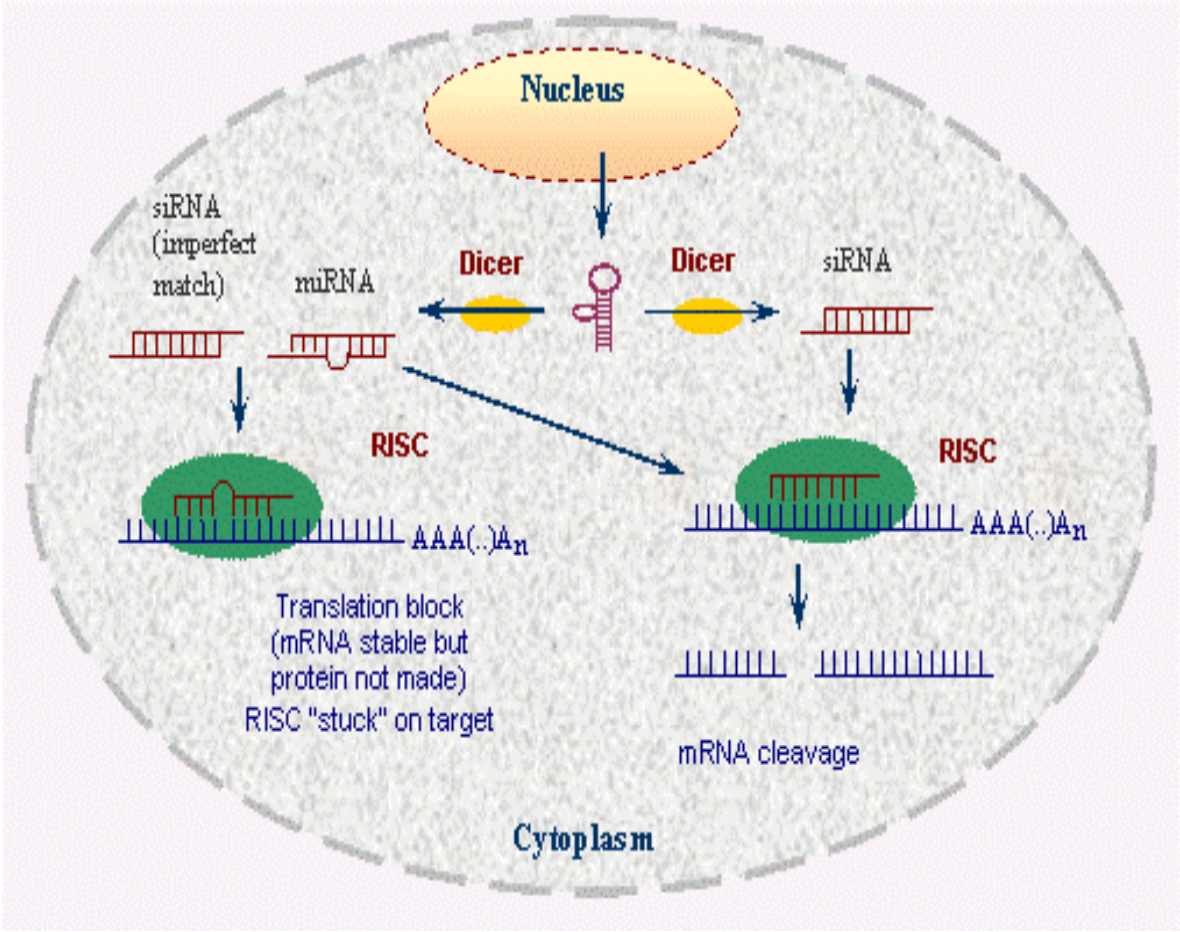
RNA INTERFERENCE

RNA interference (RNAi) refers to homology-dependent gene silencing mechanisms initiated by **Dicer**-mediated production of small interfering RNAs (siRNAs) and microRNAs (miRNAs) in eukaryotic organisms



Science. 2021 Jul 9;373(6551):231-236. doi: 10.1126/science.abg2264.

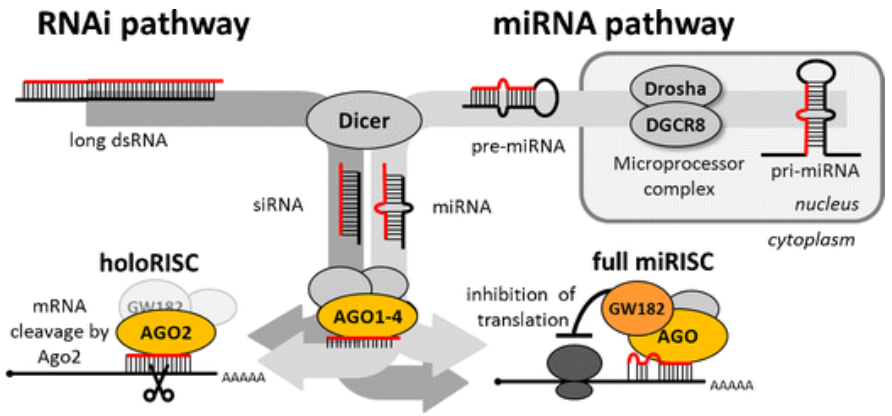
An isoform of Dicer protects mammalian stem cells against multiple RNA viruses

Enzo Z Poirier [1], Michael D Buck [2], Probir Chakravarty [3], Joana Carvalho [4], Bruno Frederico [2], Ana Cardoso [2], Lyn Healy [5], Rachel Ulferts [6], Rupert Beale [6 7], Caetano Reis E Sousa [1]

DICER

The Dicer enzyme is a member of the ribonuclease (RNase) III family. It is most well known as the endonuclease that functions in the RNA interference (RNAi) pathway to cleave long double-stranded RNA (dsRNA) molecules into short dsRNA molecules, known as small RNAs, including microRNA (miRNA) and small interfering RNA (siRNA).

Dicer's endonuclease function is not only involved in small RNA biogenesis, but also in the processing of other endogenous and exogenous substrates.

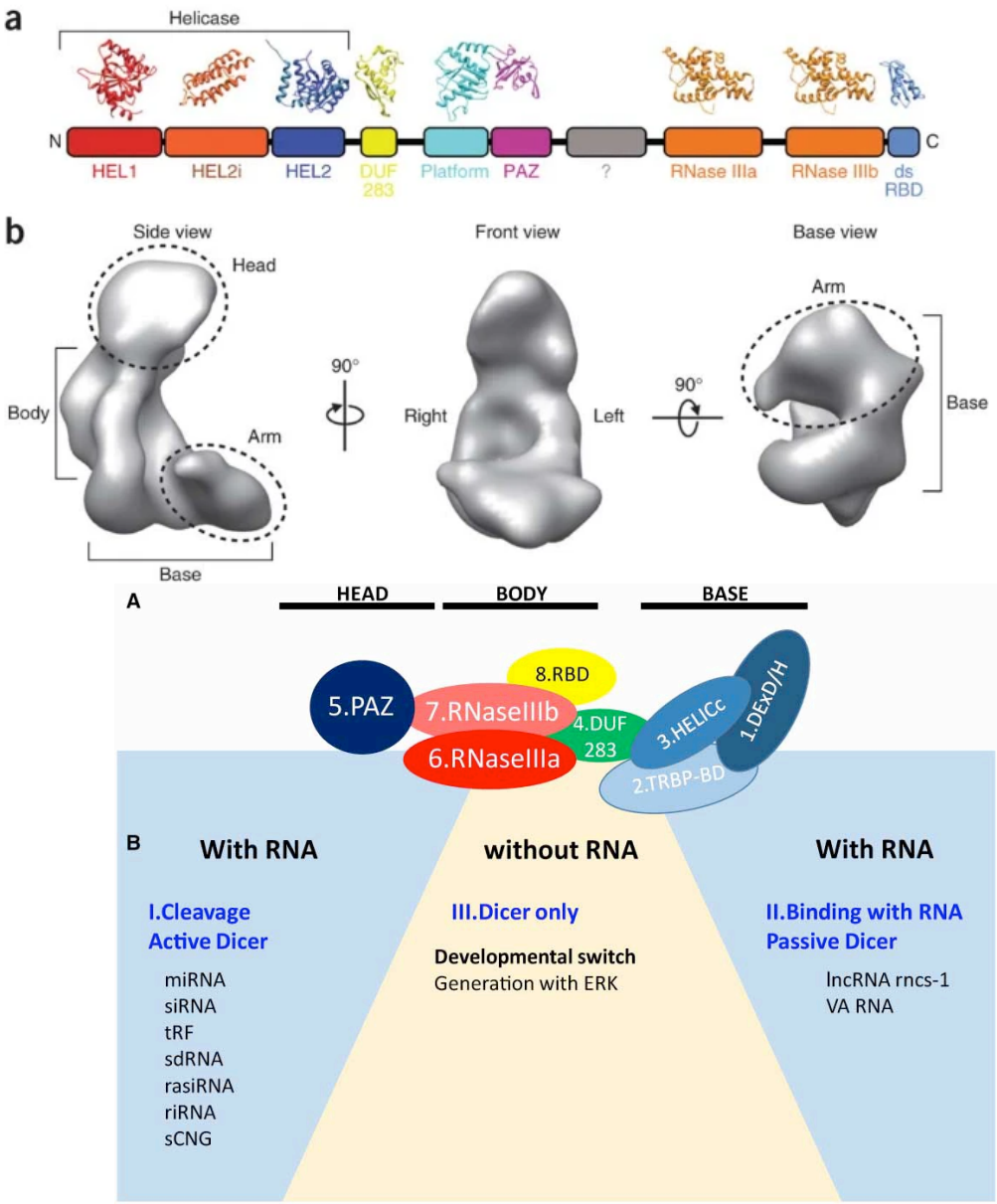


DICER STRUCTURE

- Helicase domain included with DExD/H, TRBP-BD and HELICc.
- DUF283, PAZ (Piwi/Argonaut/Zwille) domains.
- RNase IIIa and IIIb domains, and dsRNA-binding domain (RBD).

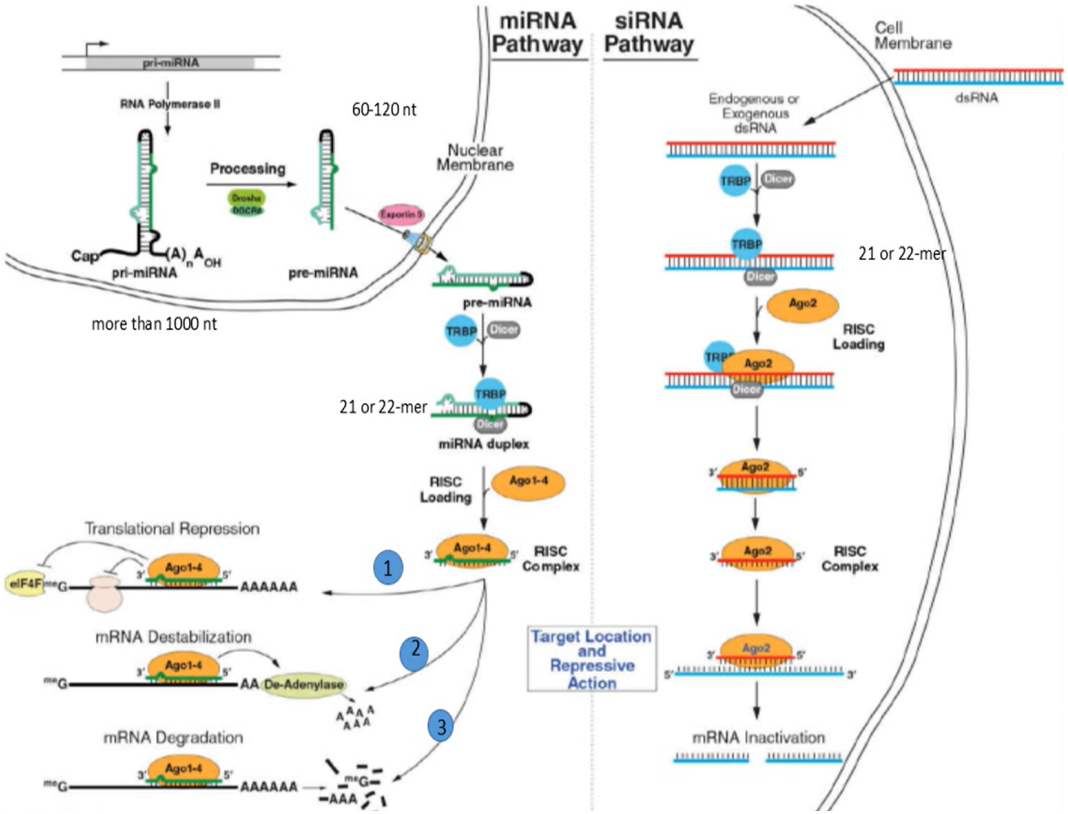
PAZ domain has an extra loop enriched in basic amino acids, which changes the electrostatic potential and molecular surface of the pocket.

RNase IIIa and **IIIb** domains form the **catalytic core** of Dicer; each domain is thought to be responsible for the cleavage of one strand of the dsRNA substrate.



DICER FUNCTIONS IN RNAi PATHWAY

1. dsRNAs, derived from the nucleus or exogenous, form a pre-RISC with Dicer → dsRNA is then cleaved by Dicer and others dsRNA binding proteins.
2. One of the two strand of miRNA duplex is incorporated into an AGO protein to form the RISC → the incorporated strand (guide-strand) is typically the strand with the less stable 5' end, the other is degraded.
3. The activated RISC will either guide the sequence-specific degradation of complementary RNAs or inhibit the translation of complementary target mRNAs by post-transcriptional gene silencing.



DICER'S ROLES IN PHYSIOLOGY

Dicer is an essential enzyme for the maintenance of physiology due to its pivotal role in several processes, and its loss or aberrant expression contributes to the development of sever human diseases (psoriasis, ankylosing spondylitis, rheumatoid arthritis and multiple sclerosis).

Reduced expression of Dicer is also associated with poor prognosis in some types of lung, breast, skin, endometrial, and ovarian cancer. In contrast, Dicer is overexpressed in metastatic lesions of prostate cancer, and is increased in Burkitt lymphoma.

Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing

Chryssa Kanellopoulou,[1,4] Stefan A. Muljo,[2,4] Andrew L. Kung,[1] Shridar Ganesan,[1] Ronny Drapkin,[1] Thomas Jenuwein,[3] David M. Livingston,[1,5] and Klaus Rajewsky[2,6]

[1]The Dana-Farber Cancer Institute, Department of Cancer Biology and [2]The CBR Institute for Biomedical Research and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115, USA; [3]Research Institute of Molecular Pathology, The Vienna Biocenter, A-1030 Vienna, Austria

Dicer is the enzyme that cleaves double-stranded RNA (dsRNA) into 21–25-nt-long species responsible for sequence-specific RNA-induced gene silencing at the transcriptional, post-transcriptional, or translational level. We disrupted the *dicer-1* (*dcr-1*) gene in mouse embryonic stem (ES) cells by conditional gene targeting and generated Dicer-null ES cells. These cells were viable, despite being completely defective in RNA interference (RNAi) and the generation of microRNAs (miRNAs). However, the mutant ES cells displayed severe defects in differentiation both in vitro and in vivo. Epigenetic silencing of centromeric repeat sequences and the expression of homologous small dsRNAs were markedly reduced. Re-expression of Dicer in the knockout cells rescued these phenotypes. Our data suggest that Dicer participates in multiple, fundamental biological processes in a mammalian organism, ranging from stem cell differentiation to the maintenance of centromeric heterochromatin structure and centromeric silencing.

[*Keywords*: RNA interference; microRNA; heterochromatin silencing; DNA methylation]

Supplemental material is available at http://www.genesdev.org.

Received August 11, 2004; revised version accepted December 14, 2004.

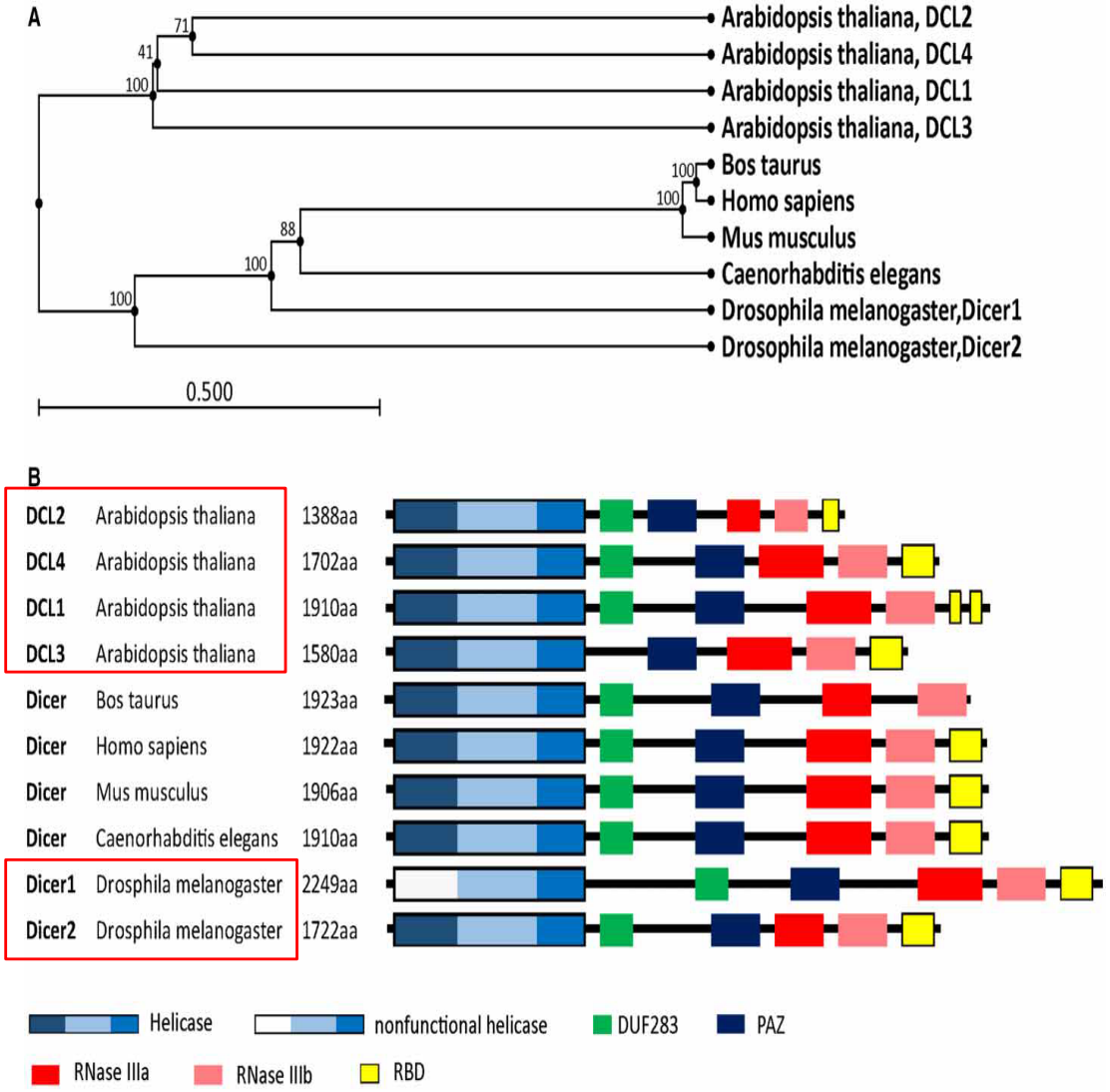
AN EVOLUTIONARY SIGHT AT DICER HOMOLOGS

Dicer probably arose from an early eukaryotic origin.

The evolutionary phylogenetic tree of animal Dicers shows that **an ancient duplication gave rise to Dicer1 and Dicer2 genes** very early in metazoan evolution.

In Drosophila melanogaster, dmDcr-1 is dedicated to the miRNA pathway while dmDcr-2 performs antiviral RNAi.

Vertebrates and nematodes possess a **single Dicer** that generates both siRNA and miRNAs while most invertebrates **express two Dicer proteins**.



dsRNAs VIRAL INFECTION AND DICER

The basic mechanism of mRNA production are similar in most, if not all, viruses having dsRNA genomes.

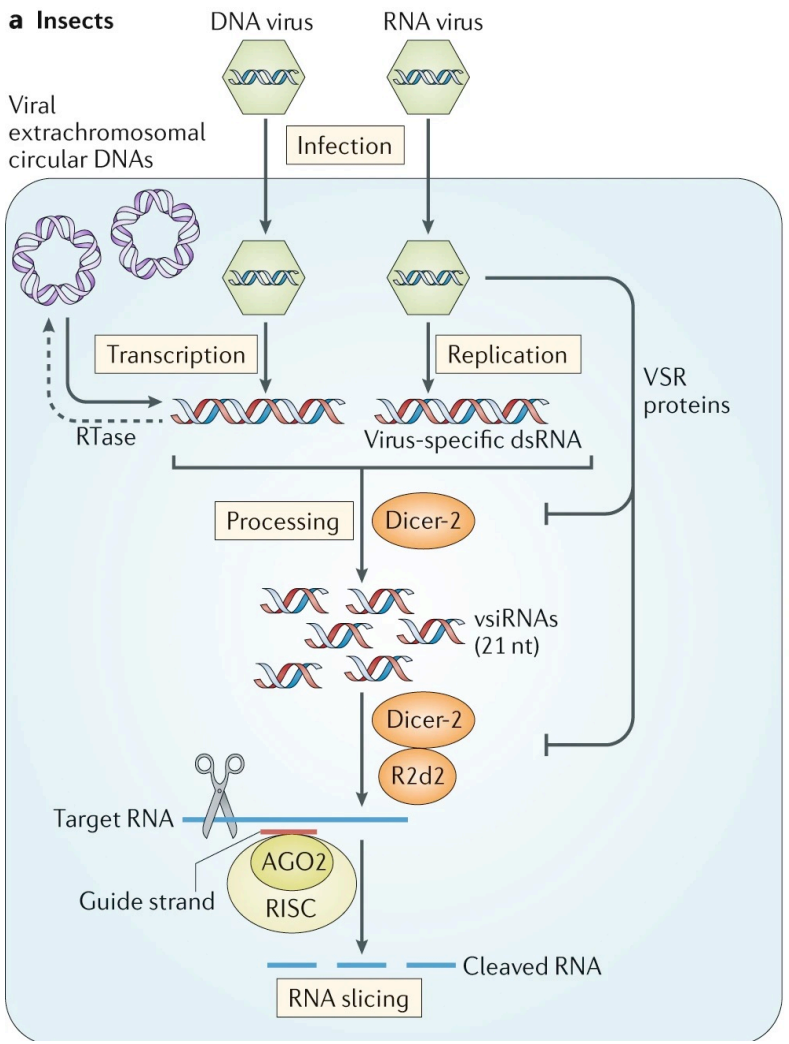
Genome replication by dsRNA viruses occurs in subviral particles.

These subviral particles, also called the cores, have an intact viral capsid that encloses the viral genome and RNA-dependent RNA polymerase molecules.

For complex, multi-layered dsRNA viruses, the core is derived from the virion by removing outer capsid proteins during entry.

Progeny cores are assembled from mRNAs, **which are then replicated inside the particle to generate the dsRNA genome (Dicer's substrates)**

Viruses with dsRNA genomes face a particular challenge in that host cells do not produce proteins which can transcribe from a dsRNA template



ANTIVIRAL RNAi

Antiviral RNAi is distinct from dsRNAi:

1. the origin of the substrate dsRNA (viral RNA vs endogenous sources)
2. the RNAs targeted by the RISC (viral RNA vs host cell mRNA).

Antiviral RNAi therefore depends on the efficient production of viRNAs from viral dsRNA and the efficient targeting of viral RNA by the RISC machinery.

Plant and invertebrate cells utilize mostly RNA interference (RNAi) for cell-intrinsic immunity to viruses

Several studies support a predominant antiviral function of the RNAi pathway in these organisms.

EVIDENCE OF ANTIVIRAL RNAi IN LOWER ORGANISMS

" In fact, so widespread and potent is this defence response (RNAi), it has driven most, if not all, plant viruses to evolve viral suppressors of RNA silencing (VSRs) that attenuate or completely inhibit this process "

Review > Nat Rev Microbiol. 2013 Nov;11(11):745-60. doi: 10.1038/nrmicro3120.

RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence

Nathan Pumplin [1], Olivier Voinnet

Affiliations + expand

PMID: 24129510 DOI: 10.1038/nrmicro3120

Abstract

RNA silencing is a central regulator of gene expression in most eukaryotes and acts both at the transcriptional level through DNA methylation and at the post-transcriptional level through direct mRNA interference mediated by small RNAs. In plants and invertebrates, the same pathways also function directly in host defence against viruses by targeting viral RNA for degradation. Successful viruses have consequently evolved diverse mechanisms to avoid silencing, most notably through the expression of viral suppressors of RNA silencing. RNA silencing suppressors have also been recently identified in plant pathogenic bacteria and oomycetes, suggesting that disruption of host silencing is a general virulence strategy across several kingdoms of plant pathogens. There is also increasing evidence that plants have evolved specific defences against RNA-silencing suppression by pathogens, providing yet another illustration of the never-ending molecular arms race between plant pathogens and their hosts.

Many plant (and insect) viruses encode viral suppressors of RNAi (VSRs) that interfere with the RNAi pathway.

EVIDENCE OF ANTIVIRAL RNAi IN LOWER ORGANISMS

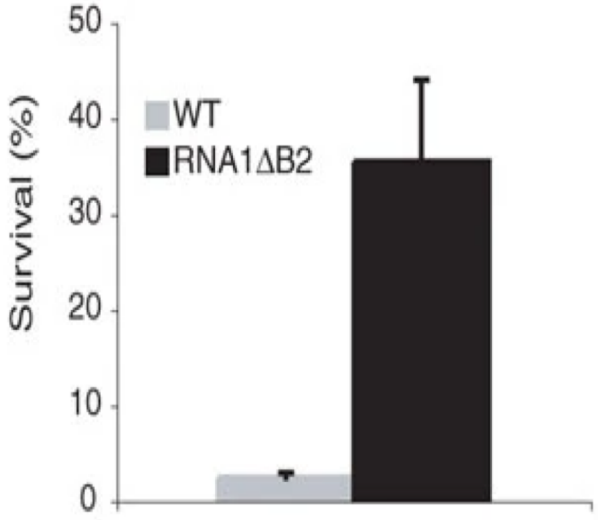
› Nat Immunol. 2006 Jun;7(6):590-7. doi: 10.1038/ni1335. Epub 2006 Mar 23.

Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila

Delphine Galiana-Arnoux [1], Catherine Dostert, Anette Schneemann, Jules A Hoffmann, Jean-Luc Imler

Affiliations + expand

PMID: 16554838 DOI: 10.1038/ni1335



Transgenic fly lines carrying chromosomally integrated constructs expressing FHV vs transgenic fly lines expressing a variant of FHV genome containing two-point mutations that disrupted the open reading frame of B2.

" ....successful infection of drosophila with FHV (member of a nodaviridae dsRNA viruses) was strictly dependent on expression of the B2 protein: an inhibitor of Dicer"

Inactivation of key components of the RNAi pathway results in a decrease of survival in hosts organisms

EVIDENCE OF ANTIVIRAL RNAi IN LOWER ORGANISMS

"During a virus infection in plants, the accumulation of 21-nt double-stranded siRNAs is observed in local and systemic tissues…."

J Virol. 2005 Jun;79(12):7812-8. doi: 10.1128/JVI.79.12.7812-7818.2005.

Plant virus–derived small interfering RNAs originate predominantly from highly structured single–stranded viral RNAs

Attila Molnár [1], Tibor Csorba, Lóránt Lakatos, Eva Várallyay, Christophe Lacomme, József Burgyán

Affiliations + expand

PMID: 15919934 PMCID: PMC1143663 DOI: 10.1128/JVI.79.12.7812-7818.2005

Free PMC article

Abstract

RNA silencing is conserved in a broad range of eukaryotes and includes the phenomena of RNA interference in animals and posttranscriptional gene silencing (PTGS) in plants. In plants, PTGS acts as an antiviral system; a successful virus infection requires suppression or evasion of the induced silencing response. Small interfering RNAs (siRNAs) accumulate in plants infected with positive-strand RNA viruses and provide specificity to this RNA-mediated defense. We present here the results of a survey of virus-specific siRNAs characterized by a sequence analysis of siRNAs from plants infected with Cymbidium ringspot tombusvirus (CymRSV). CymRSV siRNA sequences have a nonrandom distribution along the length of the viral genome, suggesting that there are hot spots for virus-derived siRNA generation. CymRSV siRNAs bound to the CymRSV p19 suppressor protein have the same asymmetry in strand polarity as the sequenced siRNAs and are imperfect double-stranded RNA duplexes. Moreover, an analysis of siRNAs derived from two other nonrelated positive-strand RNA viruses showed that they displayed the same asymmetry as CymRSV siRNAs. Finally, we show that

Viral infections lead to the accumulation of Dicer-dependent virus-derived siRNAs (viRNAs) that originate from dsRNA viral replication intermediates and are homologous to viral RNA sequences

VERTEBRATES RELY ON THE PROTEIN-BASED INTERFERON (IFN)-DRIVEN INNATE IMMUNE SYSTEM

Plants and invertebrates lack an IFN system and rely on antiviral RNAi to defend against viruses.

In contrast, vertebrates have adopted the IFN system for cell-intrinsic antiviral defence and are **thought to have abandoned antiviral RNAi** even though they have retained the RNAi machinery and utilize it for miRNA generation and function in gene silencing.

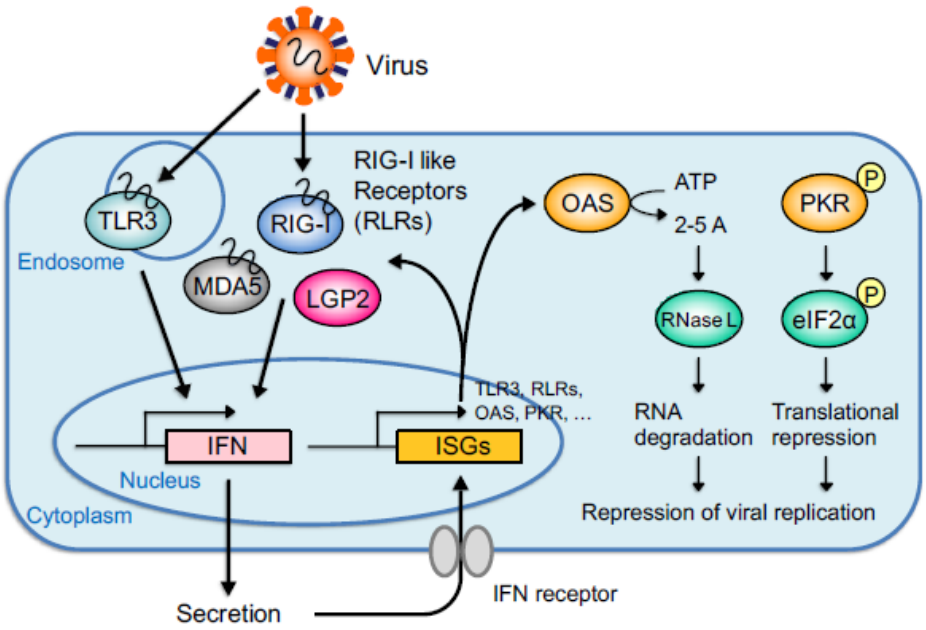
IFN RESPONSE DIRECTED BY EXOGENOUS VIRAL dsRNAs IN VERTEBRATES

Viral RNAs are detected by toll-like receptor 3 (TLR3) or retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Activated TLR3 or RLRs transfer signals to downstream molecules, inducing IFN production.

The secreted IFN is recognized by the IFN receptor on the cell surface, inducing the expression of IFN-stimulated genes (ISGs).

20-50-oligoadenylate synthetase (OAS) or protein kinase R (PKR) activates RNase L or phosphorylates eIF2 to carry out **RNA degradation or translational repression (antiviral state).**

Activation of the IFN response represses viral replication while limiting damage to the cell.

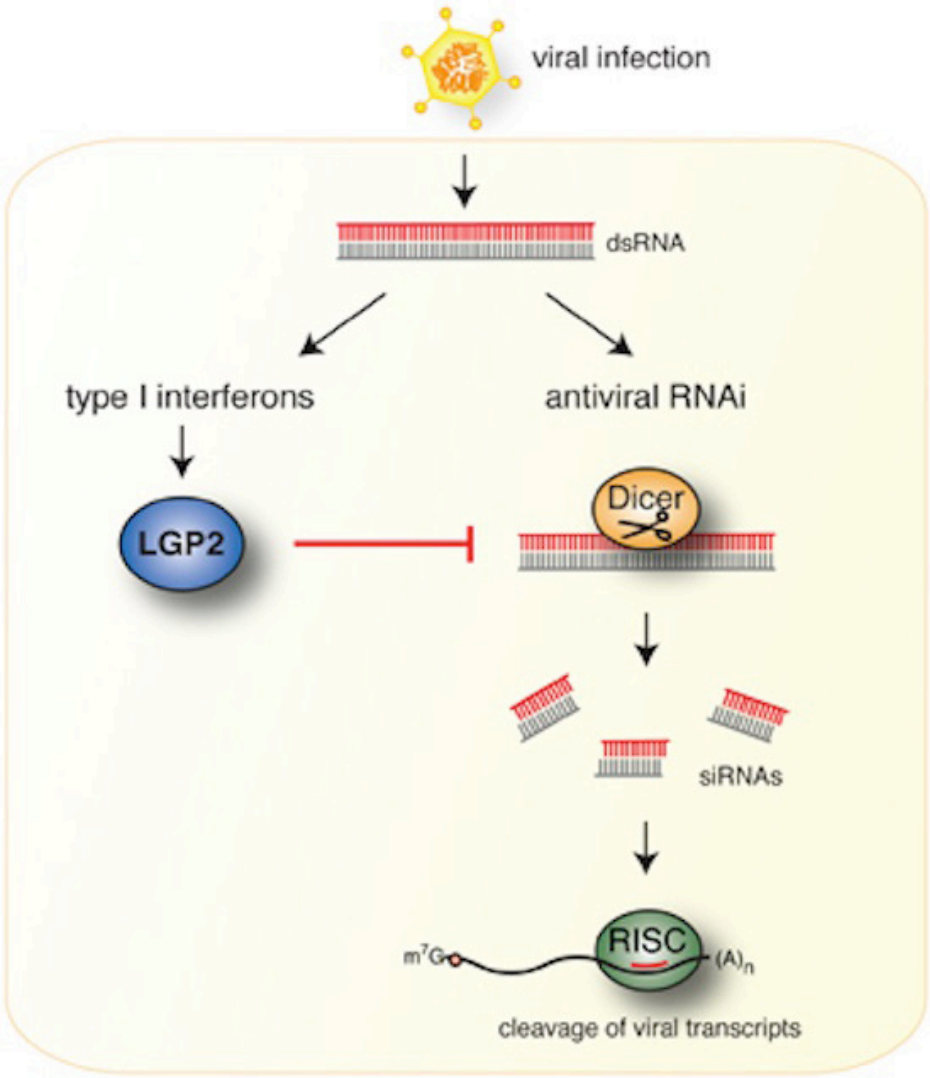


ANTAGONISM BETWEEN IFN AND RNAi IN VERTEBRATES

The IFN system actively inhibits dsRNAi in part through induction of LGP2, which binds Dicer and inhibits processing of long dsRNA into siRNAs.

LGP2 interact with the Dicer cofactor TRBP (HIV TAR RNA-binding protein) and inhibit the processing of a subset of TRBP-bound miRNAs.

Perhaps, **inhibition of Dicer and RISC is essential for effective stimulation of the IFN pathway, in part by preventing loss of dsRNA substrates for RLR activation**.

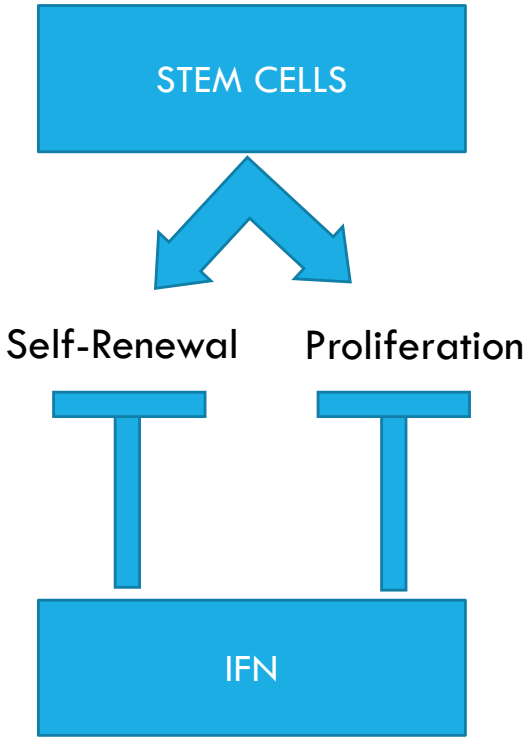


MAMMALS STEM CELLS: A NICHE FOR ANTIVIRAL RNAi?

RNAi may be important in cellular niches in which the induction of or the response to IFN is limited.

One of those niches might be stem cells.

Pluripotent stem cells are refractory to IFN might be due to the fact that self-renewal is incompatible with the anti-proliferative effects and pro-apoptotic effects of the cytokines induced by IFN.



Antiviral RNAi would constitute a mechanism to protect the integrity and function of tissue stem cells in the face of virus infection and thereby contribute to tissue maintenance, repair and regeneration.

DOES RNAi HAVE A RELEVANT ROLE IN ANTIVIRAL RESPONSE IN MAMMALS?

An isoform of Dicer protects mammalian stem cells against multiple RNA viruses

Enzo Z. Poirier[1]*, Michael D. Buck[1], Probir Chakravarty[2], Joana Carvalho[3]†, Bruno Frederico[1], Ana Cardoso[1], Lyn Healy[4], Rachel Ulferts[5], Rupert Beale[5,6], Caetano Reis e Sousa[1]*

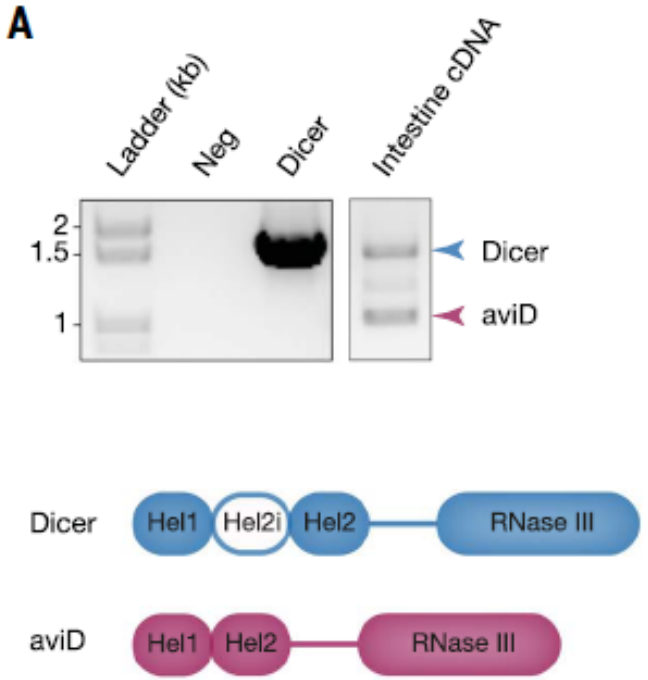
Science. 2021 Jul 9;373(6551):231-236. doi: 10.1126/science.abg2264.

A SINGLE PRODUCT FROM MAMMALS DICER GENE?

Mammals possess a **single DICER** gene with one canonical protein product, which cleaves pre-miRNA but processes exogenous dsRNA poorly.

By performing a PCR on total cDNA from mouse small intestine, it was identified an alternatively spliced in-frame transcript of Dicer missing exons 7 and 8.

In silico translation of this transcript resulted in a truncated Dicer protein in which the central Hel2i domain of the N-terminal helicase segment is absent



(A). Dicer PCR amplicons using vehicle (Neg), a plasmid coding for Dicer, or mouse small intestine cDNA templates

A truncated form of Dicer can be produced from the Dicer gene in mice: aviD (antiviral Dicer) .

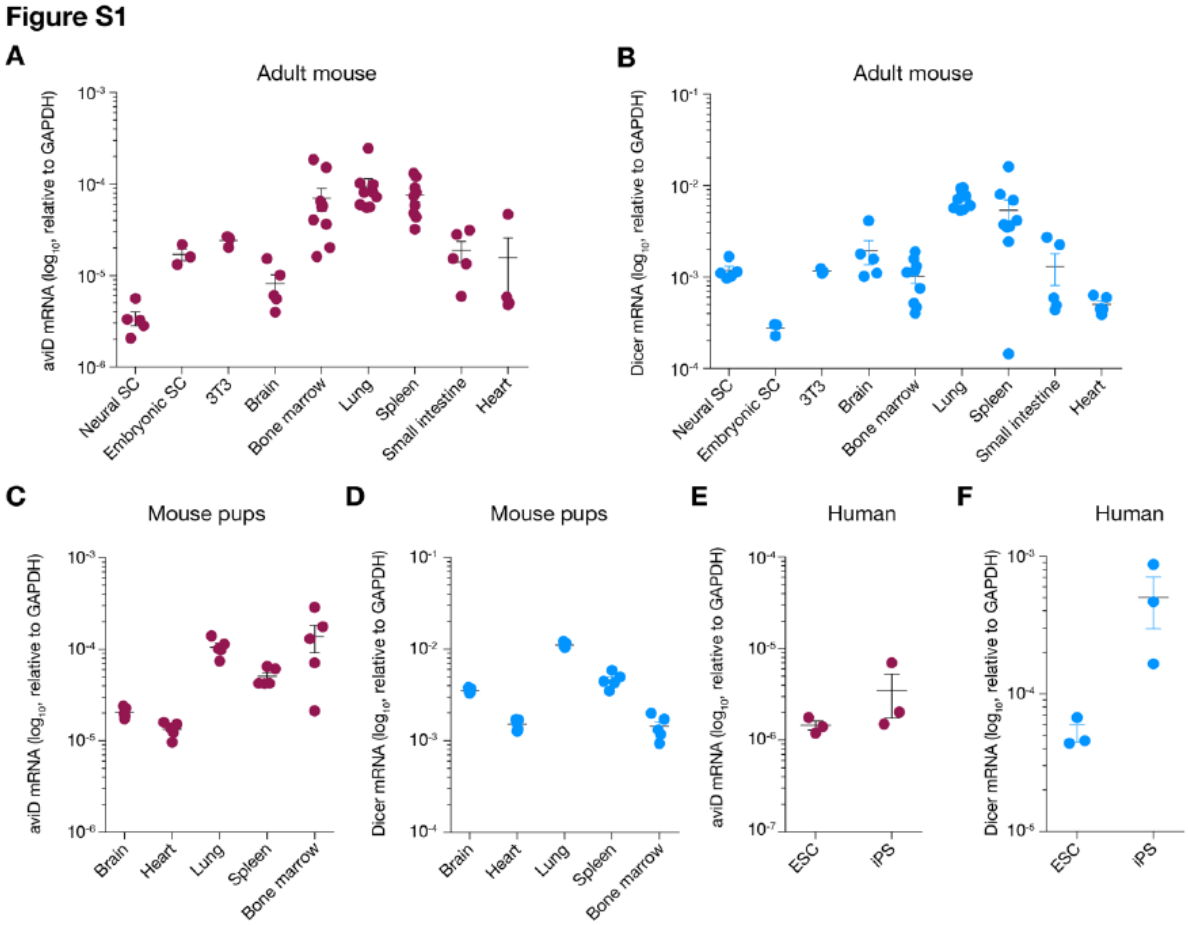
DETECTION OF aviD mRNA IN MICE AND HUMANS

(**A**) aviD or (**B**) Dicer mRNA was measured by RT-qPCR in the indicated mouse cell line or tissues from 6-10 weeks old C57BL6/J mice.

(**C**) aviD or (**D**) Dicer mRNA was measured by RT-qPCR in the indicated organs from 1 week-old mouse pups.

(**E**) aviD or (**F**) Dicer mRNA was measured by RT-qPCR in the indicated human cell line.

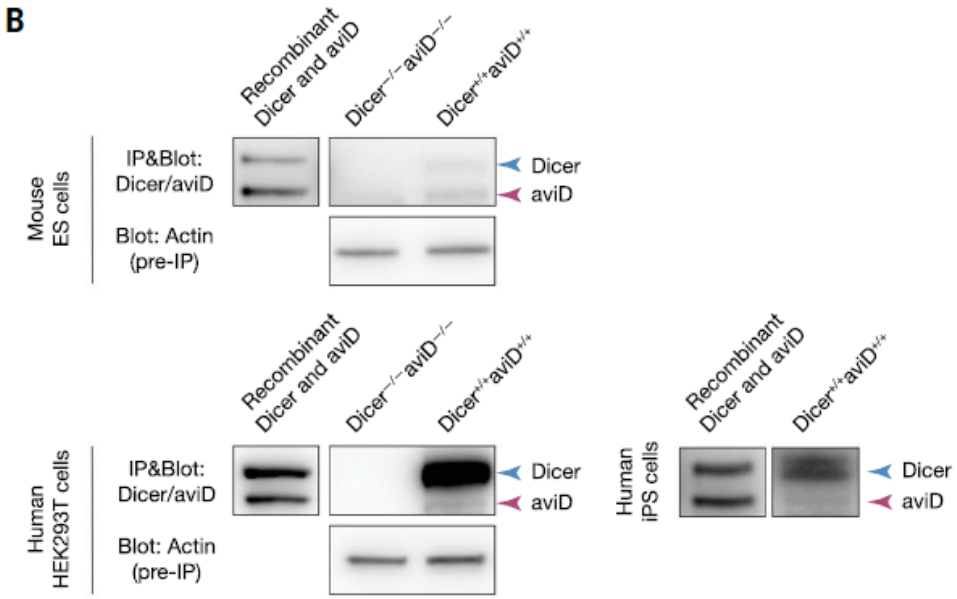
The **AVID** and **DICER** transcripts were found in the indicated cell types in mice and humans.



DETECTION OF aviD IN MOUSE ES, HUMAN IPSCs AND HEK293T CELLS

Dicer+/+aviD+/+, Dicer—/—aviD—/— mouse embryonic stem cells and "NoDice" HEK293T Dicer—/—aviD—/—were provided by other laboratories.
To complement Dicer—/—aviD—/— HEK293T cells or ES cells, sequences encoding Dicer or aviD were subcloned into a pSBbi-GH plasmid.

Immunoblots from wild-type Dicer+/+aviD+/+ or Dicer–/–aviD–/– mouse ES cells, HEK293T cells, or Dicer+/+aviD+/+ human iPSC lysates before (pre-IP) or after immunoprecipitation with a Dicer/aviD-specific antibody.
Recombinant Flag-tagged Dicer and aviD were included as controls.

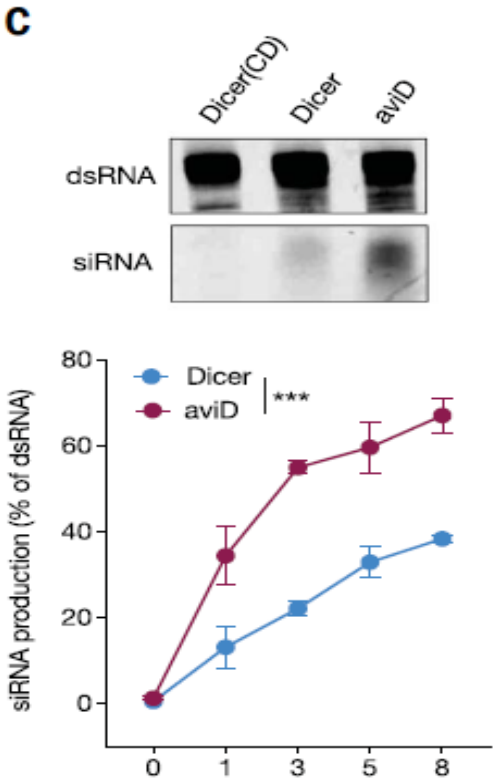


The low presence of aviD in mouse ES cells, human iPSCs and HEK-293T was demonstrated by an IP using an antibody that dually recognizes Dicer and aviD.

IN VITRO DICING ASSAY

Recombinant Flag-tagged Dicer, Dicer catalytically deficient [Dicer(CD), used as a negative control], and aviD were incubated with synthetic Cy5-labeled dsRNA.

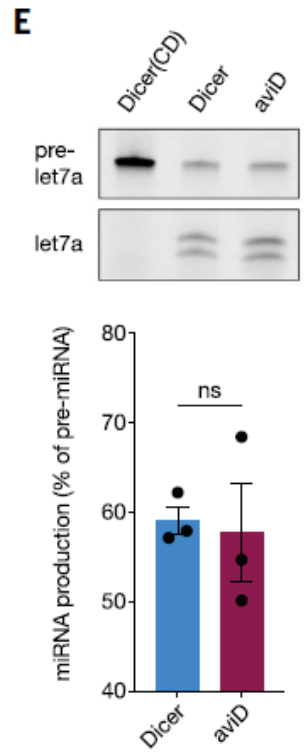
The reactions were resolved on a denaturing polyacrylamide gel and visualized by Cy5 in-gel fluorescence, and Dicer versus aviD cleavage was quantitated by densitometry.



Recombinant aviD produced about twice as much siRNA from synthetic dsRNA as did recombinant Dicer in an in vitro dicing assay.

DO aviD AND DICER PRODUCE miRNA WITH THE SAME EFFICIENCY?

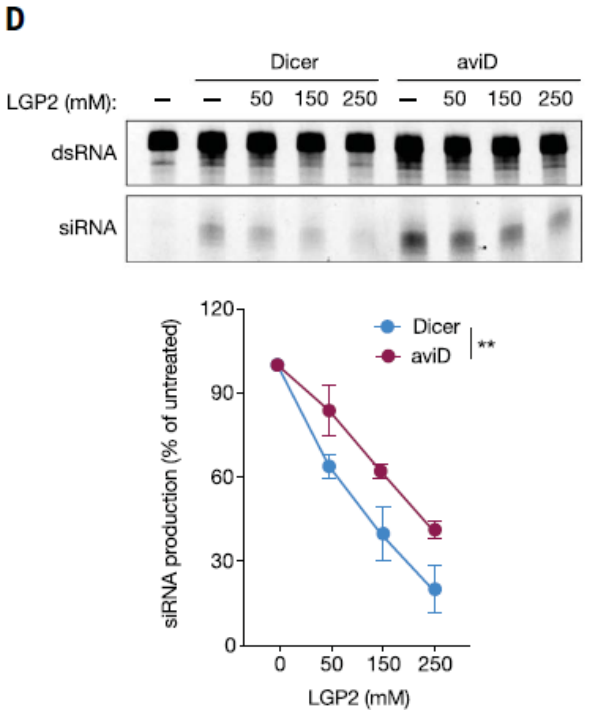
Immunopurified Flag-tagged Dicer, Dicer(CD), and aviD were incubated with let-7a pre-miRNA.
The reactions were resolved on a denaturing polyacrylamide gel and visualized by Cy5 in-gel fluorescence, and Dicer versus aviD cleavage was quantitated by densitometry.



In contrast to dsRNA cleavage, both Dicer and aviD generated equivalent amounts of let-7a miRNA from pre-miRNA

HOW DO aviD AND DICER RESPOND TO IFN-MEDIATED INHIBITION?

Increasing concentrations of recombinant LGP2 were added to the in vitro dicing reaction as in (C) and incubated for 3 hours at 37°C. After densitometric quantitation, the siRNA amount was normalized to the amount of siRNA produced in a reaction without LGP2.



aviD was more resistant to LGP2, an ISG product that inhibits dsRNA cleavage by Dicer and is partly responsible for IFN-mediated inhibition of antiviral RNAi in differentiated mammalian cells.

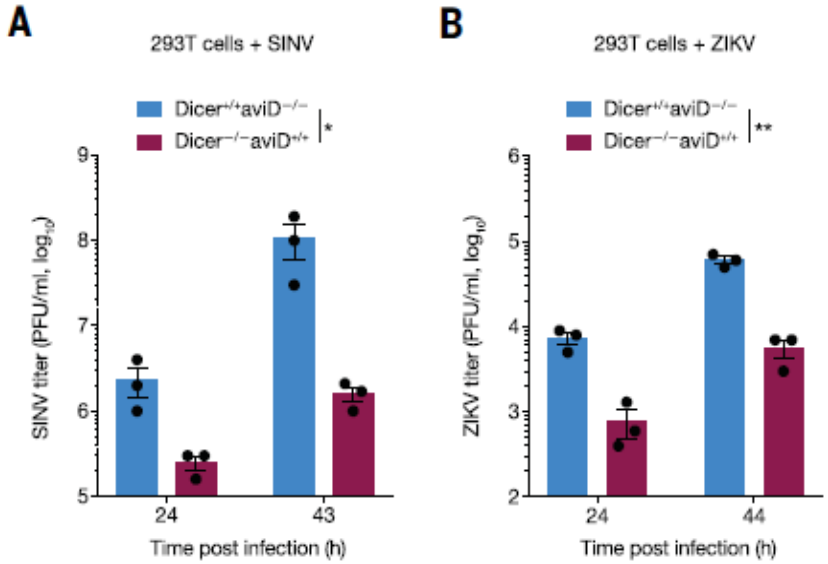
AVID PROCESSES dsRNA MORE EFFICIENTLY THAN CANONICAL DICER

Loss of the Hel2i domain **does not impair the ability of aviD to process miRNA precursors** but confers **enhanced capacity to dice dsRNA into siRNAs,** a hallmark of Dicers involved in antiviral RNAi.

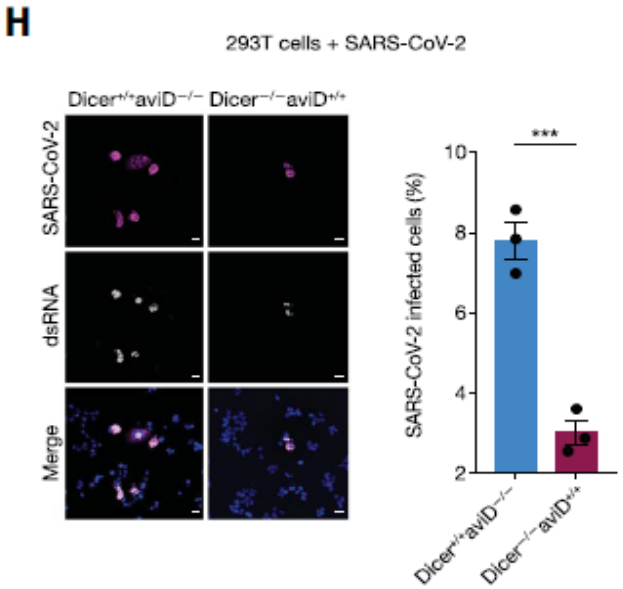
Together, these data suggest that **the helicase domain of Dicer limits its catalytic activity for long dsRNA.**

CAN aviD MEDIATE ANTIVIRAL RNAI IN STEM CELLS?

HEK293T "NO DICE" cells complemented with Dicer or aviD were infected with SINV (A) or ZIKV (B)



Immunofluorescence of Dicer–/–aviD+/+ Dicer+/+aviD–/– HEK293T cells expressing ACE2 infected with SARS-CoV-2 and stained for SARS-CoV-2 N protein and dsRNA.

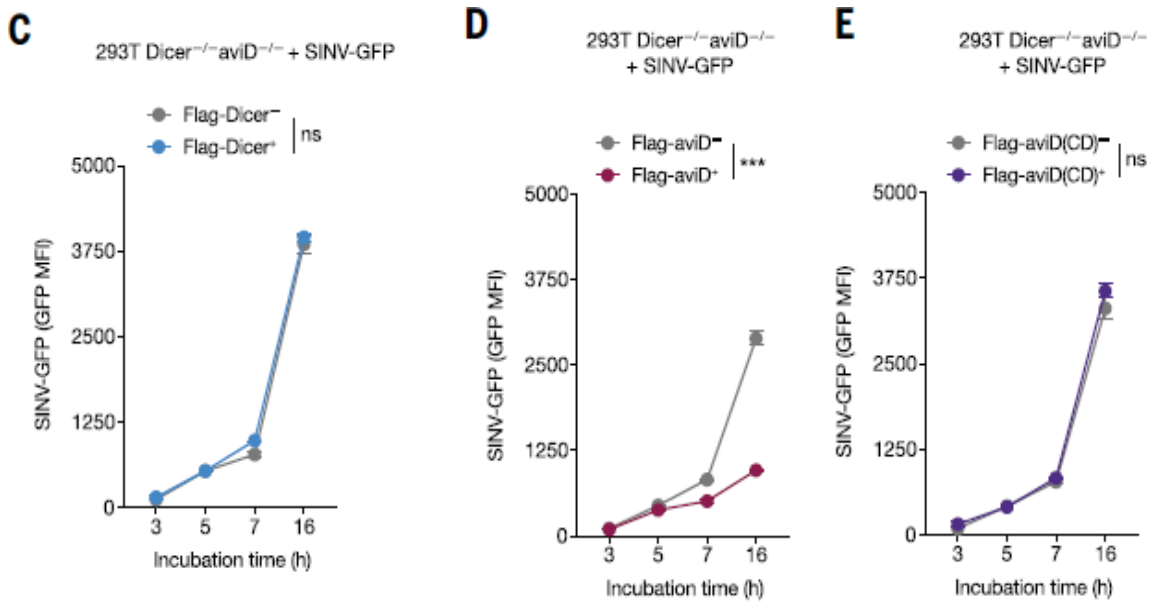


Cells expressing only aviD displayed lower production of SINV and ZIKV virus progeny than did cells that only expressed Dicer.

ARE THERE ANY DIFFERENCES BETWEEN aviD AND DICER IN ANTIVIRAL RESPONSE?

HEK293T Dicer–/–aviD–/– cells induced by doxycycline to express Flag-Dicer (C), aviD (D), or aviD(CD) (E) were infected with SINV-GFP.

Flow cytometry was used to monitor the expression of Dicer/aviD (via anti-Flag staining) and SINV replication (via GFP fluorescence).

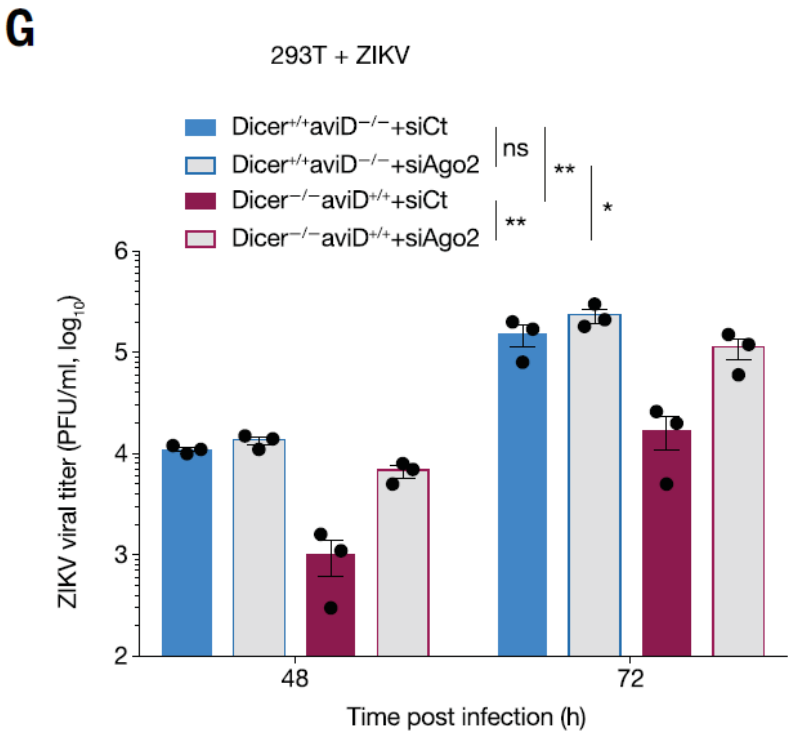


aviD but not Dicer induction impaired SINV-GFP viral replication over time, that is dependent on its catalytic domain, consistent with a role in RNAi.

IS RNAI PATHWAY INVOLVED IN DEPLETION OF VIRAL PARTICLES?

Mammals encode four Ago proteins, all of which can mediate miRNA-driven gene silencing. However, only Ago2 possesses endonuclease activity to mediate target "slicing" in antiviral RNAi.

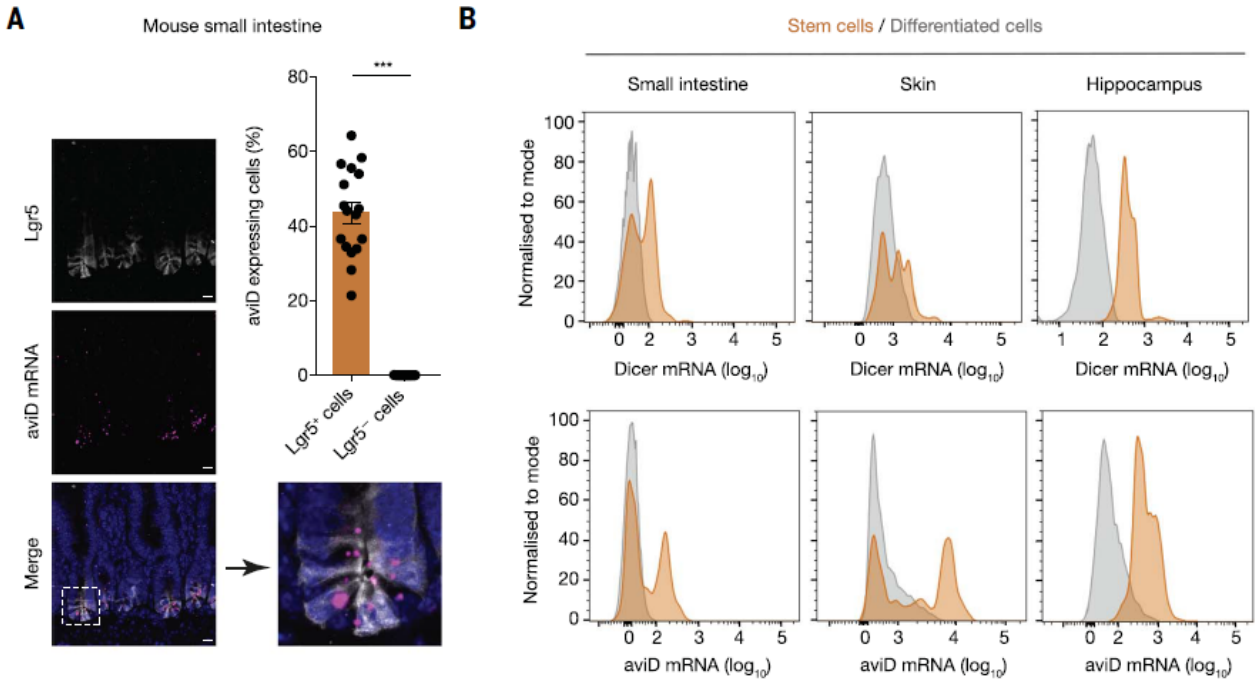
Dicer+/+aviD–/–or Dicer–/–aviD+/+ HEK293T cells were transfected with siRNA targeting Ago2 (siAgo2) or with control siRNA (siCt) and infected with ZIKV at MOI of 0.1.



Silencing Ago2 in Dicer–/–aviD+/+ cells rescued ZIKV particle production to levels like those in Dicer+/+aviD–/– cells treated with control or Ago2 siRNA.

IN WHICH CELLS IS AVID EXPRESSED?

aviD or Dicer mRNA was measured by cytometry in stem (Lgr5+) or differentiated (Lgr5–) cells from small intestine or skin isolated from Lgr5-GFP reporter mice or in stem or differentiated cells from hippocampus distinguished by the presence or absence of Sox2 mRNA, respectively.

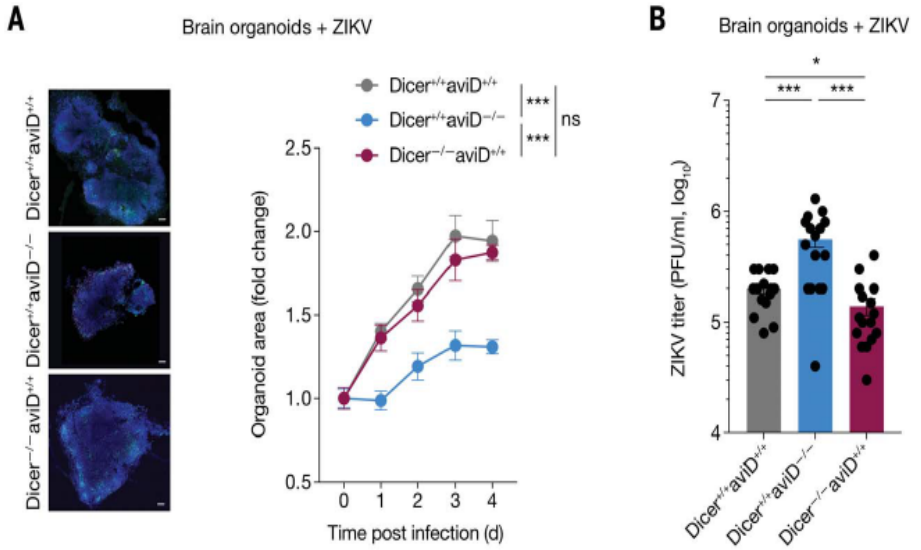


So, unlike Dicer mRNA, aviD mRNA was lost when the cells were made to differentiate.

DOES aviD COUNTERACT ZIKV-INFECTION IN BRAIN ORGANOIDS?

(A).Individual Dicer+/+aviD+/+, Dicer+/+aviD–/–, or Dicer–/– aviD+/+ brain organoids were infected with ZIKV, and organoid area was monitored. Immunofluorescent stanining and confocal microscopy were used to identify stem cells by Sox2 expression (green) and infected cells by ZIKV glycoprotein expression (magenta).

(B).Production of viral particles from ZIKV-infected organoids was determined by transferring individual organoids into fresh medium at day 3 after infection and collecting the supernatant 24 hours thereafter to determine viral content.

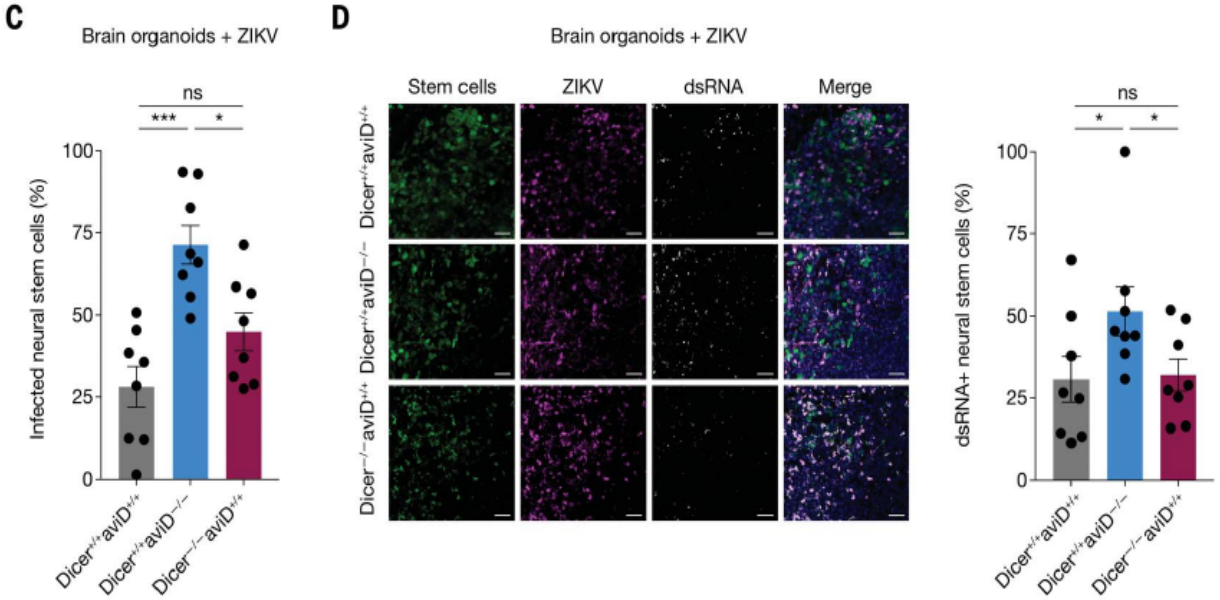


Upon infection with ZIKV, Dicer+/+aviD–/– organoids **grew more slowly** than Dicer+/+aviD+/+ and Dicer–/–aviD+/+ organoids and **produced more infectious** viral particles.

DOES aviD COUNTERACT ZIKV-INFECTION IN BRAIN ORGANOIDS?

(C).Percentage of ZIKV-infected stem cells was measured 4 days after infection by immunofluorescence on organoid sections.

(D). dsRNA in infected stem cells was visualized by immunofluorescence on organoid sections after 4 days of infection

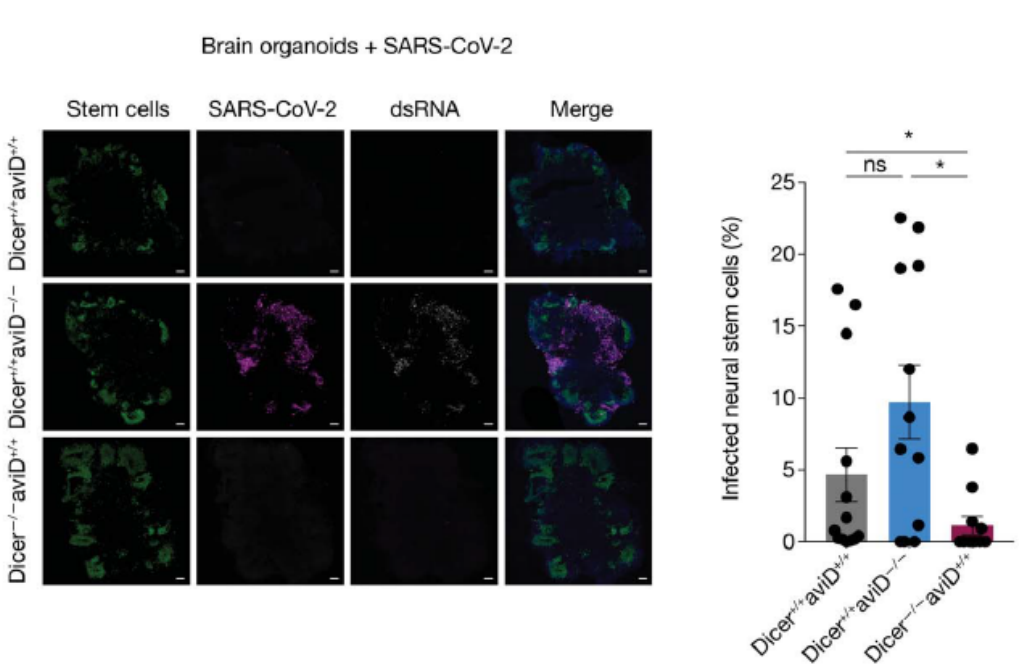


Sox2+ stem cells in Dicer+/+aviD–/– organoids displayed **increased infection** with ZIKV and accumulate more viral dsRNA.

DOES ANTIVIRAL RNAi ALSO EFFECTS ON SARS-CoV2 INFECTED CELLS?

Dicer+/+aviD+/+, Dicer+/+aviD–/–, or Dicer–/–aviD+/+ brain organoids expressing ACE2 were infected with SARS-CoV-2. Percentage of infected stem cells was determined by immunofluorescence on sections stained for the stem cell marker Sox2 (green) and for the SARS-CoV-2 N protein (magenta).

The absence of aviD in Dicer+/+aviD–/– organoids correlated with an increase in the percentage of virally infected stem cells as well as loss of viral siRNA production.



aviD can protect adult stem cells from SARSCoV-2 virus infection by orchestrating an **antiviral RNAi response**.

CONCLUSIONS

- DICER gene can generate an alternative transcript that encodes aviD, a truncated Dicer.
- Mammals like plants or insects, can produce at least two Dicer proteins, one of which is superior at initiating antiviral RNAi.
- aviD protects mouse and human stem cells against RNA virus infection and compensates in part for stem cell hypo-responsiveness to innate IFNs.

Antiviral innate immunity in mammals is therefore a composite of pathways that are tailored to the differentiation status of the cell and that display complementarity as well as redundancy !!!

FUTURE PERSPECTIVES

- The existence of a new mammalian antiviral immunity mechanism provides opportunities that may lead to a better understanding of mammalian immunology.
- Further studies are clearly needed to disentangle the complex web that regulates dsRNAi in mammals and to understand its ability to act as a cell-intrinsic mechanism of antiviral defence.
- An aviD-specific knockout mouse will help to delineate the nonredundant contributions of these distinct strategies.