Small RNAs and gene expression regulation

Roles for small RNAs in gene expression regulation

Three classes of small RNA can regulate genes by targeting transcripts in the cytoplasm. These are:

- **microRNAs (miRNAs)**, which are hairpin-derived RNAs with imperfect complementarity to targets and that cause translational repression;

- **small interfering RNAs (siRNAs)**, which have perfect complementarity to targets and cause transcript degradation;

- **PIWI-intereacting RNAs (piRNAs)**, which target transposon transcripts in animal germ lines.

RNA interference (RNAi)

- RNAi uses short antisense RNA to to degrade cytoplasmic mRNA by **post-transcriptional gene silencing (PTGS)**.

- RNAi pathways exist also in the nucleus where they can repress target genes at the transcriptional level by guiding epigenetic modification of chromatin: **transcriptional gene silencing (TGS).**

- In the nucleus RNAi pathways can also activate trancritpion by a mechanism known as **RNA activation (RNAa)**

- PTGS and TGS protects against viral infection, prevents transposon mobilization and regulates endogenous genes.

The Nobel Prize in Medicine 2006 to Andrew Fire and Craig Mello

"RNA interference – gene silencing by double-stranded RNA"

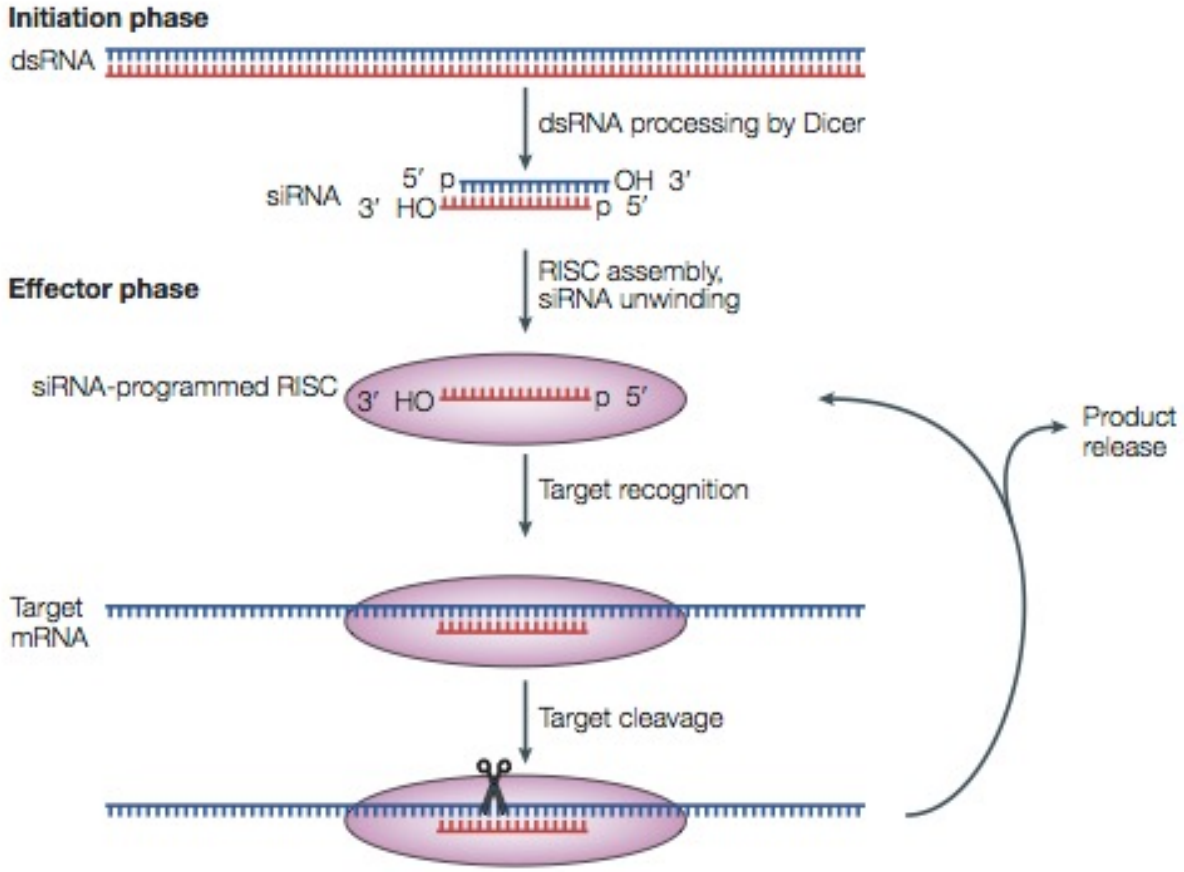
Nature. 1998 Feb 19;391(6669):806-11.

Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.

Fire A[1], Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC.

What is RNA interference (RNAi)?

RNA interference (RNAi) uses dsRNA to guide the destruction of specific mRNAs



The discovery of RNAi

Cosuppression

The overexpression of the **CHS (Chalcone synthase)** gene in petunia leads to lack of flower pigmentation instead of increasing it.

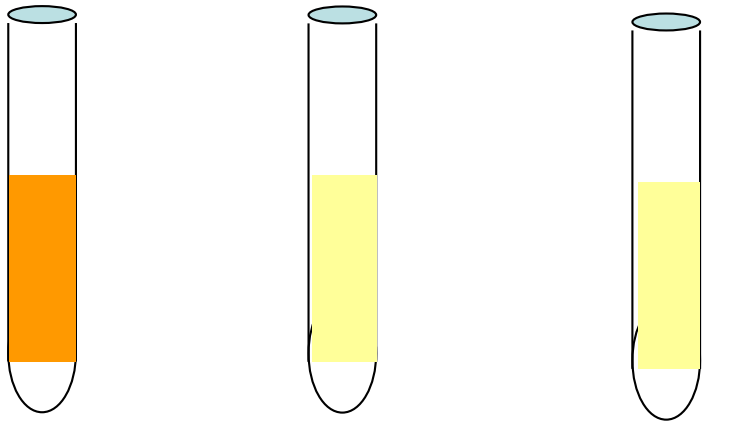
Napoli et al., (1990)
Jorgensen et al., (1996)

The transgene causes the suppression of both the exogenous and endogenous genes

The discovery of RNAi

Quelling

In *Neurospora crassa* the introduction of a transgene causes the silencing of the homologous endogenous gene **albino-1** (al-1) coding for a protein of the carotenoid biosynthetic pathway.



wt *al-1* wt + transgene al-1

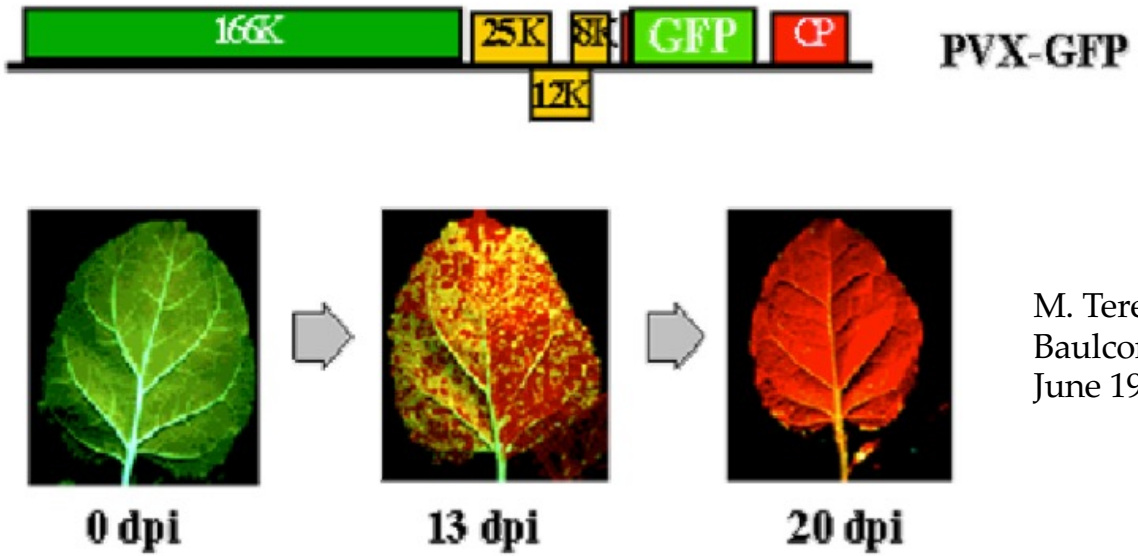
Romano e Macino (1992)
Cogoni et al., (1996)

The transgene causes the *suppression* of both the exogenous and endogenous genes

The discovery of RNAi

Virus-induced gene silencing (VIGS)

Viral infection can silence both viral genes and endogenous genes sharing complementarity



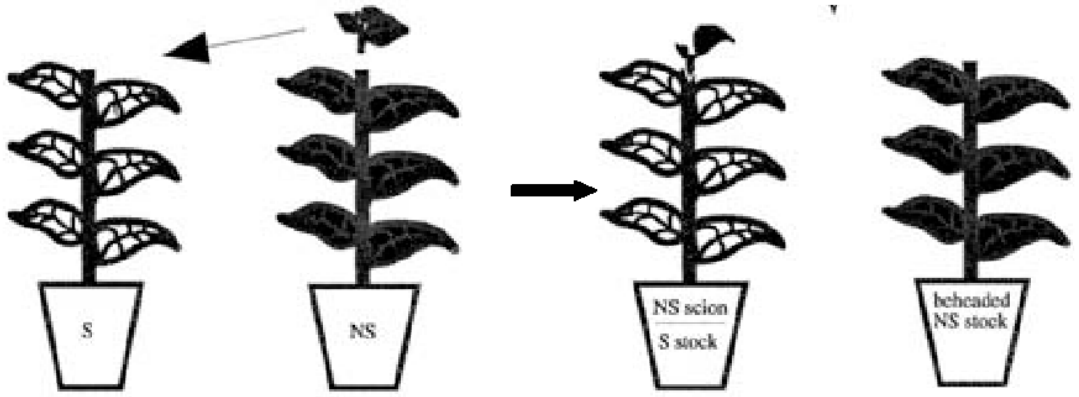
M. Teresa Ruiz, Olivier Voinnet, and David C. Baulcombe.The Plant Cell, Vol. 10, 937–946, June 1998

GFP expressing transgenic plant infected with GFP expressing potato virus X (PVX)

A mobile signal transmits gene silencing

In *Neurospora,* gene silencing can be transferred from nucleus to nucleus into heterocarotic strains (Cogoni et al., 1996)

In **plants**, PTGS is induced by a side shoot that is grafted onto a silent plant (Palauqui et al., 1997)



Nature 391, 806 - 811 (19 February 1998)

Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

RNA interference (RNAi)

2006 RNA interference

- Discovered by accident
- An extremely useful tool for researchers

Shared the Nobel Prize in Physiology or Medicine for their work on RNA interference in the nematode worm *C. elegans*, which they published in 1998

Accidental Discovery of RNAi

... **Goal:** Silence mRNAs with an antisense RNA

... The unc-22 gene encodes a myofilament protein.

... The reduction of unc-22 activity is known to cause severe contractions.

Accidental Discovery of RNAi

... Goal: Silence mRNAs with an antisense RNA

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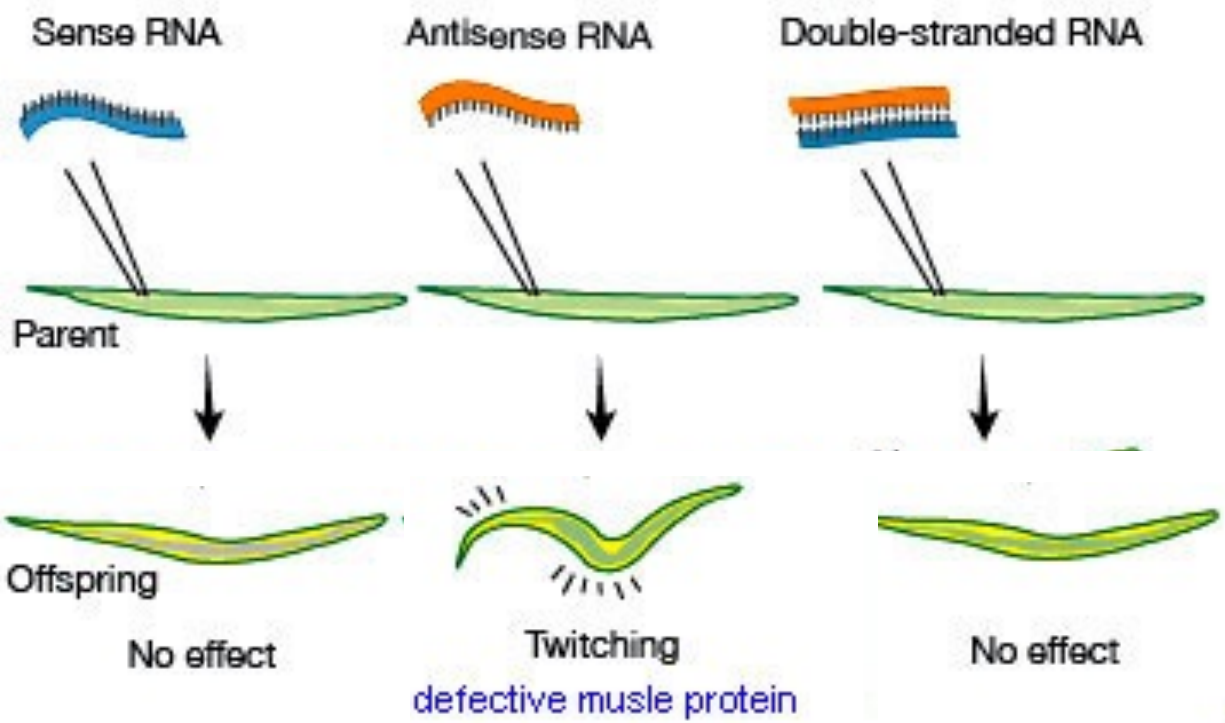

EXPECTED: The phenotypic effect is observed after the injection of ssRNA antisense to the unc-22 mRNA into the gonads of C. elegans..

Accidental Discovery of RNAi

... **Goal:** Silence mRNAs with an antisense RNA

... The unc-22 gene encodes a myofilament protein.

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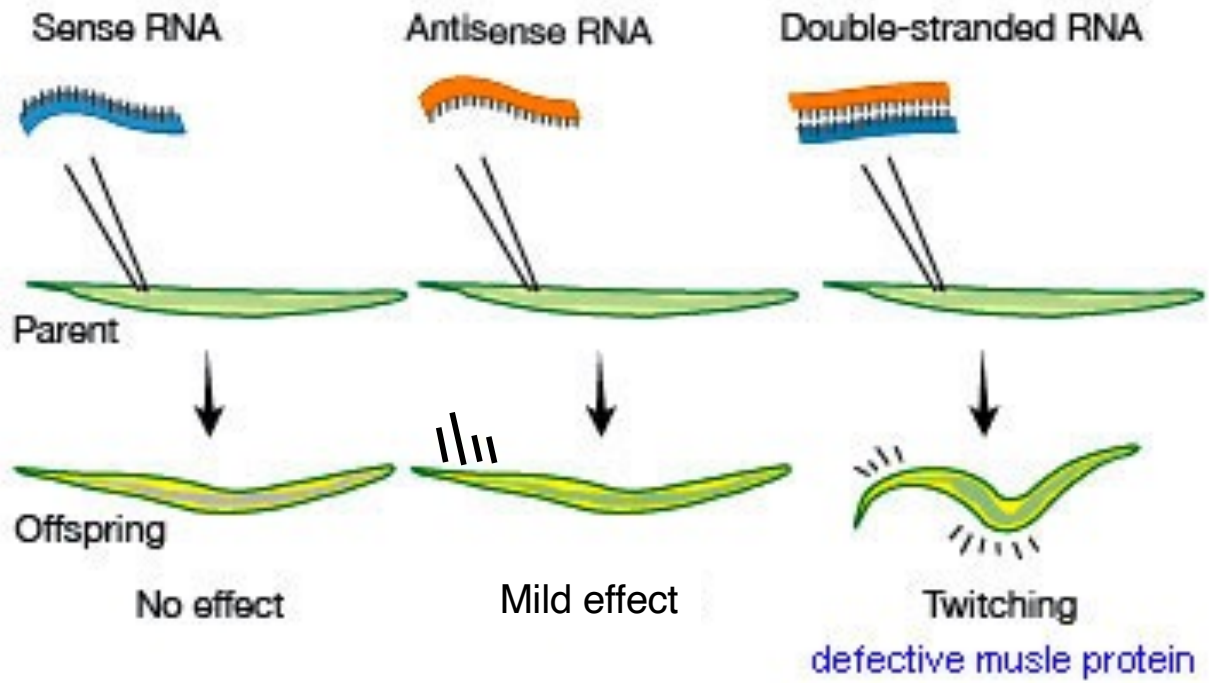


OBSERVED: The phenotypic effect is observed after the injection of dsRNA (unc-22) into the gonads of C. elegans. A very mild effect is observed after the injection of ssRNA antisense.

In 1998, A. Fire and C. Mello (Nobel Prize in Medicine 2006) working on C. elegans discovered that not only by inserting a transgene, but also by inserting the gene in antisense or directly a double-stranded RNA (dsRNA) activates a gene silencing of the corresponding gene.

dsRNA induces PTGS

- Guo and Kemphues (1995), use antisense RNA to study the function of the par-1 gene in *Caenorhabditis elegans*. As expected, the injection of antisense abolishes par-1 expression.

- Fire and Mello (1998), inject dsRNA into *Caenorhabditis elegans* and get a much more effective mute than with antisense alone.

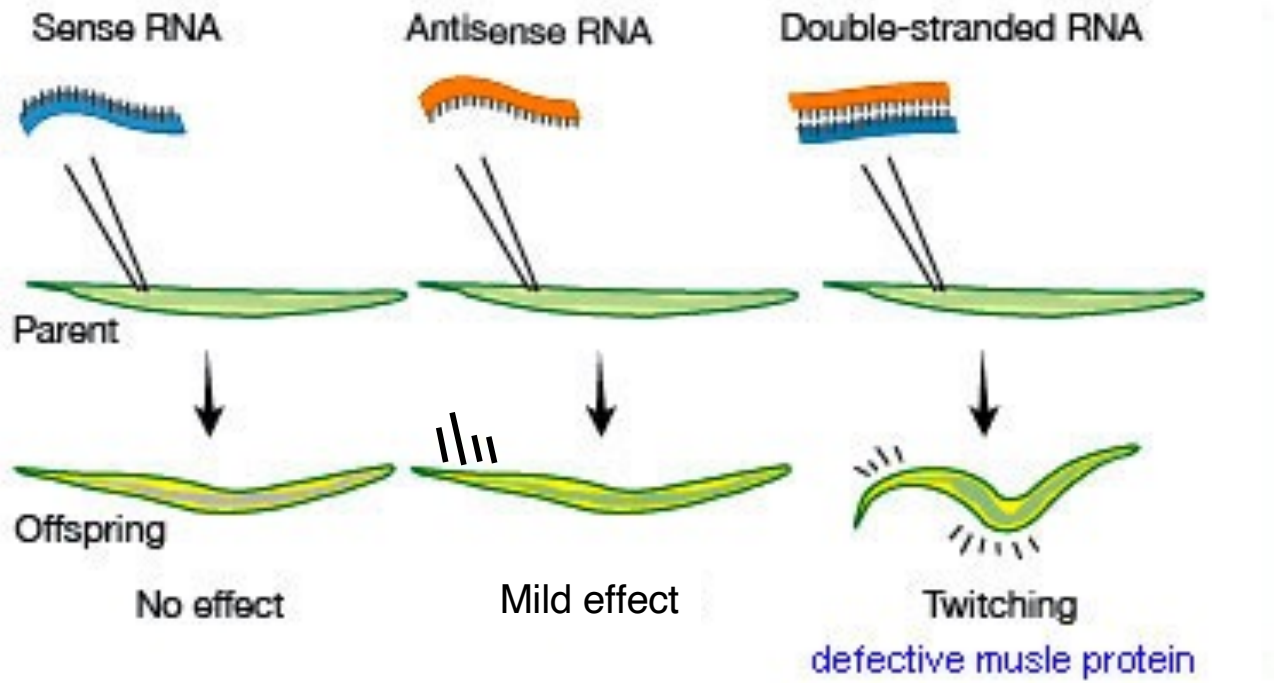
The observation came from what was intended by the authors to be a negative control!

Accidental Discovery of RNAi

... **Goal:** Silence mRNAs with an antisense RNA

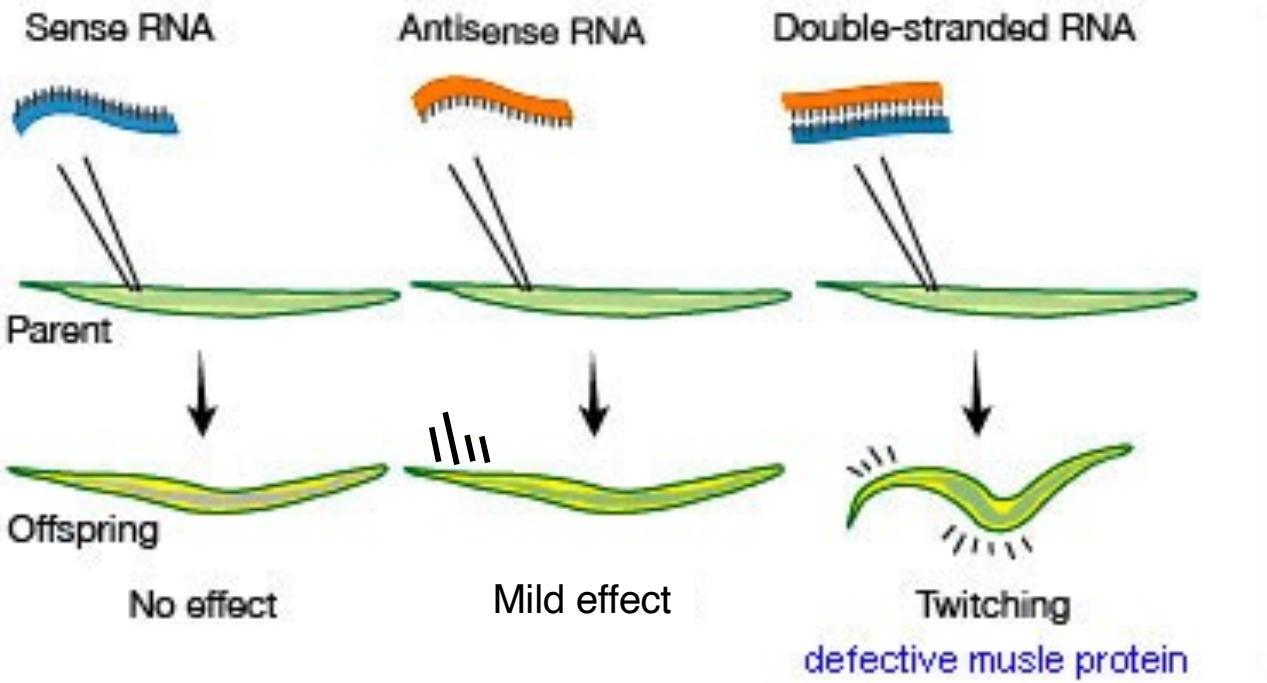
... The unc-22 gene encodes a myofilament protein.

... The reduction of unc-22 activity is known to cause severe contractions.



Scoperta accidentale dell'RNAi

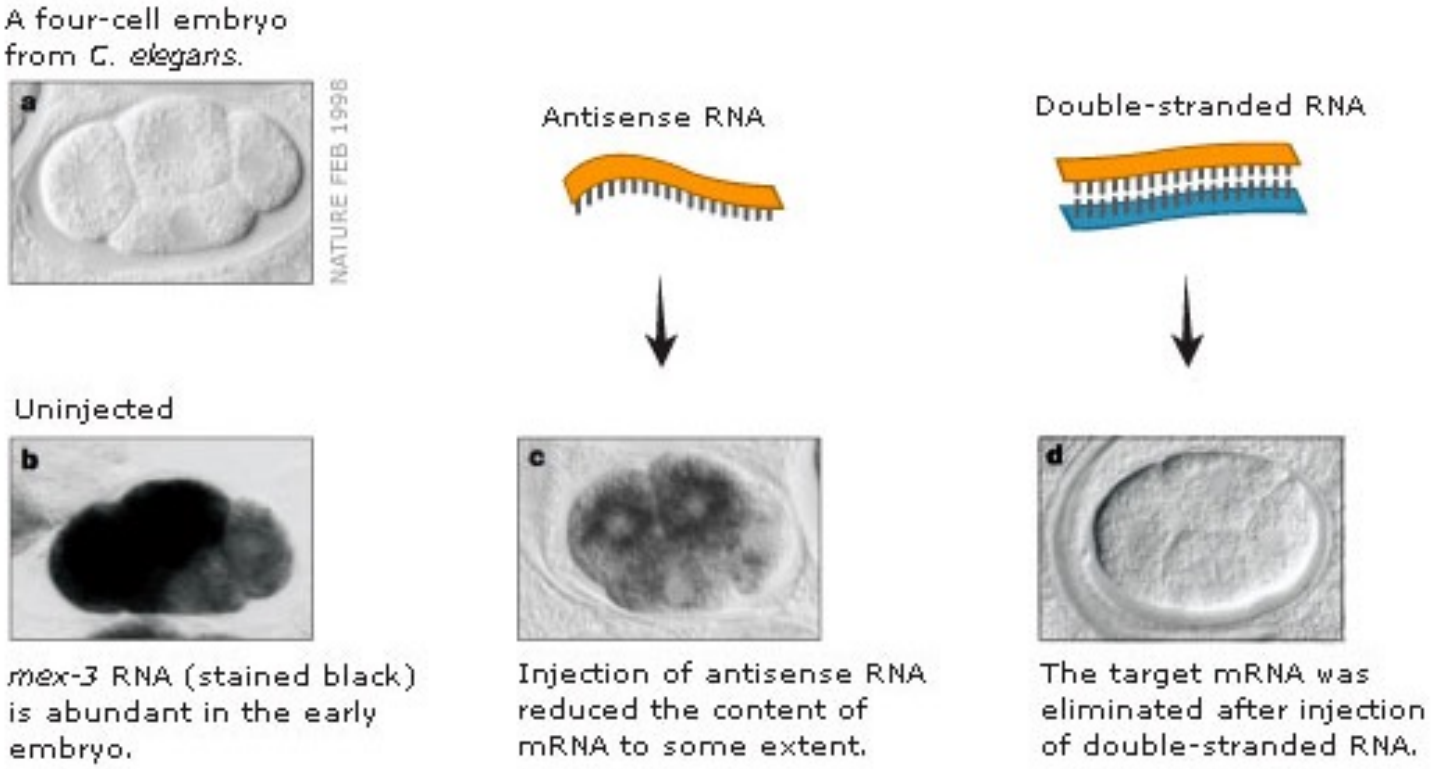
- Goal: silenziare l'mRNAs con un RNA antisenso
- Il gene *unc-22* codifica per una proteina del miofilamento.
- La riduzione dell'attività di *unc-22* è nota causare severe contrazioni.



The term RNA interference was coined on this occasion. C. elegans was a useful experimental system because the evolutionary origin of all the cells of this organism is known and it is possible to inject RNA into embryos at early stages and observe the changes compared to the model during development.

Experiments of Fire and Mello in 1998

•Injection of dsRNA (sense and anti-sense strands) in C. elegans
•Silencing more efficient than the injection of the anti-sense alone
•The dsRNA must include the exons; introns and the promoter do not work in silencing
•The silencing is due to the degradation of the target mRNA
•Only a few dsRNA molecules per cell were sufficient to silence the expression of the homologous gene. The phenomenon is transferred from one cell to another
•RNAi is applicable to many different transcripts



Important characteristics of RNA-mediated silencing

PTGS, quelling and RNAi have common critical components:

1.They are induced by dsRNA

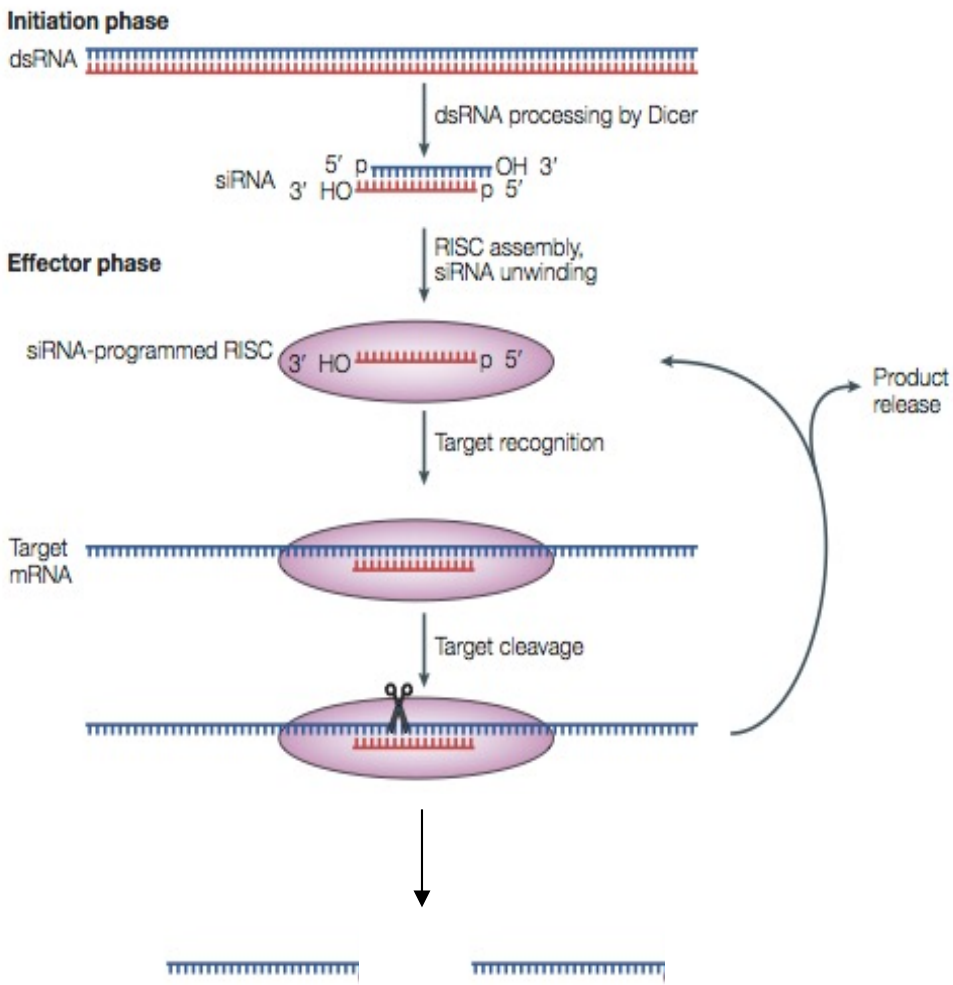
2.The target is degraded in a sequence-specific manner

3.The degradation machinery requires a set of proteins that are similar in structure and function in most organisms.

The general pathway of RNAi

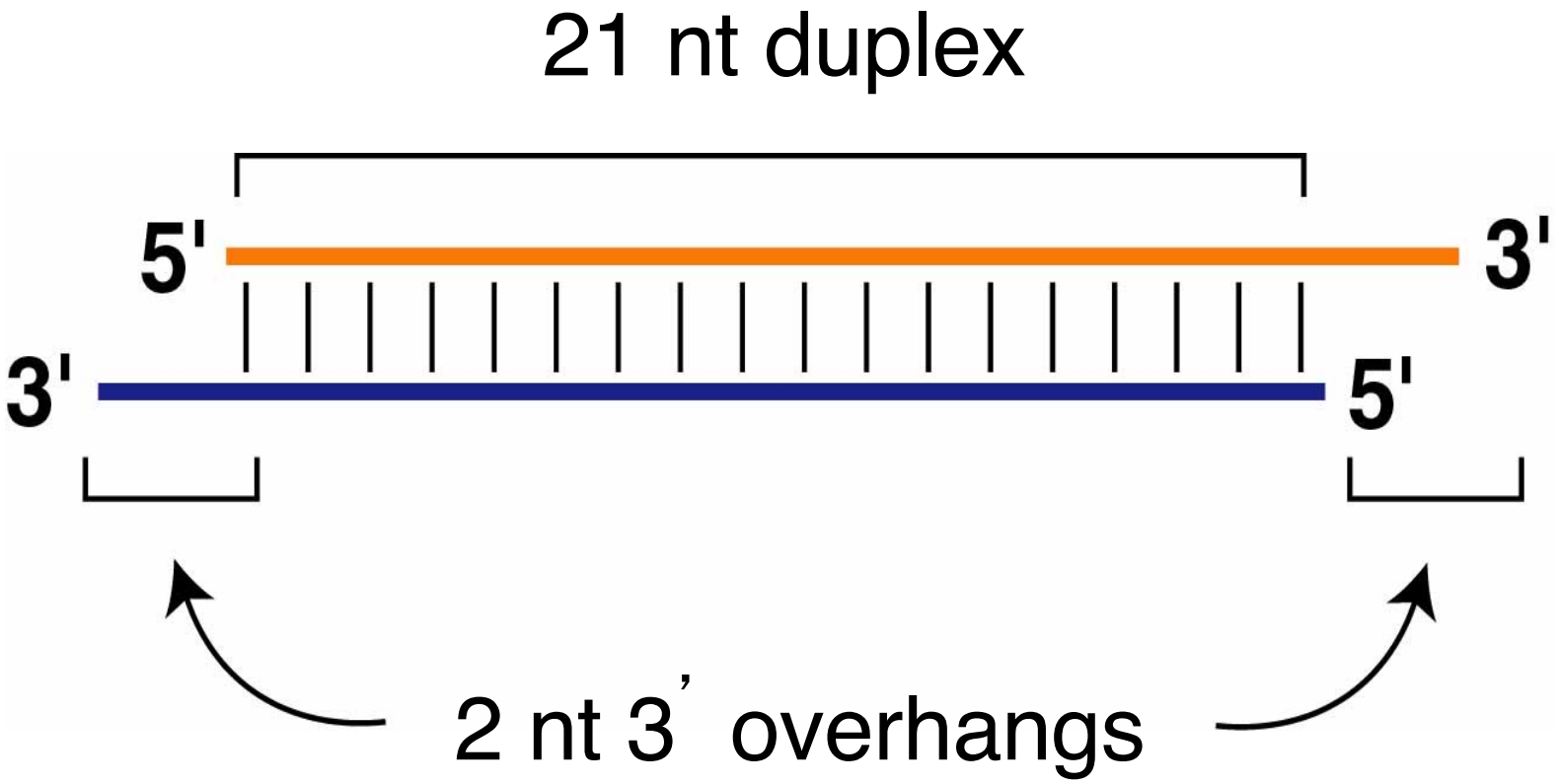
Initiation phase: the ribonuclease-III enzyme Dicer cleaves (ds)RNA molecules into 21–23-nt short interfering (si)RNA duplexes. siRNAs bear 5′-phosphate groups and 2-nt 3′-overhangs, both of which are important for subsequent siRNA-induced silencing complex (siRISC) assembly.

Effector phase: the siRNA becomes unwound and assembles into RISC. The activated effector complex recognizes the target by siRNA–mRNA base pairing, and then cleaves the mRNA strand with its endoribonuclease activity.

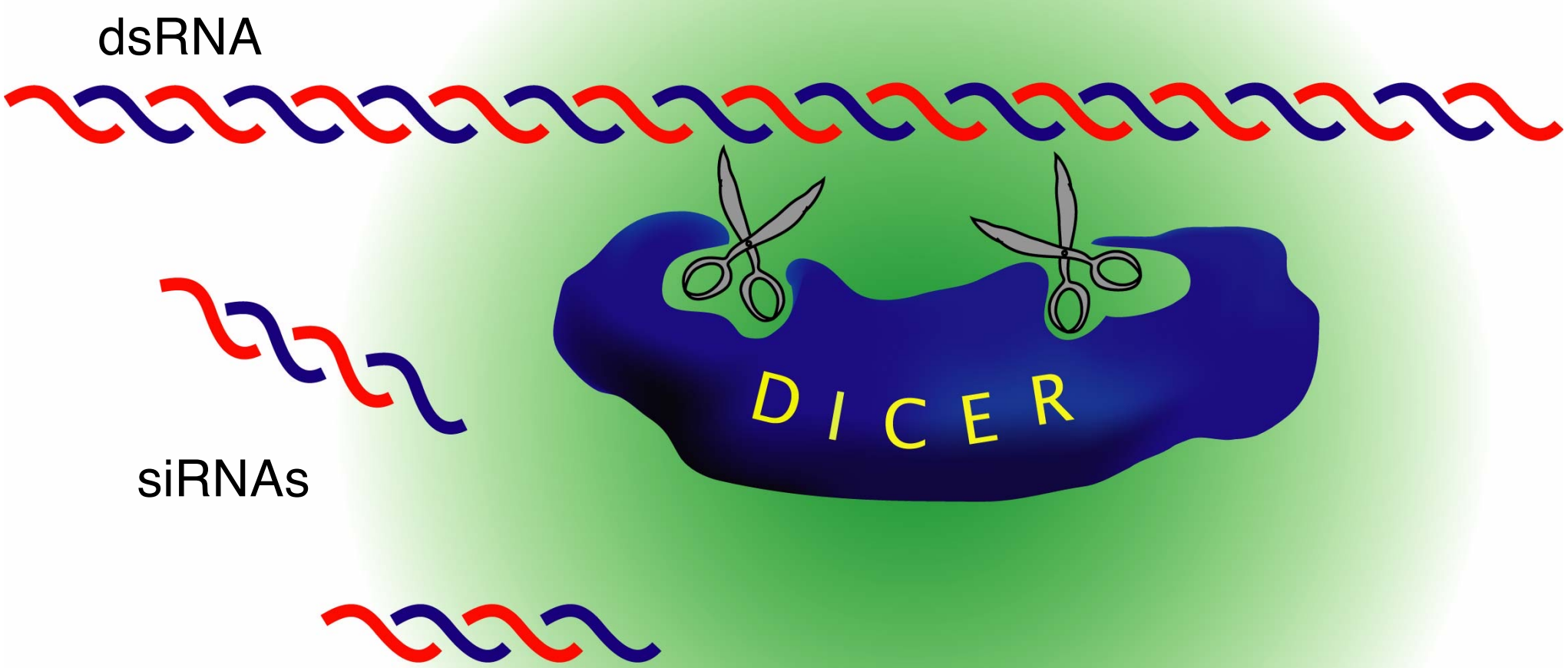


RNA degradation

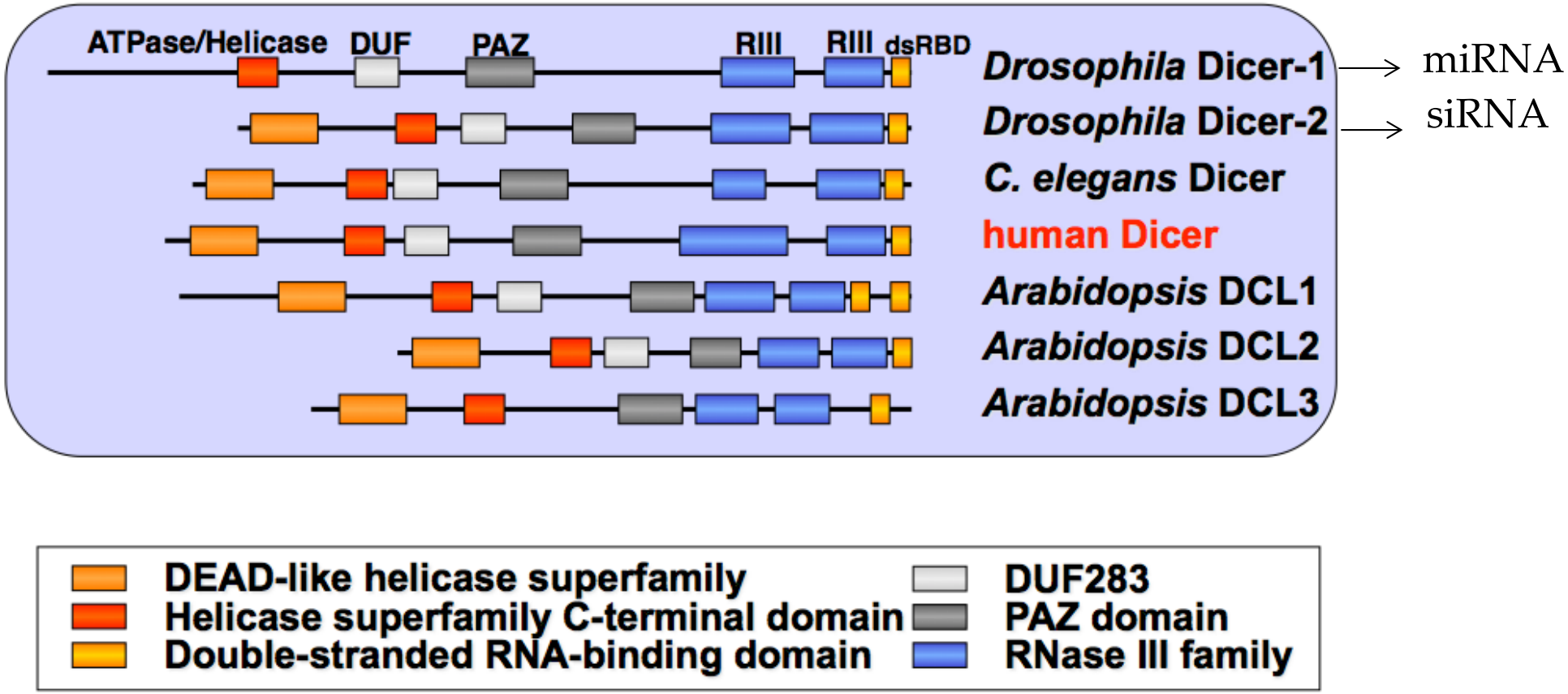
siRNAs have a well-defined structure



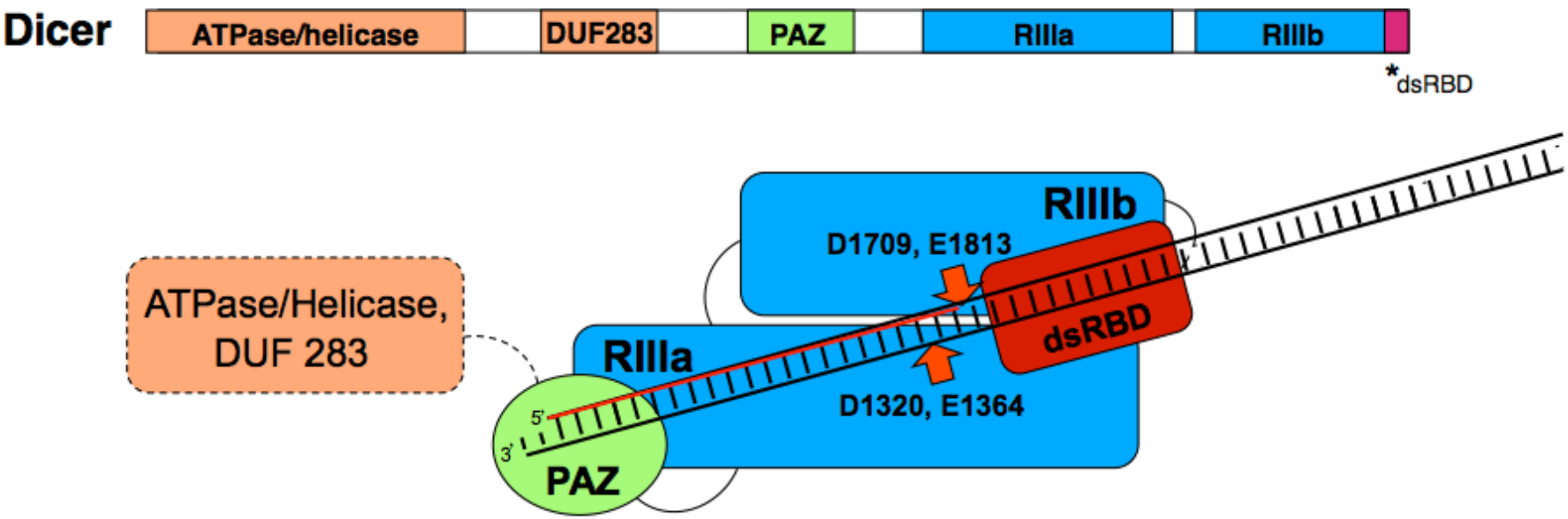
Dicer has two RNase III domains



Dicer: multi-domain nucleases involved in RNAi and microRNA biogenesis



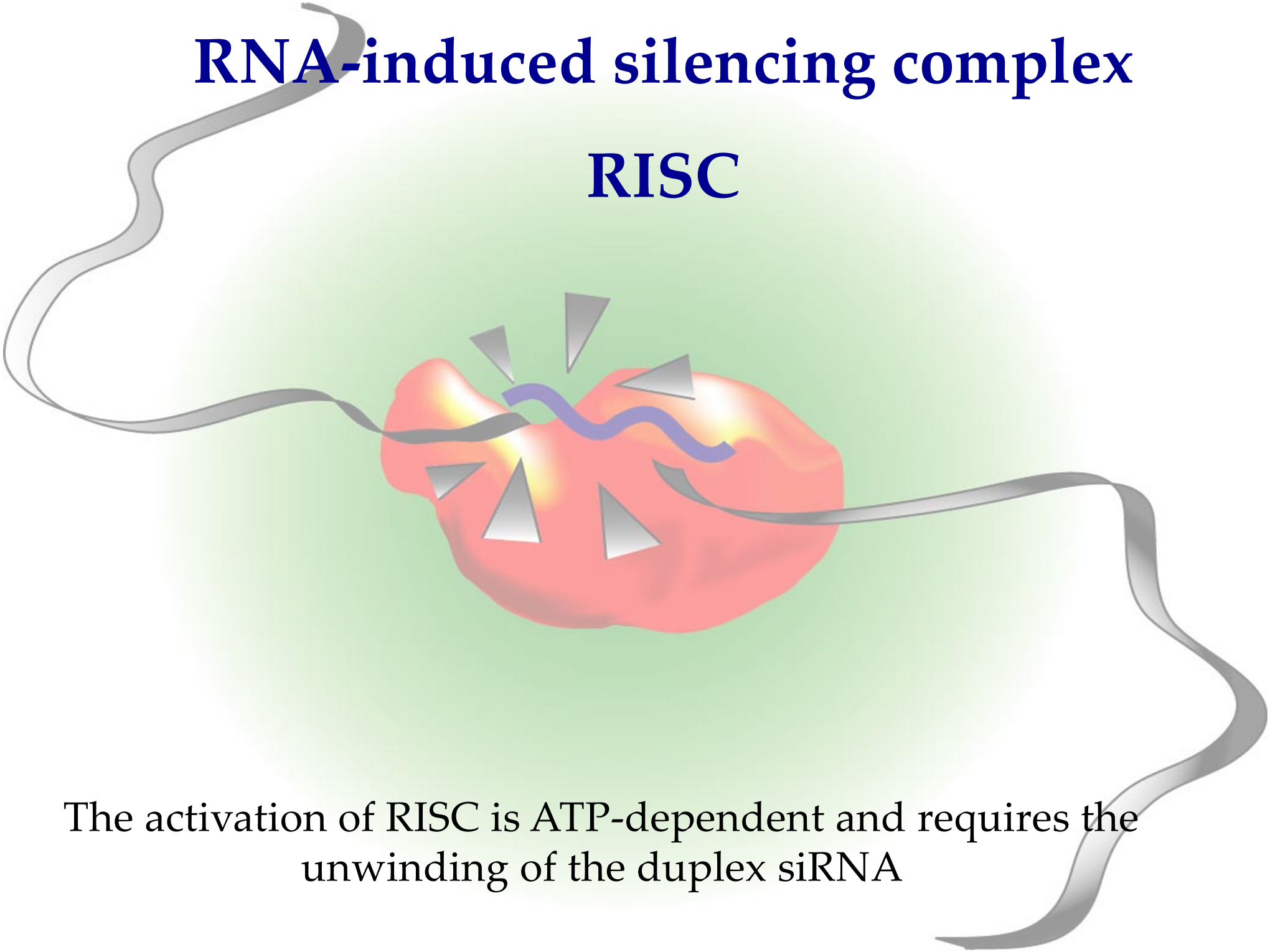
dsRNA processing by Dicer



- Dicer functions as a *monomer* (i.e., intra-molecular dimer).
- PAZ domain recognizes the 3'- overhang end.
- Dicer has a *single* processing center, with two independent catalytic sites.
- Each RNase III domain cuts one RNA strand in a *polar* way

RNA-induced silencing complex

RISC



The activation of RISC is ATP-dependent and requires the unwinding of the duplex siRNA

Genes involved in PTGS

- **RDE-1** (C. elegans), **QDE-**2 (N. crassa), **AGO-1** (A. thaliana) -> **PPD proteins**
  -They share two conserved domains: PAZ and PIWI
  - Mutants and have developmental defects and RNAi is abolished
  - Human homologue **eIF2C2** (**AGO2**)

- **RDE-4** (*C. elegans*) -> **dsRNA binding domain**

- **MUT-7** (*C. elegans*) -> **RNaseD domain**
-
- **QDE-3** (*N. crassa*) , MUT-6 (*C.elegans*) -> **RNA helicase**

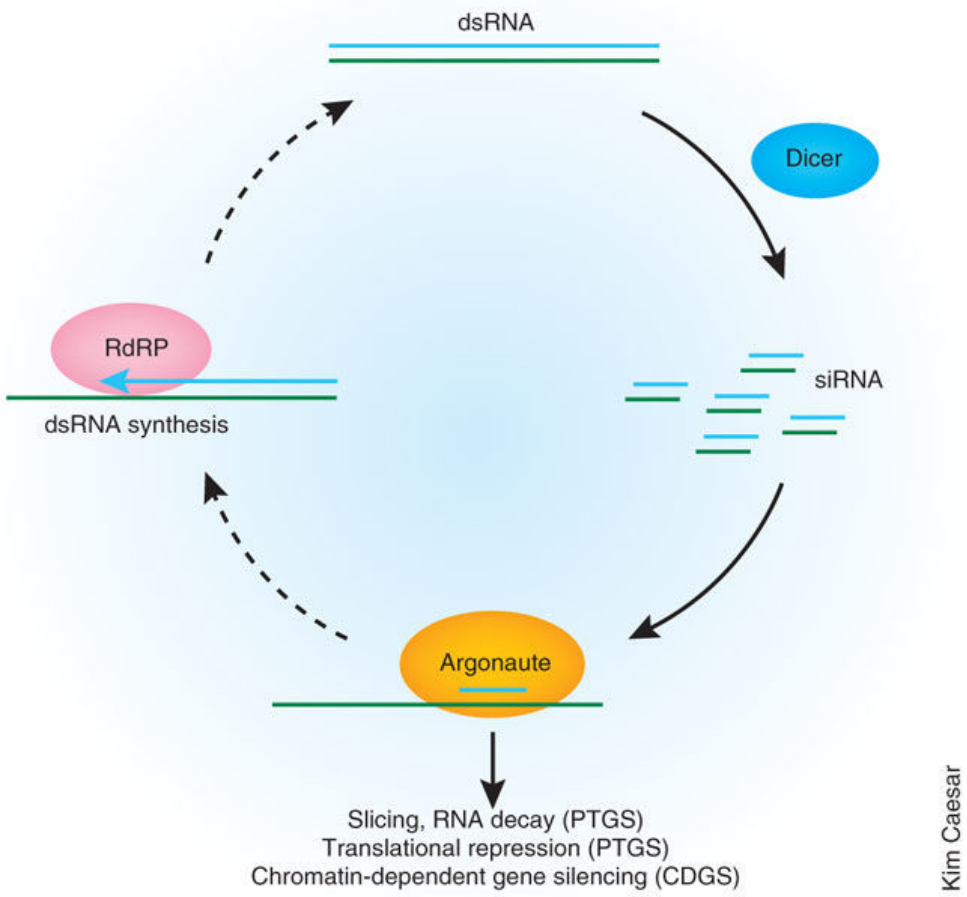
- **QDE-1** (*N. crassa*) , EGO-1 (*C.elegans*) -> **RdRP (RNA dependent RNA polymerase)**

RNA Dependent RNA Polymerase (RdRP)

RdRP found in plants, C. elegans and Drosophila m. but not in mammals

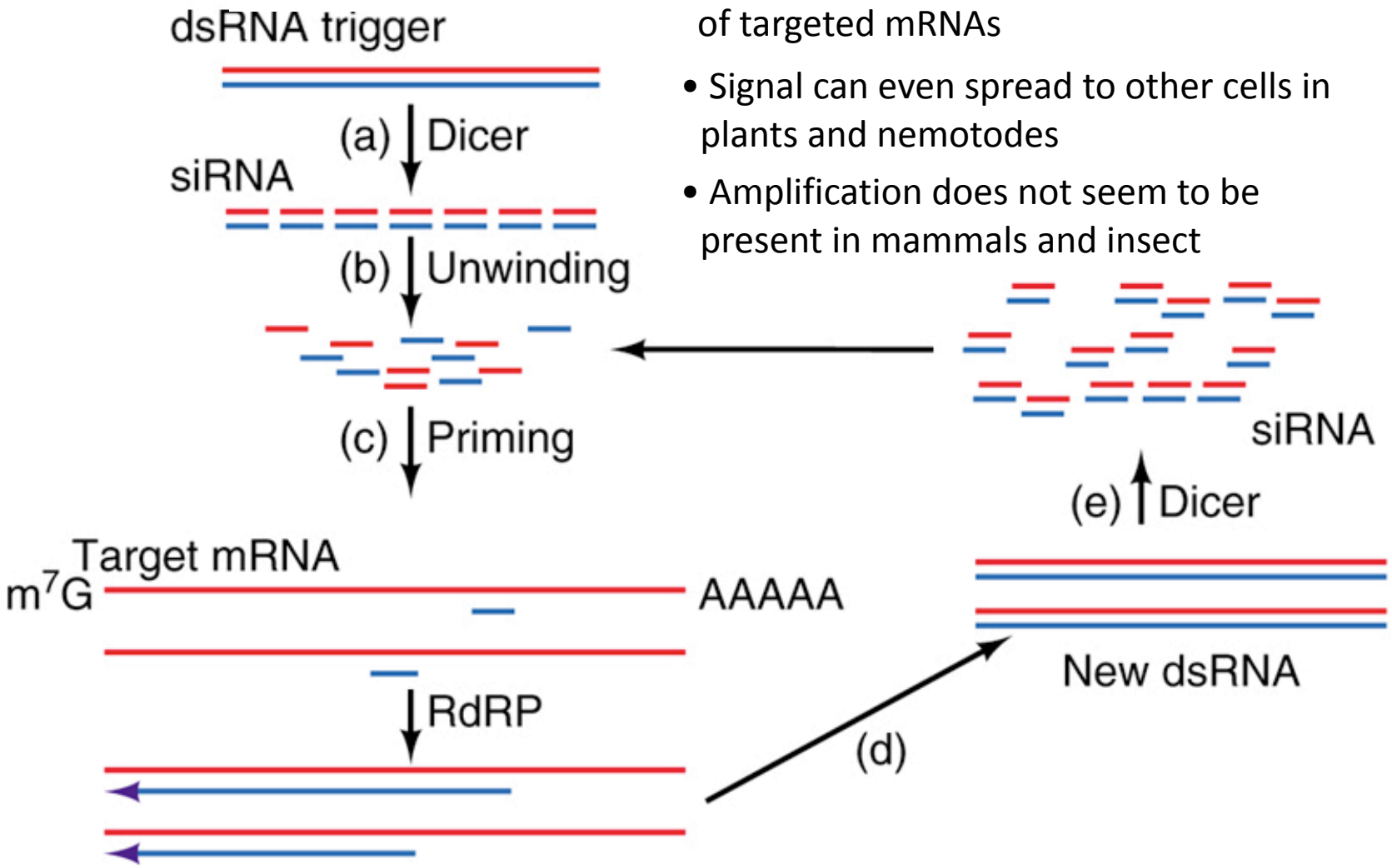
Copies one strand and resynthesizes dsRNA ex novo. The siRNAs function as primers on the messenger and the dsRNA is amplified

Can explain the duration of the phenomenon because it is responsible for the amplification of the siRNAs



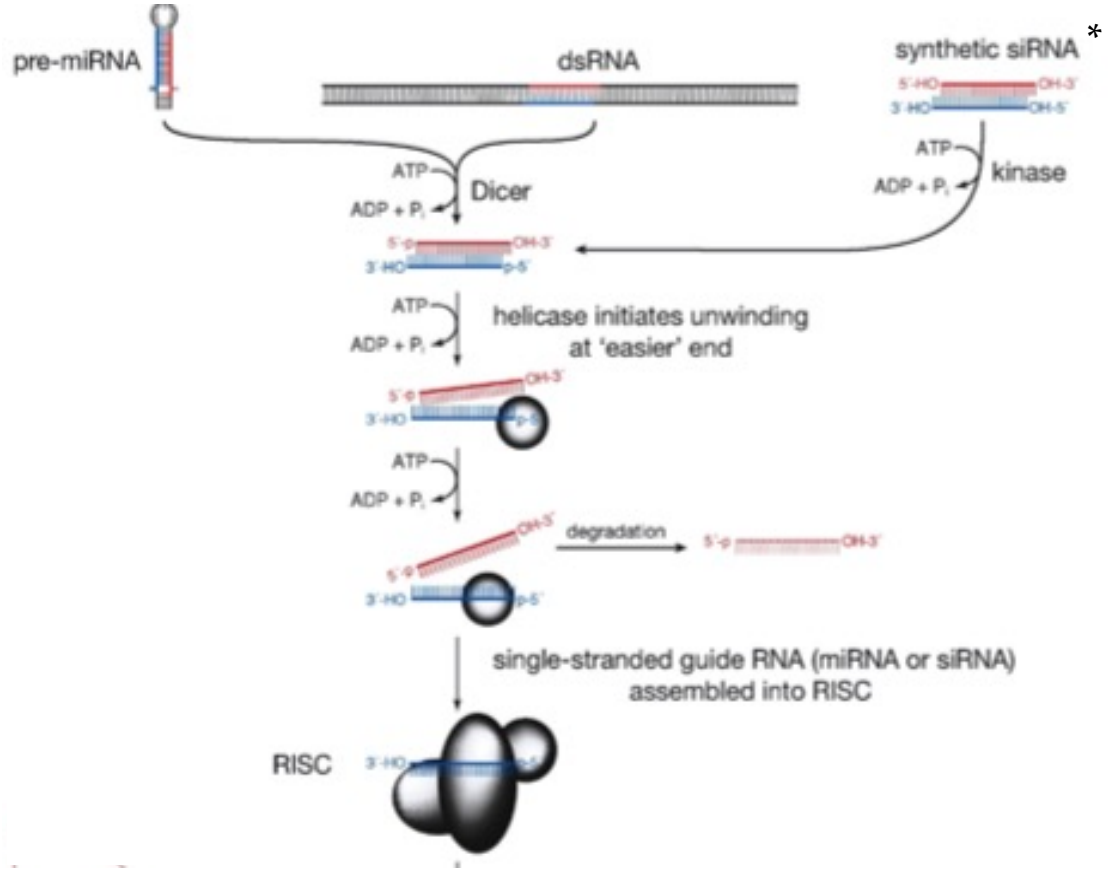
In Some Organisms, siRNA Signal Is Amplified and Spread

- New siRNAs appear against other regions of targeted mRNAs
- Signal can even spread to other cells in plants and nemotodes
- Amplification does not seem to be present in mammals and insect



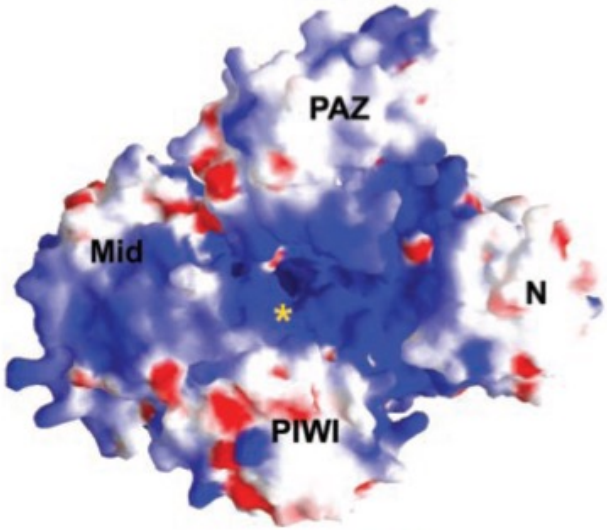
Asymmetry in the Assembly of the RNAi Enzyme Complex

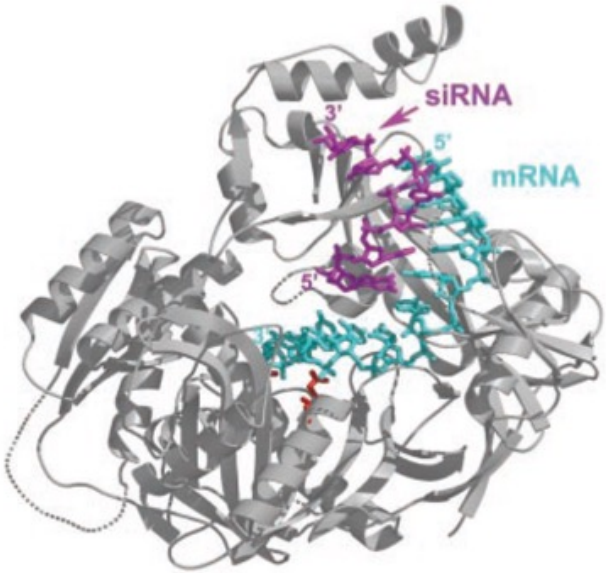
The two strands of an siRNA duplex are not equally eligible for assembly into RISC. Rather, both the absolute and relative stabilities of the base pairs at the 5′-ends of the two siRNA strands determine the degree to which each strand participates in the RNAi pathway.

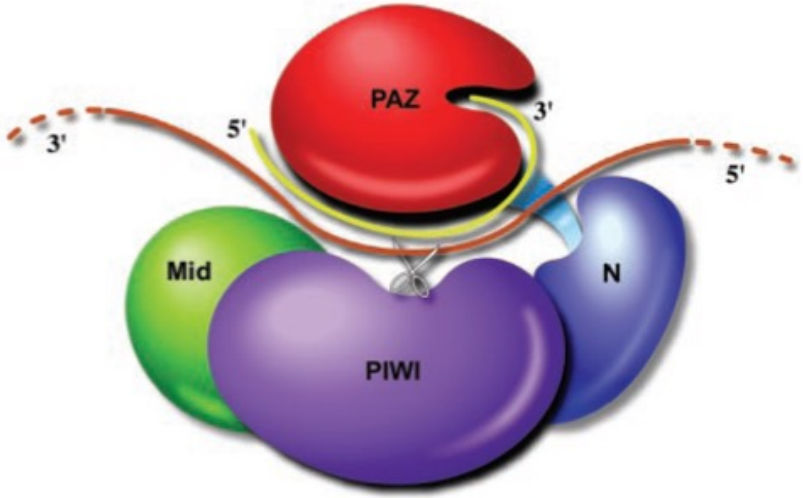


* Nucleotide can be chemically modified to get higher stability and strand specificity

The PIWI domains of Ago proteins harbour the endonuclease activity of RISC

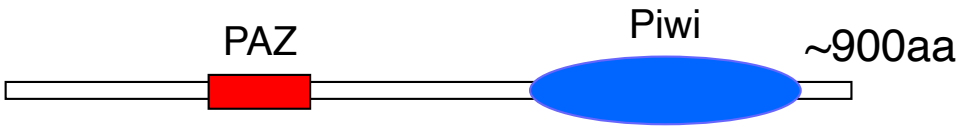


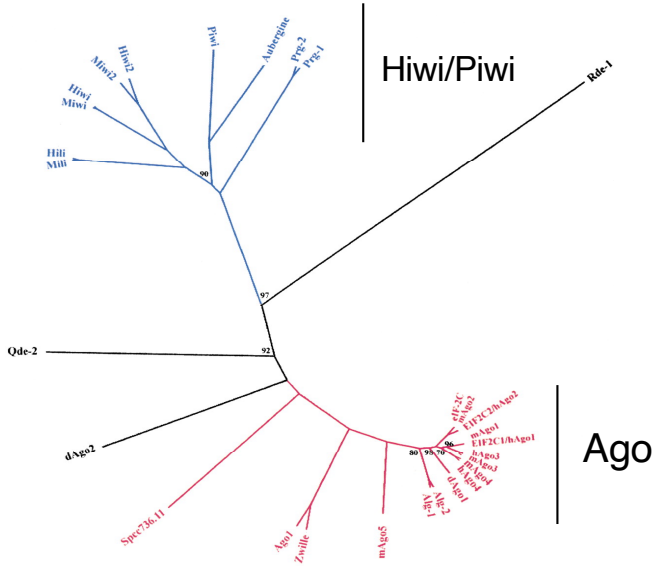




- **PAZ domain** anchors the 3'-ends of small RNAs
- **PIWI domain** is structurally similar to RNase H and can cleave complementary RNAs
- **The MID domain** anchors the 5' end of the small RNA

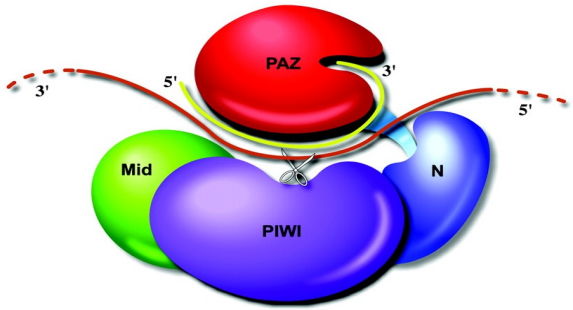
Family of Argonaute proteins





Carmell & Hannon, 2002

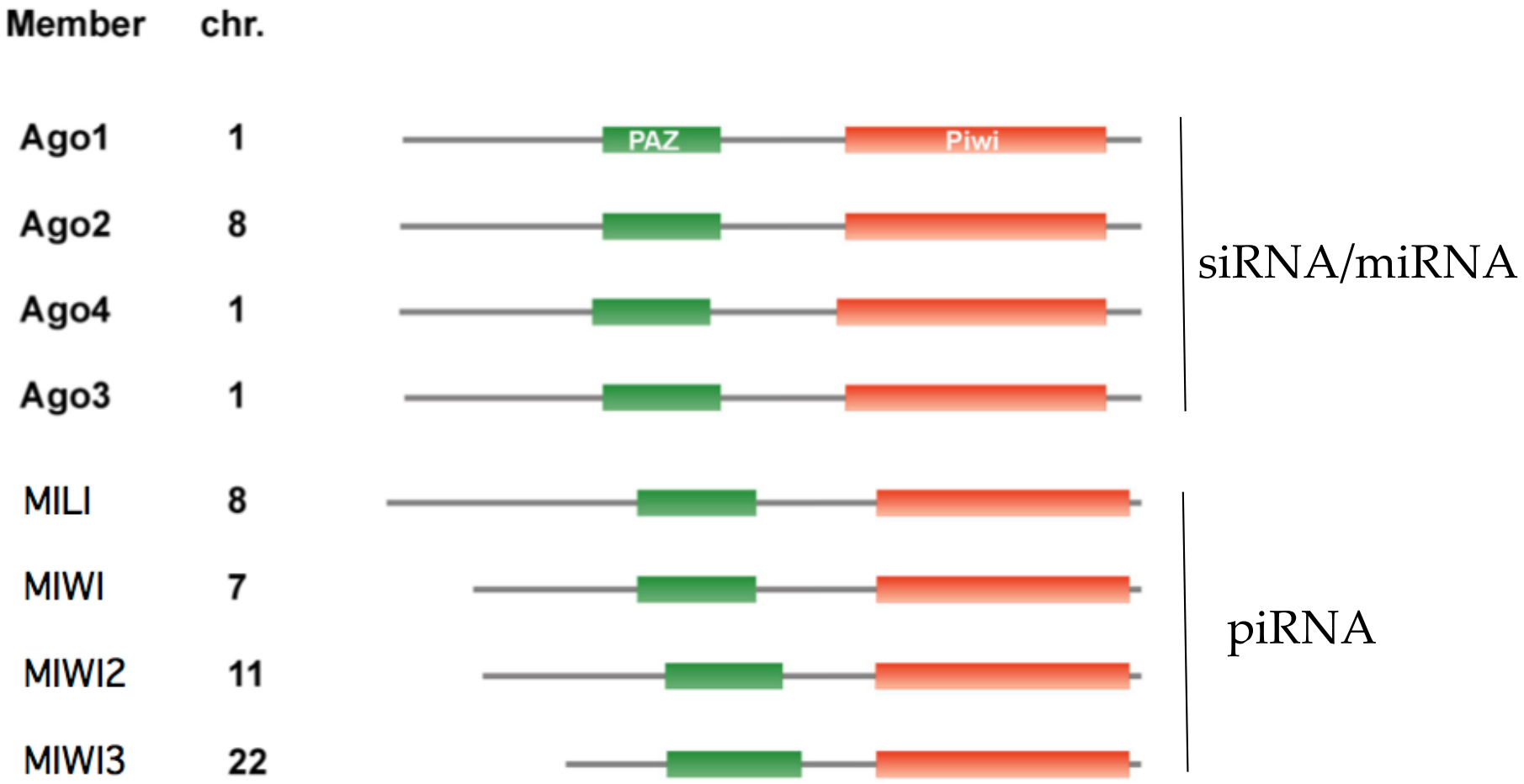
Ago proteins in different organisms	
Mammals	8
Drosophila	5
C. elegans	27
S.pombe	1
Archea	1



Role in RNAi/miRNAs:
- components of RISC/miRNPs
- bind siRNAs/miRNAs

Mammals:
- 4 Argonautes (Ago1-4)
- Ago2 is a "Slicer" (Piwi ~ RNaseH)

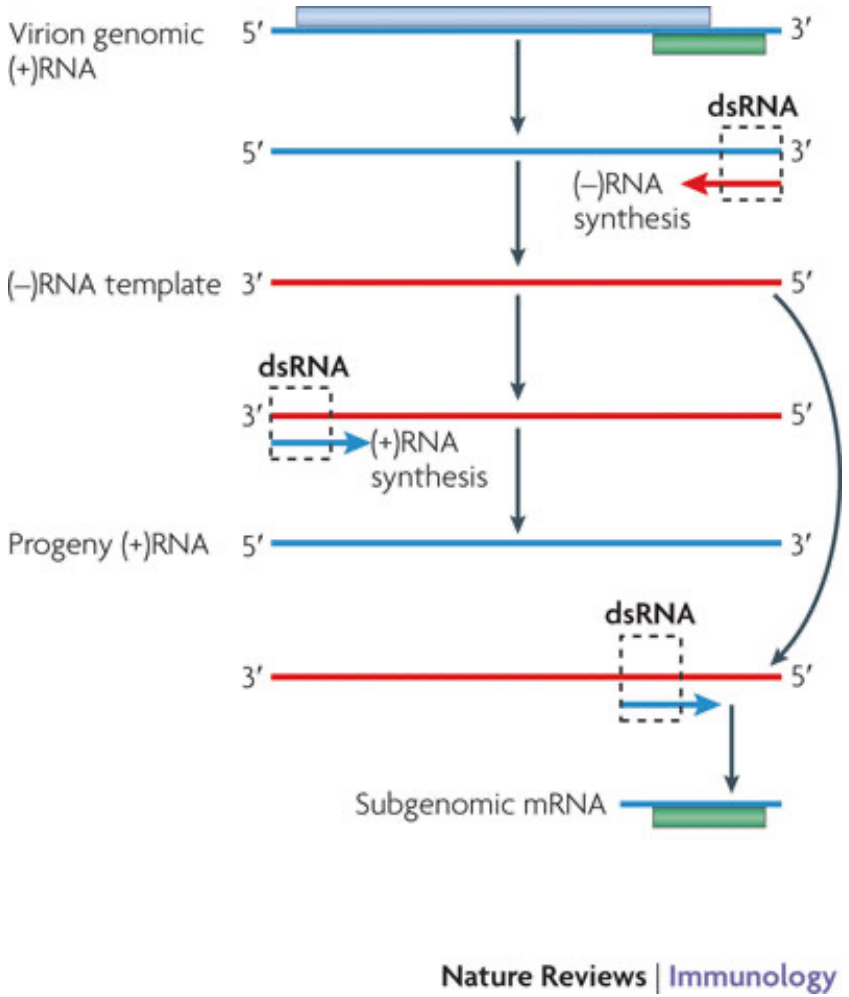
The human Argonaute protein family



RNA-based antiviral immunity

- The replication cycle of a positive-strand RNA virus includes multiple steps that yield double-stranded RNA. In addition, many organisms (plants, nematodes, insects) use viral RNA as template for **RNA-dependent RNA polymerase (RdRP)** to produce dsRNA.

- RsdRP are also involved the amplification of antiviral response.

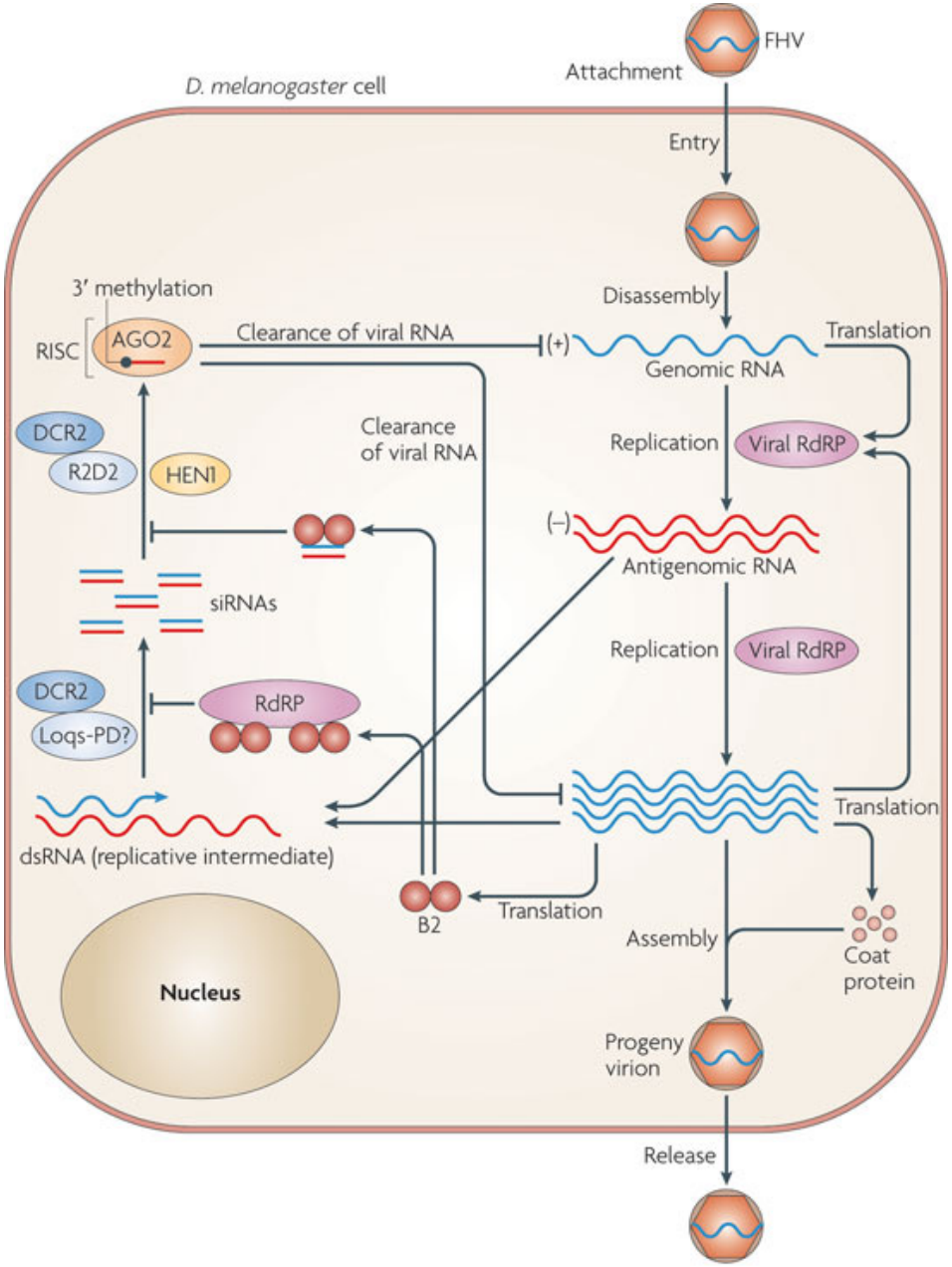


Nature Reviews | Immunology

RNA-based antiviral immunity

D. melanogaster

Following entry and uncoating of flock house virus (FHV) virions, the genomic positive-strand RNA ((+)RNA) serves as a template for the synthesis of antigenomic negative-strand RNA ((−)RNA). The resulting dsRNA formed between the 5′-terminal nascent progeny (+)RNA and the (−)RNA template is recognized by Dicer 2 (DCR2) and cleaved into siRNAs, thereby triggering **RNA-based antiviral immunity**. As a counter-defence, FHV encodes a viral suppressor of RNA silencing, the B2 protein.



Published online: November 4, 2016

Article

SOURCE DATA | TRANSPARENT PROCESS | OPEN ACCESS

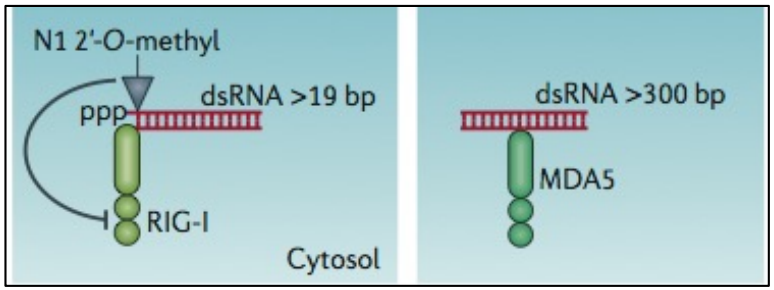
THE EMBO JOURNAL

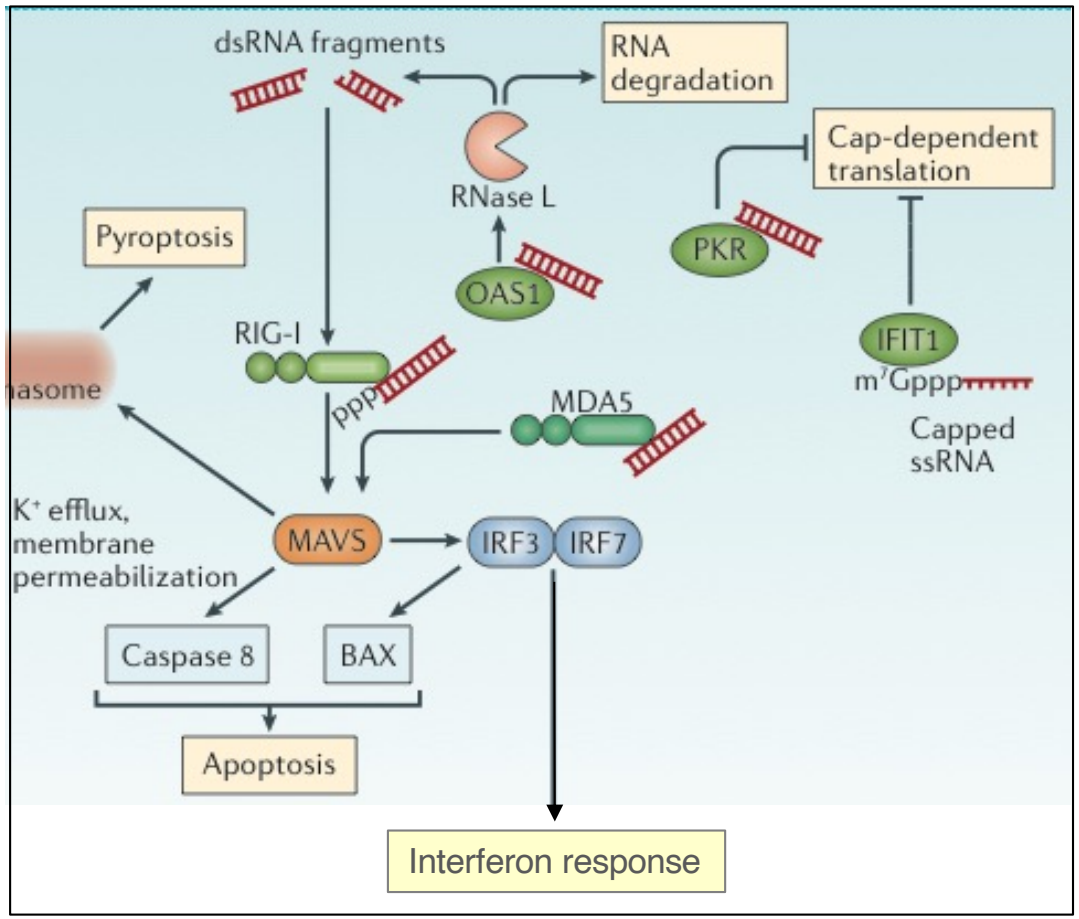
Inactivation of the type I interferon pathway reveals long double-stranded RNA-mediated RNA interference in mammalian cells

Pierre V Maillard[1,**], Annemarthe G Van der Veen[1], Safia Deddouche-Grass[1,†], Neil C Rogers[1], Andres Merits[2] & Caetano Reis e Sousa[1,*]

Transfection with long dsRNA specifically vaccinates IFN-deficient cells against infection with viruses bearing a homologous sequence.

Long dsRNAs are toxic for mammalian cells





dsRNAs must be long 21 nt in order to minimize interferon response in mammalian cells

2017 nature

LETTER

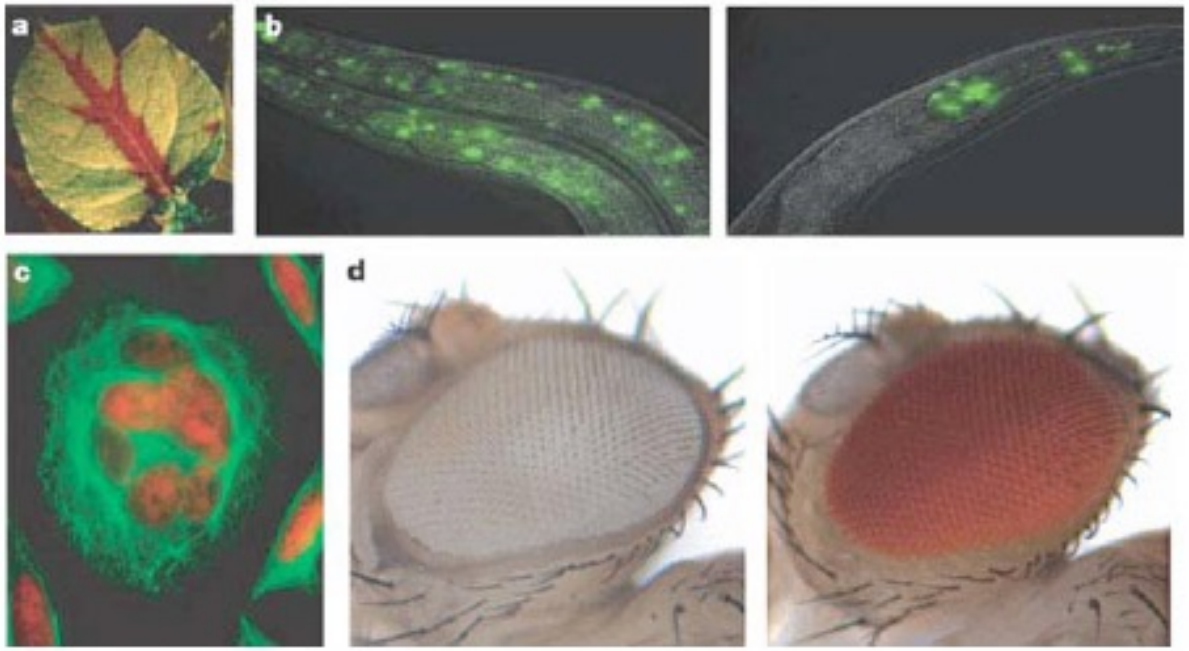
doi:10.1038/nature22990

RNase III nucleases from diverse kingdoms serve as antiviral effectors

Lauren C. Aguado[1]*, Sonja Schmid[1]*, Jared May[2], Leah R. Sabin[3], Maryline Panis[1], Daniel Blanco-Melo[1], Jaehee V. Shim[4], David Sachs[5], Sara Cherry[3], Anne E. Simon[2], Jean-Pierre Levraud[6] & Benjamin R. tenOever[1]

In contrast to the DNA-based viruses in prokaryotes, the emergence of eukaryotes provided the necessary compartmentalization and membranous environment for RNA viruses to flourish, creating the need for an RNA-targeting antiviral system[1,2]. Present day eukaryotes employ at least two main defence strategies that emerged as a result of this viral shift, namely antiviral RNA interference and the interferon system[2]. Here we demonstrate that Drosha and related RNase III ribonucleases from all three domains of life also elicit a unique RNA-targeting antiviral activity. Systemic evolution of ligands by exponential enrichment of this class of proteins illustrates the recognition of unbranched RNA stem loops. Biochemical analyses reveal that, in this context, Drosha functions as an antiviral clamp, conferring steric hindrance on the RNA-dependent RNA polymerases of diverse positive-stranded RNA viruses. We present evidence for cytoplasmic translocation of RNase III nucleases in response to virus in diverse eukaryotes including plants, arthropods, fish, and mammals. These data implicate RNase III recognition of viral RNA as an antiviral defence that is independent of, and possibly predates, other known eukaryotic antiviral systems.

RNAi: a tool for inhibithing gene expression in a sequence specific way



Hannon, G (2002) Nature 418, 244-251

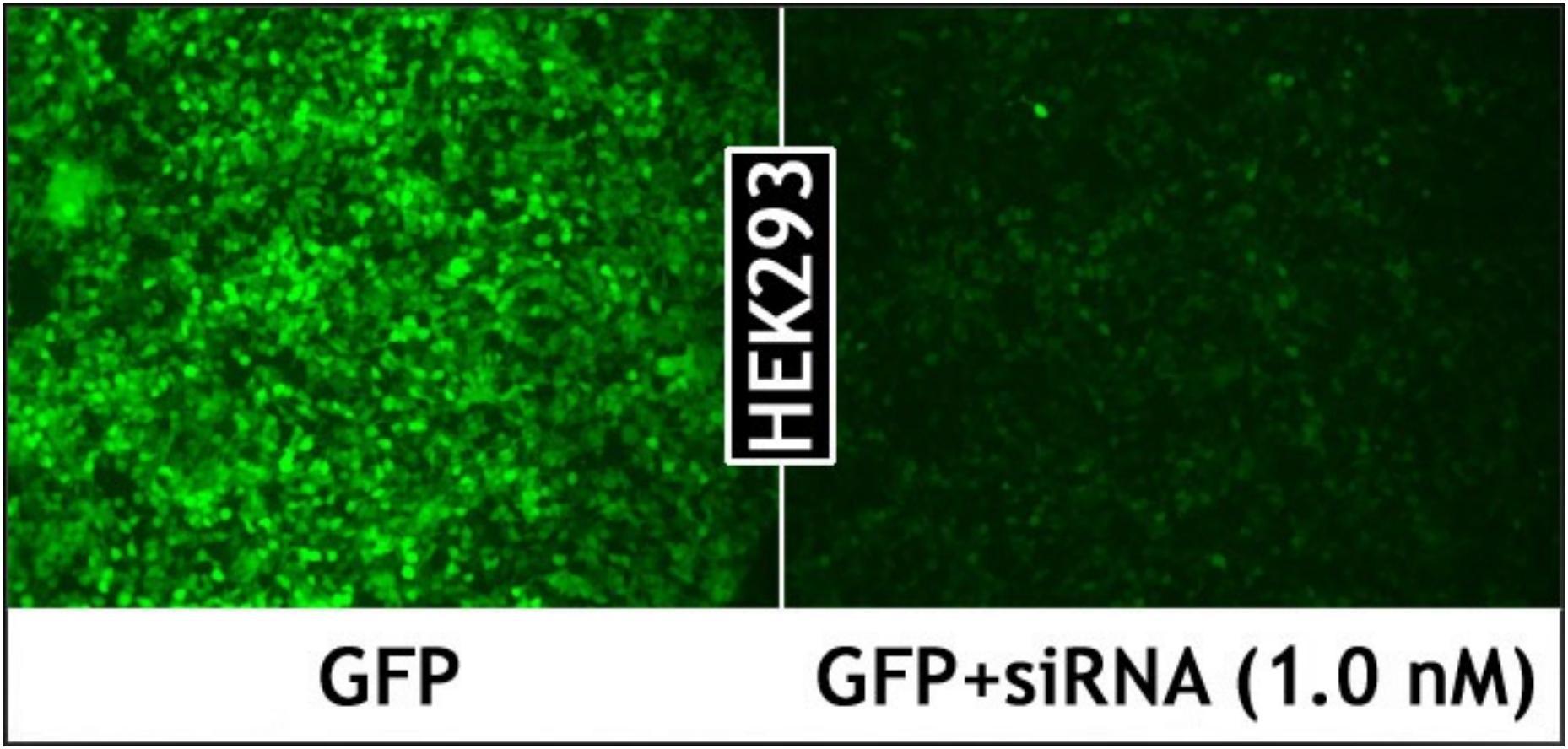
siRNAs as a tool for studying gene function

Short double-stranded RNAs are synthesized with a sequence complementary to the mRNA of interest and introduced into a cell or organism, where they are recognized by the RISC complex and activate the RNAi process.

Effect: drastic decrease in the expression of the target gene and by studying the effects of this decrease, the physiological role of the gene product can be deduced.

Since RNAi does not completely abolish gene expression, it is called a knockdown technique.

RNAi knockdown experiments can be used to study the functions of genes *in vivo*



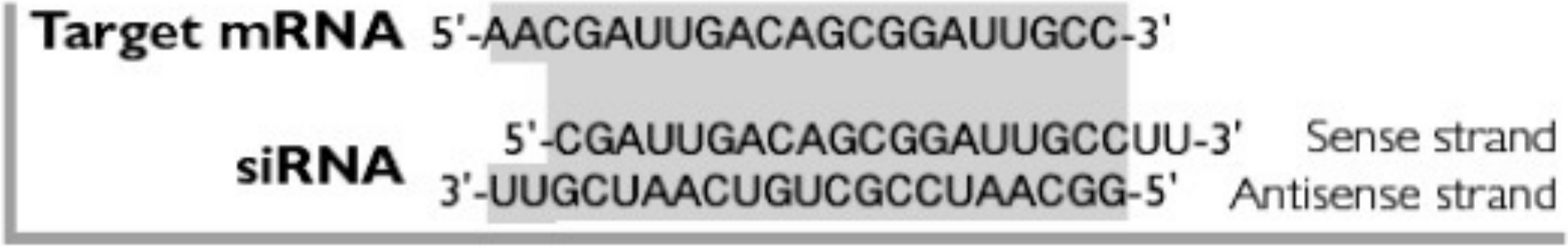
SYSTEMATIC GENOME-WIDE SCREENS OF GENE FUNCTION

Anne E. Carpenter and David M. Sabatini

By using genome information to create tools for perturbing gene function, it is now possible to undertake systematic genome-wide functional screens that examine the contribution of every gene to a biological process. The directed nature of these experiments contrasts with traditional methods, in which random mutations are induced and the resulting mutants are screened for various phenotypes. The first genome-wide functional screens in *Caenorhabditis elegans* and *Drosophila melanogaster* have recently been published, and screens in human cells will soon follow. These high-throughput techniques promise the rapid annotation of genomes with high-quality information about the biological function of each gene.

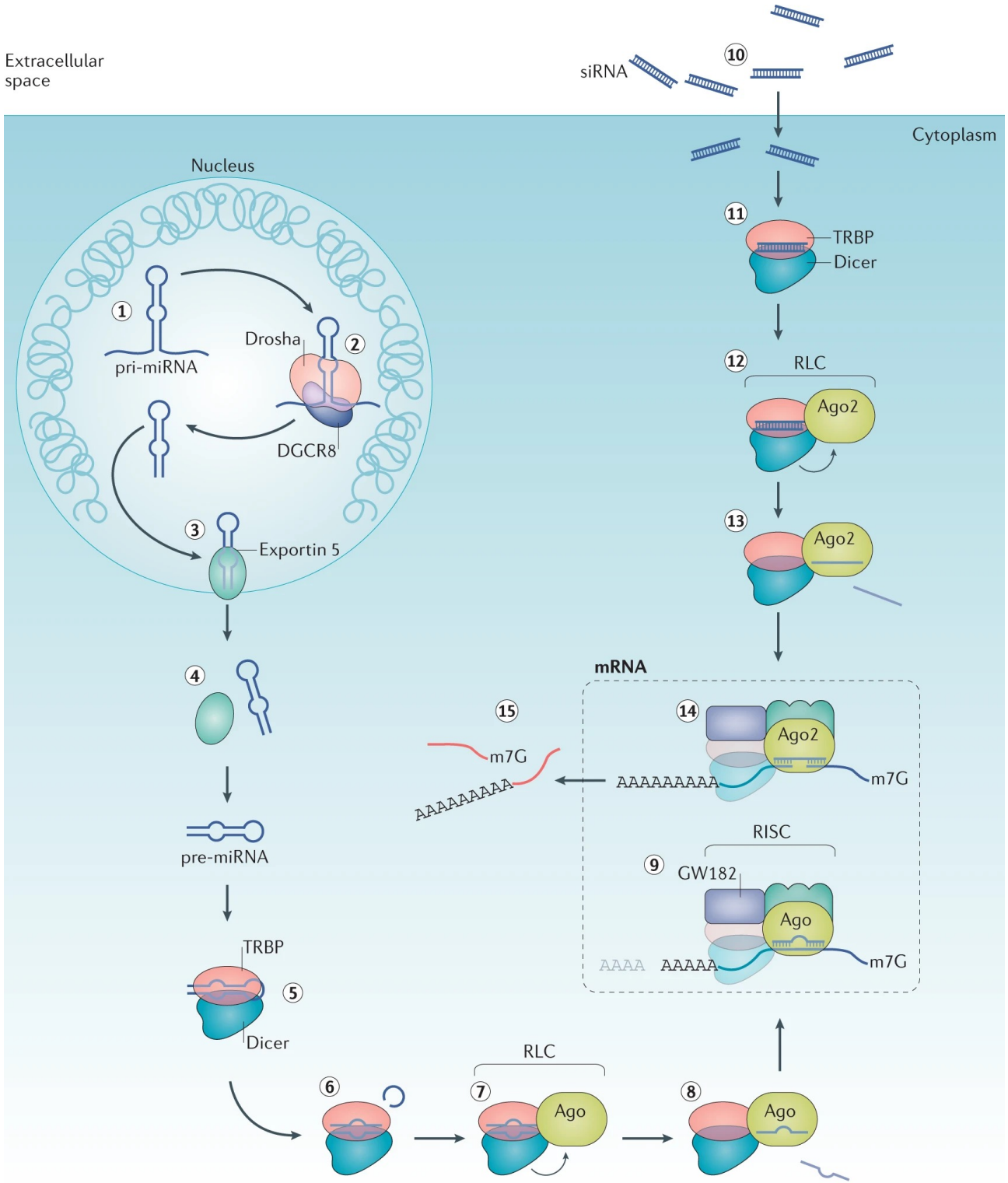
siRNA design

- 21-23nt
- 2-nt 3' overhangs (**UU overhangs**)
- G/C content: 30-50%.
- No base pair mismatch



- Synthesized siRNA should not target introns, the 5'and 3'-end untranslated regions (UTR)
- Sequences within 75 bases of the start codon (ATG)

- BLAST : eliminate any target sequences with significant homology to other coding sequences.

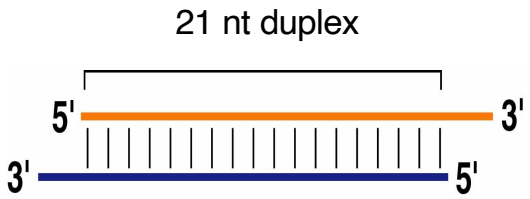


(1) primary microRNA (miRNA) transcripts (pri-miRNA) are transcribed in the nucleus (2) and cleaved by the Microprocessor complex (Drosha–DGCR8) to produce short hairpin RNAs (shRNAs) called pre-miRNA. (3) Exportin 5 binds and transports the pre-miRNA to the cytoplasm (4) where it nd binds with Dicer and TAR RNA-binding protein (TRBP). (6) Dicer cleaves the terminal loop of pre-miRNA (7) and induces formation of an RNA-induced silencing complex (RISC)-loading complex (RLC) with an Argonaute (Ago1–Ago4) protein. (8) A guide strand (antisense) is selected and loaded into Ago1–Ago4 and the passenger (sense) strand is discarded. (9) The mature RISC can regulate gene expression by inhibiting mRNA translation and promoting mRNA degradation. Argonaute, GW182 and the guide strand are essential for the mRNA-silencing activities of RISC. (10) Synthetic small interfering RNAs (siRNAs) enter the cytosol via endocytosis followed by rare endosomal escape events. (11) siRNAs then interact directly with the cytosolic RNA interference (RNAi) enzymes (Dicer and TRBP) (12) to form the RLC via Dicer-mediated (13) and undergo strand selection to produce mature RISC. (14) siRNA guide strands usually have full complementarity to a single target mRNA to induce potent and narrowly targeted gene silencing. (15) Ago2 is particularly important for RNAi therapeutics as it has intrinsic slicer activity to efficiently cleave mRNA targets.

How to provide siRNAs to a cell?

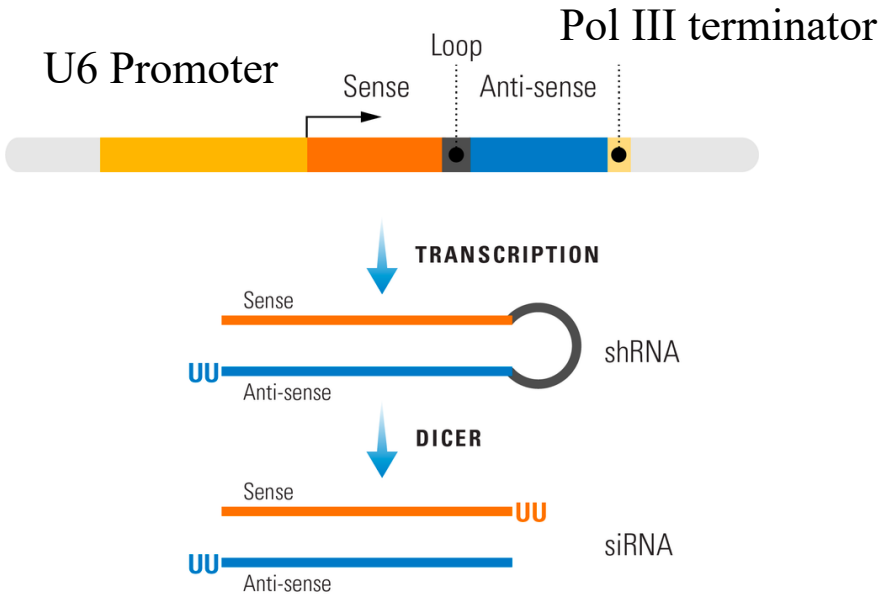
Sinthetic siRNAs

+ easy
+ many target sequences can be tested
- **but** expensive
- short-term effect (depends on the cell type)



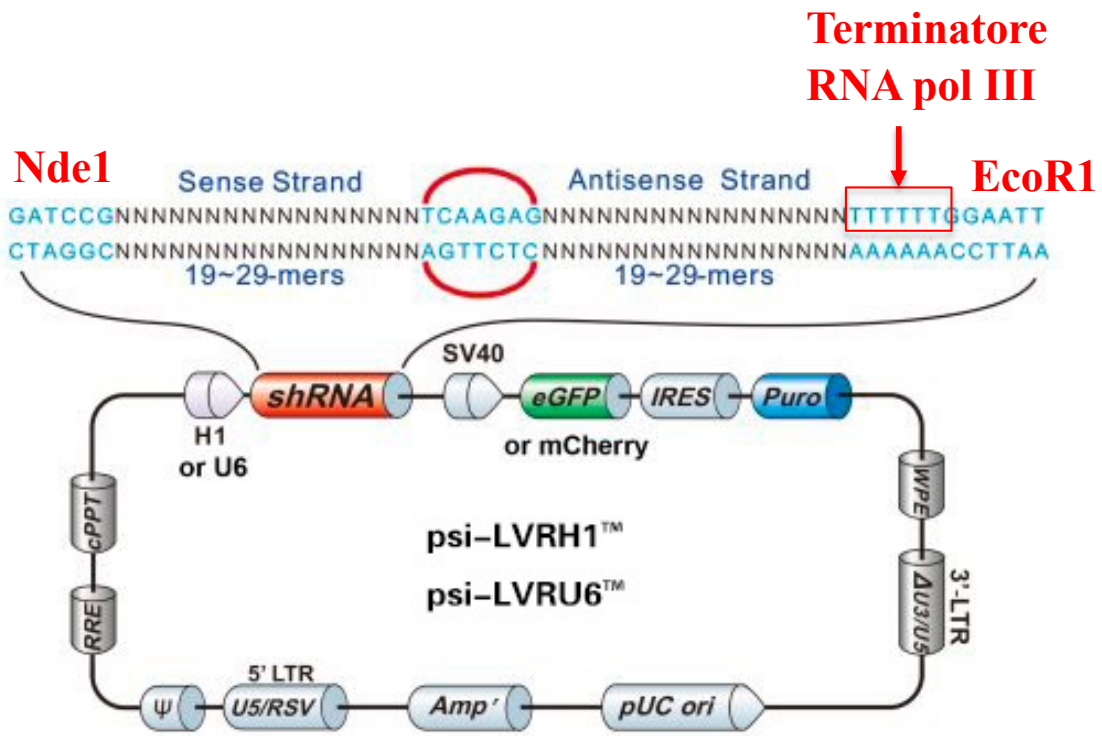
DNA vectors

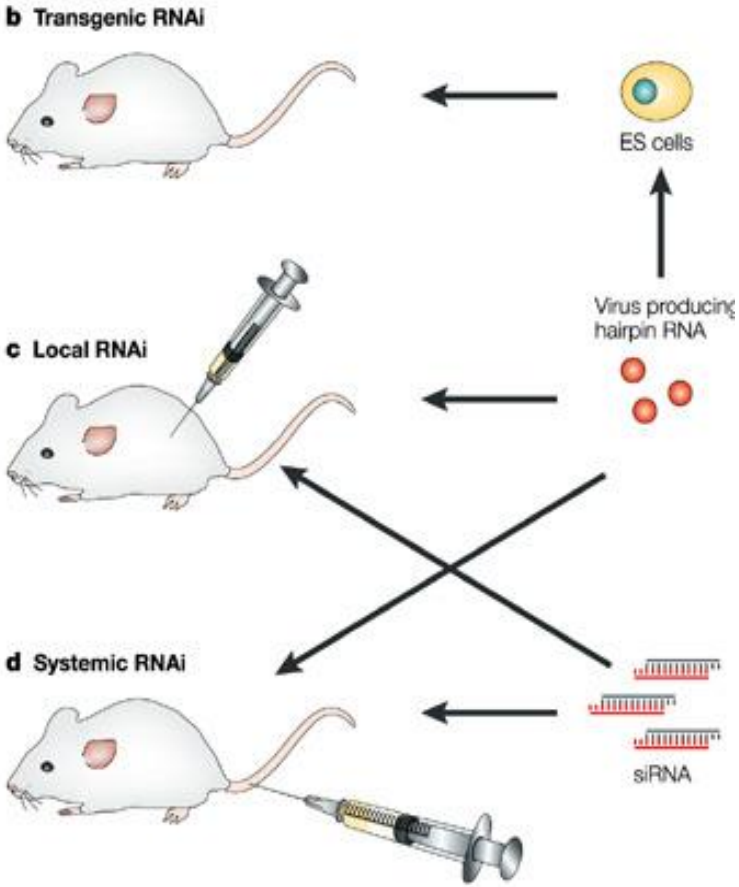
- more laborious
+ inexpensive
+ long-term effect



RNA Pol III promoter and terminator

A method for the stable expression of short interfering RNA in vivo: viral vector





Long term silencing of the target gene

siRNA in Biotechnology

Development of plants that produce low levels of natural toxins so that they can be introduced into the food chain.
The cotton seed is a particularly protein-rich and energetic product that has now become part of livestock feed, especially for dairy cows. However, these seeds contain a toxic product, gossypol, making them unusable. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in 1 the production of gossypol, without affecting the production of the enzyme in other parts of the plant where gossypol is important in preventing damage from plant parasites.

Reduction of allergen levels in tomato plants and reduction of carcinogen precursors in tobacco plants.

Recent study of a new anti-mosquito.
Nanoparticles are used to administer double-stranded RNA to mosquito larvae at the time of feeding. RNA interference has been used to silence the gene responsible for the production of chitin, the main component of the exoskeleton in insects, crustaceans, and arachnids. Since dsRNA dilutes quickly, it cannot theoretically be administered directly and for this reason, the use of nanoparticles is resorted to.

Therapeutic use of RNAi

- Hematological Diseases:
  - Alteration due to reduction, mutation, or absence of gene function

- Oncology:
  - Inhibit oncogenes
  - Increase the efficacy of chemotherapy and radiotherapy

- Infectious Diseases - Targeting Viruses (HCV and HIV):
  - Inhibit cellular and viral factors
  - Target the Reverse Transcriptase RNA, inhibit viral replication
  - Induce resistance to viral infection in infected organisms