stimulating our cells, many of the experiments were done simultaneously with the Dicer cells that had the $p16^{INK4A}$/$p19^{ARF}$ locus deleted. The results were the same, and data from Dicer c/c $p16^{INK4A}$/$p19^{ARF}$ are not shown in this dissertation.

Upon characterization of my cells, I determined that Dicer-deficient cells are truly deficient in a variety of ways. The loss of miRNA maturation was expected and became the evidence used to determine the knockout or reconstituted status of my cells. Reconstitution of the Dicer KO cells with hsDicer

or ΔHelicase Dicer did result in differing amounts of protein being expressed. However, in future chapters, the phenotype observed for both the full length and ΔHelicase Dicer were identical, suggesting that, at least for the role of Dicer in controlling the IFN response, the helicase domain is dispensible and having additional Dicer protein may not affect the overall maturation of miRNAs. Impacting my studies, I found that some of the innate immune receptors and adaptors were expressed at low levels in the KO cells and a subset of them could not be rescued with expression of ectopic Dicer. While Let-7 maturation suggests that Dicer reconstitution rescued mature miRNA formation, Let-7 is a miRNA that is constitutively expressed. A low copy-number inducible miRNA may not be fully rescued in the same manner and may be responsible for the lack of rescue seen with LGP2, and IFIT3. While these two genes were the only ones that showed statistical significance between the WT and the reconstituted cells, it is unclear how the levels of these genes impacts the response. Other genes like IFI205, MX1 and IFI204 showed variation in how well they were rescued after the addition of Dicer, and while the raw numbers do not support significance based on the universally supported $p<.05$ scheme, there can still be a biological relevance to the depletion of these genes. IFI204, STING and MX1 all showed a marked decrease in their reconstituted form compared to wild-type, but had p values between .05 and .1. Again, it is unclear how these differences affect function however, it is clear that Dicer null cells are deficient in a variety of innate immune receptors and adaptors and that reconstituting Dicer may not

rescue all miRNA expression when that expression is independent of the temporal and stimulated status of the cells when they are reconstituted.

MATERIALS AND METHODS

Dicer WT and KO MEFs

Dicer1$^{c/c}$ were created as previously described in the laboratory of Dr. Stephen Jones.[208] These conditional knockout mice have loxp sites flanking exons 15-17 of both *Dicer1* alleles. The exons encode the PAZ domain, which is required for Dicer to bind to the terminal end of the dsRNA, whether it is linear or the stem of a pre-miRNA. Following Cre recombinase expression by transducing the cells with a Cre-expressing adenovirus, exons 15-17 are excised and all downstream exons, including those that encode the RNase III domains, are placed in an incorrect translational frame, effectively eliminating protein expression. As previously described, knocking out *Dicer1* induces p19^{ARF} – p53 mediated senescence. In order to relieve the cells of p53 mediated senescence, the *Dicer1* conditional mice were crossed to p53 null mice to generate a line of mice that were *Dicer1*$^{c/c}$ p53 KO. To generate murine embryonic fibroblasts (MEFs), pregnant mice were sacrificed on day E13.5 and the embryos were dissociated and cultured. A line of MEF cells was established after adherence of the dissociated cells. The *Dicer1*$^{c/c}$ p53$^{-/-}$ MEFs were transduced with Ad5CMV-Cre Adenovirus vector from the Gene Transfer Vector Core at the University of

Iowa. The cells were cloned using limiting dilution and assayed by PCR and Northern blot. Three lines were established; *Dicer1* WT p53 KO, *Dicer1* Het p53 KO and *Dicer1* KO p53 KO. MEFs were maintained in DMEM supplemented with 10% FBS 2 mM glutamine and 2 mM penicillin/streptomycin.

Virus

Sendai virus, Cantell strain, was purchased from ATCC (Cat# VR-907). MEFs were infected at 80 hemagglutination units [HA/mL] and harvested at 8 hours for RNA, which was then processed for the deep sequencing libraries.

Northern Blot

Total RNA was isolated from cells lysed in TRIzol (ThermoFisher scientific #15596026) by extraction using acidified phenol:chloroform (ThermoFisher scientific #AM9720). The total RNA was resuspended in water and re-extracted using MirVana lysis/extraction buffer and miRNA homogenate additive (Life Technologies #AM1560). Following this second phenol:chloroform extraction, large RNA (>200 nt) was precipitated using 1/3 volume of ethanol and low speed centrifugation (2500g) at room temperature for 4 minutes. The remaining small RNA was precipitated with a 0.7 volume chilled isopropanol precipitation followed by a high speed centrifugation (16,000g) at 4° C for 10 minutes. The precipitated small RNAs were resuspended in water, quantified, and 4 µg of RNA was loaded in each lane of a 15% polyacrylamide gel (National Diagnostics #EC-833). The

gels were run, transferred to a positively charged nylon membrane, and non-specific hybridization was blocked by incubating the blot in ULTRA-Hyb oligo prehybridization buffer (Life Technologies Cat#AM8663). Starfire Probes against miRNA Let-7 were purchased (Integrated DNA Technologies) and the probes were labeled by α-P^{32} dATP in a polyA dependent manner. The probes were hybridized overnight, washed with 2X and 0.5X SSC wash buffer, and exposed to a phosphoimager plate.

Deep sequencing

Note: Sequences are listed in a table at the end of this subsection. RNA was extracted by phenol:chloroform and size selected on a 15% polyacrylamide gel. RNA between 18-30nt was ligated on the 3' end to the **3' adaptor** using T4 RNA ligase II, truncated (NEB #M0242S). The RNA was size selected on a 15% polyacrylamide gel again and RNA between 39 and 51nt was selected. The RNA was then ligated on the 5' end to the **5' adaptor** using T4 RNA ligase (NEB #M0204S) and bands between 65 and 77nt were size selected on a 10% polyacrylamide gel (National Diagnostics #EC-833). The RNA was then reverse transcribed using Superscript III reverse transcriptase (ThermoFisher scientific #18080044) and the **RT primer**. The newly synthesized cDNA was amplified using the following primers: **cDNA forward, cDNA reverse** The cDNA was amplified in 20 cycles and the resulting PCR products were gel purified on a 1% agarose gel. The fresh PCR products were then TOPO cloned into a pCR-Blunt-

TOPO vector (ThermoFisher scientific #K2800-02) and transformed into competent cells. Twenty colonies were selected and the inserts were amplified by PCR using the primers **M13R** and **M13F**. The inserts were then sequenced and analyzed to ensure library validity. Once the libraries were confirmed, the libraries were processed on a Solexa Genome Analzyer (Illumina, San Diego, CA)

Analysis was done by mapping sequences to the mouse genome (mm9) and allowing up to 1 mismatch. Sequences were annotated based on predicted miRNAs in miRBase release 17. [219-224]

Oligo Name	Sequence
3’ adaptor	5’ -AppCTGTAGGCACCATCAAT/ddC/- 3’
5’ adaptor	5’ –GUUCAGAGUUCUACAGUCCGACGAUC- 3’
RT primer	5’ –ATTGATGGTGCCTACAG- 3’
cDNA forward	5’-AATGATACGGCGACCACCGACAGGTTCAGACTTCTACAGTCCGA- 3’
cDNA reverse	5’ –CAAGCAGAAGACGGCATACGAATTGATGGTGCCTACAG- 3’
M13R	5’ –CAGGAAACAGCTATGAC- 3’
M13F	5’ –TGTAAAACGACGGCCAGT- 3’

Dicer reconstitution

The human *DICER1* gene sequence was obtained from NCBI gene bank (Gene ID: 23405). The gene was codon optimized by Dr. Jeremy Luban’s laboratory, and synthesized by Genscript USA Inc. The delta helicase mutant

was created by synthesizing the codon optimized human *DICER1* with amino acids 2-601 deleted. The start codon led directly into the sequence immediately following the helicase domain. The resultant codon optimized human *DICER1* gene was cloned into pAIP (pALPS-IRES-Puromycin S deaminase), an HIV-1 based transfer vector with *DICER1* expression under the control of the spleen focus-forming virus long terminal repeat (LTR). The generation of pAIP has been described previously. [225] Viruses were produced by transfection of 293T cells using TransIT-LT1 (Mirus #MIR2300), according to the manufacturer's instructions. For three-part vector systems, the following DNA ratio was used: 4 parts transfer vector : 3 parts packaging plasmid : 1 part envelope. The viral supernatant was collected 48 h after transfection, filtered through a sterile 0.45 µm syringe filter (Millipore #SLGP033S), and stored in 1mL aliquots at -80 °C. The frozen virus stocks were titered by measuring the reverse transcriptase (RT) activity present in the viral supernatant using qRT-PCR.

Nanostring

RNA from cells was collected and purified using the RNeasy Plus RNA purification kit (Qiagen, Cat #73404) according to manufacturer's specifications. RNA was quantified by UV absorbance on NanoDrop. Once quantified the RNA was hybridized overnight at 65^{o} C to a custom probe set covering 50 mouse innate immunology related genes. The labeled RNA was prepared and run on a NanoString Prep station and bioanalyzer. Data was normalized and analyzed

using nSolver v2.5. Counts were normalized to the geometric mean of GusB and HPRT as housekeeping genes.

CHAPTER III: Dicer depletion leads to a disparate Type I IFN response.

ABSTRACT

The regulation of the innate immune response is a complex process involving the activation of signaling pathways, transcriptional regulation, post-transcriptional regulation, and translational regulation, among others. miRNAs and other small RNAs can function in a trans-activating and post-transcriptional manner to initiate expression of, or suppress translation of, a variety of innate immune receptors and adaptors. Here I explore the role that Dicer depletion has on the initiation of the type I IFN response following a variety of stimulants. Intriguingly, Dicer depletion results in a near-universal loss of type I IFN expression regardless of the type of stimulant used. However, Sendai virus infection produces the opposite phenotype, where higher levels of IFN are produced after Dicer depletion. Here I report the results of IFN induction in Dicer null cells and hypothesize the various mechanisms that may be responsible.

INTRODUCTION

Viral infection gives rise to a variety of common indicators of infection, one of the most prevalent indicators being the presence of foreign nucleic acids in the cell. Viral genomes can have many possible nucleic acid configurations. RNA viruses can have positive, negative, or ambisense single-stranded RNA genomes as well as double-stranded RNA genomes. DNA viruses can be single- or double-stranded, or in the case of *Hepadnaviridae*, a genome consisting of single and double-strandedness.[216] Since mammalian cells contain some of the same types of nucleic acid configurations, primarily dsDNA and ssRNA, it is vital that the host antiviral defense mechanisms include a way to differentiate and detect nucleic acids that either do not exist in mammalian cells or are detected outside of the expected compartment that the nucleic acid would normally reside in. As discussed in Chapter I, these foreign nucleic acids activate a variety of receptors including the RIG-I like helicases: RIG-I, MDA5 and LGP2, and various DNA receptors including DAI, IFI16, cGAS/STING, and RNAPol III/RIG-I amongst others. These receptors are responsible for detecting a variety of ligands that are seen as foreign, including dsRNA (MDA5), 5' pppRNA (RIG-I), modified di-nucleotides(cGAS/STING), dsDNA (cGAS/STING, DAI, IFI16, RNAPolIII/RIG-I), as well as normal cellular ligands that are out of their normal location, such as genomic DNA in the cytosol of cells.[28,32,38,48,68,70,73,81,226] We are interested in

how the deletion of Dicer can alter these pathways and alter the downstream production of type I IFN

In order to regulate the expression of type I IFNs, multiple methods of gene expression regulation are employed, including transcriptional and translational regulation. One form of post-transcriptional control that has been heavily implicated in the innate immune response is the microRNA pathways. [176,227,228] miRNAs have been shown to heavily regulate the innate immune response. Eliminating certain miRNAs has deleterious effects on signaling, proper activation and maturation of the adaptive immune response[175,189,229], and in a few cases, direct suppression of a viral infection. [193] Complicating the role of miRNAs in general is that there are different expression profiles for miRNAs depending on the type of host cell. Additionally, miRNA expression differs as the cell undergoes maturation with some miRNAs only expressed after a cell has fully differentiated and some expressed only during the developmental stages. Here I explore the role of Dicer and its ability to process miRNAs upon the activation of the IFN response in murine embryonic fibroblasts (MEFs).

To test this, I used several synthetic ligands including the RNA ligands; poly I:C and 5'ppp RNA and the DNA ligands; poly dA:dT, HSV 60mer, and VACV 70mer. Additionally I used several viruses: EMCV, VSV and Sendai. EMCV and VSV are reported in Chapter IV, and Sendai is reported in this chapter.

Sendai virus[u] is a paramyxovirus that encodes its own RNA-dependent RNA polymerase (RdRp). During viral replication, the newly synthesized RNA is quickly bound by the viral nucleoprotein N, which prevents detection of the 5' ppp RNA by RIG-I. The N gene is the located on the 5' end of the (+) RNA, ensuring that it is the first viral protein generated during a viral infection. [216] Sendai virus is also known for having a RdRp that is error-prone during replication initiation. It often initiates runs along the genome before falling off, thus generating quantities of small 5'ppp RNAs that are too short to support any protein translation. These RNAs are known as defective interfering (DI) particles, due to their ability to promote detection by RIG-I and lead to IFN production that interferes with a successful viral infection. Various strains of Sendai virus have varying degrees to which they generate DI particles and thus activate the interferon response. The Cantell strain of Sendai virus has long been known to generate large quantities of DI particles, to the point where the DI particles interfere with viral replication by activation of the immune response as well as acting as ribosomal decoys, where no productive protein production is generated from them. [217,218] For this reason, the Cantell strain of Sendai has long been used as a method to induce interferon. Unless otherwise specified, the Cantell strain of Sendai was used for experiments.

[u] Sendai virus is also known as parainfluenza virus 5

RESULTS

Dicer1 KO cells generate more IFN and IFN-related chemokines following Sendai virus infection

I stimulated Dicer wild-type and Dicer knockout cells with medium or Sendai virus [80HA/mL] and assayed the supernatants by ELISA after 16 hours. While neither cell type showed any appreciable interferon production at basal levels, the Dicer knockout cells consistently produced more interferon protein than their wild-type counterparts following Sendai infection. **(Fig. 3.1a)** In order to ascertain if the increase in interferon was seen at the mRNA level as well as the protein level, I isolated RNA from Dicer wild-type and Dicer knockout cells over several time-points and measured interferon induction normalized to total ribosomal 18s RNA. Intriguingly, I found that interferon mRNA levels stayed fairly consistent between the wild-type and knockout cells, with only one time point showing a significant increase in message levels in the knockout cells. Whether or not this increase during a single time point can account for the increase in protein production remains unclear.**(Fig. 3.1b)**

To assess if the increase in IFN protein made a difference in downstream activation of IFNAR and upregulation of ISGs, I looked at supernatant levels of the IFN dependent chemokine CCL5/RANTES. Again, I observed that RANTES production was significantly higher in the Dicer KO MEFs compared to WT. **(Fig. 3.1c)** When replication levels of Sendai were observed, I noticed very mild

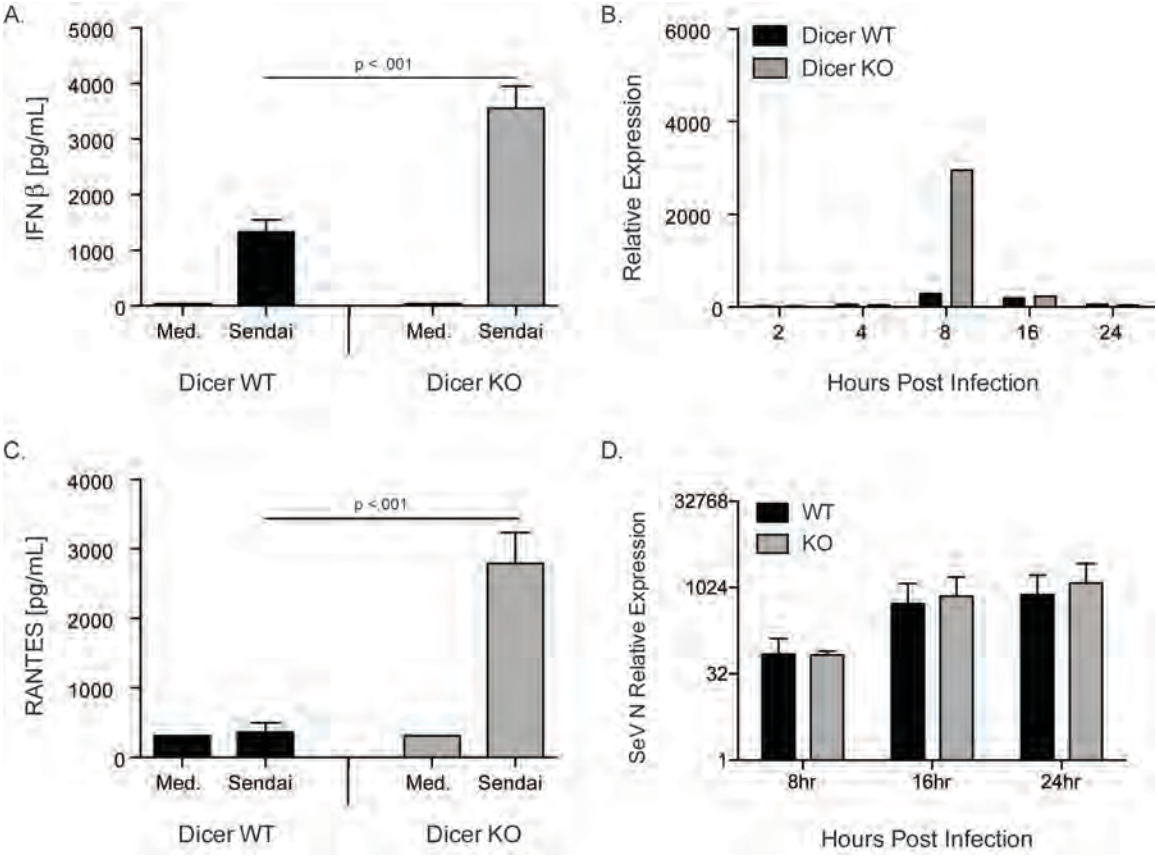

Figure 3.1 IFNβ protein production is enhanced in *Dicer1* KO cells following Sendai virus infection

(A.) *Dicer1* WT and KO cells were infected with Sendai virus [80HA/mL] for 16 hrs and the supernatants were assayed by IFNβ ELISA or **(C.)** RANTES ELISA. Data shown as mean of three biological replicates. **(B.)** *Dicer1* WT and KO cells were infected with Sendai [80HA/mL] and RNA was isolated at the time points indicated. *IFNβ* mRNA was detected by qPCR and normalized to 18s RNA and compared to steady state normalized *IFNβ* mRNA at time 0. Data shown as representative of three biological replicates. **(D.)** Sendai replication was monitored at the times indicated by extracting RNA and measuring the Sendai nucleoprotein RNA relative to 18s. Results are average of two biological replicates. replication of the virus with no discernible difference between Dicer WT and KO cells. The general replication defect of the Cantell strain has been observed before. **(Fig. 3.1d)**

replication of the virus with no discernible difference between Dicer WT and KO cells. The general replication defect of the Cantell strain has been observed before. **(Fig. 3.1d)** While most of the experimentation thus far had been in the Dicer WT and KO p53-/- cells, I also explored Sendai infection in primary macrophages taken from a Dicer conditional KO, ESR1-Cre + mouse. This mouse was not used much in experimentation due to the quick senescence in rapidly dividing cells, but as macrophages do not divide rapidly, they could be used for infection studies. By adding tamoxifen, the ESR1-Cre drove recombination of the loxP sites flanking Dicer and Dicer was excised. **(Fig 3.2a)** When infected with Sendai virus, the primary mouse macrophages showed a phenotype identical to the MEFs, with CCL5/RANTES significantly increased in the Dicer C/C ESR1-Cre cells that received tamoxifen and were depleted of Dicer. There was no difference between Dicer C/C and Dicer C/C ESR1-Cre when no tamoxifen was added to the system. **(Fig. 3.2b)**

Several factors may influence mRNA stability and levels, so I wanted to see if IFNβ transcription was affected in the absence of any coding region regulation as well as 5' and 3' untranslated region regulation. To test this, I used siRNAs to knockdown Dicer in HEK293 cells. HEK293 cells were transfected with 10nM of either a non-silencing (NS) siRNA or an siRNA specific against Dicer. Due to the fast growing nature of the HEKs, the knockdown was transient and I chose to use 48 hours as the endpoint for our assays. **(Fig. 3.3a)** I transfected HEK293 cells with a firefly luciferase reporter gene under control of

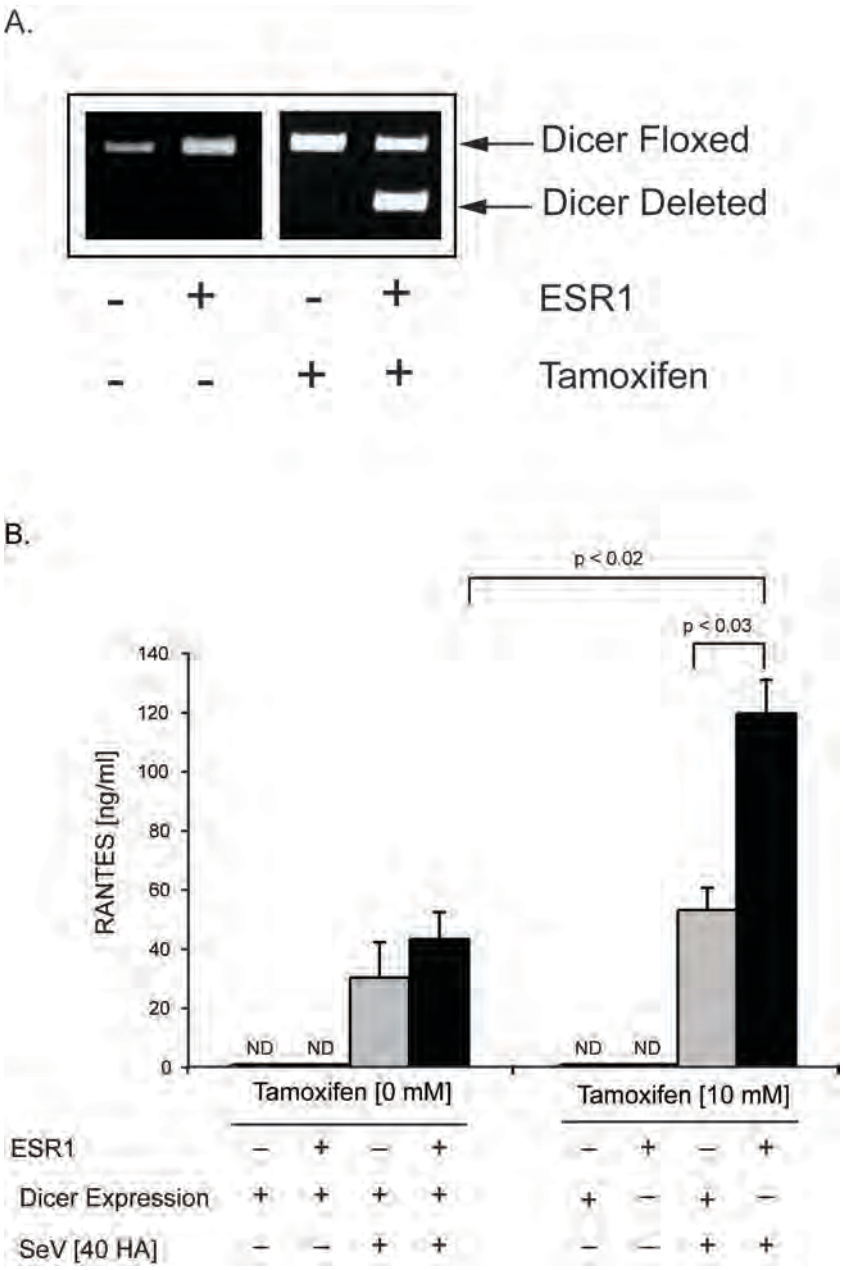

Fig. 3.2.) Bone Marrow Derived Macrophages (BMDMØ) from Dicer Conditional ESR1-Cre Knockout Mice Produce More of the IFN-related chemokine RANTES

(A.) Bone marrow cells were isolated from the femur and treated with L929 supernatants for 10 days. BMDMØ were given media or media with Tamoxifen and DNA and protein were isolated 48 hours later. Knockout was confirmed by PCR **(B.)** 48 hours post tamoxifen treatment, Dicer c/c BMDMØ were infection with 40 HA units of Sendai virus. Supernatants were assayed by ELISA 24 hours after infection.

the IFNβ promoter. I then stimulated the cells with medium or Sendai virus and measured total firefly luciferase values that were normalized to cellular protein content. When compared to medium or a non-silencing siRNA control, HEK cells that were knocked down for Dicer showed increased levels of interferon beta reporter activation when compared to medium or non-silencing siRNA controls. **(Fig. 3.3b)** To test whether this response was interferon specific and not a broad response to Dicer depletion, I decided to look at NF-κB promoter-luciferase reporter activity in the system. Sendai virus is a poor activator of the pathways that lead to NF-κB activation so I tested the cells with medium, Sendai and respiratory syncytial virus, which is known to activate the NF-κB response through TLR4 and TLR2. In our system, Dicer depletion had no effect on NF-κB reporter luciferase activity. **(Fig. 3.3c)** To see if the Sendai phenotype extended to a primary human line, I took human peripheral blood mononuclear cells (PBMCs) and transfected NS or Dicer specific siRNAs into them. Following Sendai infection of the human PBMCs, IFNα was measured in the supernatants and a significant increase of IFNα was observed in the PBMCs that were knocked down with Dicer. **(Fig. 3.4)**

***Dicer1* KO MEFs fail to produce IFN in response to stimulation with immuno-stimulatory nucleic acids.**

To examine how the loss of Dicer would impact the expression of type I IFN, I assayed the Dicer WT and KO MEFs' ability to generate IFN following

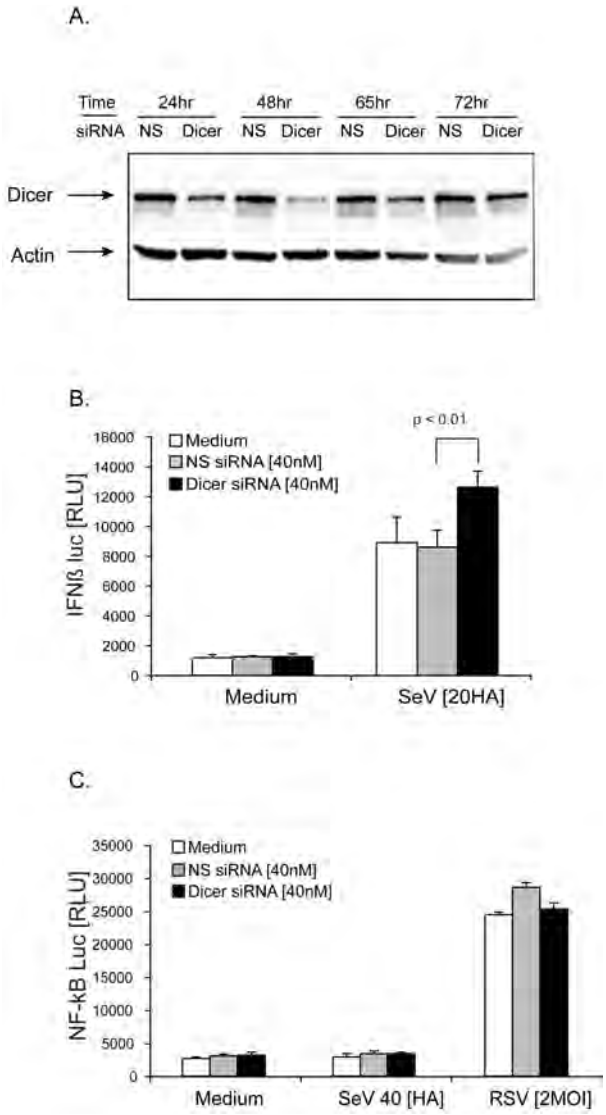

Figure 3.3) Dicer Knockdown Increases Sendai induced IFNβ transcription
HEK293FT cells were transfected with Lipid, a control non-silencing siRNA pool or a siRNA pool against Dicer (siDicer). 24 hours later, the cells were transfected with 80ng IFNβ-luciferase reporter **(A.)** Western blot of Dicer protein in HEK 293FT cells 24, 48, 65, and 72 hours post transfection with control or Dicer siRNAs. Actin blot shown as loading control. 48 hours was chosen as optimal Dicer knockdown. **(B.)** 24 hours post siRNA transfection, cells were transfected with IFNβ luciferase reporters. They were then infected with 20HA units of Sendai virus 24 hours after that. IFNβ-luciferase activity was measured 16 hours following infection. Luciferase activity is shown as relative light units (RLU) and have been normalized to total protein content in each well, determined by microBCA assay. **(C.)** 24 hours post siRNA transfection, cells were transfected with NF-κB luciferase reporters. They were then infected with medium, Sendai virus, or Respiratory Syncytial Virus and assayed after an additional 24 hours.

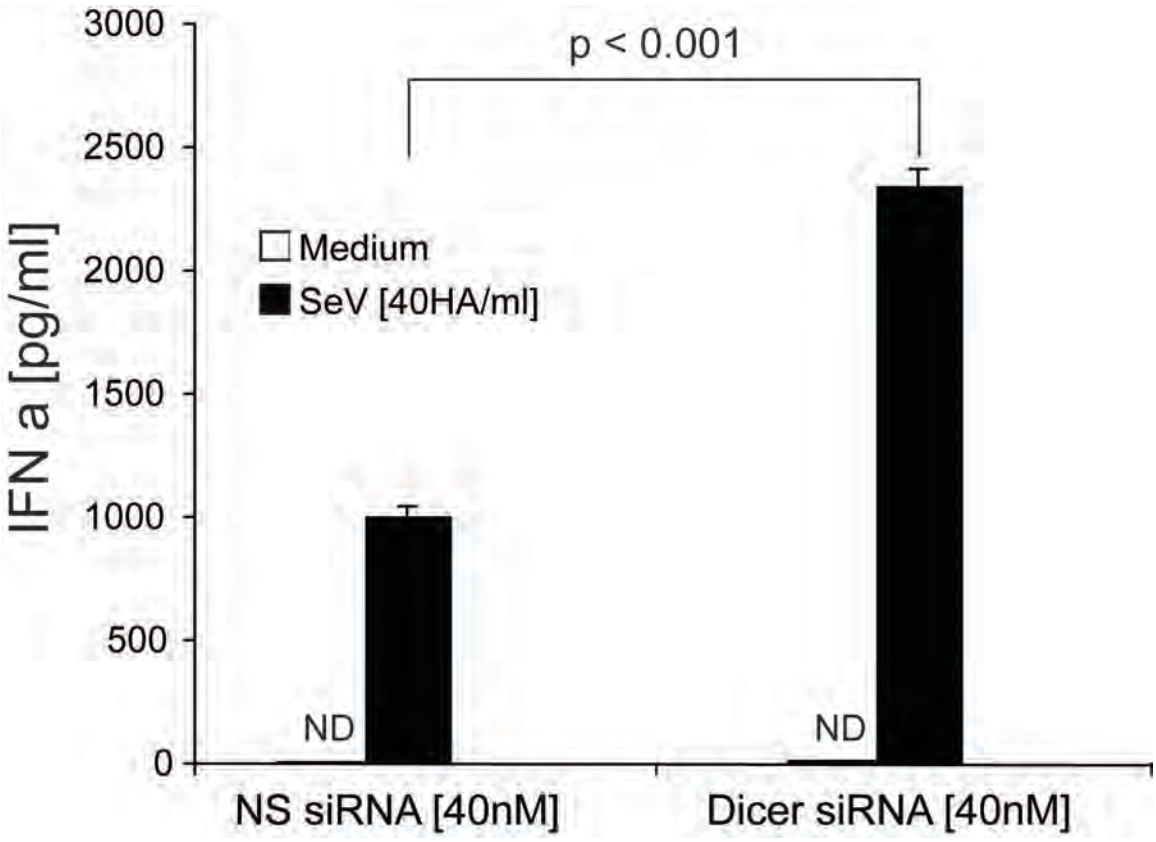

Fig. 3.4 Human PBMC's infected with Sendai express more IFNα after Dicer knockdown.

Human PBMC's were harvested, counted and plated. 24 hours later, non-adherent cells were removed and the cells were transfected with the indicated siRNAs. 48 hours later, the cells were stimulated with Sendai virus and supernatants were collected 16 hrs later. IFNa measured by ELISA.

stimulation with a variety of artificial ligands that activate different receptors. polydA:dT is a synthetic DNA ligand that is able to activate multiple receptors depending on the cell type. The RNA Polymerase III can transcribe AT-rich DNA into its RNA counterpart, which is then detected by RIG-I.[70] Additionally, IFI16 and its presumed mouse counterpart IFI204 can bind to the naked dsDNA and cooperatively assemble into filaments in order to initiate signaling to STING and ultimately IFN or to activate NLRP3 inflammasomes. [230] When I transfected poly dA:dT into our Dicer WT and KO MEFs, I observed a robust IFNβ protein induction in the wild-type cells while IFNβ protein induction was absent in the KO cells. **(Fig. 3.5a)** The downstream IFN inducible chemokine RANTES was also assayed by ELISA and a similar result was observed, with the KO cells failing to secrete RANTES. **(Fig. 3.5b)** A time course of the transfected cells revealed a strong and early induction of IFNβ mRNA transcription in the Dicer WT cells that tapered off over time, while the Dicer KO response was inconsequential. **(Fig. 3.5c)**

Since poly dA:dT is an entirely synthetic ligand, I then transfected in oligomers derived from herpes simplex virus (HSV60) and vaccinia Virus (VACV70) which had previously been shown to induce IFN signaling.[68] While both the Dicer WT and Dicer KO MEFs induced IFN in response to the positive control (Sendai virus) only the Dicer WT cells generated a protective IFN response when transfected with the immuno-stimulatory nucleic acids. **(Fig. 3.5d)** To test RNA based immune activation in the MEFs, I transfected the

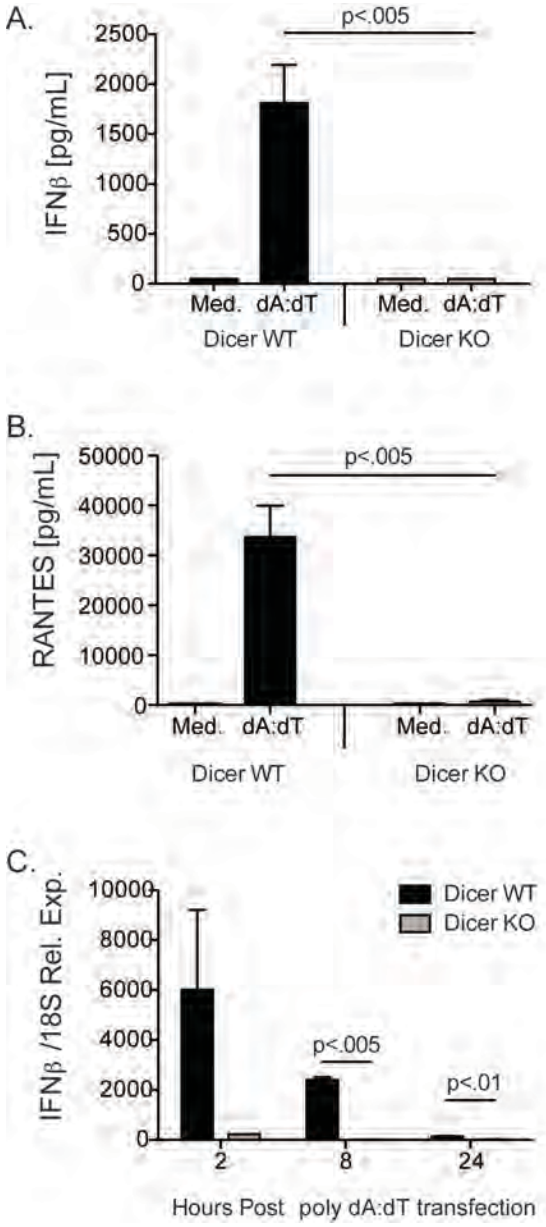

Fig 3.5 a-c. Dicer depletion results in loss of nucleic acid stimulated IFN induction.

Dicer WT and KO cells were transfected with medium or poly dA:dT [600ng/mL] and supernatants were collected after 8 hours and tested for **(A.)** IFNβ and **(B.)** RANTES production. Results are average of three biological replicates. **(C.)** WT and KO cells were transfected with medium or poly dA:dT [600ng/mL] and RNA was isolated at the indicated time points. *IFNβ* mRNA was normalized to 18s expression and graphed relative to medium stimulated cells. Results are average of three biological replicates.

synthetic dsRNA branched nucleic acid ligand poly I:C, which has repeatedly been shown to induce an IFN response through MDA5/MAVS.[32,33] While both the WT and KO cells responded to the polyI:C stimulation, the RANTES response was significantly higher in the WT cells compared to KO cells. **(Fig. 3.5e)** Attempts to detect IFNβ protein by ELISA were unsuccessful, as the induction of IFN was too low to accurately quantify in our cell types. The synthetic ligand 5'ppp RNA has been described to be a ligand for RIG-I based induction of IFN; however in our hands, the induction was low.[28,29] Regardless, when IFNβ mRNA levels were measured two hours after induction with HSV-60mer, poly I:C and 5'ppp RNA, a general trend was observed where the Dicer KO cells failed to robustly respond to any of the ligands. **(Fig 3.5f)**

Exogenous IFN fails to rescue ISG induction by nucleic acid stimulation.

Since I had observed that certain receptors were down-regulated in Dicer KO cells **(see Chapter II Fig. 2.4)**, I assessed whether exogenous IFN would drive expression of the receptors and allow a normalized response when stimulated with nucleic acids. I pre-stimulated Dicer WT and KO cells with medium or IFN and then transfected the cells with poly dA:dT or 5'ppp.[v] I isolated the RNA and analyzed it via Nanostring and observed a general defect in ISGs upregulation in the Dicer KO cells. Even so, the effect was more

[v] It should be noted that the medium and IFN only data is the same as presented in Chapter II. It is repeated here to compare to the data following stimulation with poly dA:dT and 5'ppp RNA.

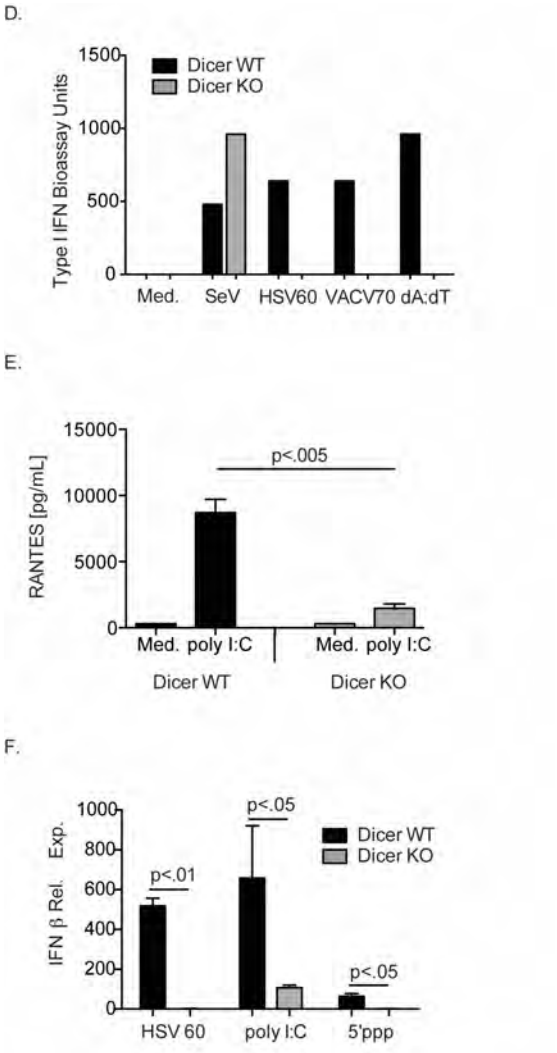

Fig 3.5 d-f. Dicer depletion results in loss of nucleic acid stimulated IFN induction.

(D.) WT and KO cells were stimulated with Sendai virus [80HA/mL] or transfected with medium, HSV60mer [500mM], VACV70mer [500mM], or poly dA:dT [600ng/mL] for 8 hours and supernatants were harvested. The supernatants were serially diluted onto NCTC929 cells for 24 hours and then challenged with VSV [1000pfu/mL]. The lowest dilution that protected 50% of the cells was recorded. Results are representative of three biological replicates. **(E.)** WT and KO cells were transfected with medium or poly I:C [2ug/mL] and supernatants collected and assayed for RANTES after 8 hours. Results are average of three biological replicates. **(F.)** Cells were transfected with medium, HSV60mer [500nM], poly I:C [2ug/mL], or 5'ppp RNA [1ug/mL] and RNA was collected after 2 hours. *IFNβ* expression was normalized to *GusB* expression and displayed relative to *IFNβ* induction in medium-treated cells. Results are average of three biological replicates.

noticeable in MEFs that had been pre-stimulated with medium instead of IFN. This suggests that while the Dicer KO cells still showed a general inability to respond to poly dA:dT and 5'ppp, this defect could be partially overcome by driving expression of the receptors with exogenous IFN. **(Fig. 3.6 a-d.)**

Dicer reconstitution fails to rescue poly dA:dT induced IFN and many anti-viral receptors.

By reconstituting Dicer into the KO MEFs we could add back the ability to process constitutive and inducible miRNAs into the cells. Transduction of Dicer KO cells with a lentivirus containing either a full length or helicase-deleted codon optimized version of human Dicer resulted in the full reconstitution of miRNA expression, as measured by Let-7 expression. **(Data shown in Chapter II Fig. 2.5)** Despite this, the reconstituted Dicer KO cells showed little actual IFNβ or RANTES produced following transfection with poly dA:dT, while the cells continued to respond normally to the control, Sendai virus. **(Fig. 3.7a-c.)**

DISCUSSION

The loss of Dicer potentially impacts hundred or thousands of genes, depending on the miRNAs expressed in the particular cell in question and the status of any external stimulants the cell may be receiving. I wanted to assess what impact the loss of miRNAs had on type I IFN responses to a variety of

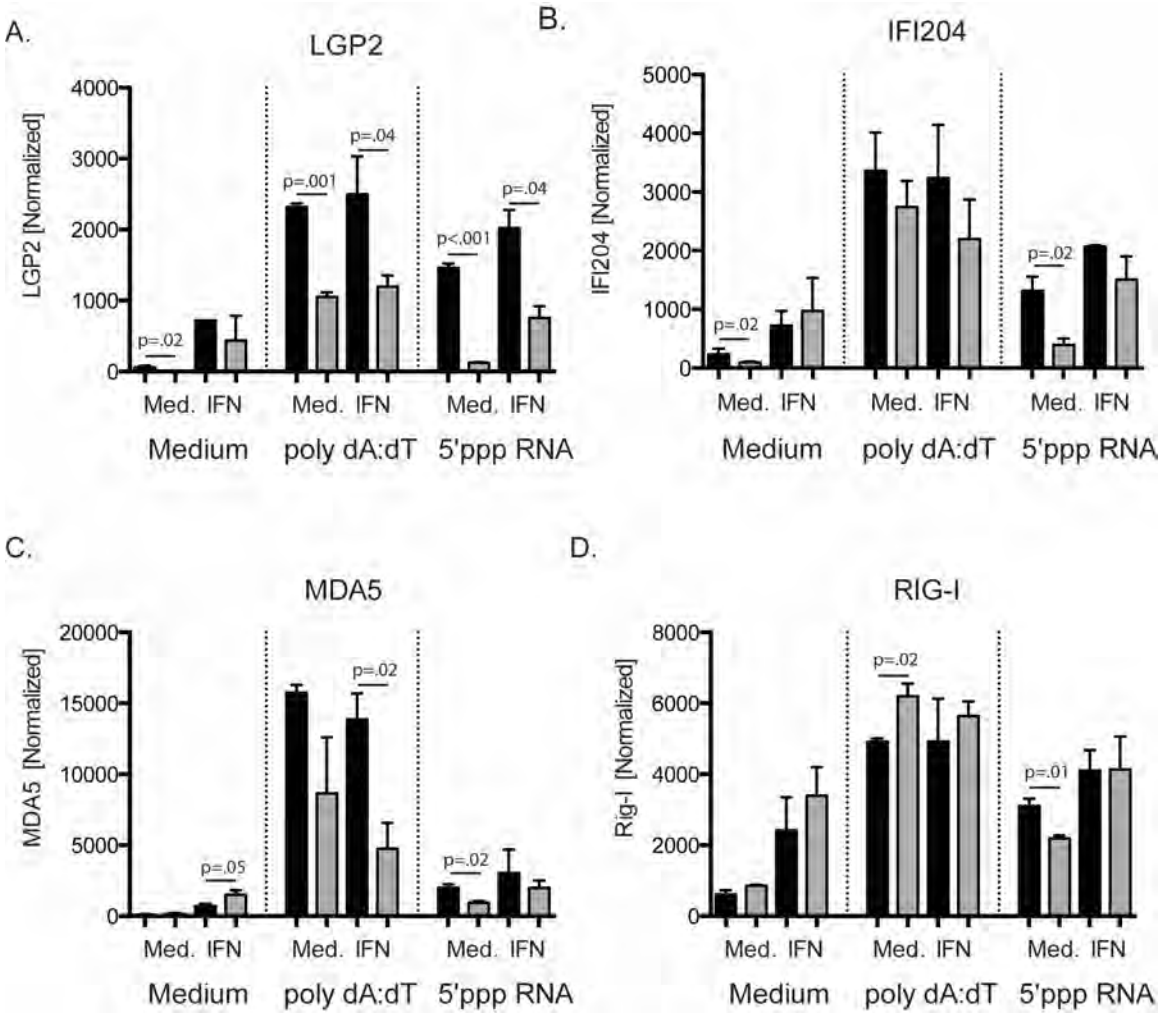

Fig. 3.6 Exogenous IFN can drive receptor expression in Dicer WT and KO cells and rescue some nucleic acid based stimulation

Dicer WT and KO cells were pre-stimulated with either medium or IFN [100U/mL] for 1 hour. They were then transfected with poly dA:dT [600ng/mL] or 5'pppRNA [1ug/mL] and RNA was isolated after an additional 6 hours. The RNA was quantified and hybridized to a custom nanostring codeset. Selected ISGs include **(A.)** *LGP2*, **(B.)** *IFI204*, **(C.)** *MDA5*, and **(D.)** *RIG-I*. Results are normalized to the geometric mean of *GusB* and *HPRT* expression and are the average of two biological replicates.

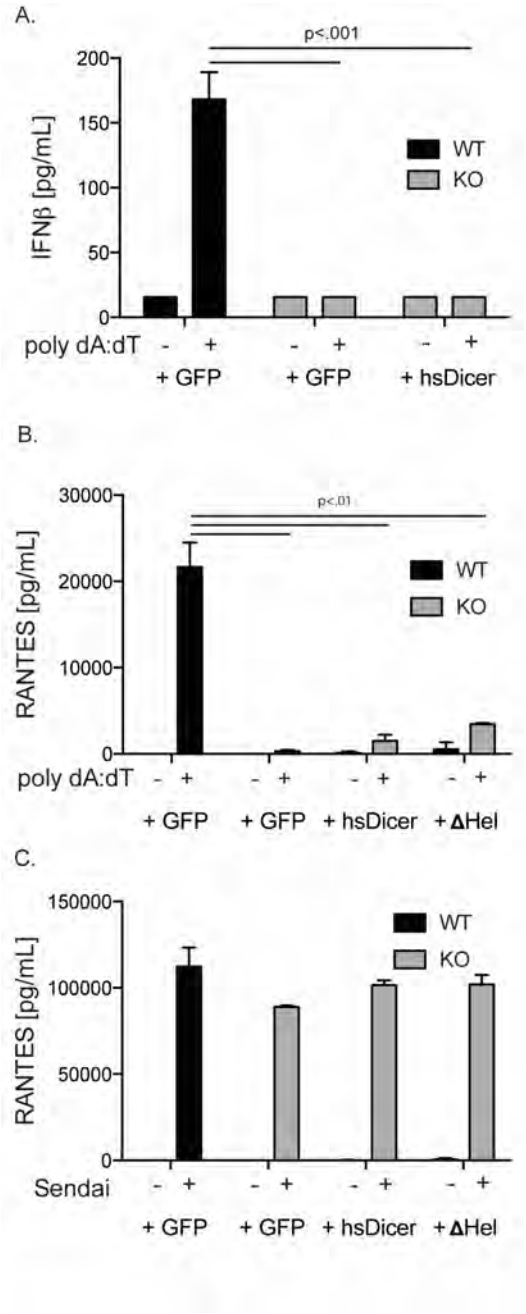

Fig. 3.7 Dicer reconstitution fails to rescue poly dA:dT induced IFN expression.

Dicer KO cells were reconstituted with human Dicer or ΔHelicase Dicer. The cells were transfected with **(A., B.)** poly dA:dT [600ng/mL] or infected with **(C.)** Sendai [40HA/mL] and supernatants were collected after 8 hours, (poly dA:dT) or 16 hours (Sendai). Supernatants were measured for **(A.)** IFNβ or **(B.)** RANTES by ELISA.

stimulants. Intriguingly, we saw that a disparate response arose, depending on the type of stimulant used. The Cantell strain of Sendai virus is a potent interferon inducer, in large part due to the high number of ~600 nt 5'ppp DI particles it creates. It is detected predominately through a RIG-I mediated mechanism, but recent studies have implied that IFIT1 can also detect 5'ppp RNAs and can cause a decrease in Influenza replication, despite seemingly having no ability to trigger downstream IFN activation. [55] IFIT1 is expressed at slightly higher levels in Dicer KO cells, but given their lack of signaling, it appears unlikely that this would result in the higher levels of IFN observed. Interestingly, Sendai virus has been shown to upregulate miR203, which in turn downregulates IFIT1. [231] Thus, the increased levels of IFIT1 may be due to the dysregulation of this miRNA. A different, potential mechanism for the observed IFN induction may lie in the loss of IFITM3 expression in Dicer KO cells. **(For ref, see Chapter II Fig. 2.4)** IFITM3 is significantly reduced in Dicer KO cells and is important for the restriction of several viruses, including Influenza, West Nile virus and dengue. [59,60] By restricting pore formation, IFITM3 acts as a barrier to the initial entry of the virus to the cell. [232] It may be that IFITM3 reduction in the Dicer KO cells allows more immune-stimulatory RNA to enter the cell and drive higher IFN levels. However, this is countered by the fact that we did not see increased viral replication in our cells. However, it is possible that there were more DI particles in KO cells that were undetectable by the qPCR methodology we employed. Complicating our observations was that we also saw a decrease in IFIT3

expression in Dicer KO cells, and IFIT3 has been implicated in increasing RIG-I signaling by engaging MAVS and TBK1 and enhancing signaling. [233] All told, with so many receptors and adaptors having increased or decreased expression in the KO cell, it is difficult to pinpoint a precise cause for the observed increase in IFN.

Surprisingly, Sendai virus infection was the only stimulant we observed with increased IFN expression. The non-viral stimulants all showed a marked decrease in signaling, regardless if they were RIG-I, MDA5, or DNA sensor agonists. Since RIG-I and MDA5 expression levels remained consistent between WT and KO cells, it would appear that some other mechanism is at play. One possibility for increased Sendai signaling would be due to the down-regulation of IFIT3 and IFITM3. The reduction in expression may result in increased 5'ppp RNA in the Sendai infected Dicer KO cells. With regards to the loss of poly I:C and 5'ppp RNA signaling, the most obvious potential culprit would be the loss of LGP2, since LGP2 is essential for MDA5 signaling[47-49] and has also been shown to be important in RIG-I signaling.[48] Previous reports have shown a loss of MDA5 dependent signaling when LGP2 is knocked out, suggesting they act in concert to initiate dsRNA induced IFN signaling.[49,234] Further investigation of LGP2 reconstitution is warranted to ascertain its importance to signaling in a Dicer null environment.

DNA sensing is largely ablated in a Dicer null cell. Poly dA:dT, HSV60mer, and VACV70mer all failed to induce measurable amounts of IFN or

RANTES. In MEFs, poly dA:dT can stimulate IFN through direct activation of DNA sensors but can also be transcribed by RNA pol III to create intermediate RNA species that activate IFN through RIG-I. [71] While RIG-I expression appears normal in Dicer null cells, the ability to stimulate IFN through a RIG-I ligand such as 5'ppp RNA is diminished, which suggests any RNA intermediates created from poly dA:dT may share a similar fate. Additionally, the IFI16 ortholog IFI204 is diminished in Dicer null cells; thus, any direct DNA sensing through IFI204 would be impaired as well. These redundant methods of initializing IFN induction are both diminished in Dicer null MEFs, which may explain why no induction of IFN is observed. Additionally, if IFI204 is a transcriptional activator like its human ortholog IFI16 is, then the loss of IFI204 may result in greater defects than just loss of DNA recognition. The loss of transcription initiation by IFI204 could have far-reaching effects on downstream signaling after all ligands, DNA, RNA, and others included.

By adding exogenous IFN we induced receptor expression in the Dicer WT and KO MEFs. While this did not rescue the observed phenotype entirely, it did drive some of the receptor expression high enough that upon further stimulation with poly dA:dT or 5'ppp RNA, the responses were normalized. In particular, IFN induction largely rescued 5'ppp RNA activation of RIG-I, MDA5, and IFI204 expression and partially rescued LGP2 expression.

Intriguingly, reconstituting Dicer into the Dicer null cells did not rescue the poly dA:dT phenotype. While this may seem counterintuitive considering that the

reconstitution rescued miRNA expression, the Dicer KO cells had been without miRNAs for some time and it is possible that a key developmental and time-sensitive miRNA may have been lacking early during the cell's growth. Additionally, considering that various innate immunity receptor and adaptor proteins were already down-regulated in the Dicer KO cells, and that many miRNAs are not constitutively expressed, the miRNAs responsible for altering the expression of the receptors may not have been upregulated until stimulation, which may then render them too late to be able to alter the production of IFN. In this case, a certain threshold of receptor and adaptor would need to be reached before the rescue of signaling could occur.

Altogether, it's difficult to come up with a set pattern of how adding Dicer back into cells that had been lacking Dicer alters the expression of cytosolic nucleic acid receptors. This is likely due to each receptor or adaptor being under the regulation of different miRNAs and transcription factors that are expressed at different times and under different stimulations.

It is clear that deleting Dicer has deleterious effects on the ability of a MEF to respond to nucleic acid agonists. However, since any given cell has multiple signaling pathways and receptors for various types of nucleic acid ligands, it's difficult to draw broad conclusions. For example, if poly dA:dT is being recognized by IFI204 and STING but also being transcribed by RNA Pol III into an RNA that can be detected by RIG-I or the IFIT proteins, it would be difficult to tease out which dysregulated receptor in the Dicer KO is responsible for the loss

of IFN induction. Still, this study highlights the importance of miRNA regulation of innate immune responses and further work should be done to determine which miRNAs impact the expression of these various cytosolic nucleic acid receptors.

MATERIALS AND METHODS

Cells

Dicer WT and KO MEFs were created and maintained as outlined in **Chapter II Materials and Methods**. HEK293T cells were maintained in Dulbecco's modified eagle medium (DMEM) with 10% fetal calf serum 100U/mL penicillin/streptavidin and 10mM glutamine.

Murine bone marrow derived macrophages were isolated from Dicer C/C ESR1-Cre + mice. The mice were sacrificed and bone marrow was flushed from both legs. Cells were resuspended in 5ml of red cell lysis buffer. After red blood cell lysis, remaining cells were spun down and resuspended in 120 ml of DMEM with10%FCS, 10mM glutamine, 100U/mL penicillin/streptavidin and 20% L929 supernatant containing recombinant mouse colony stimulating factor. The cells were plated and 3 days later, non-adherent cells were removed. Fresh medium with L929 supernatant was added as needed and on day 7, the cells were subjected to 10uM tamoxifen. Tamoxifen treatment resulted in depletion of Dicer from Dicer C/C ESR1-Cre + cells.

Human peripheral blood mononuclear cells (PBMCs) were obtained by harvesting whole blood and spinning through a lymphocyte separation media gradient. (Cellgro # 25-072) Lymphocytes were extracted from the interphase and the cells were plated. Non-adherent cells were removed the following day and adherent cells were scraped and counted.

ELISAs

Secreted IFNβ and RANTES from infected cell culture supernatants were quantified by ELISA. RANTES was assayed using R&D systems DuoSet ELISA (R&D Systems, Cat# DY478) and IFNβ was assayed using a PBL murine IFN ELISA kit (PBL Assay Sciences, Cat# 42400-2). IFNα was assayed using a PBL human IFNα ELISA kit (PBL Assay Sciences, Cat# 41100-1). All ELISAs were run according to manufacturer's specifications albeit in half the normal volume.

Quantitative PCR and Nanostring

RNA from cells was collected and purified using the RNeasy Plus RNA purification kit (Qiagen, Cat #73404) according to manufacturer's specifications. RNA was quantified by NanoDrop and 500 ng of RNA was transcribed to DNA, amplified, and quantified in an MJ Research thermal cycler using QuantiTect One-Step SYBR green RT-PCR kit (Qiagen Cat# 204243). The following primers were purchased from Qiagen as QuantiTect Primer Assays: (IFNβ – Cat #QT00249662, GusB – Cat #QT00176715, 18S – Cat# QT02448075,). Sendai

virus N gene was amplified by **SeV-N-F** and **SeV-N-R** Dicer depletion in BMDMO was assayed using the primer sets: **Exon 14F**, **Exon 16F**, and **Exon 18R**.

Oligo name	Sequence
SeV-N-F	5'-TGAAGTTAGACAGGATTTTAGGG-3'
SeV-N-R	5'-GGCCGGGGATAACAGCACC-3'
Exon 14F	5'-CCAAGATGCAGTGATCATTCC-3'
Exon 16F	5'-CCATTGGTGCCAAGACAATG-3'
Exon 18R	5'-CAGGCTCCACTCCCTAAC-3'

RNA from cells was collected and purified using the RNeasy Plus RNA purification kit (Qiagen, Cat #73404) according to manufacturer's specifications. RNA was quantified by UV absorbance on a NanoDrop. Once quantified the RNA was hybridized overnight at 65^{o} C to a custom probe set covering 50 mouse innate immunology related genes. The labeled RNA was prepared and run on a Nanostring Prep station and bioanalyzer. Data was normalized and analyzed using nSolver v2.5. Counts were normalized to the geometric mean of the housekeeping genes GusB and HPRT.

siRNA knockdown

siRNAs were purchased as a SMARTpool set of four siRNA from Dharmacon. Dicer siRNAs (Dharmacon L-003483) and negative control

(Dharmacon D-001810-10) were transfected into HEK293 or human PBMCs at 10μM using Dharmafect 4 according to the manufacturer's specifications.

Western Blot

Cell lysates were prepared in Passive Lysis Buffer (Promega E1941) and centrifuged to pellet cell nuclei. Clarified lysates were denatured by boiling samples in 1X Laemmli buffer and run on 10% SDS-PAGE gels until the leading dye band reached the bottom of the gel. Gels were transferred to PVDF membranes using a semi-dry transfer apparatus for 45 minutes at 25V. Following transfer, PVDF membranes were blocked with 3% BSA and probed with a mouse monoclonal antibody against Dicer (Clongene 13D6) followed by a polyclonal HRP conjugated anti-mouse antibody (Sigma A9044). Actin was detected using a rabbit polyclonal antibody against β-Actin (Abcam ab8227) and a goat HRP-conjugated anti-rabbit antibody (Vector PI-1000). The blot was developed in Pierce SuperSignal West Dura Extended substrate (Thermo Scientific #34075).

Luciferase Assays

The IFNβ promoter and NF-κB promoter were cloned into pGL4 firefly luciferase reporter plasmids (Promega #E6651). IFNβ or NF-κB reporters were transfected using GeneJuice according to manufacturer's specifications (EMD Millipore #70967). Twenty-four hours post transfection, cells were stimulated and

lysates were collected after an additional 16 hours. Dual-luciferase reporter assay kit (Promega #E1980) was used to determine total luciferase activity. Luciferase activity was normalized to total protein content using a microBCA kit(ThermoFisher Scientific #23235).

IFN Bioassay

NCTC 929 cells were plated at 50,000 cells/well in a 96 well plate. Supernatants from stimulated cells were irradiated in a Stratalinker 2400 to eliminate any infectious virus or crosslink any remaining immunostimulatory nucleic acids. Supernatant was serially diluted onto the NCTC929 cells for 24 hours. After 24 hours, the medium was replaced and the NCTC929 cells were challenged with 1000 pfu/mL of VSV-Indiana strain. After an additional 24 hours, cells were monitored for cytopathic effect. IFN protective titers were determined as the dilution of supernatant where fewer than 50% of the cell monolayers were still intact.

CHAPTER IV: Loss of *Dicer1* negatively regulates EMCV viral production.

ABSTRACT

Dicer depletion has been shown to impact the replication of many viruses. DNA viruses that encode viral miRNAs tend to be negatively impacted by the loss of miRNA biogenesis machinery, while some RNA viruses, primarily those that naturally infect flies or plants have shown to have increased replication following the loss of Dicer. Considering that miRNAs also play a major role in the regulation of innate immunity, understanding how the absence of Dicer affects viral replication is key to our understanding of what role, if any, Dicer plays in regulating viral infection. Here we report for the first time that Dicer depletion in a mammalian system results in negative regulation of a RNA virus: EMCV. Dicer depletion results in ~100-fold loss of virus production due to aberrant binding of the virus to the cell.

INTRODUCTION

A successful viral infection requires a series of steps to occur. First, the virus must bind to their target cell via a receptor and enter through membrane fusion, endocytosis, or capsid/genome injection. Next, the virus must replicate its genomic material in the appropriate cellular compartment. Concurrent to or post-replication, the viral genes are transcribed and translated to create the necessary structural and non-structural proteins required for the assembly of new virions. Lastly, the virions assemble and the virus sheds either through lytic or non-lytic release of the virions. Successfully targeting any of these stages can result in a significant loss to viral production and so both host cellular defenses and human anti-viral therapeutic strategies look to disrupt these processes.

The picornavirus, encephalomyocarditis virus (EMCV) is a model virus for studying multiple pathways and diseases. It can cause encephalitis, myocarditis, and diabetes in a host of species, but is generally considered to have little morbidity in human infection (reviewed in[235]). In the laboratory, EMCV infection is used to stimulate the dsRNA sensing pathways as it strongly induces IFN in an MDA5/MAVS dependent manner.[38] Like other picornaviruses, EMCV is a small non-enveloped virus. The EMCV genome is a small, roughly 7.8kb positive-sense RNA genome. As it is positive sense, the RNA from the virus itself is infectious (reviewed in[235]). EMCV RNA has a covalently linked small viral protein called VPg on the 5', followed by a 5' UTR which contains the Internal Ribosome

Binding Site (IRES).[236] The IRES itself is roughly 450 nt with complex secondary structure and allows for immediate translation of the viral genome into a polypeptide.[237] EMCV encodes 13 proteins, 4 structural and 9 non-structural, which include the viral protease and RNA-dependent RNA polymerase. EMCV viral entry is mediated through an unknown mechanism. Two sialoglycoproteins have been reported as being receptors for EMCV: Vascular cell adhesion molecule 1 (VCAM-1) and Sialylated glycophorin A. Glycophorin A is found on human blood cells and has been shown to have an impact on EMCV binding, but it is dispensable for infection.[238] VCAM-1 was found to control viral entry on murine vascular endothelial cells, but EMCV infection of cells that do not express VCAM-1 has also been observed.[239] Once bound, the viral genome enters the cell in an acidification independent manner[240], but it is not fully understood. The positive-sense infectious RNA is immediately translated in the cytoplasm upon entry. The polyprotein that results from translation is primarily processed by the viral protease 3C.[241] Polyprotein processing liberates the various non-structural proteins and the RNA-dependent RNA polymerase, which are required for replication. Negative strand synthesis occurs in an unknown manner and uses the initial positive strand RNA genome as the template. The negative strand then acts as template for additional positive strand synthesis which can be used for translation and genome packaging.[235] During this phase however, replication intermediates provide the dsRNA[242] that is essential for initiating MDA5/MAVS based IFN activation.[38] Once the genomes have been replicated and the

structural proteins cleaved, virion assembly occurs in the cytosol. Newly synthesized VPg capped RNA is loaded into the icosahedral capsid and virus escapes through membrane permeabilization and cell lysis with 7 to 10 hours. [243,244] Here I describe the role of Dicer in EMCV reproduction in a Dicer null environment.

RESULTS

Dicer1 KO cells exhibit reduced IFN β production following EMCV infection.

miRNAs have been shown to be involved in a large cross-section of gene regulation, and miRNAs in the immune response have been reported to alter everything from cytokine production to the differentiation of adaptive cell lineages. To assess the impact of losing a vital component of the miRNA machinery during viral infection, I infected *Dicer1* WT and *Dicer1* KO cells with encephalomyocarditis virus (EMCV) at a multiplicity of infection (MOI) of 0.1. *Dicer1* WT cells responded readily, producing Interferon-β (IFNβ) protein following a 16 hour infection **(Fig 4.1a)**. *Dicer1* KO cells failed to respond as robustly, producing a significantly reduced amount of IFNβ. To determine if this was related to a difference in IFNβ mRNA levels, I measured IFNβ mRNA by qPCR throughout the course of infection. **(Fig. 4.1b)** As the infection progressed, IFNβ mRNA was produced in both samples, albeit at a greater level

in the WT cells. Thus at both the mRNA and protein levels, *Dicer1* KO cells fail to respond robustly to the presence of an EMCV infection.

IFNβ is a vital signaling cytokine for the stimulation of Interferon Stimulated Genes (ISGs) and can directly influence the production of other effector chemokines and cytokines, such as CCL5/RANTES. When measured by ELISA, the amounts of CCL5/RANTES protein in the *Dicer1* WT supernatants exceeded the amount from *Dicer1* KO cells, suggesting that the increased levels of IFN β seen in the WT samples was due to increased IFN β protein production and not simply less uptake of IFN β by the culture **(Fig. 4.1c)**. To assess if the IFN and CCL5/RANTES defect could be overcome by infection with additional virus, I infected *Dicer1* WT and KO cells with MOIs of 0.1, 1, and 10 **(Fig. 4.1d)**. At high MOIs, I measured a very limited CCL5/RANTES response in the *Dicer1* WT cells, whereas the *Dicer1* KO cells produced a robust response. However, observation of the cells revealed that the *Dicer1* WT monolayers had been obliterated by the virus, while the *Dicer1* KO cells were alive and healthy. Since *Dicer1* KO cells are able to produce CCL5/RANTES at a high MOI of infection while maintaining a healthy monolayer, this suggests that the failure to produce a cytokine response may be due to an issue with virus replication rather than a failure of the cellular viral response.

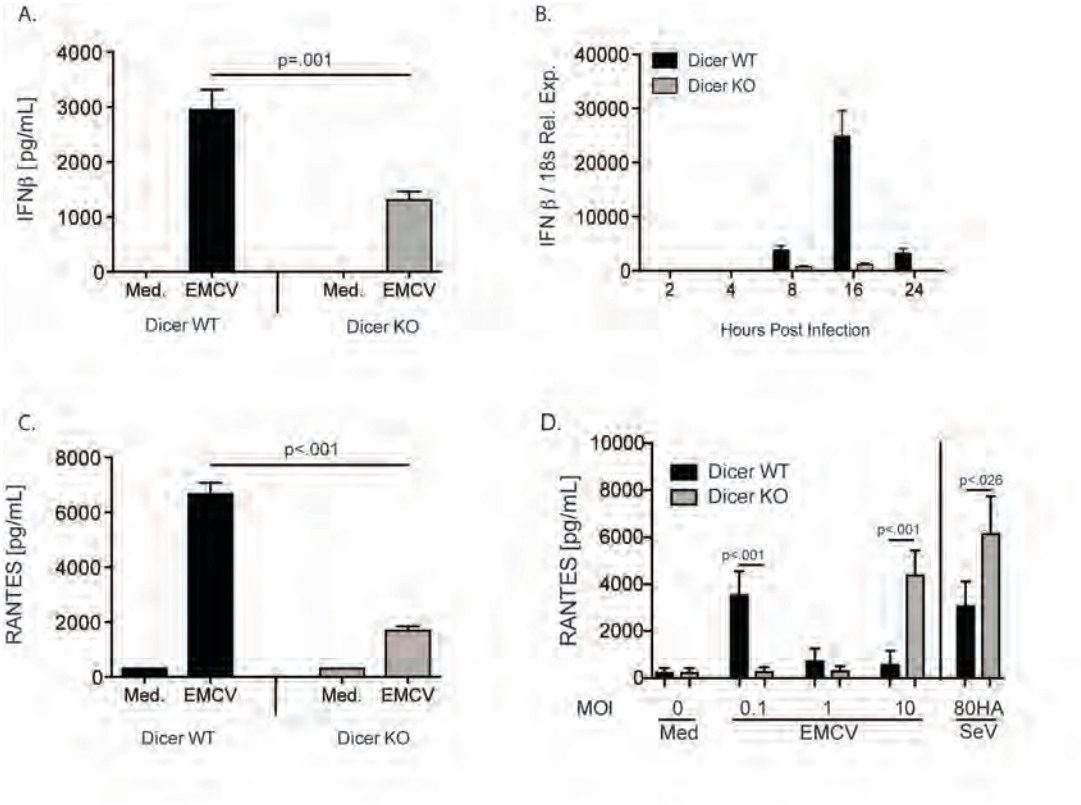

Figure 4.1 IFNβ and RANTES protein production is diminished in *Dicer1* KO cells following EMCV infection.
(A.) *Dicer1* WT and KO cells were infected with EMCV for 16 hrs and the supernatants were assayed by IFNβ ELISA or **(C.)** RANTES ELISA. Data shown as the mean of four biological replicates. **(B.)** *Dicer1* WT and KO cells were infected with EMCV and RNA was isolated at the time points indicated. *IFNβ* mRNA was detected by one-step RT-qPCR and normalized to 18s RNA and compared to steady state normalized *IFNβ* mRNA at time 0. Data shown is representative of three biological replicates. **(D.)** *Dicer1* WT and KO cells were infected with different MOIs of EMCV and supernatants were collected after 16 hours and assessed for CCL5/RANTES protein by ELISA. Sendai was added as a control. Data shown as mean of four biological replicates.

***Dicer1* KO cells do produce IFNβ in response to VSV infection.**

In order to ascertain if the IFNβ defect was universal in nature, I decided to infect the *Dicer1* WT and KO cells with the rhabdovirus vesicular stomatitis virus (VSV). VSV grows well in a variety of cell types and while it does not induce a strong immune response it is easily monitored for cytopathic activity. Contrary to what was seen with EMCV, VSV infection led to a moderate production of IFNβ in both *Dicer1* WT and KO cells. **(Fig. 4.2a)** While the low level of IFNβ did not induce further downstream effector molecules such as RANTES, IFNβ mRNA could be detected in both *Dicer1* WT and KO cells although a significantly reduced amount of IFNβ mRNA was detected in the KO cells. **(Fig. 4.2b)**

These results indicate that the phenotype observed in the Dicer KO cells was specific to EMCV infection and was not recapitulated in the VSV infection model.

EMCV virus production is defective in *Dicer1* KO cells.

Because IFNβ production was affected so drastically in the KO cells, I explored the possibility that EMCV viral infection may be altered between the two cells types. *Dicer1* WT and KO cells were infected with various MOIs and after 16 hours, the supernatants were plaqued onto BHK-21 cells. Intriguingly, the number of EMCV plaque forming units was the same between Dicer WT and Dicer KO cells at an MOI of 10, whereas if the MOI dropped to .1, a 2 log difference could be seen in the amount of productive virions produced. At low

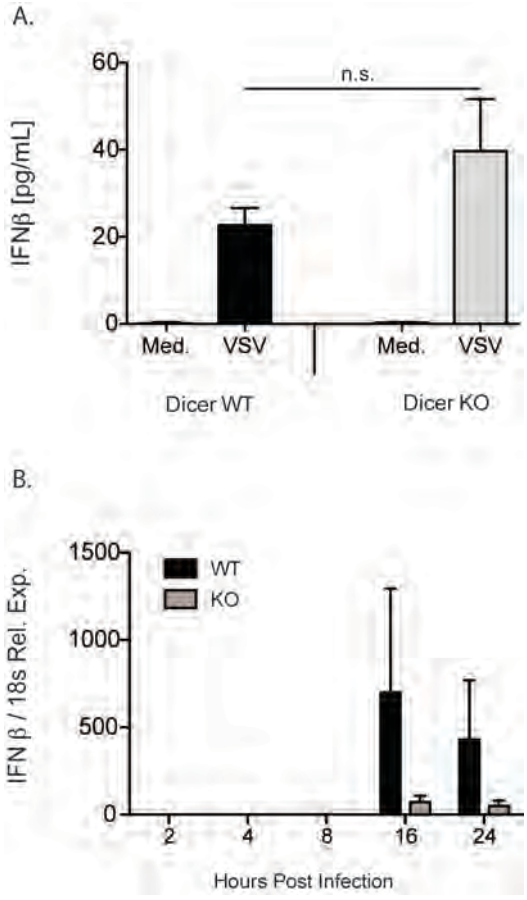

Figure 4.2 IFNβ protein and RNA production is unaffected in *Dicer1* KO cells following VSV infection.
(A.) *Dicer1* WT and KO cells were infected with VSV for 16 hrs and the supernatants were assayed by IFNβ ELISA. Data shown as the mean of four biological replicates. **(B.)** *Dicer1* WT and KO cells were infected with VSV. RNA was isolated at the time points indicated. *IFNβ* mRNA was detected by one-step RT-qPCR and normalized to 18s RNA and compared to steady state normalized *IFNβ* mRNA at time 0. Data shown is representative of three biological replicates.

MOI infections, EMCV in *Dicer1* KO cells failed to replicate at levels seen in WT cells. In contrast, when infected with VSV, equivalent amounts of virus were recovered from infected cell supernatants, suggesting Dicer has a virus-specific impact on EMCV infection. **(Fig. 4.3a)** Visual confirmation revealed that WT cells infected with EMCV at an MOI of 10 were dead, while the KO cells maintained a monolayer. The obliteration of the monolayer of *Dicer1* WT cells may have impacted the absolute number of PFUs produced. Since *Dicer1* KO cells are able to produce a cytokine response at high MOIs (as seen in **Fig. 4.1d**), this suggests that the 2 log defect in virion production is to blame for the loss of cytokine response in the *Dicer1* KO cells, and that the loss of cytokine response can be overcome by adding 2 log more virus. To assess if the virus replication was affected by Dicer depletion we monitored viral RNA replication by detecting EMCV RNA over time using qPCR. We infected Dicer MEFs with EMCV for 1 hour, followed by multiple washes to remove unbound virus. As the infection continued, EMCV replication in *Dicer1* WT cells far outpaced replication in the *Dicer1* KO cells **(Fig. 4.3b.)** However, this difference in EMCV RNA levels may be due to virus infection steps that occur prior to replication of the RNA. There are multiple stages of the virus life cycle where Dicer may be impacting the viral growth including: binding, entry, replication, transcription, translation, assembly and release. Since plaque assays measure multi-cycle viral kinetics, I assessed the ability of EMCV and VSV to infect *Dicer1* WT and KO cells in a single cycle assay. I infected equal numbers of *Dicer1* WT and KO cells with EMCV or VSV

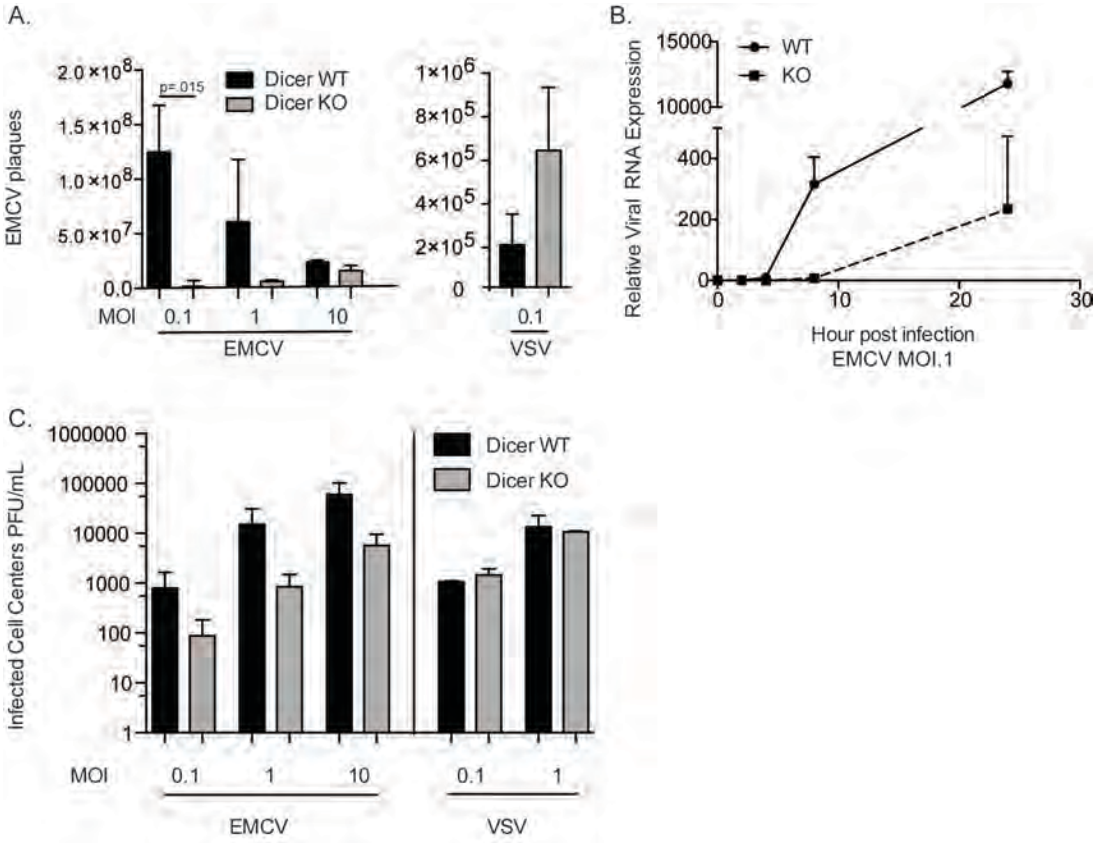

Figure 4.3 EMCV fails to efficiently infect *Dicer1* KO cells.
(A.) Plaque Assay: *Dicer1* WT and KO cells were infected with EMCV at 10, 1, or 0.1 MOI or VSV at 0.1MOI for 16 hours and supernatants were harvested and serially diluted on BHK-21 cells. After 1 hour absorption, the cells were washed and a 1X-MEM 2% agarose overlay was added. Monolayers were fixed and stained with 4% formaldehyde-5%crystal violet and plaques were counted after 2 days. Data shown as mean of three biological replicates. **(B.)** Viral RNA Replication: *Dicer1* WT and KO cells were infected with EMCV at .1 MOI and after 1 hr, the cells were washed multiple times and each well was harvested at the indicated time points. RNA was isolated and EMCV specific RNA was detected using qRT-PCR. EMCV RNA was normalized to *GusB*. Data shown as the mean of two biological replicates. **(C.)** Infected Cell Center: *Dicer1* WT and KO cells were infected with EMCV or VSV at varying MOIs and allowed to absorb for 1 hr. After 1 hr, a series of washes removed unbound virus and the cells were trypsinized and serially diluted into cultures of suspended BHK-21 cells. The mixed cultures were plated and allowed 2 hours to adhere, before the medium was removed and a 1X MEM-2% agarose overlay was added. Monolayers were fixed and stained with 4% formaldehyde-5% crystal violet and plaques counted after 2 days. Data shown as the mean of three biological replicates.

and allowed the virus to absorb for 1 hr. After trypsinization of the cells, I mixed serially diluted Dicer MEFs into BHK-21 cells and plated the mixed cell population. The cells were allowed to adhere for 2 hours and then overlaid with 2% Agar-MEM. Since the BHK-21 cells grow EMCV and VSV similarly between the two cultures, any difference in plaque formation would arise from the initial single round of infection in the Dicer MEFs. After two days, we counted plaques and observed that while there was no difference in the growth of VSV plaques, EMCV plaques showed a similar two-log reduction of PFU in the cultures that were seeded with *Dicer1* KO cells. In both WT and KO seeded BHK-21 monolayers, EMCV and VSV plaque size was similar, regardless of the genotype of the initially infected cell. **(Fig. 4.3c)**

Reconstitution of Dicer rescues EMCV virus production and IFN production.

Dicer is involved in regulating many genes through its function as a miRNA processor. Given that a prolonged loss of miRNAs may have unforeseen effects on the overall fitness of the cell, I wanted to reconstitute Dicer and test whether this rescued the EMCV infection defect. I reconstituted expression of Dicer in the KO cells using a codon-optimized version of human *DICER1*. I transduced *Dicer1* WT and KO cells with a lentivirus based vector containing GFP, full length human *DICER1* (hsDicer), or a truncated *DICER1* missing the helicase domain (ΔHelicase) as reported in **Chapter II Fig. 2.5**. I infected

reconstituted *Dicer1* WT and KO cells with EMCV and VSV and after 16 hours, assayed their supernatants for IFNβ and PFUs. **(Fig 4.4a and 4.4b)**. IFNβ protein levels were raised significantly following rescue of the KO cells with both human Dicer (hsDicer) and ΔHelicase Dicer, compared to KO cells that received control GFP. The ΔHelicase reconstituted *Dicer1* KO cells in fact showed a marked increase in IFNβ production over even *Dicer1* WT cells. Reconstitution with both Dicer variants led to a complete rescue of EMCV virion formation with the reconstituted KO cells produced roughly 3 log more virus than the KO + GFP cells. VSV PFU's remain unchanged regardless of the status of Dicer **(Fig 4.4c)**.

***Dicer1* is critical for EMCV binding to MEFs.**

While Dicer reconstitution rescued the observed phenotype, the mechanism remained unclear. In order to ascertain what aspects of a productive viral infection were being compromised in a *Dicer1* KO cell, I tested the ability of EMCV to bind to the surface of the WT and KO MEFs. I incubated virus with WT and KO MEFs at 4^{o} to inhibit internalization of the virus. Following extensive washing, the total amount of virus bound to the cells was assayed by quantification of viral RNA. My viral binding assay showed that EMCV is unable to bind to a *Dicer1* KO cells at a level comparable to WT. EMCV binding was restored when the *Dicer1* KO cell was reconstituted with either hsDicer or ΔHelicase Dicer. **(Fig 4.5a.)** If binding is truly a bottle-neck in EMCV infection, then skipping the binding and entry step entirely should alleviate the phenotype.

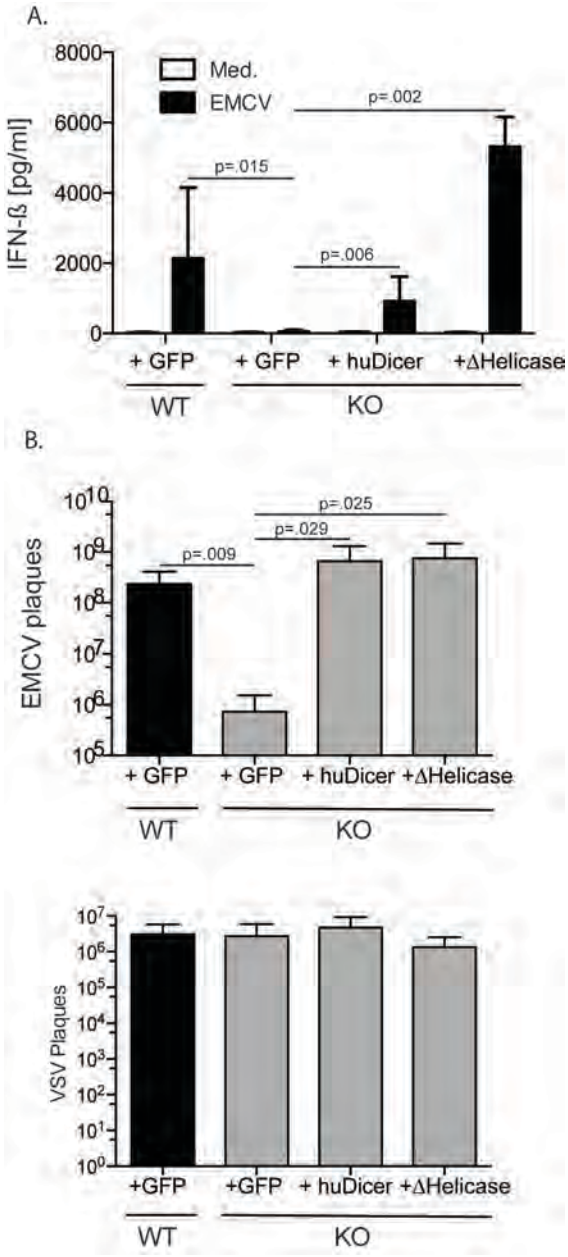

Figure 4.4 IFNβ production and viral growth can be rescued by exogenous *DICER1* expression.
Dicer1 WT and KO cells were transduced with a lentivirus that expressed GFP, human *DICER1* (hsDicer) or *DICER1* missing the helicase domain (ΔHelicase). *Dicer1* WT, KO and rescued KO cells were infected with EMCV [.1 MOI] or VSV [.1 MOI] After 16 hours, the supernatants were assayed for **(A.)** IFN**β** protein production, **(B.)** EMCV plaque number, or **(C.)** VSV plaque number. Data shown as mean of six biological replicates for IFN and three biological replicates for plaque assay data.

To test this hypothesis, I isolated whole positive sense EMCV RNA from virions and transfected the RNA into *Dicer1* WT and KO cells. The cells were subsequently washed and diluted into BHK-21 cells. This assay was identical to the one in **Fig 4.3c**, however instead of infecting the cells, the RNA transfection allowed me to bypass the physical interaction between virion and cell. I observed no statistical difference in the number of plaques between the *Dicer1* WT and KO cells following transfection of viral RNA. **(Fig. 4.5b)** As binding appears to be a major cause of the EMCV infection defect, I looked at the only known EMCV receptor in mice: Vascular cell adhesion molecule 1 (VCAM1).[239] It is found predominantly on vascular endothelial cells and while it has been shown to allow entry of EMCV into otherwise non-permissive cells, it is not the only receptor for EMCV, as cells that express no VCAM1 can still become infected. When I compared levels of *Vcam1* between Dicer WT and KO cells, I observed no difference in expression of the gene by qPCR, suggesting an alternative receptor is responsible for EMCV infection in MEFs. **(Fig. 4.5c)**

DISCUSSION

Small RNAs have proven to be an integral part of gene regulation systems in a variety of eukaryotes, from the miRNA regulated control of mRNA to the siRNA regulation of exogenous foreign RNA. However, its dual-role as a both a system of cellular maintenance and cellular defense mechanism has raised

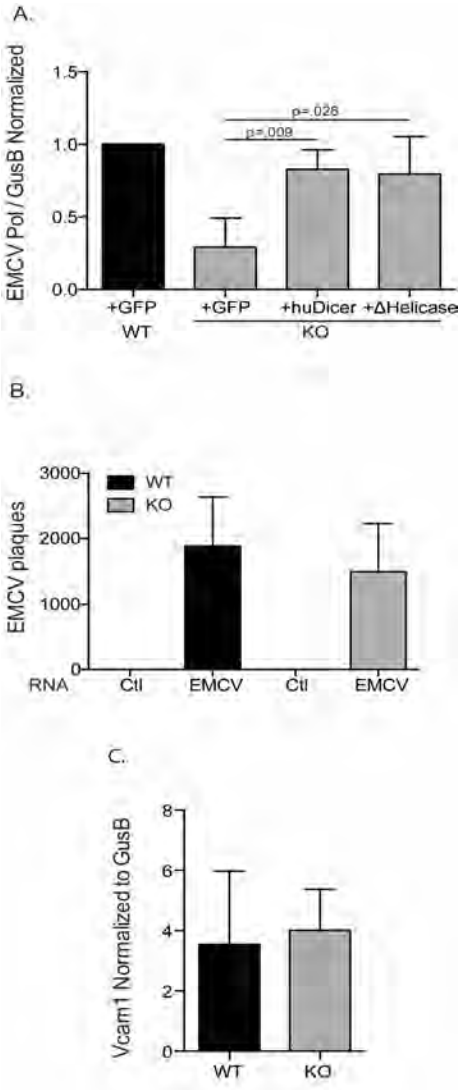

Figure 4.5. EMCV binding is reduced in *Dicer1* KO cells.
(A.) *Dicer1* WT and KO cells were shifted to 4^0 C and incubated with EMCV at an MOI of 10. After two hours, cells were washed three times in cold PBS and lysed for RNA extraction. EMCV RNA copy number was measured by qPCR and normalized to *GusB* expression. Data shown as the mean of three biological replicates. **(B.)** *Dicer1* WT and KO cells were transfected with 100ng of full length EMCV viral genomic RNA extracted from virions. After 1 hour, the cells were washed, trypsinized and diluted into BHK-21 cells. After 2 hours, the cells were overlaid with 2% Agarose-MEM. Monolayers were fixed and stained with 4% formaldehyde-5%crystal violet and plaques were counted after 2 days. Data shown as the mean of three biological replicates. **(C.)** *Vcam1* expression was quantified in RNA extracted from unstimulated *Dicer1* WT and KO cells by qPCR. *Vcam1* expression was normalized to *GusB*. Data shown as the mean of three biological replicates.

questions regarding what role Dicer and other proteins involved in the small RNA pathways may have and what kind of cross-talk may exist between its multiple effector functions. With regards to small RNA regulation of viral RNA, most efforts have focused on the role of small RNA pathways in suppressing viral replication [201,202,245-247] and conversely, the RNAi inhibitors encoded by many viruses. [248-254] The data presented here suggests that certain viruses may in fact, have adapted to the presence of RNAi machinery and require that machinery for efficient replication.

The disparate IFN response to EMCV between WT and KO cells can be attributed to differences in replication of EMCV in the two cell types. It takes roughly 100 fold more EMCV in the *Dicer1* KO cells to generate an equivalent IFNβ response as the *Dicer1* WT cells. As observed, when equivalent MOIs of EMCV were used to infect WT and KO cells, the KO cells consistently produced roughly 100 fold less virus than the WT cells. When the EMCV RNA was quantified over time, EMCV RNA was observed in much greater quantities in the *Dicer1* WT cells, which is expected given the large difference in virus output observed. The infected cell center assay is a useful assay to look for the efficiency in establishing a primary infection in both WT and KO cells. Since the cells are mixed with uninfected BHK-21 cells, plated, and overlaid with agarose within 3 hours of infection, any virus spread in the BHK-21 cells must come from an initially infected *Dicer1* WT or KO cell. Since EMCV produced approximately a log more infected cell centers in *Dicer1* WT cells at every MOI, it is apparent

that the true effective difference between Dicer WT and KO cells during a single cycle of EMCV replication is a roughly 10-fold reduction in infected cells, rather than the 100-fold difference in recovered virus observed through multiple round kinetics in the plaque assay. VSV had no problem spreading into the uninfected BHK-21 cells, regardless of whether it initially infected a *Dicer1* WT or KO cell, again suggesting that this particular defect is seen with EMCV, but not all viruses.

While EMCV showed a marked decrease in its ability to replicate and produce progeny virus in *Dicer1* KO cells, the phenotype was completely reversed upon the addition of Dicer back into the cells. As the cells were once again able to generate mature miRNAs **(Chapter II Fig 2.5)**, the amount of virus generated in the rescued cells approached that of the WT cells. Once viral replication had been restored, the expression of IFNβ and RANTES was also restored, suggesting that the production of these cytokines was dependent on the replication of the virus and matched what had been observed earlier; when *Dicer1* KO cells are given a high MOI of initial virus, they are able to produce an immune response.

Intriguingly, when the *Dicer1* KO cells were rescued with helicase-deleted Dicer, more IFNβ was observed, suggesting that the helicase-deleted Dicer processes either the mature miRNAs or the EMCV genome in a manner separate from full-length Dicer. Some evidence of alternative dicing efficiency has been observed before. When the helicase domain of Dicer was deleted, increased

efficiency of dsRNA processing was observed, suggesting that the helicase domain served as an auto-inhibitory domain that may be conformational changed upon the binding of various partners.[163] However, more recent reports have suggested the helicase domain plays a vital role in distinguishing dsRNAs with a terminal loop, allowing Dicer to distinguish miRNAs from other RNAs.[169,255] Additionally, a recent report has described a second isoform of *Dicer1* that is predominately expressed in murine oocytes.[170] This isoform of *Dicer1* is lacking part of the helicase domain and preferentially processes RNAs that are derived from long double-stranded regions of RNA, rather than the canonical stem-loop structure of a pre-miRNA. This isoform difference may explain why mouse oocytes have robust RNAi responses to long dsRNA,[170,201] while failing to efficiently process miRNAs[172] yet somatic cells tend to preferentially cleave pre-miRNAs[169,255] and not process long dsRNA, where the long dsRNA instead triggers the IFN response.[34]

My results show that a loss of Dicer leads to decreased sensitivity to infection with EMCV due to an inability of EMCV to bind to the surface of a *Dicer1* KO cell. While EMCV binding is reduced in a *Dicer1* KO to approximately 25% of binding to a Dicer WT, this effect is compounded through each subsequent round of infection, which could explain the 2 log difference seen in PFU formation. By using the positive sense EMCV RNA and transfecting it directly into cells, we were able to abrogate the 10-fold difference in plaque formation that we observed when using an infection model, providing further

evidence that the binding phase of the viral infection cycle was disrupted in *Dicer1* KO cells. While I did test for differences in VCAM1 expression in my cells, since expression was identical it is unlikely that VCAM1 is acting a major binding receptor in the MEFs. VCAM1 is not the only receptor for EMCV, since VCAM1 deficient cells can still be infected, so it is possible that another cellular adhesion molecule may act as a redundant, or in this case, primary receptor for EMCV.

Together, these results strongly suggest that EMCV requires the presence of an active miRNA processing pathway in order to replicate efficiently. When the ability to process miRNAs was removed, EMCV replicated poorly and subsequently led to a reduced IFN response to infection. While this is not the first time that Dicer depletion has impacted viral replication, previous reports have focused on DNA viruses that encode their own viral miRNAs and thus require processing of their miRNAs to replicate at WT levels. [193,256-258] For RNA viruses, the current literature has focused on increased viral growth in the absence of Dicer in order to focus on Dicer as a potential anti-viral mechanism (for review, see [259]). This report joins one other report indicating that an RNA virus requires a functional miRNA pathway to replicate efficiently.[260] This data suggests that the pro or anti-viral role of mammalian Dicer may be more complicated than previously thought and some RNA viruses may have adapted to the presence of the miRNA pathway directly or indirectly.

MATERIALS AND METHODS

Cells and Virus

Baby hamster kidney-21 cells and MEFs were maintained in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum, 2 mM glutamine and 100U/mL penicillin/streptomycin. Encephalomyocarditis virus (EMCV) strain 2887A-EGFP was a gift from L. Bakkali-Kassimi, ANSES, Maisons-Alfort, France and was grown on BHK-21 cells. MEFs were infected with EMCV at MOIs of 10,1, and 0.1, depending on the experiment. In all cases, cells were infected with EMCV for one hour and then washed, with samples taken at 2,4,8,16,and 24 hours for RNA, 16 hours for protein and viral titer. Vesicular stomatitis virus (VSV) Indiana strain was previously described were grown on BHK-21 cells and infections were done the same as EMCV.

ELISAs

Secreted IFNβ and RANTES from infected cell culture supernatants were quantified by ELISA. Supernatant was first irradiated using a UV stratalinker 2400 (Stratagene) to inactivate residual EMCV and VSV and samples were run neat or diluted, depending on the cytokine being assayed. RANTES was assayed using R&D systems DuoSet ELISA (R&D Systems, Cat# DY478) and IFNβ was assayed using a PBL murine IFN ELISA kit (PBL Assay Sciences,

Cat# 42400-2). All ELISAs were run according to manufacturer's specifications, with the exception that volumes were halved.

Quantitative PCR

RNA from cells was collected and purified using the RNeasy Plus RNA purification kit (Qiagen, Cat #73404) according to manufacturer's specifications. RNA was quantified by NanoDrop and 500 ng of RNA was transcribed to DNA, amplified and quantified in a MJ Research thermal cycler using QuantiTect One-Step SYBR green RT-PCR kit (Qiagen Cat# 204243). The following primers were purchased from Qiagen as QuantiTect Primer Assays: (IFN β – Cat #QT00249662, GusB – Cat #QT00176715, 18S – Cat# QT02448075, Vcam1 - QT00128793) EMCV 3D polymerase specific product was amplified using **EMCV3D-F** and **EMCV3D-R**

Oligo name	Sequence
EMCV3D-F	5'-GGGATCAGCTTTTACGGCTTT-3'
EMCV3D-R	5'-TGCATCCGATAGAGAACTTAATGTCT-3'

Virus Assays

For plaque assays, BHK-21 cells were plated at 400,000 cells per well in a 6 well plate. *Dicer1* WT and KO MEFs were incubated with EMCV or VSV at an MOI of 10,1, or 0.1 for 16 hours and the supernatants were serially diluted onto the BHK-21 cells for 1 hour. Monolayers were washed and overlaid with 2%

Agarose – MEM. Monolayers were fixed and stained 2 days later with 4% Formaldehyde – 5% Crystal Violet and plaques quantified.

Infected Cell Center assays were performed as follows. *Dicer1* WT and KO were plated at 500,000 cells/well in a 12 well plate in 1 mL of medium. After 2 hours, the medium on the *Dicer1* WT and KO cells was removed and replaced with 500uL of medium. EMCV and VSV were added to the *Dicer1* WT and KO cells at an MOI of 0.1, 1 and 10 (EMCV only). After 1 hour absorption, the cells were washed twice in 1X PBS to remove unattached virus. The cells were then trypsinized in 0.5mL Trypsin-EDTA for 2 minutes at 37^{o}C. Trypsin activity was quenched by adding 0.5mL of complete media to each well. The cells were removed from the plate, with a final concentration of 500,000 cells in 1 mL of media. The cells were first diluted 100 fold and then serially diluted into 500,000 suspended BHK-21 cells per well. The BHK-21 plus infected MEFs were plated and allowed to adhere for 2 hours before the medium was removed and an agar overlay was added to the monolayer. Transfected Cell Center assays were performed following the above method, using viral RNA isolated using QIAamp Viral RNA purification kit (Qiagen #52904). The RNA was transfected at 100ng/well with .5µl of Lipofectamine 2000 (ThermoFisher scientific #11668027). After 1 hour, the cells were washed, trypsinized and diluted into BHK-21 cells as described above.

Viral binding assays were performed using plated Dicer MEF cells that were shifted to 4^{o}C for 30 min, followed by the addition of 10 MOI of EMCV or 1

MOI of VSV on ice. The cells were incubated at 4^{o}C for 2 hours, after which the cells were washed three times with cold PBS, lysed for RNA extraction, and the genes of interest were amplified by qPCR.

CHAPTER V: Discussion

The role of Dicer in regulating anti-viral immunity in mammals has been extensively investigated and hotly contested since RNAi and miRNAs were first discovered. The dual role that Dicer plays in both anti-viral defense and gene regulation via miRNAs makes it an important candidate for involvement in anti-viral immunity, directly or indirectly. While in plants and flies Dicer has differentiated these two roles into distinct Dicer proteins,[155,261,262] nematode Dicer is able to perform the dual-role of RNAi and miRNA regulation in a single protein.[158] Despite this, the evidence suggests that in chordates the role of Dicer has evolved to primarily process miRNAs for gene regulation[169,170,255], while the ability of Dicer to act as a direct anti-viral protein may have been largely supplanted by the development of the type I IFN pathways. The few reported instances of direct Dicer cleavage of viral RNA have largely been when the virus itself encodes a miRNA, [263] or in cells where the IFN response has not developed, such as oocytes and embryonic stem cells.[201,202]

Predicted and confirmed miRNAs in mammalian cells number in the hundreds to thousands depending on the organism. However, several factors complicate our ability to examine the role of any given miRNA in modulating host activity. First, there is the host cell type; the miRNAs expressed by a MEF are different from those expressed in an ES cell or a T cell. Second, there is the context in which miRNA expression is being measured; whether the cell is in steady-state or is being stimulated, and what type of stimulant is engaging the cell. Third, there is the promiscuity involved in the miRNA targeting. Since any

given miRNA can have tens to thousands of potential, computationally defined targets, the physiologically relevant function of a miRNA depends on the amount of miRNA expressed, the amount of miRNA loaded into an argonaute and which argonaute is favored, as well as the availability of the target mRNA. Additionally, it has been reported that certain mRNAs can alter the length of their 3' UTR in order to become more or less susceptible to regulation by post-transcriptional mechanisms. [264-266]

DICER NULL MEFS HAVE IMPAIRED NUCLEIC ACID DETECTION

As discussed in Chapter II, there are several Dicer null models that have been created.[180,181,254,267] The data generated from these various models has not always been consistent. As an example, in a report by Paramesewan et al., dengue virus infection of Huh7 cells generated very low levels of dengue specific vsRNAs, whereas in a report by Bogerd et al., dengue infection in HEK293 cells produced no Dicer dependent vsRNAs.[180,256] Additionally, in the same report by Bogerd et al., they saw no difference in dengue replication regardless of Dicer status, while a report by Kakumani et al. reported that dengue replication was increased when Dicer, Drosha, Ago1, and Ago2 were downregulated.[254,256] As another example, Bogerd et al. and Backes et al saw no difference in VSV replication in Dicer null cells compared to WT, while Otsuka et. al. saw an increase in VSV production following the depletion of Dicer.[181,193,256] Backes et.

al. argued that small RNAs play no role in VSV replication by engineering an artificial virus that encoded a small RNA antagonist. While the antagonist did affect miRNA maturation, it had no impact on VSV replication, thus they proposed that VSV replication was independent of small RNAs.[181] When it came time to look at a Dicer null cell, they compared VSV growth patterns between Dicer WT and Dicer KO cells, however they used WT MEFs and KO ES cells. These are considerably different cell types, where the ES cells produce no immune response[203] and employ a different isoform of Dicer compared to somatic cells.[170]

Considering that these are just a few examples, it is clear that the current literature regarding Dicer's role in antiviral immunity is incomplete. A few important notes should be considered here. The Bogerd report relied on depleting Dicer in HEK293 cells, which are an immortalized embryonic kidney cell. HEK cells are very limited in the induction of innate immune activation. HEK cells do not express TLR2, TLR4, MDA5 or LGP2 and have other immune deficits.[32,46,268,269] Also the Bogerd, Parameswan and Kakumani studies did not report innate immune activation or IFN induction, thus any differences in virus replication that they observed are not necessarily due to Dicer interacting with the viral RNA, but may instead have to do with differing amounts of IFN produced by their Dicer depleted cells. The MEF model employed in my studies also shares caveats. A line of fibroblasts is not an entire immune system and only represents one potential type of cell that is infected. Thus the conclusions drawn

from these MEF studies should be recognized as MEF specific and adapting the conclusions observed here to other cells and systems should be done with caution. The major strengths of this system however are as follows:

1. The cells are drawn from the same lineage. The Dicer KO cells are Dicer C/C cells where the Dicer was deleted, thus the WT and KO cells share a common ancestry.
2. The cells support a complete innate immune pathway. Unlike HEKs, or ES cells, the MEFs express a wide range of proteins involved in pathways that detect various types of PAMPs and nucleic acids.
3. The cells are complete Dicer knockouts. While some data was confirmed in Dicer knockdowns of other cells, the main data here was produced from true Dicer knockouts.
4. The cells were reconstituted with Dicer and the reconstitutions rescued some of the phenotypes.

While no model is perfect, these Dicer MEFs have proven a capable system for studying the induction of the type I IFN response by a variety of ligands.

MICRORNAS: THE REPRESSOR OF MY REPRESSOR IS MY ACTIVATOR

For decades, a standard practice in immunology was to identify a gene that appears to play a role in the immune response, by a pull-down, screen, or

array, and then study its role by knocking out the gene in a mouse model and examining the phenotype post infection. The TLR genes, RIG-I, MDA5, LGP2, and others were all researched by this method and the results helped establish the role of each of these proteins in innate immunity. The issue, of course, with using the same approach for looking at Dicer's role in innate immunity is that knocking out Dicer knocks out the regulation of thousands of genes via miRNAs. As previously discussed in Chapter III, determining the contribution of any particular miRNA in regulating innate immunity in a Dicer knockout is a daunting effort.

What is intriguing, however, is the generalized loss of nucleic acid detection in a Dicer knockout cell. 5'ppp RNA, poly I:C, poly dA:dT and a variety of immuno-stimulatory DNAs all fail to induce an IFN response in Dicer null cells. Considering that the canonical behavior of miRNAs is to repress translation of a protein, and Dicer null cells are lacking miRNAs, this suggests that the miRNAs responsible may actually be repressing a repressor of the innate immune response. One potential model of innate immune receptor regulation by miRNA is as follows: **(Fig. 5.1)**

1. Innate immune receptors are maintained at steady-state at low levels by a counteracting repressor.
2. Upon activation of innate immunity in the cell, either primary engagement via PRR, or secondary engagement via IFNAR, a miRNA is rapidly upregulated.

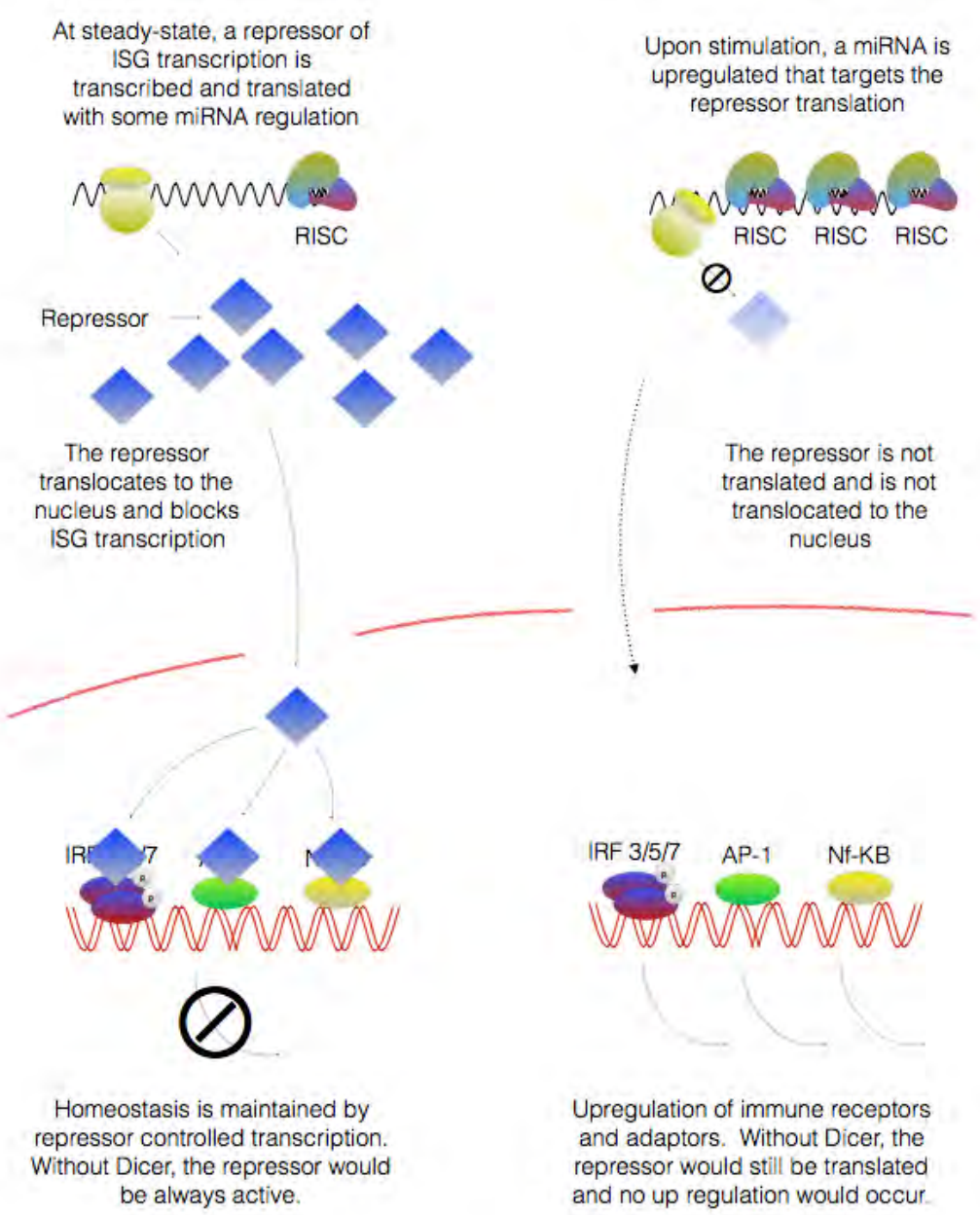

Fig. 5.1 Potential model of miRNA repression of repressors.
A miRNA that targets a nuclear repressor or co-repressor of ISGs is maintained at steady state levels, thus driving a level of repressor that allows modest ISG expression. Upon stimulation, the miRNA is unregulated and suppresses translation of the repressor, resulting in increased expression from the ISG promoter. Without Dicer, no regulation of the repressor exists, and it can suppress all transcription of the ISGs it is bound to.

3. This miRNA represses translation of the repressor.
4. Innate immune receptors are then upregulated in the cell.

As an example, the nuclear receptor co-repressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT)[w] are proteins that reside in the nucleus and actively repress other DNA-binding transcription factors that rely on hormonal activation.[270,271] They have both been shown to be active co-repressors of both NF-κB responsive genes and AP-1 responsive genes. The AP-1 transcription factor is downstream of TAK1 kinase signaling and is vital for the initiation of several anti-viral response genes downstream of PRR engagement, including IFNβ.[272,273] Thus, NCoR and SMRT are able to co-repress AP-1 activation sites until they themselves are repressed, leading to increased activation of AP-1 and its downstream effector genes. In humans, at least, NCoR is able to be targeted by several confirmed or predicted miRNAs, including Let-7b-5p.[274] While this remains to be confirmed experimentally, it provides a potential framework for understanding how a loss of miRNAs can lead to a repression of signal.

Alternatively, the non-canonical role of small RNAs as activators of transcription (RNAa) may also play a role here, where small, Dicer-dependent RNAs may act to stimulate expression of anti-viral receptors or adaptors, and this pathway may be lost in Dicer null cells. In Chapter II, I discussed a paper by

[w] SMRT also known as NCOR2

Chiang et. al. that described a series of “miRNAs” that had been computationally determined, but could not be experimentally validated.[215] One such miRNA, mmu-miR-1186, was then later determined to be a small activating RNA that bound to the promoter region of cyclin B1 and drove its expression.[275] Another miRNA, miR373, was reported to drive enrichment of RNA Pol II on the promoter regions of genes whose promoter matched the sequence of miR373.[152] Driving enrichment of RNA Pol II resulted in a 5-fold increase in targeted gene expression, when miR373 was introduced into the cells.[152] Therefore, given our current understanding of how small RNAs can repress or activate transcripts, there are multiple possible mechanisms by which the loss of Dicer-dependent small RNAs could cause the loss of IFNβ expression observed.

SENDAI VIRUS INDUCED IFN

As noted earlier, Sendai virus infection is the exception to the general failure of Dicer KO cells to produce an IFN response. Despite the evidence **(Chapter III, Fig. 3.1, 3.2, 3.3, and 3.4)** suggesting this is a consistent phenotype, a mechanism for how Sendai induces more IFN in a Dicer null environment has not been established. What has been established is that IFN is produced more readily at the protein and RNA levels, and that Dicer depletion results in more transcription at the IFNβ promoter, regardless of the mRNA downstream of the promoter. We observed this despite seeing no difference in

the limited replication of the virus between Dicer WT and KO cells. This result may be due to the uniqueness of the Sendai virus used to induce IFN. Sendai Cantell is grown in chicken eggs and allantoic fluid is harvested to prepare viral stocks. The virus isolated from allantoic fluid strongly induces IFNβ due to the number of DI copybacks produced.[217] At the same time, full-length genome replication and protein production are impaired compared to other strains of Sendai.

Intriguingly, Sendai Cantell virus passaged one time through LLC-MK2, a rhesus macaque cell line, and purified from plaques, no longer creates DI copyback fragments and fails to induce IFN activation.[217] This suggests that the selection pressure to eliminate DI copyback dsRNAs arises in IFN competent cells (LLC-MK2) and not the chicken embryos which have active small RNA processing pathways. [276,277] While we saw no evidence of dsRNA processing by Dicer in our deep sequencing, there remains the possibility that some function of Dicer, even if it is simply binding and sequestering DI copyback dsRNAs, may play a role in limiting their visibility to the cytosolic innate immune receptors. Andersson et. al. reported that in adenovirus infected cells, Dicer became so over-encumbered with adenovirus VA RNAs that it failed to continue processing other small RNAs.[253] Contrary to our studies, the binding of adenovirus VA RNA did lead to the production of Dicer-dependent small RNAs, whereas we did not observe any Dicer mediated, Sendai-specific, small RNAs in our cells. This suggests that the processing seen in other systems, such as with adenovirus VA

RNA may rely on either the sheer amount of RNA presented to Dicer, or may rely on a secondary structure that is a more preferred substrate to mammalian Dicer than pure dsRNA.

Another potential mechanism for increased Sendai-induced IFNβ in Dicer null cells relates to the loss of LGP2 expression in Dicer null MEFs. Several reports have shown that over-expression of LGP2 can result in diminished RIG-I signaling.[46,278,279] Examinations of LGP2 KO mouse models has resulted in conflicting results, with LGP2 KO mice created by Venkataraman et. al. showing increased RIG-I based responses and diminished MDA5 responses.[47] Meanwhile, a separate LGP2 KO mouse created by Satoh et. al. revealed a positive role for LGP2 in both RIG-I and MDA5 signaling.[48] While questions remain as to the true role of LGP2 in RIG-I induced signaling, reconstituting Dicer null cells with ectopic LGP2 may address the increase in Sendai induced IFNβ expression. It may be that without LGP2 to sequester Sendai RNA, more RNA is readily available to drive RIG-I based recognition in the Dicer null MEFs.

DICER AS AN ANTI-VIRAL

Evidence that Dicer has a direct role in anti-viral immunity is currently lacking. However, ample evidence suggests that Dicer, as a pre-miRNA processor, does play a vital role in viral innate immunology. While the general small RNA anti-viral role appears to have been superseded by IFN in chordates,

there remains the notion that in certain circumstances, Dicer can directly cleave and produce small RNAs that impact viral fitness.[201,202] Much has been made about the fact that viruses encode dsRNA antagonists that disrupt RNAi; several viruses have been shown to encode proteins or RNA that disrupt RNAi and Dicer processing *in vivo*. [248-252] However, the extent to which these proteins and RNA directly target Dicer and the RNAi pathway is less clear. The proteins described in these reports sequester dsRNA and thus protect the viral genome or replication intermediates from being detected by the host innate immune response. In the case of adenovirus VA RNA, the sheer amount of VA RNA caused competition for Exportin 5 and Dicer and thus inhibited microRNA biogenesis.[248] Besides the dual reports by Maillard and Li[201,202], Dicer has not been shown to produce viral derived siRNAs that function to suppress viral growth in chordates. The Maillard report may prove to be the exception to the rule because they used murine oocytes and embryonic stem cells, which lack IFN and express the shorter, dsRNA specific Dicer[O,x] and this may just provide the perfect conditions for RNAi.[201] The report by Li et. al. is more complicated in its interpretation. They infected baby hamster kidney cells (BHK-21) with a positive sense ssRNA virus, nodamuravirus, which expresses a potent RNAi inhibitor. They only saw vsiRNA accumulation when the RNAi inhibitor protein B2 was expressed. Additionally, they detected vsiRNA accumulation when a nodamura ΔB2 virus was used to infect baby suckling mice. WT nodamura virus

[x] Dicer[O] is the Oocyte version

killed newborn suckling mice, while nodamura ΔB2 virus was non-lethal. ΔB2 virus resulted in approximately 1,000 fold less viral RNA, but did show an accumulation of 22nt vsiRNAs. Adult suckling mice cleared both WT and ΔB2 virus without issue by invoking an IFN response.[202] Of note, neither the Maillard nor Li reports showed that any of the vsiRNAs generated had actual activity against the viral genome. Many questions remain from these studies:

1. Why is RNAi seemingly limited to oocytes, ESCs, or newborn mice?
2. Is RNAi only detected *in vivo* when viruses are stripped of their ability to encode an RNAi inhibitor?
3. Since many viruses do not encode RNAi inhibitors, why aren't vsiRNAs detected with these viruses?
4. Is RNAi unique to viruses such as nodamuravirus, which is transmitted by mosquitos and thus has to deal with the active arthropod RNAi defenses?

One of the first concepts that must be considered is that the role of Dicer in viral RNA processing can vary. Mice express an isoform of Dicer ($Dicer^{O}$) that is efficient at processing linear dsRNA,[170] in lieu of expressing the isoform ($Dicer^{S}$)[y] that efficiently processes miRNAs, thus leading to a generalized loss of miRNA expression in mouse oocytes.[172,280] When the Drosha binding partner DGCR8 is depleted from mouse oocytes, the cells expressed no phenotype,[280] despite DGCR8's essential role in miRNA processing providing additional

[y] $Dicer^{S}$ being the somatic version

evidence that miRNAs are not a major factor for gene expression in oocytes.[110] Humans, however, do not express an alternative isoform like Dicer^{O}.[170] In fact, human ESC and oocytes do express miRNAs, albeit a different subset from the miRNAs expressed in somatic cells. [281-283] Thus the presence of virus derived, Dicer dependent small RNAs may be due to the unique features of Dicer^{O}, but cannot be extrapolated to other mammalian systems.

The role of RNAi inhibitors is complicated, since many RNAi inhibitors sequester RNA,[249-251] thus making them adept at inhibiting RNAi as well as evading the IFN response. Whether viral RNAi inhibitors directly target the RNAi response or if it is the result of an off-target effect due to binding available RNA in the cell remains to be seen. As expected, it has been reported that RNAi targets viral replication in arthropod vectors,[284] and arboviruses encode RNAi inhibitors.[252] What is intriguing however, is that in the case of West Nile virus, the viral RNAi inhibitor is a non-coding 525 nt RNA with complex secondary structure.[252] This same region had previously been reported to be required for replication and pathogenicity.[285,286] This held true even when the IFN deficient Vero cell line was used for infection.[286] It may be that RNAs with complex secondary structure are able to act as effector molecules in currently undiscovered roles.

For somatic cell anti-viral protection, Dicer's role appears to be currently limited to processing miRNAs that regulate anti-viral immunity. However given the number of proteins involved in the RNAi and microRNA pathways, there may

still yet be a role for Dicer, Drosha, or the Argonaute proteins in direct anti-viral immunity.

DICER'S ROLE IN CONTROLLING EMCV INFECTION

The data presented in Chapter IV show a clear role for Dicer in regulating infection of MEFs by EMCV. A 100-fold reduction of virus production **(Fig. 4.3)** is a significant defect and supports the notion that the prevention of infection is perhaps more useful than modulating the intracellular response after an infection has already occurred. A significant amount of research using RNAi, siRNAs, and miRNAs has focused on creating small RNAs that directly target viral sequences. (For examples, see [245-247,287]) This research has shown promise in silencing viral genomic and mRNA and many are being adapted as potential therapeutic agents. There are obstacles to overcome, such as the appropriate specificity and delivery of the small RNA into the cell that is infected in order to reduce any potential toxicity or off-target effects (for review, see[288]). Additionally, with some viruses that have high natural variation and mutation rates, such as HIV-1, escape mutants can quickly generate to resist siRNA based therapies. [289-291] Thus, research into anti-viral therapies have expanded to include using small RNAs to target cellular proteins that serve as receptors for viruses and have a much lower natural mutation rate. In a recent example, work from the Rossi group showed that targeting the HIV-1 co-receptor protein CCR5 could inhibit

HIV-1 infection.[292] Obviously, targeting the receptor for many viruses would not work due to the importance of many receptor proteins in normal cellular function. However, this does provide an additional pathway forward for developing potential therapeutics.

The work presented here strongly suggests that Dicer impacts EMCV growth, not through direct RNAi activity, but by processing miRNAs, one of which is important for the expression of the unknown EMCV receptor. (**Fig. 4.4, 4.5)** This is not the first time that viral infection has been modulated by receptor down-regulation; miR-23b was previously reported to be induced upon RIG-I activation and targets the very low density lipoprotein receptor (VLDLR).[260] VLDLR knockdown inhibited infection with Rhinovirus 1B. Similar to my results, transfection of the infectious RNA into the cell, thus bypassing receptor engagement, resulted in equal growth rates of rhinovirus in cells regardless of miR-23b expression.[260] My results show that Dicer can have a major role in regulating the growth of a virus in mammalian cells, however it is the *absence* of Dicer and its attendant role in processing cellular small RNAs that results in the loss of viral reproduction.

FUTURE DIRECTIONS

The work presented in this dissertation suggests that a complicated regulatory network exists for miRNA regulation of nucleic acid based sensing in

MEFs. While some of the primary receptors involved in cytosolic RNA sensing remain unchanged in a Dicer null cell (RIG-I, MDA5) the downstream effector response after engagement of these receptors is severely diminished. The greatest caveat of the Dicer null model is that it becomes difficult to tease apart the individual contributions of miRNAs into the overall regulatory network. Closer examination of potential miRNA binding sites in the mRNAs of genes like IFI204 and LGP2 may yield potential candidates, However this approach seems unlikely to be informative considering that if miRNAs directly targeted their mRNAs, one would expect to see increased expression following Dicer depletion. Thus it may be more important to examine the activation of transcription of these genes; examine what transcription factors and repressors are involved in regulating transcription of their mRNA. Since siRNA and miRNA based therapies are already thought to have great potential in the treatment of disease, determining small RNAs that regulate the transcription of genes important in innate anti-viral immunity could potentially be useful as therapeutic interventions.

The most pressing issue related to the EMCV work presented in this dissertation is to determine what cellular protein is used as the EMCV receptor. Future efforts may want to focus on determining how other pathogenic viruses replicate in a Dicer null environment. The Dicer null cells allow a unique opportunity to screen individual miRNA effects by reconstituting individual or pools of miRNAs and monitoring how they impact the growth of a variety of pathogens. Considering the high rate of mutations in various viruses, therapeutic

small RNAs that target relatively static host genes to prevent viral infection may have more use than small RNAs that can quickly be rendered inactive by a single nucleic acid mutation in the target region of the pathogen genome.

CONCLUDING REMARKS

RNA was once considered a relatively unimportant messenger stuck between the genetic repository of information, DNA, and the building blocks of life, protein. RNA has been the target of an explosion of research in the last few decades as it became increasingly obvious that the DNA regions that are protein-coding are not the only important regions of genetic material. In 1972, Susumu Ohno argued that an organism, like a human, could only support a certain number of genes before the mutation rate would be intolerable. He theoretically determined an upper limit of 30,000 potential genes for a human. Recognizing that our genome was too large to only code for such a limited number of genes, he helped popularize the misnomer of “junk DNA” as the non-coding regions of DNA that were unrecognized as contributing to protein synthesis. [293] Rather amazingly, in humans, ~98% of DNA is non-protein coding. [294] In a landmark paper, the ENCODE consortium biochemically determined that at least 80% of the human genome is active in some form. [295] This non-protein coding DNA is still revealing its function, and as more work focuses on how RNA regulates other cellular processes, the nuances of gene expression will come into focus. Dicer

has proven to be integral to a large number of cellular functions due to its role as a small RNA processor. The work presented here argues that Dicer has an essential role in anti-viral innate immunity, both as a regulator of the expression of genes important for nucleic acid sensing and as a restricting factor in the ability of EMCV to infect MEFs efficiently.

In conclusion, Dicer and Dicer-like proteins can be traced back through the evolutionary timescale to the beginnings of eukaryotic life, and beyond, when one considers prokaryotic type III RNases. Therefore, one can infer that the processing of dsRNA is a key regulatory step across many of the kingdoms of life. In that context, it is clear that humans have evolved with the presence of small RNA regulation and any pathogen we face evolved in the presence of small RNA regulation as well. This body of work delineates a role for Dicer in the control of viral reproduction as well as control of the signaling pathways that allow the innate immune response to recognize the foreign from the self.

APPENDIX

The purpose of this appendix is to address the pertinent questions that remain following this body of work. As such, I will introduce the question at hand and provide my comments on the experimentation that should take place to answer the question. While this is in no way exhaustive, it will provide a framework from which future proposals may be generated.

WHERE DOES DICER AFFECT NUCLEIC ACID SENSING AND SIGNALING?

Perhaps the most extensive question is determining where Dicer is having its impact on nucleic acid sensing. Based on the data presented in Chapter III, the production of IFN and RANTES post-NA stimulation is low or lacking, regardless of the type of NA used. In order to ascertain where Dicer is working in these signaling pathways, I propose starting in the nucleus and working backwards to cytosolic sensing of the NA. This section will actually address two different approaches: exploring the signaling pathway, and looking for overall chromatin rearrangement in the cells. The reasoning behind this is that total chromatin rearrangement may cause differences in transcription at the IFN**β** promoter without it being unique to the IFN locus itself.

1.) Determine if there is a difference between Dicer WT, KO, and reconstituted cells in the engagement of RNA Pol II to DNA in the cells to look at total chromatin remodeling changes that occur in the absence of Dicer. Previous

reports have suggested Dicer can regulate chromatin and histone methylation by direct regulation via miRNAs[298,299] or other mechanism[299]. By using Pol II CHIP-SEQ, the sequences of DNA that are available for RNA Pol II transcription at the basal state can be analyzed and overall changes in open chromatin can be assessed. This will be followed up by RNA Pol II CHIP-SEQ after stimulation of the Dicer WT, KO and reconstituted cells after with Sendai virus, poly dA:dT, poly I:C, 5'ppp RNA, and exogenous IFN. All of the data generated in my system thus far suggests that Dicer WT and KO cells respond equally to the presence of exogenous IFN, so by adding it as a control stimulant, we can look at the engagement of RNA Pol II to various ISG's and they should be similar between the cells types. Sendai virus is a stimulant that activates IFN**β** transcription and production in both WT and KO cells, although the data suggests IFN**β** production is higher in Dicer KO cells. By looking at the engagement of RNA Pol II at the IFN promoter after Sendai infection, we can ascertain if the higher IFN levels are driven by more recruitment of transcription machinery. With poly dA:dT and 5'ppp RNA, very low levels, if any, of IFN are seen, thus it is important to determine if the regulation is prior to transcription or post-transcriptional. It is possible that post transcriptional modification of the IFN transcript may be different depending on the type of stimulant used, and can be assessed by adding Alpha-amanitin after stimulation and measuring the amount and stability of IFN**β** mRNA. However, I would hypothesize that in all cases, the amount of RNA Pol II on the IFN promoter would mimic the phenotype already observed at

the RNA and protein levels.

2.) Moving further upstream, I would assess the dimerization and translocation of IRF3 into the nucleus by making cytosolic and nuclear fractions after stimulation with the above agents or medium, and then determine what fraction of IRF3 is a monomer versus dimer, and how much of it translocates from cytosolic fraction to nuclear fraction by western blot. There are a series of kinases that act as signaling cascade proteins that lead from either STING, in the case of DNA, or MAVS, in the case of the RIG-I, and MDA5 RNA sensors. These can all be assessed for their phosphorylated forms and you can "walk back" up the chain of signaling proteins, however I prefer the idea of jumping a few steps ahead and then walking back down if necessary.

3.) Thus the next step I would examine would be MAVS aggregations (post poly I:C or 5'ppp RNA stimulation) or STING??? (post poly dA:dT stimulation) MAVS aggregations occur when RIG-I or MDA5 bind their ligand and aggregate their CARD domains, leading to MAVS aggregations on the mitochondria[44,45]. By staining for MAVS aggregations, you can assess an approximation of the amount of signaling that is immediately downstream of the actual ligand engagement. At this point, if you are still seeing differential MAVS aggregations in Dicer WT and KO cells, then it suggests it's the actual sensing of ligand that is impacting the downstream expression of IFN**β**. It's unlikely that it's the actual RIG-I and MDA5 engagement of ligand that is playing a role, seeing as how RIG-I and MDA5 are expressed at equivalent levels in Dicer WT and KO cells. But there is another

protein, LGP2 that can impact ligand sensing by both RIG-I and MDA5. LGP2 has a long and complicated history in RNA sensing, and more detailed information can be found in Chapter I of my dissertation. Briefly, LGP2 has been shown to help with the initiation and aggregation of MDA5 on dsRNA, thus leading to enhanced signaling to MAVS and further downstream. LGP2 has also been shown to inhibit and enhance RIG-I signaling, depending on what cells the experiments were performed in and whether it was *in vitro* or *in vivo* work. We also know that LGP2 is essentially missing in a Dicer KO cell, and does not come back following reconstitution. Thus, we will want to look at what happens if we add back LGP2.

WHAT HAPPENS WHEN YOU RECONSTITUTE DICER WT AND KO CELLS WITH LGP2, IFI204, OR IFI205?

Of the sensors that are down-regulated in Dicer KO cells, LGP2, IFI204, and IFI205 are either not reconstituted, or reconstituted in a non-significant amounts (**Fig. 2.6)**. This is significant for several reasons. As mentioned above, LGP2 can impact RIG-I and MDA5 signaling, despite its inability to signal on its own. IFI204 is an ortholog of IFI16 in humans, which has been implicated in both DNA sensing, but also in the transcription initiation of a variety of ISGs following stimulation of cells[296]. Either of these two functions may play a role in the loss of IFN production following DNA stimulation. IFI205 is a related PYHIN family member but has been suggested to play more of a role in activating ASC

mediated inflammasomes after DNA binding[300]. If the loss of IFI204 and IFI205 are responsible for a failure to sense DNA, then you never get initiation of signaling downstream of DNA only. However, if IFI204 acts like its human ortholog IFI16 and is able to regulate the transcription initiation of IFN-related genes, then the loss of IFI204 would have far reaching effects beyond just that of DNA sensing. The loss of signal from RIG-I and MDA5 mediated pathways would also be impacted by the loss of IFI204 if it plays a general role in the transcription of ISGs. Thus it is important to look at what happens in a Dicer null cells where these proteins have been reconstituted.

Using the same lentivirus-derived transduction approach as I did with Dicer, I propose making lentivirus constructs expressing a codon optimized version of LGP2, IFI204 and IFI205. The lentivirus would use a selection antibiotic so that cells that receive the various constructs could be enriched out of the population of Dicer KO cells. After the cells expressing the various constructs have been selected, the cells will be stimulated with viral and non-viral ligands to see if the addition of any of the single receptors can rescue some or all of the phenotypes observed. Of course, the rescue of a single protein may not actually rescue the phenotype if they are required to work in concert with another down-regulated gene. In addition to measuring the terminal output, such as IFN expression, I propose that a variety of assays are run in the control and reconstituted cells. For LGP2, I would run a LGP2/MDA5 aggregation assay to see if the addition of LGP2 back into the Dicer null cell rescues the detection of dsRNA. If

LGP2/MDA5 aggregation occurs, with no downstream signaling, then it would suggest that the loss of Dicer plays a role downstream of whether LGP2 is expressed or not. In the case of IFI204, I would want to explore upstream signaling of IFI204 to STING, as well as looking to see if IFI204 is sitting on the transcription start site of ISGs following stimulation. By assessing both, it may help tease out if IFI204 is having function as a DNA sensor, transcriptional activator or both. For IFI205, the limited knowledge we have of is does not suggest it would play a role in IFN induction, but rather in inflammasome activation. Still, it would be informational to ascertain if IFI205 reconstitution has any role in downstream IFN signaling.

WHAT vsiRNAS ARE GENERATED BY THE ΔHELICASE DICER?

The report by Flemr et. al. suggested that oocytes and ESCs express a helicase truncated form of Dicer that can more readily bind and process long dsRNA, such as those arising from viral infection[170]. Additionally Mailliard et. al. was able to show that in oocytes and ESCs, vsiRNAs could be detected after infection with EMCV[201]. Considering that my **Δ**Helicase Dicer could potentially mimic the alternative mouse transcript of Dicer reported by Flemr, it would be intriguing to assess whether a Dicer null cell that has been reconstituted with **Δ**Helicase and infected with EMCV would generate detectable vsiRNAs. A recent paper by Kennedy et. al. revealed that mutated human Dicer where the helicase has been deleted can generate vsiRNAs when expressed in Dicer null

cells[301]. By reconstituting the Dicer null MEFs with WT and **Δ**Helicase Dicer, and then infecting with EMCV, VSV, Sendai, and mock, we could assess any vsiRNA formation by deep sequencing. I would expect that the **Δ**Helicase Dicer will generate vsiRNAs when infected with EMCV and VSV, and Sendai may generate vsiRNAs, depending on the length and double-strandedness of the copybook DI particles that this particular strain of Sendai generates. However, I would expect that the WT Dicer reconstituted cells would not generate vsiRNA from any of the viruses, since the full-length, somatic version of Dicer selects for dsRNA with a hairpin loop[255].

WHAT RECEPTOR IS RESPONSIBLE FOR BINDING EMCV ON A MEF

According to my data, EMCV fails to bind as well to a Dicer KO MEF compared to a Dicer WT MEF. However the only defined receptor for EMCV, VCAM1, is expressed equally at the RNA level in both Dicer WT and KO cells. Thus, two possibilities arise: First, VCAM1 may be expressed equally at the mRNA level, but post-transcriptional regulation may result in different levels of protein expression. Second, since VCAM1 is not the only receptor for EMCV, a different receptor may be responsible for the difference in binding. To test for the first, I would do a western blot or fluorescent microscopy to look at total VCAM1 protein expression or surface expression, respectively. However, array data from other groups suggests VCAM1 is expressed at very low levels in MEF cells, so VCAM1 expression may be difficult to observe. I would then transduce VCAM1

into the Dicer WT and KO MEFs and see if over expression of VCAM1 leads to a rescue of the binding phenotype. To identify other potential receptors for EMCV, there are multiple approaches that could be taken. First, if there are sufficient antibodies, I would propose doing an EMCV pulldown of bound cellular proteins and using mass spectrometry to identify the peptide sequences pulled down by the anti-EMCV antibody. However, from personal experience, I know that EMCV antibodies are lacking. Another potential approach is to screen CRISPR libraries for EMCV binding and then identify which genes have an impact on EMCV binding when they are deleted. Thus, other potential receptors for EMCV can be identified and then by looking at the candidates individually for expression in my Dicer MEFs, a suitable candidate could be found. Since transfecting EMCV infectious RNA directly into the cells results in equal viral growth, (**Figure 4.5b)** I would expect that after using one of these methods to identify a potential receptor, the overexpression of that receptor in Dicer KO cells would ameliorate the EMCV growth defect in Dicer KO cells.

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