Ch4 - L3.3

RNA interference

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

Andrew Fire and Craig Mello had the Nobel prize in 2006.

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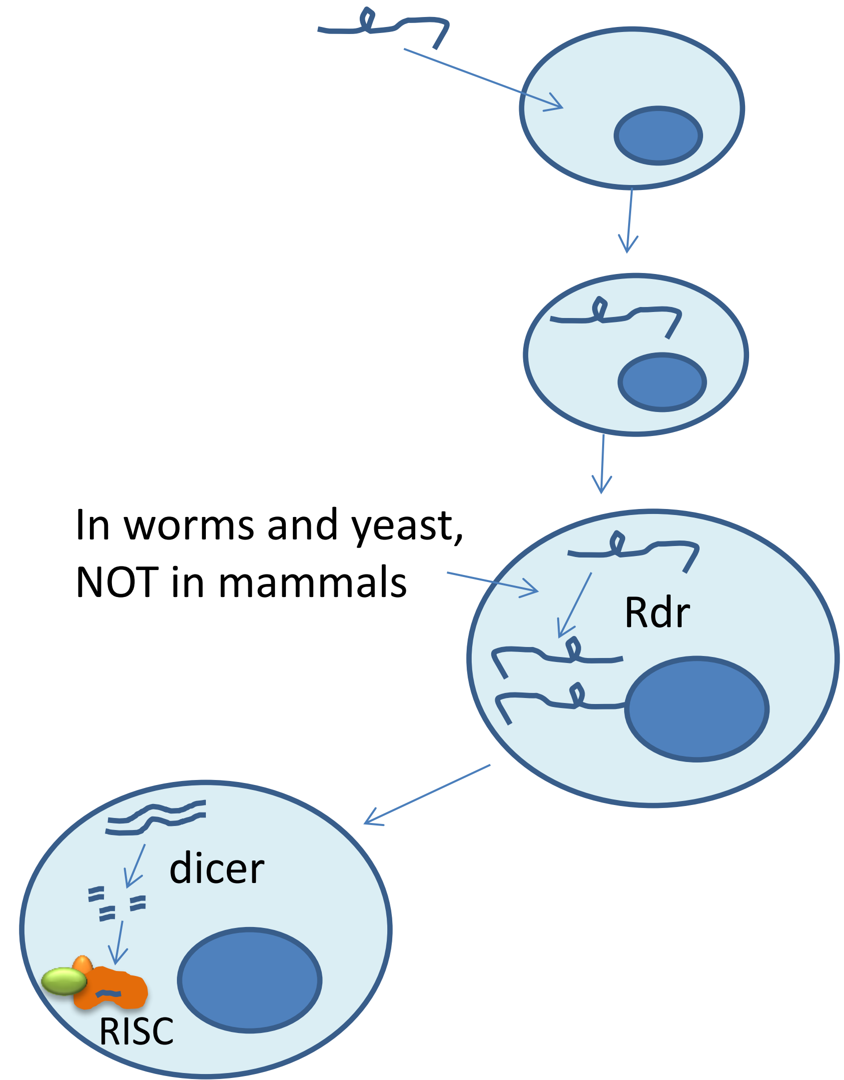
Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene[1,2]. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression[3,4]. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stochiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process.



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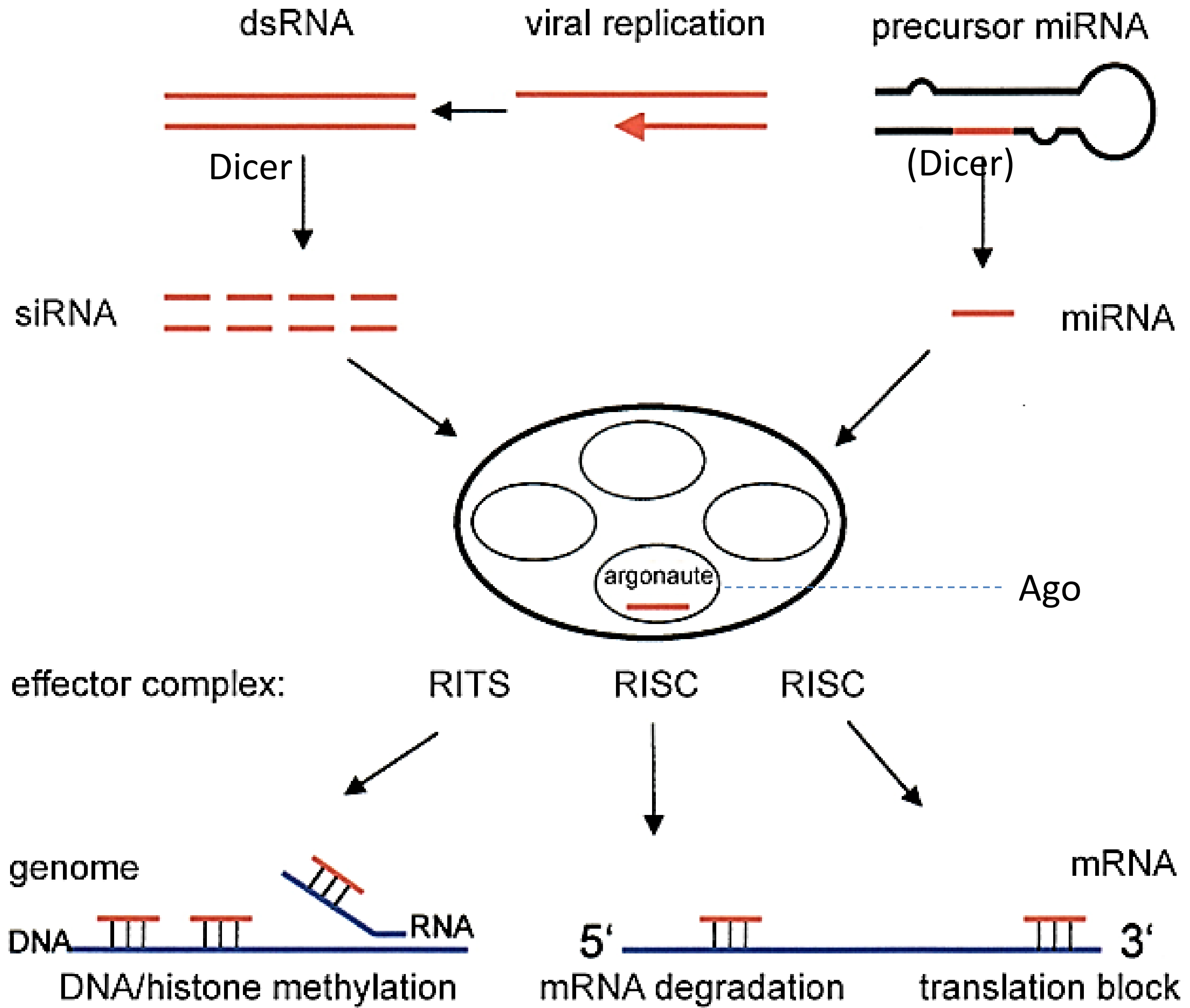
RNA interference



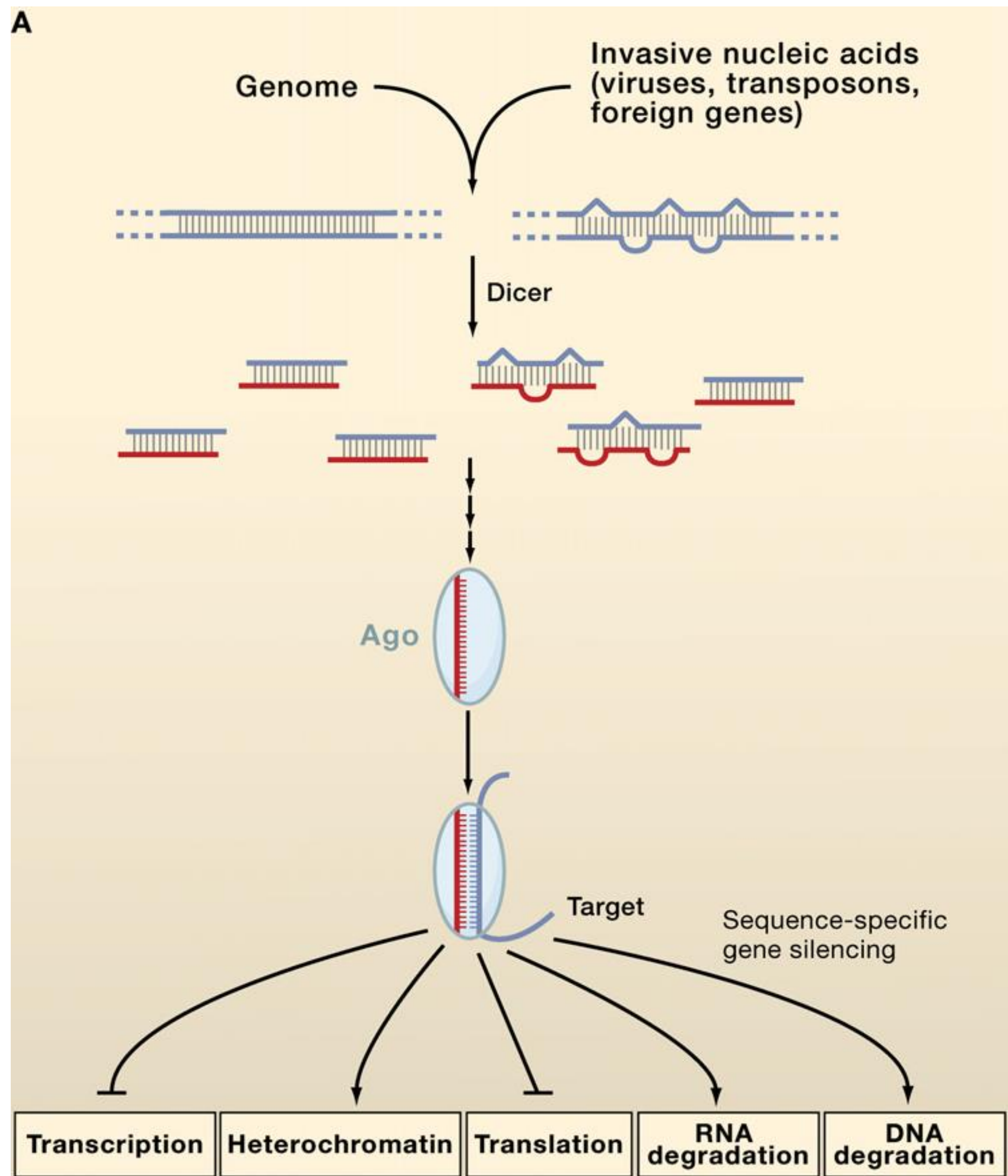
RNA interference

Endogenous and exogenous double-stranded RNAs

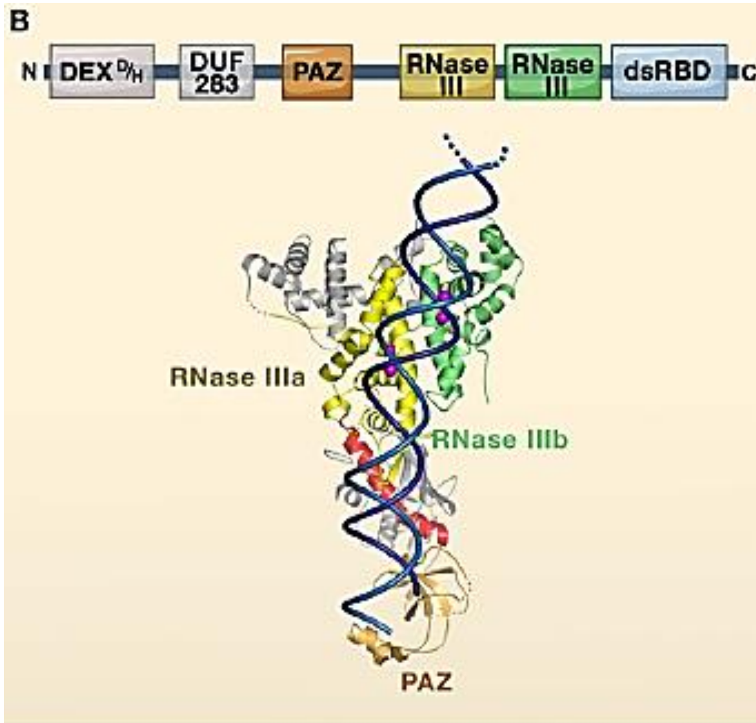
Dicer cleaves into 21-23nt pieces

Si-RISC or mi-RISC

Effect



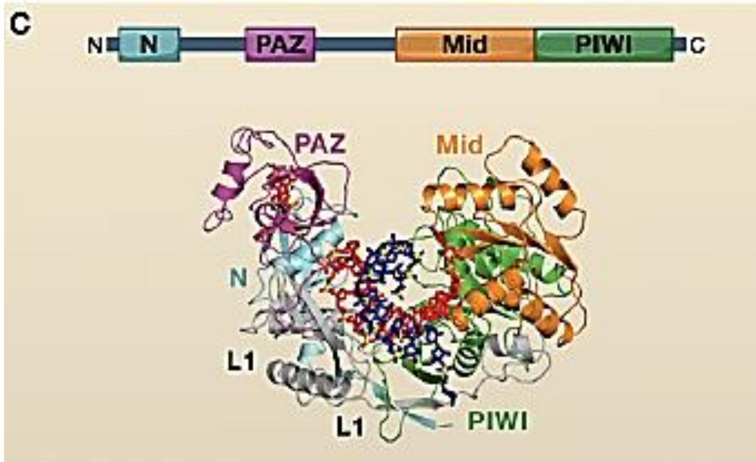
Cartew, 2009



Carthew, 2009

DICER is a RNase III enzyme.
The PAZ domain binds the RNA duplex end, then the two catalytic subunits cleave leaving **2nt 3'-overhangs (5' monophosphate).**

Mammals have a single DICER, whereas other classes have more enzymes.



Argonaute superfamily: 3 clades.

- ✓ Piwi clade: bind piRNAs
- ✓ Ago clade: bind siRNA and miRNA
- ✓ 3rd: only in Nematodes (to date)

8 Argonaute proteins (Ago) in H. Sapiens
Piwi domain has high affinity for ends with 3'-overhangs and in some case has endonucleolytic activity.
Ago enter a RISC assembly pathway: only one RNA strand stably associated, the other (passenger) is discarded.

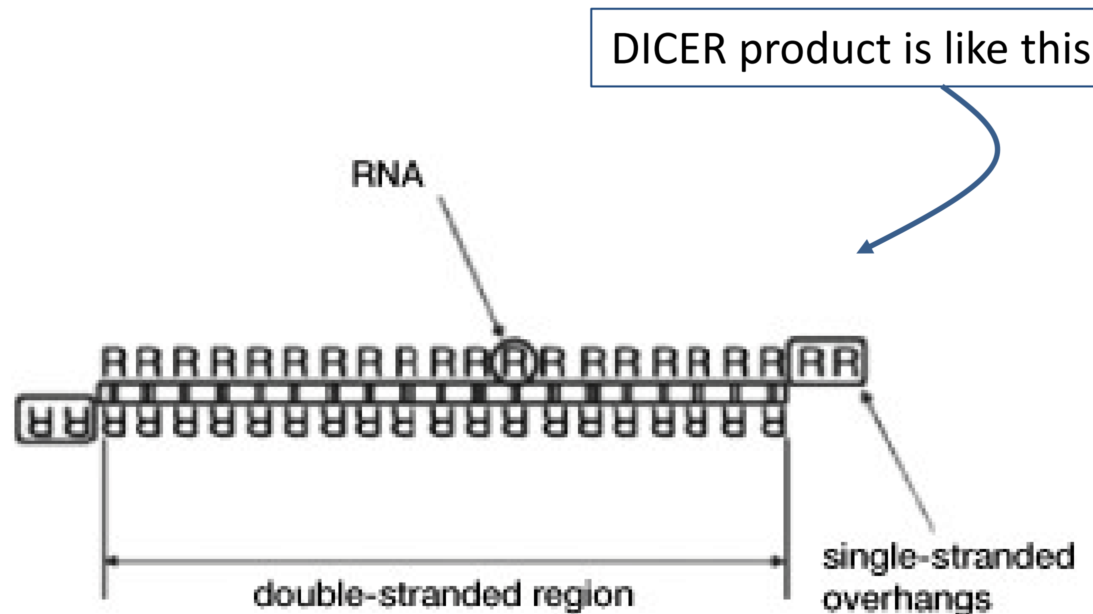
Three major classes of **silencing small RNAs**.

- Micro RNA (miRNA, also miR, *but caution for confusion with MIR, a class of transposons*)
- Small interfering RNA (siRNA)
- Piwi-dependent RNA (piRNA)

- Some new classes emerged from NGS RNA-Seq experiment, but no clear evidence of function is available to date (see next table)

In the lab:

Gene silencing using siRNA. (and shRNA)



Different short noncoding RNA classes emerged from short-RNA-seq analyses

Table 1

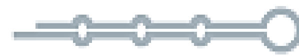
Classes of small non-coding RNAs

ncRNA	Abbreviation	Model organism(s)
microRNA	miRNA	Most eukaryotic organisms except fungi; viruses
miRNA-offset RNA	moRNA	*Ciona intestinalis*, mammals
Short hairpin-derived miRNA	shRNA-derived miRNA	*Mus musculus*
miRNA-like small RNA	milRNA	*Neurospora crassa*
mirtron	–	*D. melanogaster*, *C. elegans*, mammals
Piwi-interacting RNA	piRNA	*D. melanogaster*, mammals, *Danio rerio*, *Xenopus laevis*, *Bombyx mori*
21U-RNA	–	*C. elegans*
Endogenous small interfering RNA	endo-siRNA	*D. melanogaster*, *C. elegans*, mammals
tRNA-derived RNA fragment, tRNA-derived small RNA	tRF, tsRNA	Mammals
Promoter-associated small RNA, termini-associated small RNA	PASR, TASR	Mammals
Transcription start site-associated RNA, transcription initiation RNA	TSSa-RNA, tiRNA	Mammals, *Gallus gallus*, *D. melanogaster*
Splice-site RNA	spliRNA	Metazoans
snoRNA-derived RNA	sdRNA	Metazoans, *Arabidopsis thaliana*, *Schizosaccharomyces pombe*
QDE-2-interacting small RNA	qiRNA	*N. crassa*
Small vault RNA	svRNA	*Homo sapiens*

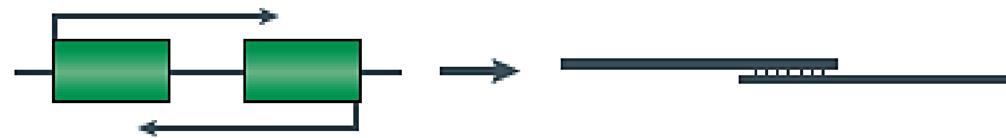
From Aalto & Pasquinelli, 2012

Endo-siRNA

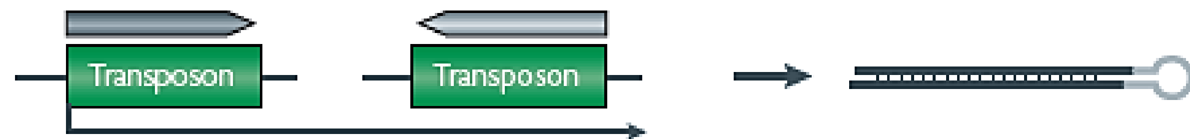
Structured loci



Convergent transcription

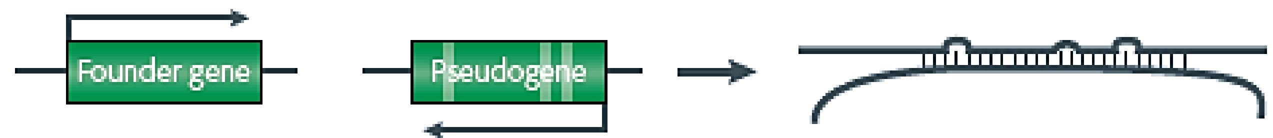


Read-through transcription of transposons in inverted orientation



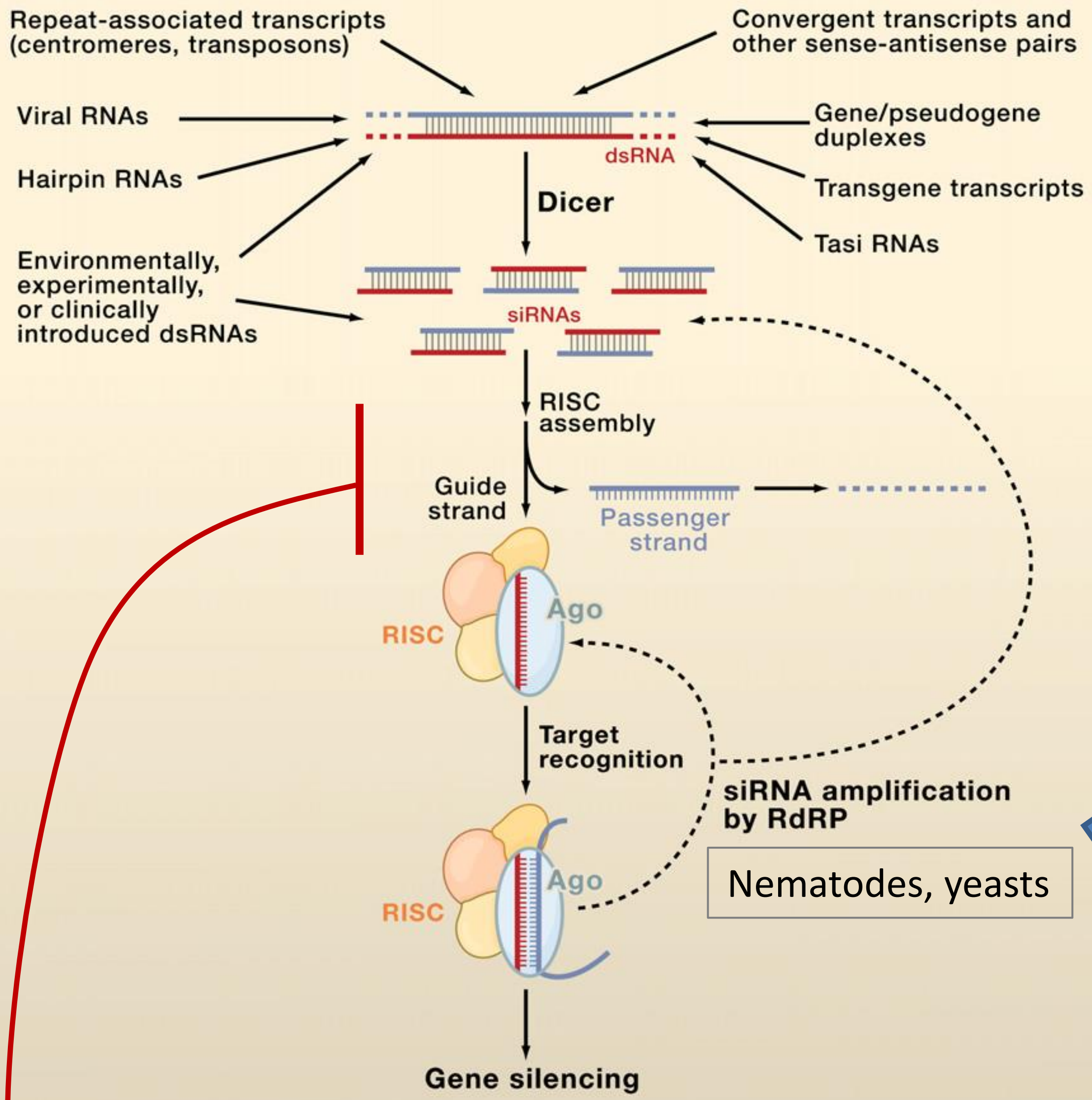
Bidirectional transcription

Trans-interaction



Duplicated and inverted pseudogene copies

Figure 3 | **Genomic sources of dsRNA triggers for endogenous small interfering RNAs (endo-siRNAs) in flies and mammals.**



siRISC assembly
Insects:
R2D2/Dicer heterodimer binds siRNA duplex
+ other factors→ RISC loading compex
+ Ago2 → pre-RISC
Ago2 cleaves the passenger strand (eliminated) → RISC

Humans:
Dicer, TRBP, Ago2 → assembling
Binds dsRNA, processes to siRNA, eliminates passenger → RISC

Which siRNA strand is selected ?
Guide strand is the one that has its 5' terminus at the less stably base-paired end.
Selection graded: siRNAs with equal base-pairing stabilities at their ends → either strand used.

piRNA are small RNAs associated to the Piwi-subfamily of Argonaute proteins.

They have other <u>specific features</u>:

- Lack of dsRNA precursors
- Independence of Dicer

Primary piRNA produced from piRNA clusters.
Processed into **24-30 nt** piRNA that associate with Argonaute Piwi clade proteins.
piRNA pairs with **transposon sense transcript** and cleave them endonucleolitically.

piRNAs were first proposed to ensure germline stability by repressing transposons

Mammalian piRNAs:
pre-pachytene and pachytene piRNAs
(according to the stage of meiosis in developing spermatocytes).

miRNA

Micro RNA are a family of small RNA that are transcribed from several locations in genomes.

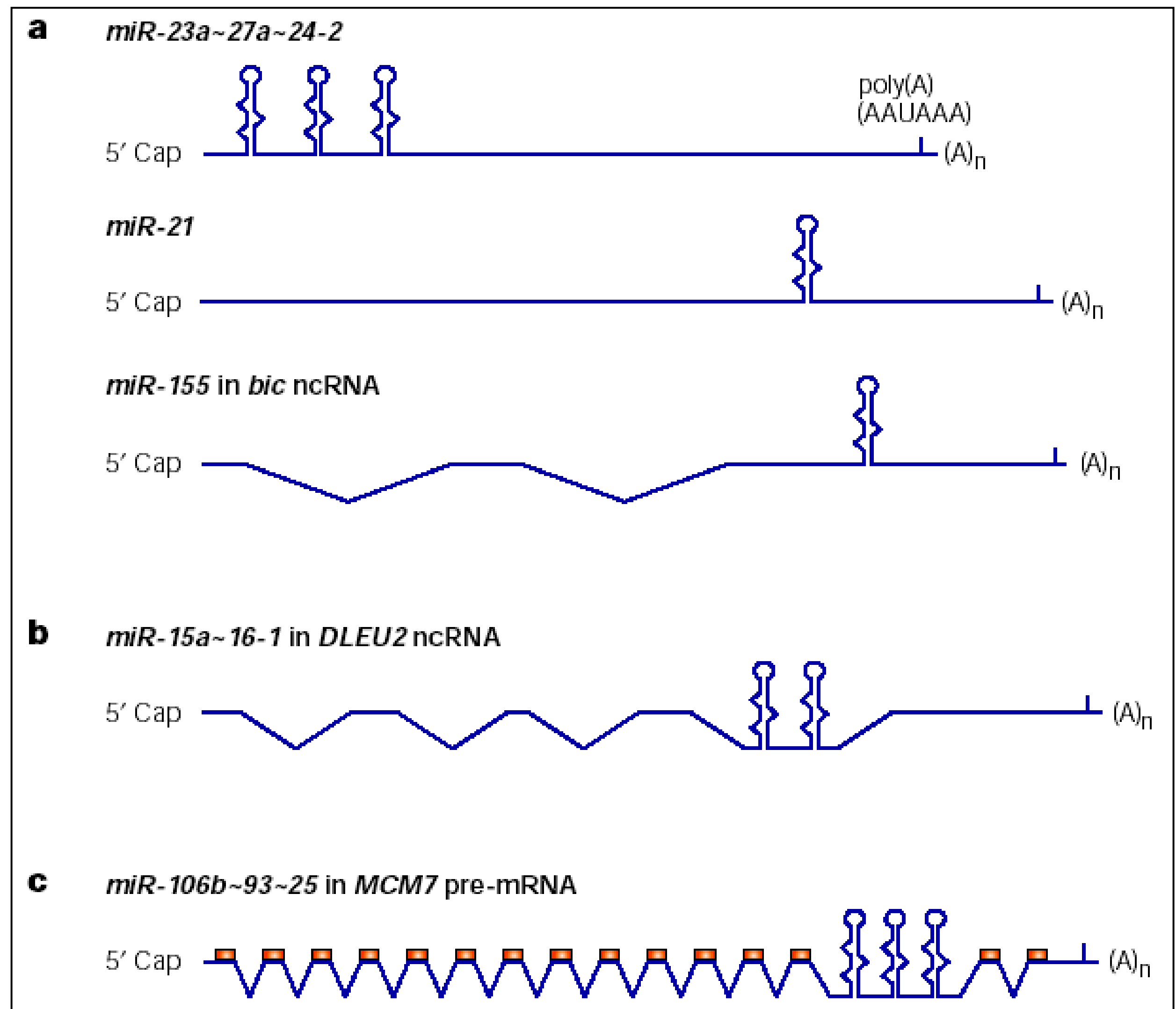
They have a typical structure, making a stem-loop structure with some mismatches in the stem

MiRBase: http://www.mirbase.org/cgi-bin/browse.pl?org=hsa

Figure 1 | **The structure of five pri-miRNAs.**

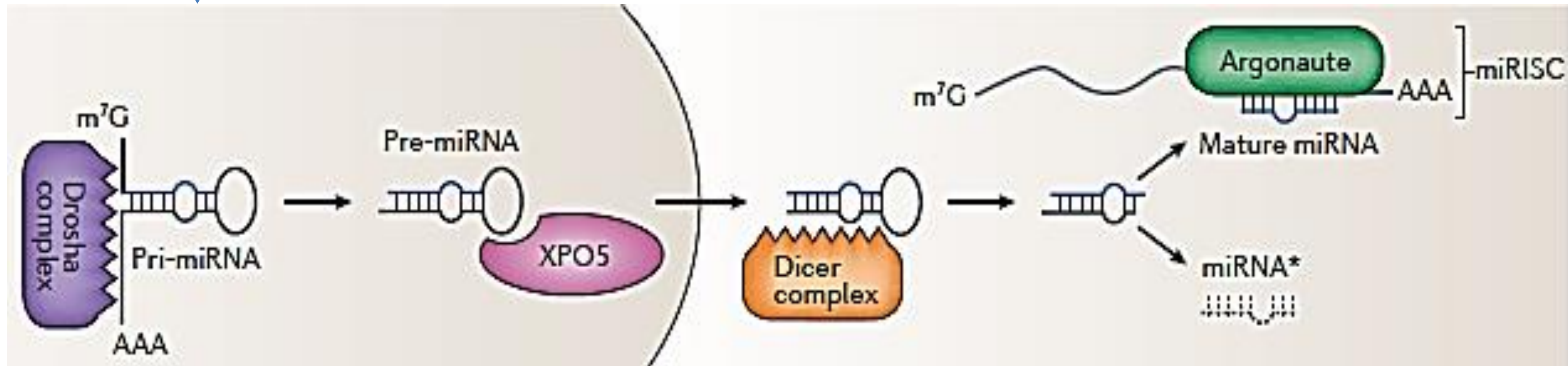
Primary transcripts that encode miRNAs, primiRNAs, contain 5’ cap structures as well as 3’ poly(A) tails. miRNAs can be categorized into three groups according to their genomic locations relative to their positions in an exon or intron.

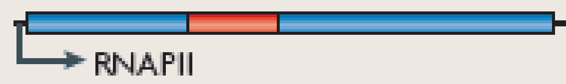
a | Exonic miRNAs in non-coding transcripts such as an *miR-23a~27a~24-2* cluster, *miR-21* and *miR-155*. *miR-155* was found in a previously defined non-coding RNA (ncRNA) gene, *bic*17.

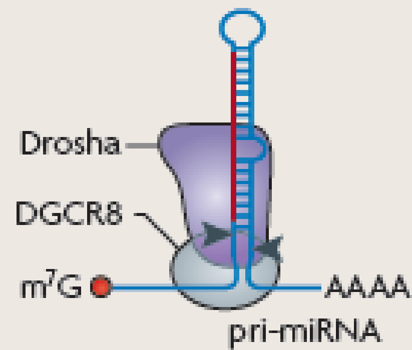


b | Intronic miRNAs in non-coding transcripts. For example, an *miR-15a~16-1* cluster was found in the fourth intron of a previously defined non-coding RNA gene, *DLEU2* (REF. 126). **c** | Intronic miRNAs in protein-coding transcripts. For example, an *miR-106b~93~25* cluster is embedded in the thirteenth intron of DNA replication licensing factor *MCM7* transcript (variant 1, which encodes isoform 1). The mouse *miR-06b~93~25* homologue is also found in the thirteenth intron of the mouse *MCM7* homologue gene15. The hairpins indicate the miRNA stem-loops. Orange boxes indicate the protein-coding region. This figure is not to scale.

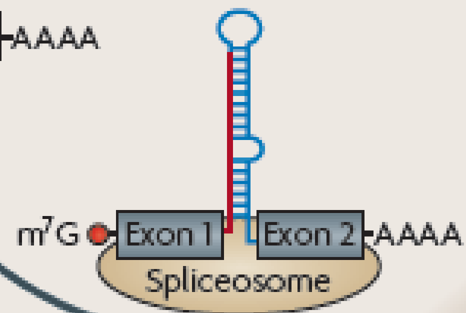
miRNA gene

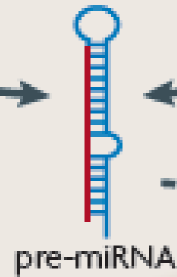

m7G
Drosha complex
Pri-miRNA
AAA
Pre-miRNA
XPO5
Dicer complex
m7G
Argonaute
AAA
miRISC
Mature miRNA
miRNA*

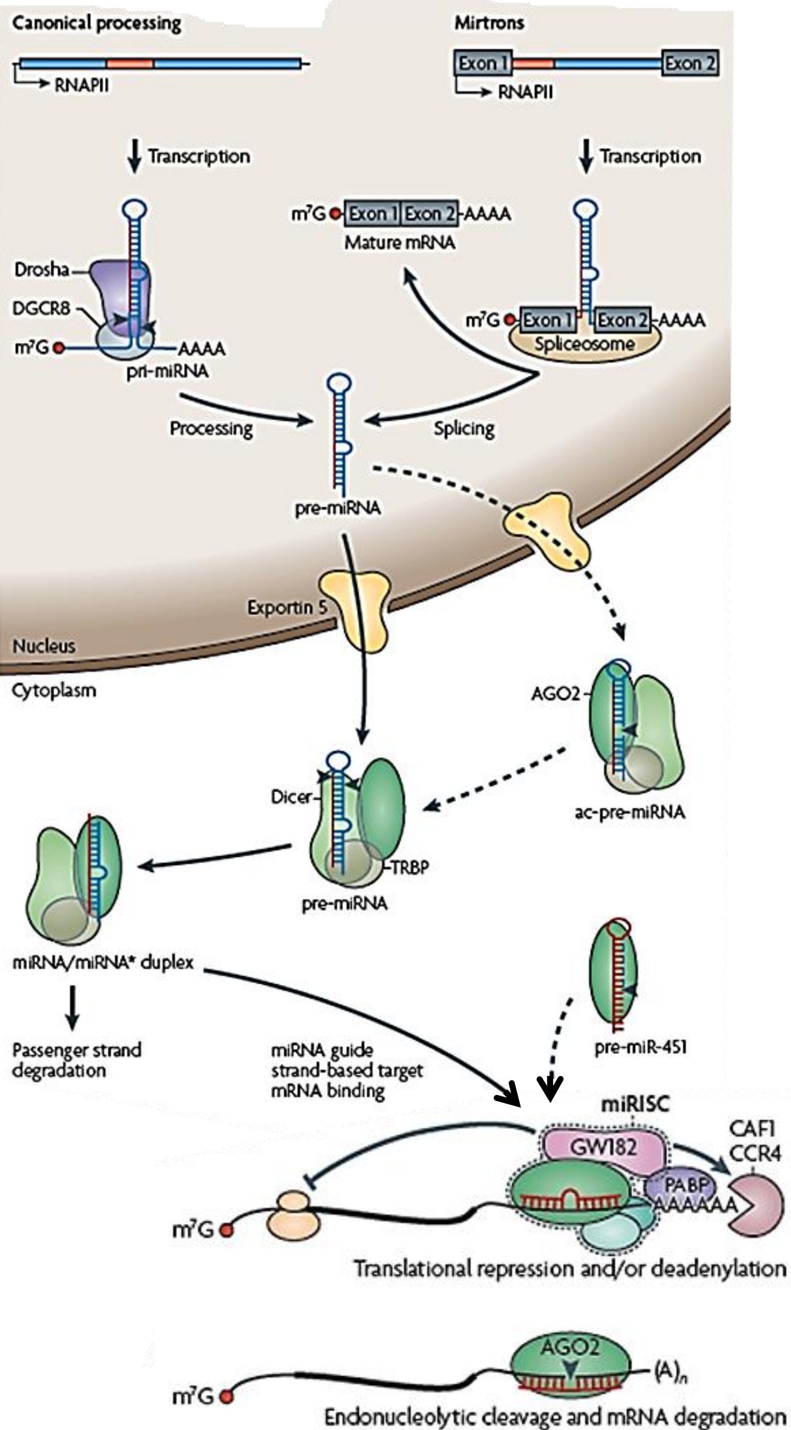

Canonical processing
RNAPII
Mirtrons
Exon 1
Exon 2
RNAPII
Transcription
Transcription


Drosha
DGCR8
m7G
AAAA
pri-miRNA

m7G
Exon 1
Exon 2
AAAA
Mature mRNA


m7G
Exon 1
Exon 2
AAAA
Spliceosome


Processing
Splicing
pre-miRNA
Exportin 5
Nucleus



MicroRNAs (miRNAs) are processed from RNA polymerase II (RNAPII)-specific transcripts of independent genes or from introns of protein-coding genes. In the canonical pathway, primary precursor (pri-miRNA) processing occurs in two steps, catalysed by two members of the RNase III family of enzymes, Drosha and Dicer, operating in complexes with dsRNA-binding proteins (dsRBPs), for example DGCR8 and transactivation-responsive (TAR) RNA-binding protein (TRBP) in mammals. In the first nuclear step, the Drosha–DGCR8 complex processes pri-miRNA into an ~70-nucleotide precursor hairpin (pre-miRNA), which is exported to the cytoplasm. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing the Drosha–DGCR8 step. In either case, cleavage by Dicer, assisted by TRBP, in the cytoplasm yields an ~20-bp miRNA/miRNA* duplex. In mammals, argonaute 2 (AGO2), which has robust RNaseH-like endonuclease activity, can support Dicer processing by cleaving the 3′ arm of some pre-miRNAs, thus forming an additional processing intermediate called AGO2-cleaved precursor miRNA (ac-pre-miRNA)70. Processing of pre-miR-451 also requires cleavage by AGO2, but is independent of Dicer and the 3′ end is generated by exonucleolytic trimming. Following processing, one strand of the miRNA/miRNA* duplex (the guide strand) is preferentially incorporated into an miRNA-induced silencing complex (miRISC), whereas the other strand (passenger or miRNA*) is released and degraded (not shown). Generally, the retained strand is the one that has the less stably base-paired 5′ end in the miRNA/miRNA* duplex. miRNA* strands are not always by-products of miRNA biogenesis and can also be loaded into miRISC to function as miRNAs. See BOX 2 for details of miRISC function. GW182, glycine-tryptophan protein of 182 kDa; m7G, 7-methylguanosine-cap; PABP, poly(A) binding protein.
(*From Krol 2010 NRG, modified*)

Examples from first discovered miRNA-mRNA pairs.
The 5’-end sequence of miRNA is the main determinant of targeting:
Continuous Watson-Crick pairing is required here (6-8 nt).
Target sequences mainly in mRNA 3’-UTR.

Example

```
HMGA2    5' CCGACAUUCAAUUUCUACCUCA 3'
                   : | :| ||||||||
let-7a   3' UUGAUAUGUUGGAUGAUGGAGU 5'

NF2      5' UACAAGAGAUUCUCCUGCCUCA 3'
             || | | | :|| ||:|||||
let-7a   3' UUGAUAUGUUGGAUGAUGGAGU 5'

E2F2     5' GUGGGUGCU-CUGGGCUGAACCA  3'
             ||  | || |||::|||| |||
miR-24   3' GACA-AGGACGACUUGACUCGGU  5'

DNMT3B   5' UGGCAAAGAAGAUGUUUUGUGGUGCACUGAG 3'
              :||||      |||:|||:|||||||||
miR-148  3'  -UGUUU......CAAGACAUCACGUGACU- 5'
```

This sequence loops out

Additional example

Note that U:G pairs are allowed in dsRNA

AAAAAAAAAAA

3'

5'

nucleus or «seed» sequence

myotrophin 3' UTR

miR-375

Blue: weak
Red: strong

Filipowich et al., 2008

Nature Reviews | Genetics

As a rule of thumb, miRNA recognize their targets using 6-8 nt at their 5'-end, followed by variable loop and some additional complementarity towards 3'-end.

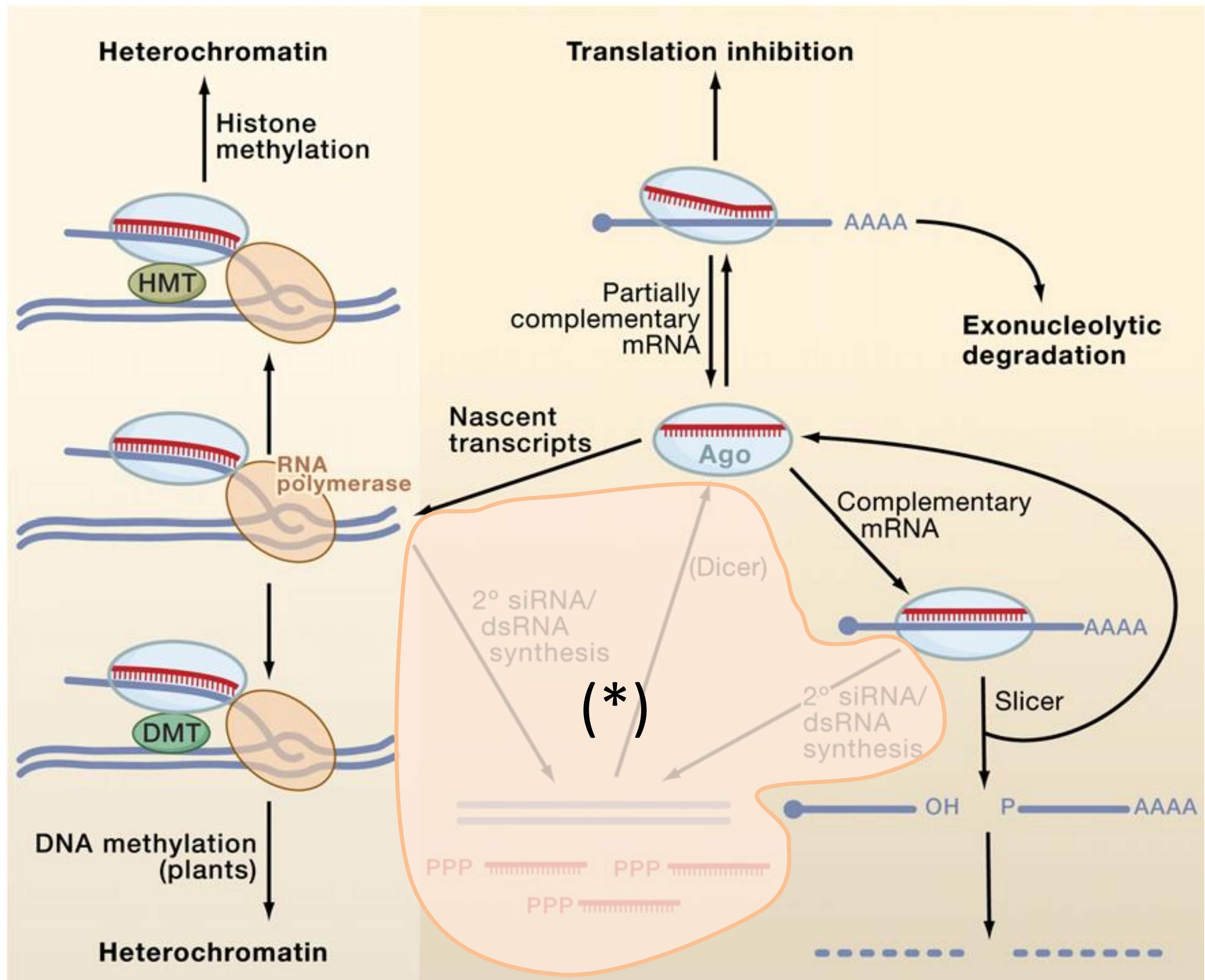
Due to this variable recognition mode, each miRNA recognizes multiple mRNA (or other RNAs) with variable affinity (can calculate from duplex stability).

The union of possible RNA targets of a specific miRNA is called «regulome».

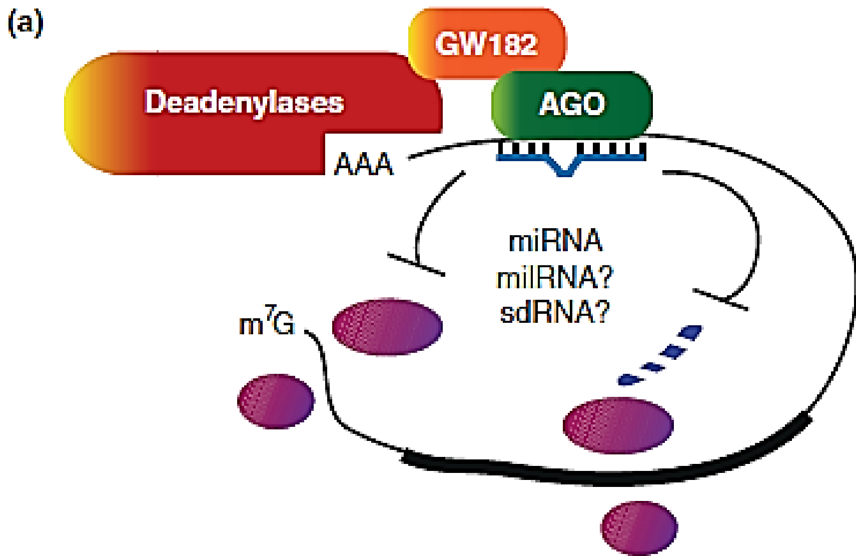
Several algorithms were developed to predict miRNA targets, and are available on websites. However, due to uncertainty of recognition modes, these algorithms work clearly in sub-optimal way.

How do small interfering RNA exert their silencing effects?

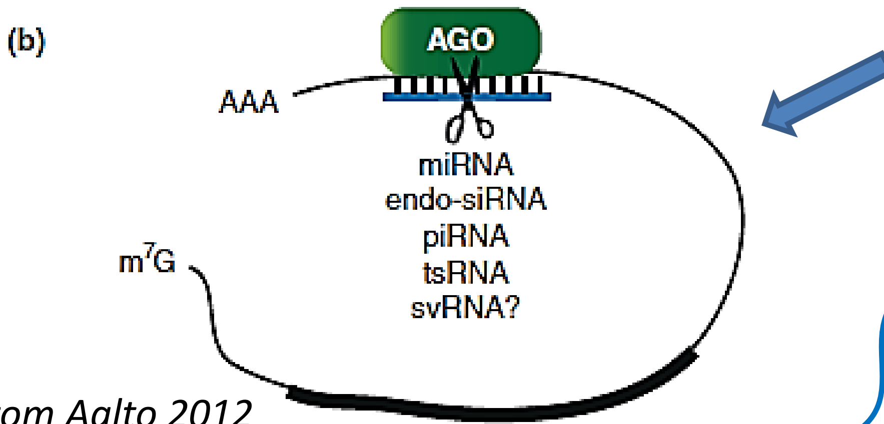
Different mechanisms



Heterochromatin
Histone methylation
HMT
RNA polymerase
DMT
DNA methylation (plants)
Heterochromatin
Translation inhibition
AAAA
Exonucleolytic degradation
Partially complementary mRNA
Nascent transcripts
Ago
Complementary mRNA
AAAA
Slicer
OH
P
AAAA
(Dicer)
2° siRNA/ dsRNA synthesis
2° siRNA/ dsRNA synthesis
(*)
PPP
PPP
PPP



miRNAs partially pair to mRNA targets leading to mRNA degradation by GW182/deadenylases bound to Argonaute (AGO);
or miRISC inhibits translation initiation;
or stalls translation elongation;
or stimulates proteolysis of nascent peptides encoded by the target mRNA.



Perfect pairing of many types of sRNAs results in cleavage of the target mRNA by **Ago2**.

From Aalto 2012

Endo-siRNAs, piRNAs and possibly many of the sRNAs derived from protein-coding regions of the genome direct DNA and histone modifications (red stars) that regulate transcriptional activity