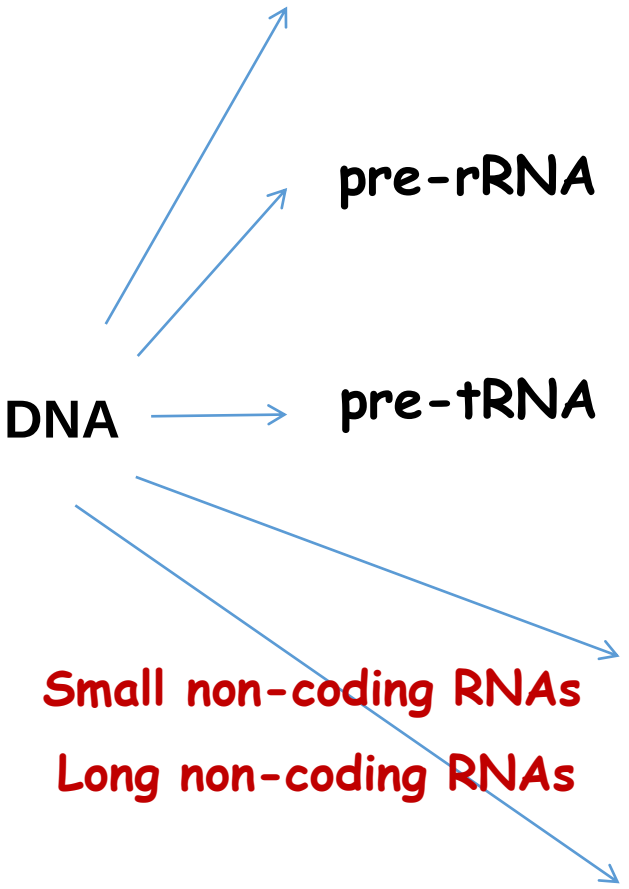
Regulatory mechanisms mediated by RNA

Functional types of RNA



hnRNA **mRNA** Translation; encodes a sequence of proteins

pre-rRNA **rRNA** Translation; part of the ribosome

pre-tRNA **tRNA** Translation; transfer / amino acids activation

Small non-coding RNAs

Long non-coding RNAs

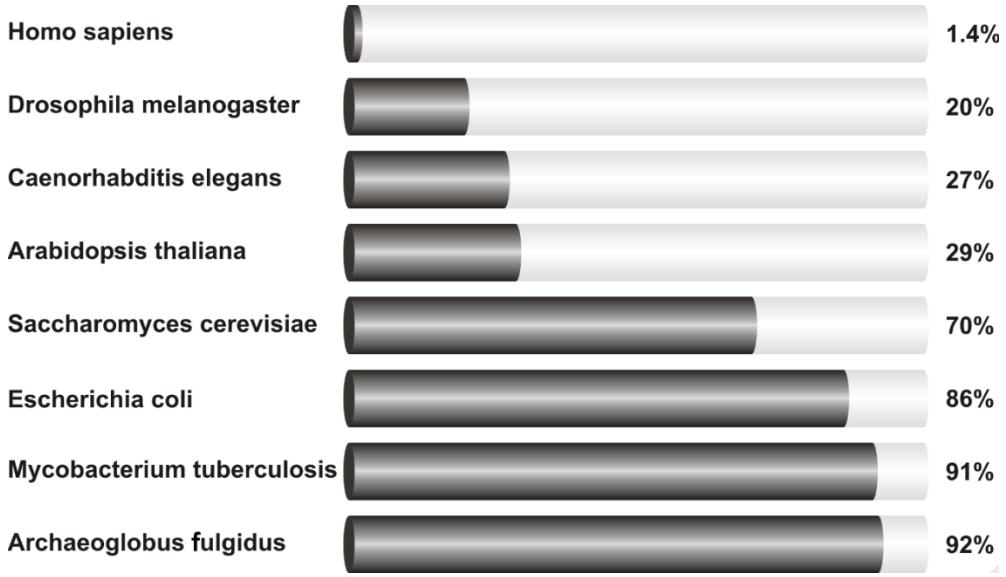
snRNA, snoRNA, ..Splicing / modification of RNA

siRNA, miRNA, … **Regulation of gene expression**

World of noncoding RNAs

encoding genes represent less than 2% of the total genome sequence
vs.
at least 90% of the human genome is actively transcribed
the more complex organism, the more it comprises non-coding RNAs

The percentage of protein-coding genes sequences in several eukaryotic and bacterial genomes.



Recent evidence suggests that the non-coding RNAs (ncRNAs) may play major biological roles in cellular development, physiology and pathologies.
NcRNAs could be grouped into two major classes based on the transcript size: small ncRNAs and long ncRNAs.

Small non-coding RNAs

miRNA
siRNA
piRNA
snoRNA
PARS
tiRNA

microRNA (miRNA)
Piwi-interacting RNA (piRNA)
small interfering RNA (siRNA)
small nucleolar RNA (snoRNAs)
tRNA-derived small RNA (tsRNA)
small rDNA-derived RNA (srRNA)
small nuclear RNA,
also commonly referred to as U-RNA

Long non-coding RNAs

lincRNA
TERRAs
T-UCR

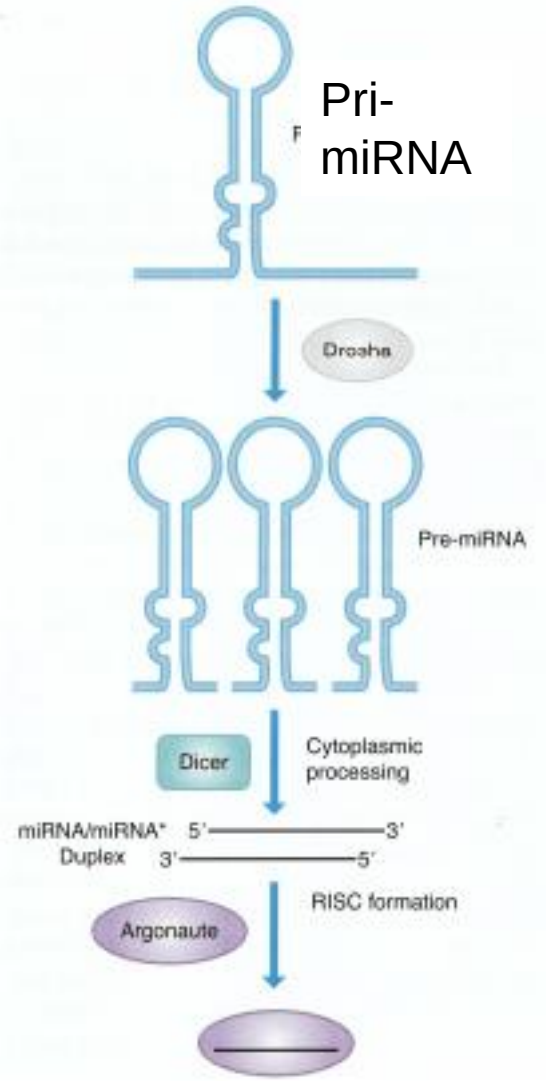
Table 1 Types of recently discovered human non-coding RNAs

	Class	Symbol	Characteristic	Disease / biological function associations
Small non-coding RNAs	MicroRNAs	miRNAs	18–25 nt; account 1–2% of the human genome; control the 50% of protein-coding genes; guide suppression of translation; Drosha and Dicer dependent small ncRNAs	initiation of various disorders including many, if not all, cancers / regulation of proliferation, differentiation, and apoptosis involved in human development
	Small interfering RNAs	siRNAs	19–23 nt; made by Dicer processing; guide sequence specific degradation of target mRNA	great potential in diseases treatment / posttranscriptional gene silencing mainly through RISC degradation mechanism; defence against pathogenic nucleic acids
	Piwi-interacting RNAs	piRNAs	26–30 nt; bind Piwi proteins; Dicer independent; exist in genome clusters; principally restricted to the germline and somatic cells bordering the germline	relationship between piRNAs and diseases has not yet been discovered / involved in germ cell development, stem self-renewal, and retrotransposon silencing
	Small nucleolar RNAs	snoRNAs	60–300 nt; enriched in the nucleolus; in vertebrate are excised from pre-mRNA introns; bind snoRNP proteins	association with development of some cancers / important function in the maturation of other non-coding RNAs, above all, rRNAs and snRNAs; miRNA-like snoRNAs regulate mRNAs
	Promoter-associated small RNAs	PASRs	20–200 nt; modified 5′ (capped) ends; coincide with the transcriptional start sites of protein- and non-coding genes; made from transcription of short capped transcripts	relationship with diseases has not yet been discovered / involved in the regulation of the transcription of protein-coding genes by targeting epigenetic silencing complexes
	Transcription initiation RNAs	tiRNAs	~ 18 nt ; have the highest density just downstream of transcriptional start sites; show patterns of positional conservation; preferentially located in GC-rich promoters	
	Centromere repeat associated small interacting RNAs	crasiRNAs	34–42 nt; processed from long dsRNAs	relationship between crasiRNAs and diseases has not yet been discovered / involved in the recruitment of heterochromatin and/or centromeric proteins
	Telomere-specific small RNAs	tel-sRNAs	~ 24 nt; Dicer independent; 2′-O-methylated at the 3′ terminus; evolutionarily conserved from protozoa to mammals; have not been described in human up to now	relationship between tel-sRNAs and diseases has not yet been discovered / epigenetic regulation
	Pyknons		subset of patterns of variable length; form mosaics in untranslated and protein-coding regions; more frequently in 3′ UTR	expected association with cancer biology / possible link with posttranscriptional silencing of genes, mainly involved in cell communication, regulation of transcription, signaling, transport,
Long non-coding RNAs	Long intergenic non-coding RNAs	lincRNAs	ranging from several hundreds to tens of thousands nts; lie within the genomic intervals between two genes; transcriptional cis-regulation of neighbouring genes	involved in tumorigenesis and cancer metastasis / involved in diverse biological processes such as dosage compensation and/or imprinting
	Long intronic non-coding RNAs		lie within the introns; evolutionary conserved; tissue and subcellular expression specified	aberrantly expressed in human cancers / possible link with posttranscriptional gene silencing
	Telomere-associated ncRNAs	TERRAs	100 bp - >9 kb; conserved among eukaryotes; synthesized from C-rich strand; polyadenylated; form inter-molecular G-quadruplex structure with single-stranded telomeric DNA	possible impact on telomere-associated diseases including many cancers / negative regulation of telomere length and activity through inhibition of telomerase
	Long non-coding RNAs with dual functions		both protein-coding and functionally regulatory RNA capacity	deregulation has been described in breast and ovarian tumors / modulate gene expression through diverse mechanisms
	Pseudogene RNAs		gene copies that have lost the ability to code for a protein; potential to regulate their protein-coding cousin; made through retrotrans-position; tissue specific	often deregulated during tumorigenesis and cancer progression / regulation of tumor suppressors and oncogenes by acting as microRNA decoys
	Transcribed-ultraconserved regions	T-UCRs	longer than 200 bp; absolutely conserved between orthologous regions of human, rat, and mouse; located in both intra- and intergenic regions	expression is often altered in some cancers; possible involvement in tumorigenesis / antisense inhibitors for protein-coding genes or other ncRNAs

RNA interference - RNAi

- **sequence-specific gene silencing mechanism triggered by double stranded RNA, on the post-transcriptional level or transcriptional level**
- inhibitory elements are small RNA molecules **(miRNAs, siRNAs….)**
- **miRNAs** generated by cleavage of **larger pre-miRNA molecules**
- nucleases **Drosha and DICER**, which are compiled into multiprotein complex **RISC** (RNA-induced silencing complex) with **proteins Argonaut**
- RNA interference is a process by which **noncoding RNA molecules** interfere (pair) with target regions of mRNA, resulting in **prevention of gene expression** of these mRNAs.
- For short, this proces is also called RNAi. We rank him among **posttransriptional mechanisms** of gene expression.

- **Most eucaryotic organisms is capable of RNA interference**, the process was first studied in the C. elegans.



RNA interference

RISC has helicase activity, thanks to which miRNA is loosened; only one chain remains associated with the complex

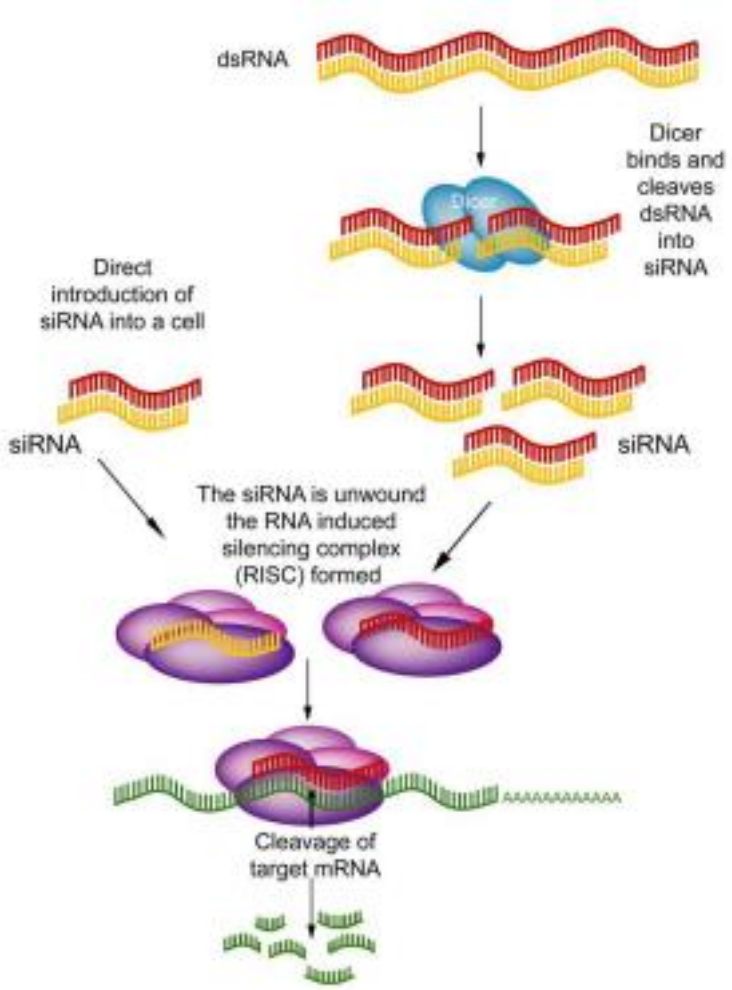
that **allows sequence-specific binding of the whole complex to the target complementary mRNA**

nuclease activity of RISC complex **cleaves the mRNA** - its degradation occurs

Originally protecting cells against viruses

common in eukaryotic cells

useful for targeted inactivation of genes: research of gene functions



Discovery of RNA interference (1998)

- silencing of gene expression with dsRNA

The Nobel Prize in Physiology or Medicine 2006

"for their discovery of RNA interference - gene silencing by double-stranded RNA"

Photo: L. Cicero

Andrew Z. Fire

Photo: J. Mottern

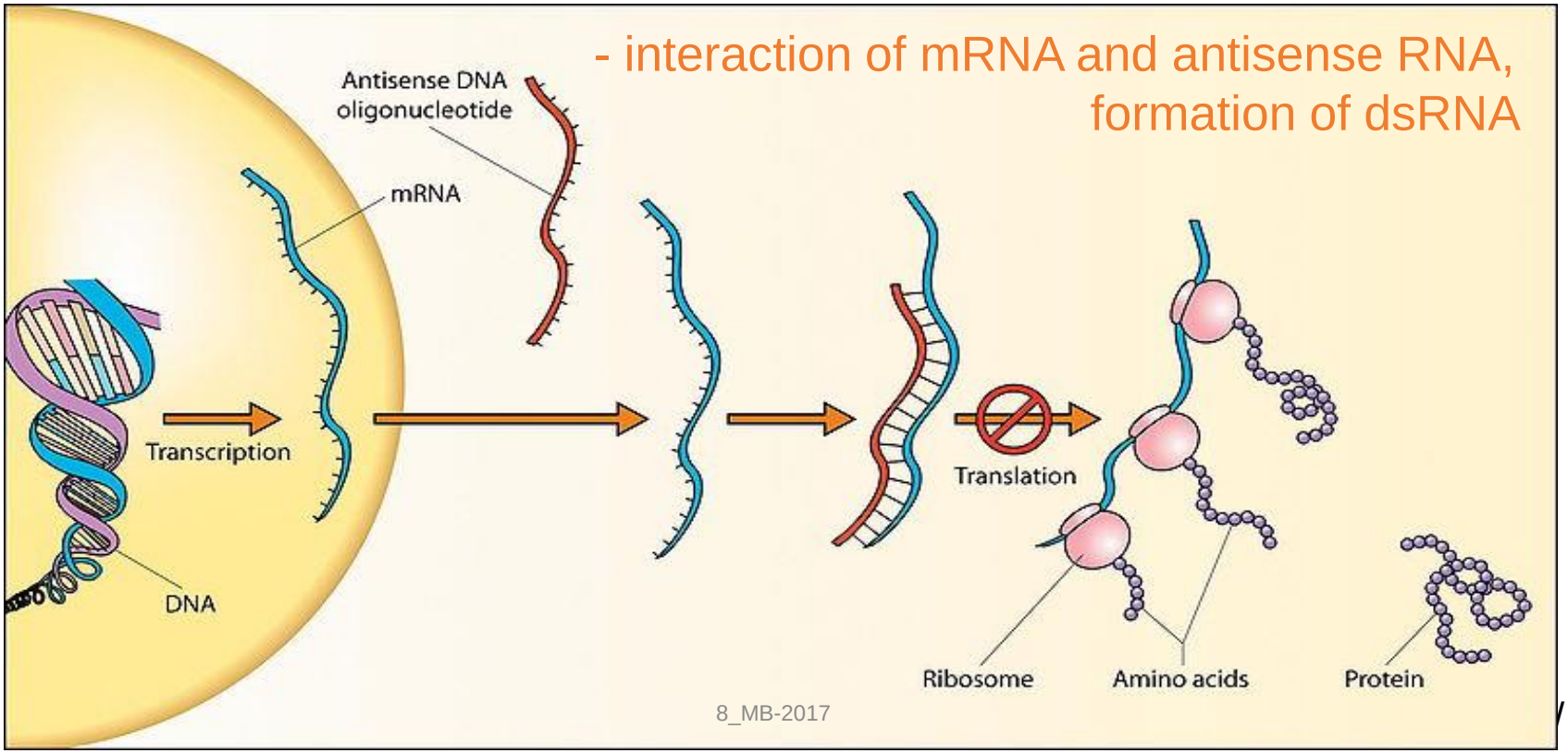
Craig C. Mello

Cenorhabditis elegans

Antisense RNA (= **RNA komplementární k mRNA**) can silence gene expression

(již počátek 80. let 20. století)

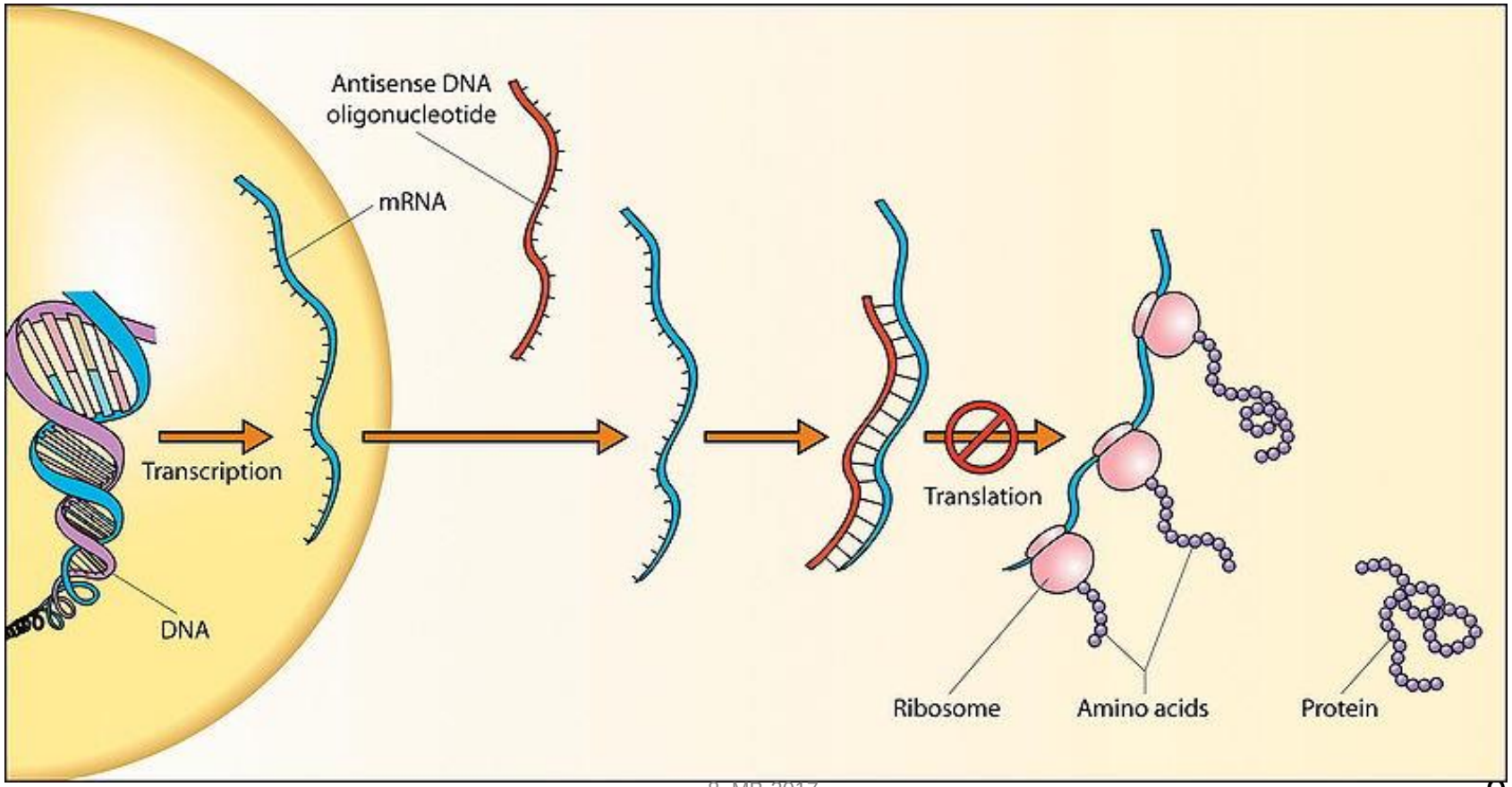
- direct introduction of antisense RNA (or transcription in reverse orientation)



What is the mechanism behind?

<u>Original (!)</u> hypotheses:

- antisense RNA mechanically prevents translation
- dsRNA is degraded (RNases)



Cosuppression in Petunia

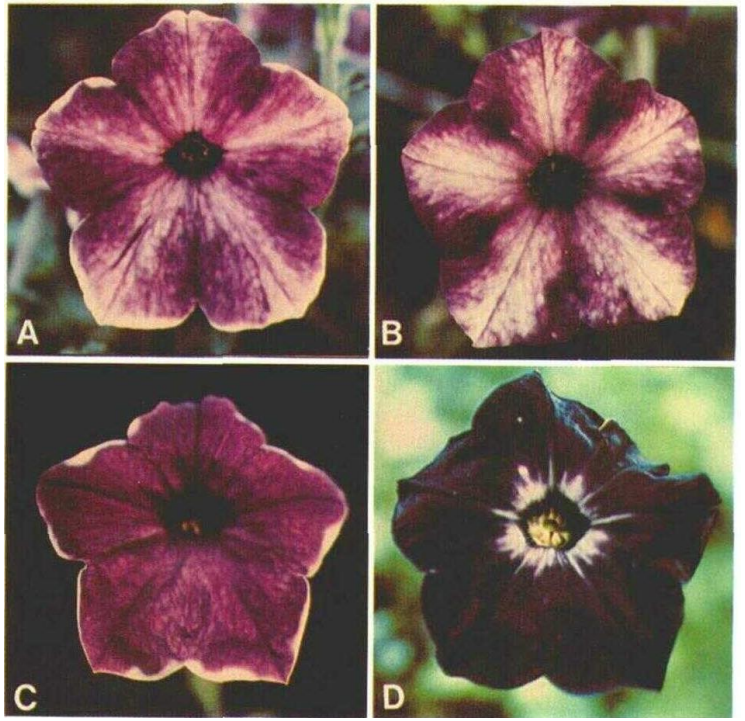
Aim: increase expression of pigment-synthetizing enzym

Result: loss of pigmentation in flower segments

Napoli et al. 1990 Plant Cell 2:279–289

Cosuppression in Petunia

Expression of antisense RNA was less efficient!!!



Napoli et al. 1990 Plant Cell 2:279–289

Mechanism see later!

What they get the Nobel prize for?


Photo: L. Cicero

Andrew Z. Fire


Photo: J. Mottern

Craig C. Mello

- making proper controls pays off!

- Introduction of even very small amount of dsRNA induce specific silencing (**antisence RNA is less efficient!**)

dsRNA has to be a <u>signal</u>!

- for sequence specific silencing

Andrew J. Hamilton David C. Baulcombe*

Small Antisense RNA

Science 286 (5441): 950-952

RNA interference (RNAi)

= silencing of gene expression mediated by small RNAs (**s**mall RNA, sRNA)
in plants predominantly - 21-24 nt

The precise role of 25-nt RNA in PTGS remains to be determined. However, because **they are long enough to convey sequence specificity** yet **small enough to move through plasmodesmata**, it is possible that **they are components of the systemic signal and specificity determinants of PTGS** (Hamilton and Baulcombe, 1999).

RNA interference (RNAi)
gene silencing at

- transcriptional level (TGS)

  (transcriptional gene silencing)

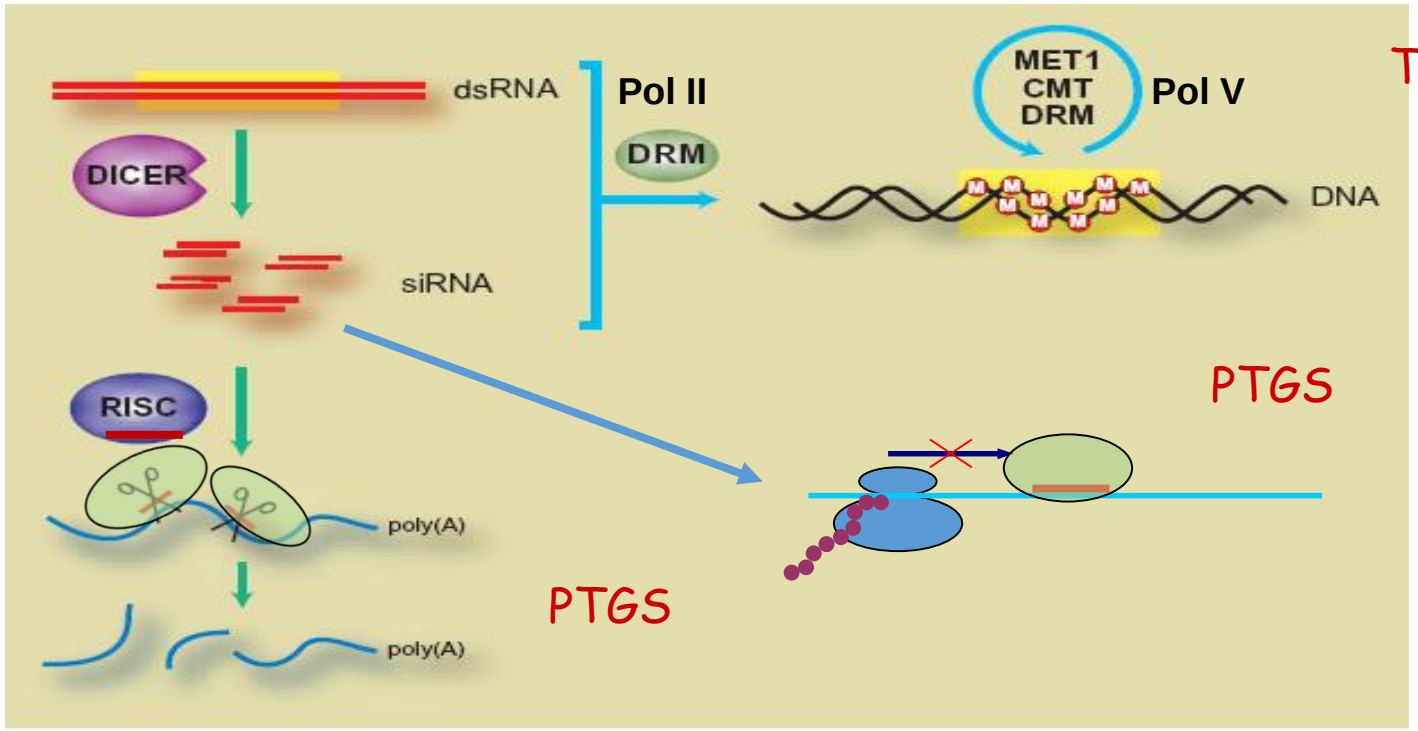
  - **induction of DNA methylation** (mRNA not formed)

- posttranscriptional level (PTGS)

  (posttranscriptional gene silencing)

  - transcript cleavage
  - block of translation

Mechanism of action of small RNA- depends on the length of sRNA, biogenesis (precursor), ...



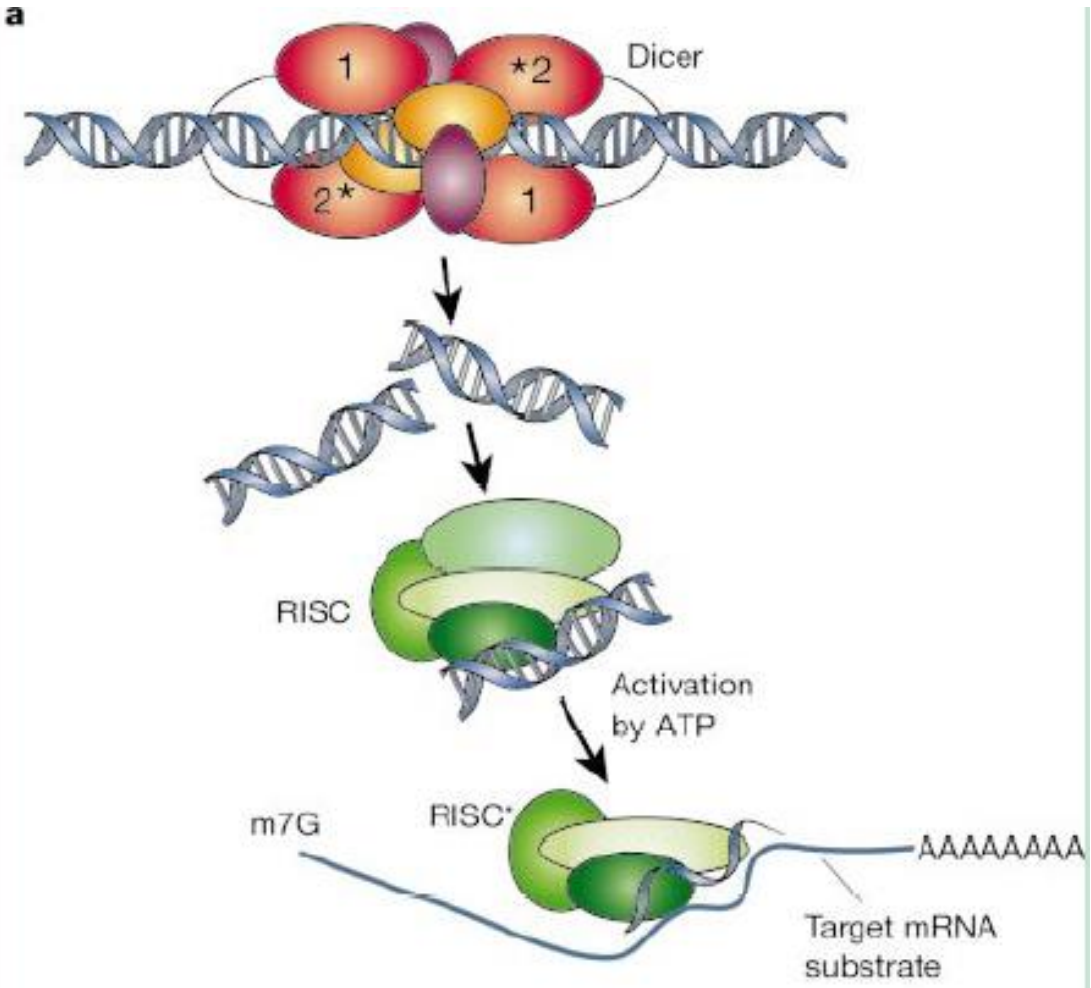
PTGS (posttranscriptional gene silencing):

- specific transcription degradation or translation blocking

TGS (transcriptional gene silencing):

- methylation of cytosines in the promoter (RdDM), heterochromatinization, inhibition of transcription factor binding

Basic mechanism of RNAi



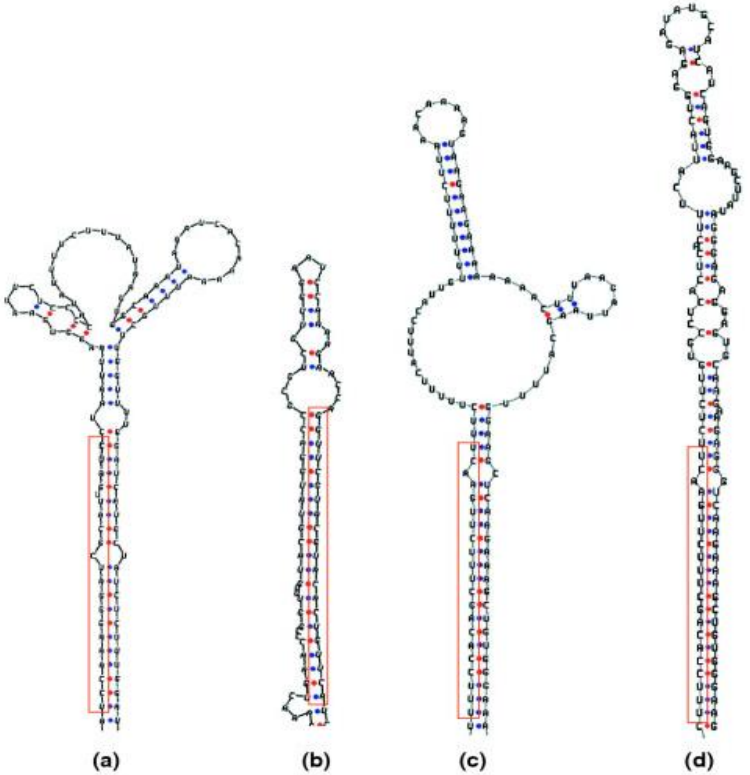
dsRNA in cell is cleaved by RNase DICER into short dsRNA fragments – sRNA

Argonaute with a single strand (from sRNA) mediates recognision of complementary sequences, which should be silenced (TGS, PTGS)

Small RNA in plants/animals

- 3’ end of sRNA methylated (HEN1) - protection
- **miRNA (micro) – from transcipts of RNA Pol II (pre-miRNA)**
  – hunderds MIR genes (*in trans*)

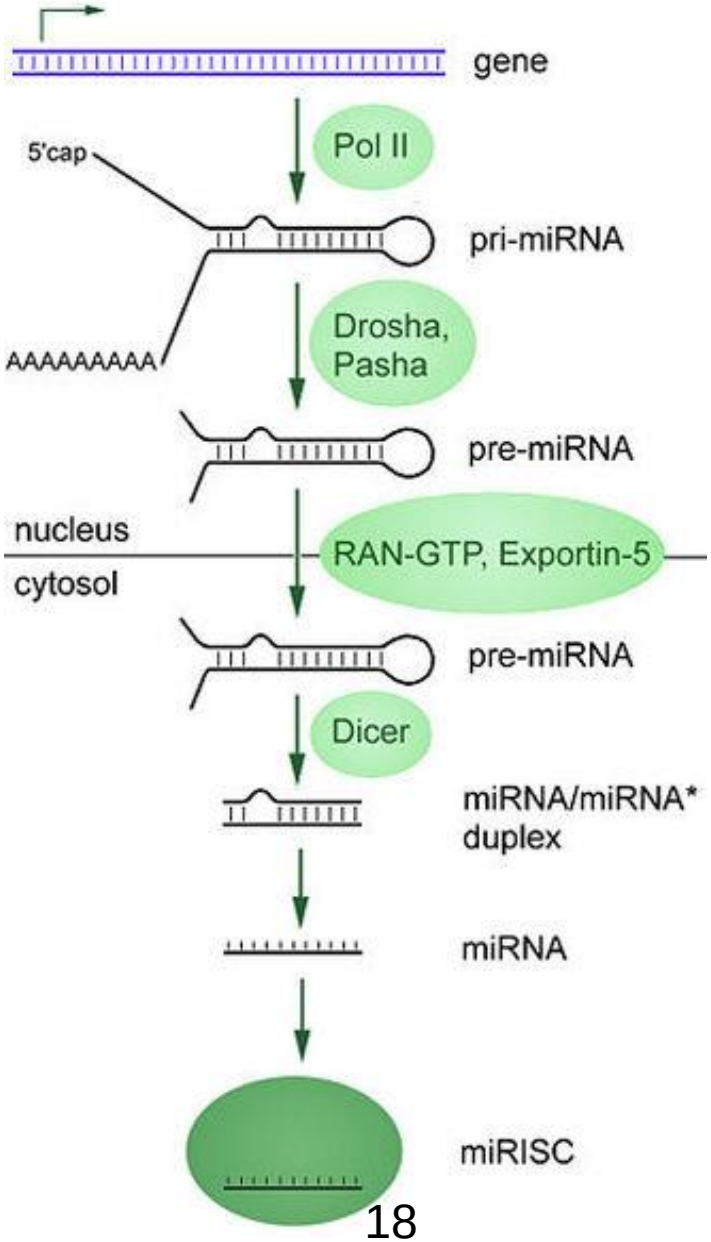
Pol II
DROSHA (Rnaze III),
PASHA (RNA binding protein),
DICER



- **siRNA (small interfering)** – from dsRNA of various origin (both internal and external – thousands types (both *in cis* and *in trans*)

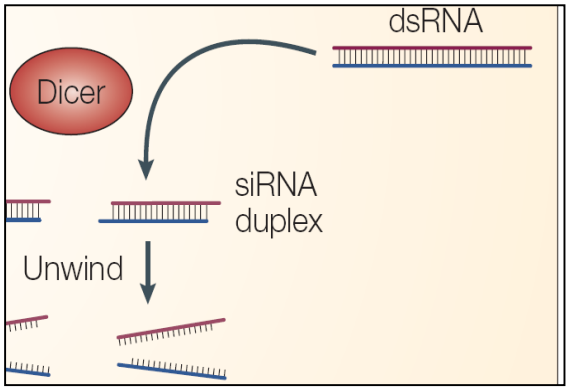
….. (+ piRNA in animals)

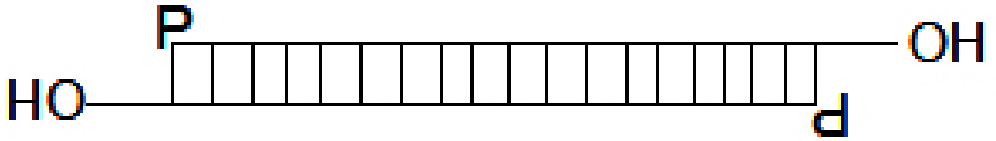
miRNA biogenesis



siRNA

• **siRNA (small interfering)** from dsRNA of various origin (both internal and external – thousands types (both *in cis* and *in trans*) ….. (+ piRNA in animals)

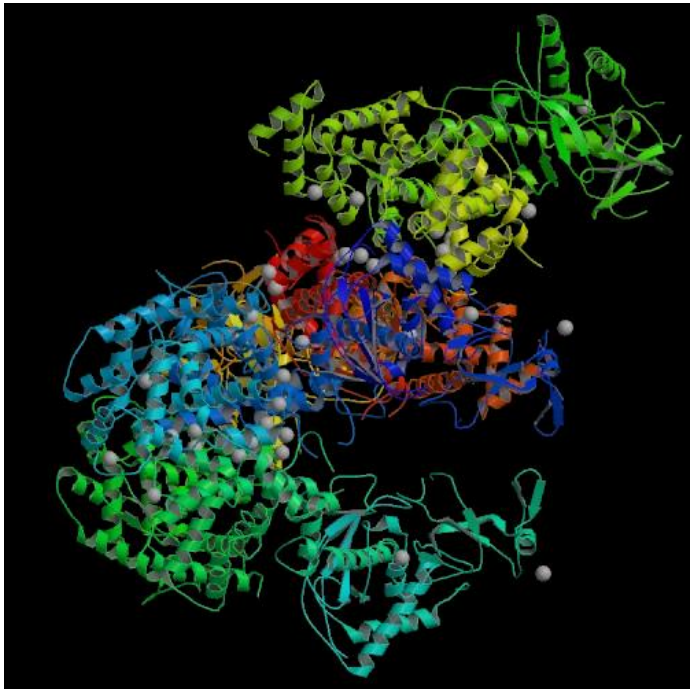




Schematic representation of a siRNA molecule: a ~19-21basepair RNA core duplex that is followed by a 2 nucleotide 3' overhang on each strand. OH: 3' hydroxyl; P: 5' phosphate.

DICER

Dicer, also known as **endoribonuclease Dicer** or **helicase with RNase motif**, is an enzyme that in humans is encoded by the *DICER1* gene. Being part of the RNase III family, Dicer cleaves double-stranded RNA (dsRNA) and pre-microRNA (pre-miRNA) into short double-stranded RNA fragments called small interfering RNA and microRNA, respectively. These fragments are approximately 20-25 base pairs long with a two-base overhang on the 3' end. Dicer facilitates the activation of the RNA-induced silencing complex (RISC), which is essential for RNA interference. RISC has a catalytic component argonaute, which is an endonuclease capable of degrading messenger RNA (mRNA).



Argonaute

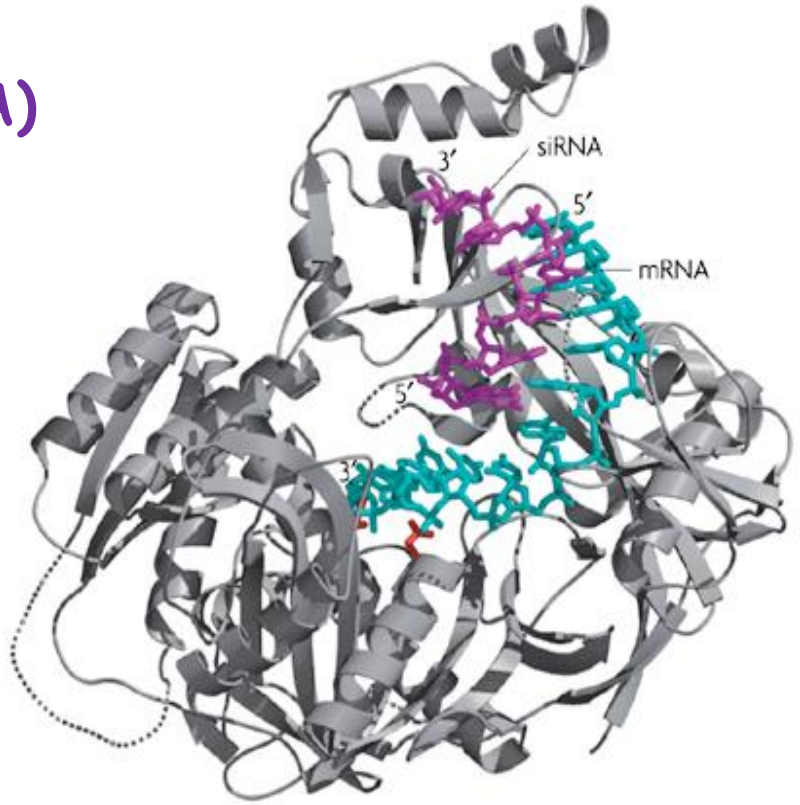
RNA binding protein (20-26 nt RNA)
- strand selection (5' nt, participation of HSP90)

- 10 genes in *Arabidopsis*

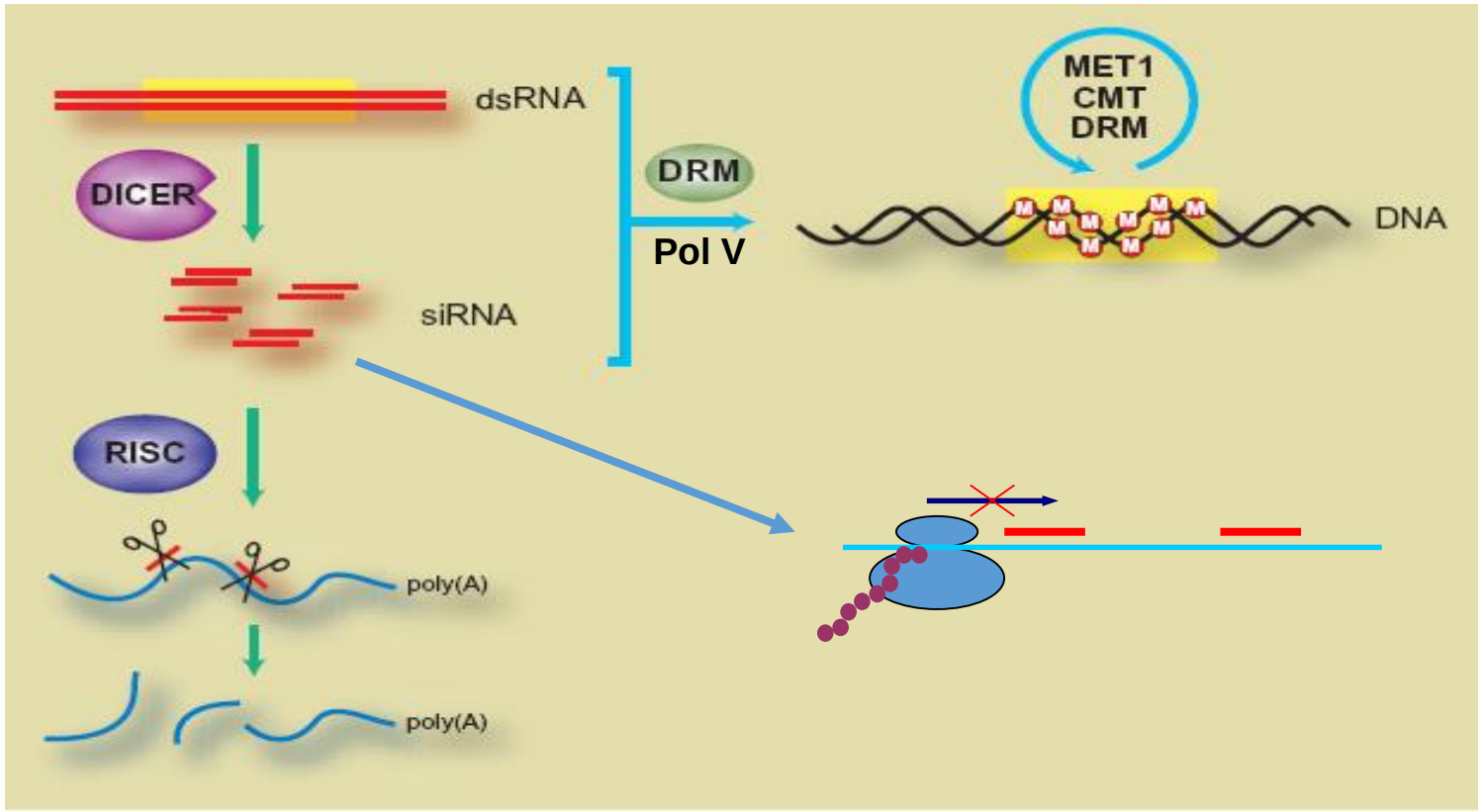
- main component of RISC (RNA induced silencing complex)

- block of translation or slicer (RNAse H-like endonuclease - PIWI doména)

- role in TGS (RdDM) (RNA directed DNA methylation)



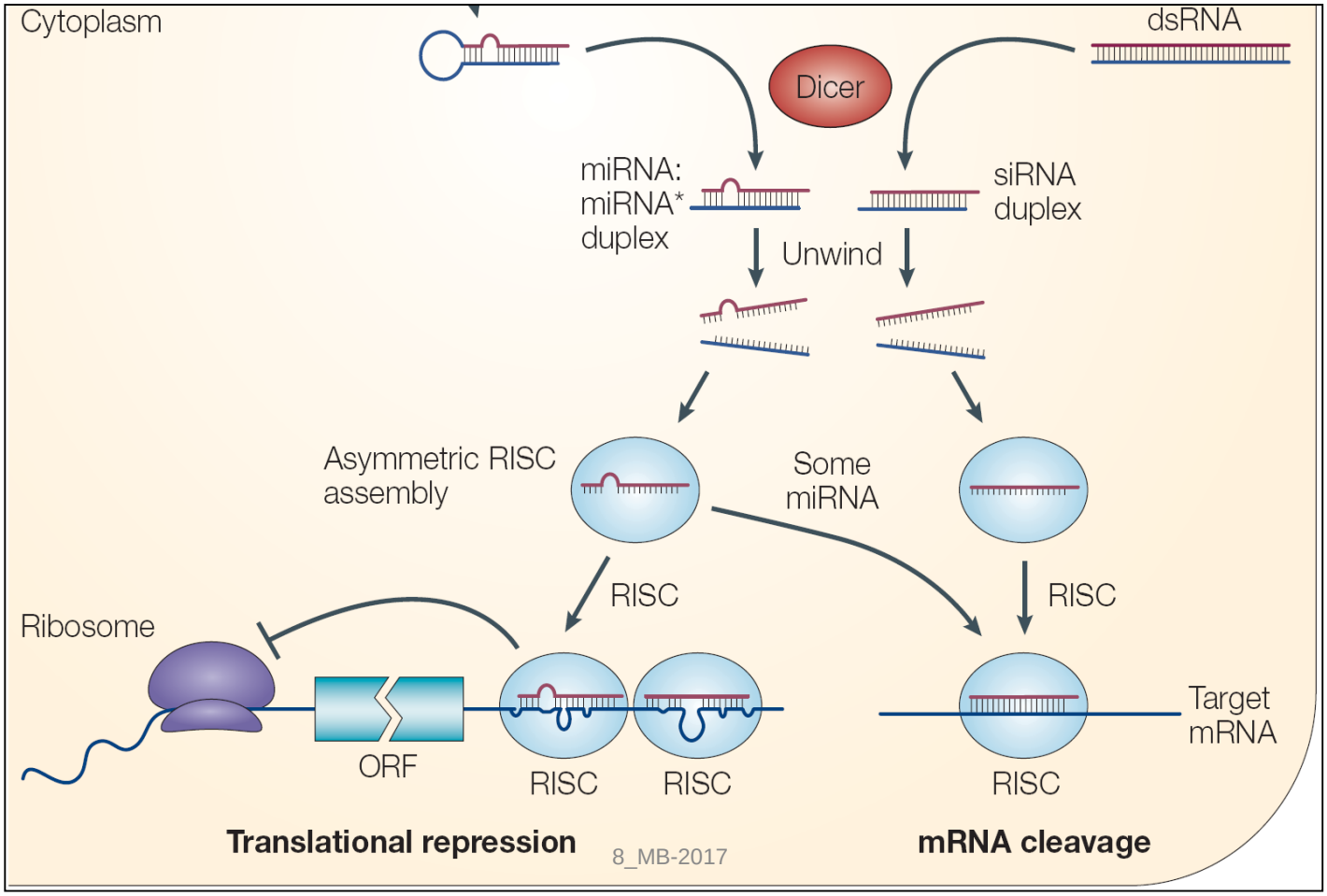
Mechanism of small RNA action - overview



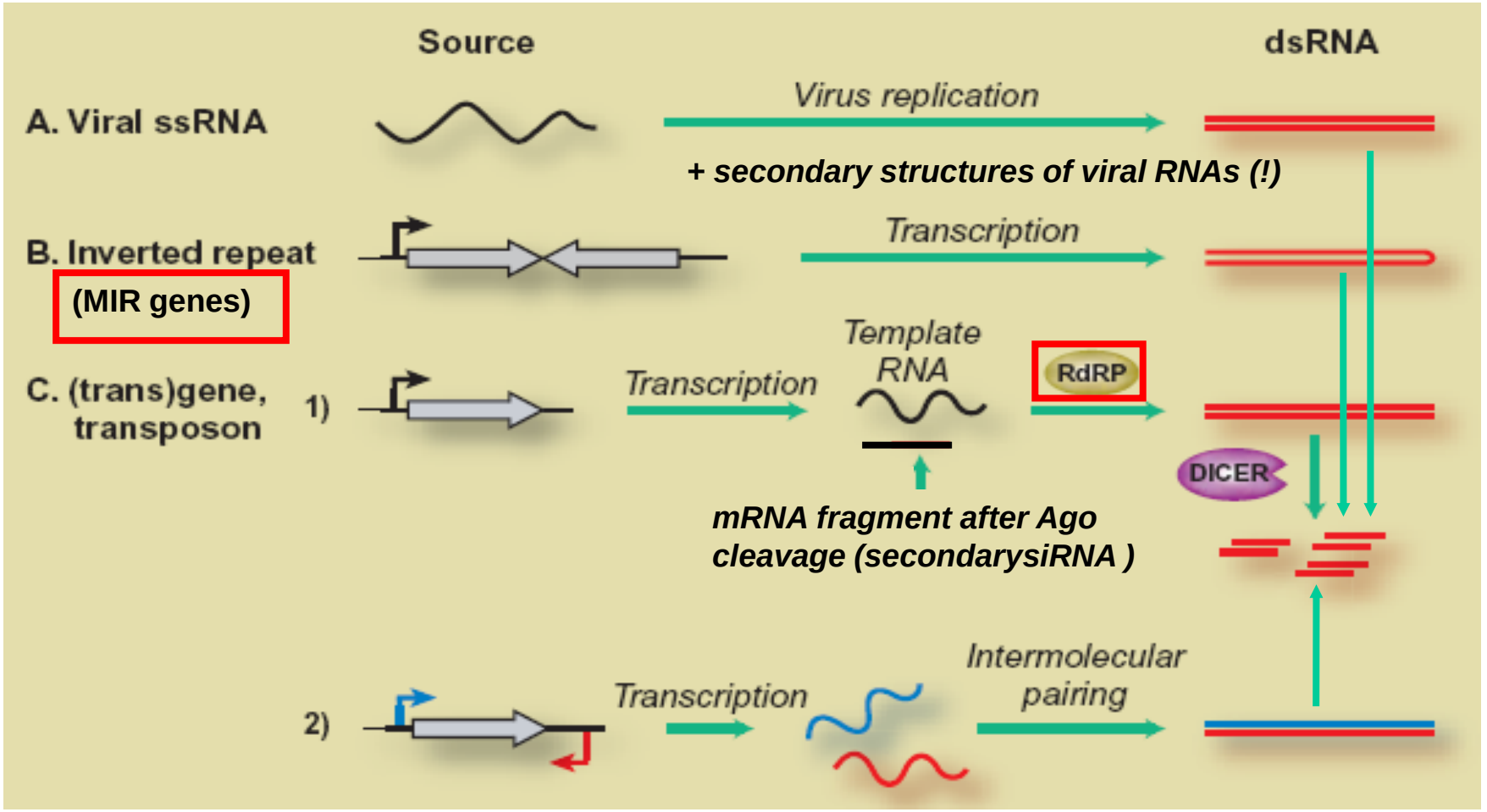
- specific cleavage of transcript
- block of translation

- methylation of promoter, heterochromatin formation
- preventing interaction of transcription factors

sRNA mode of action also depends on complementarity



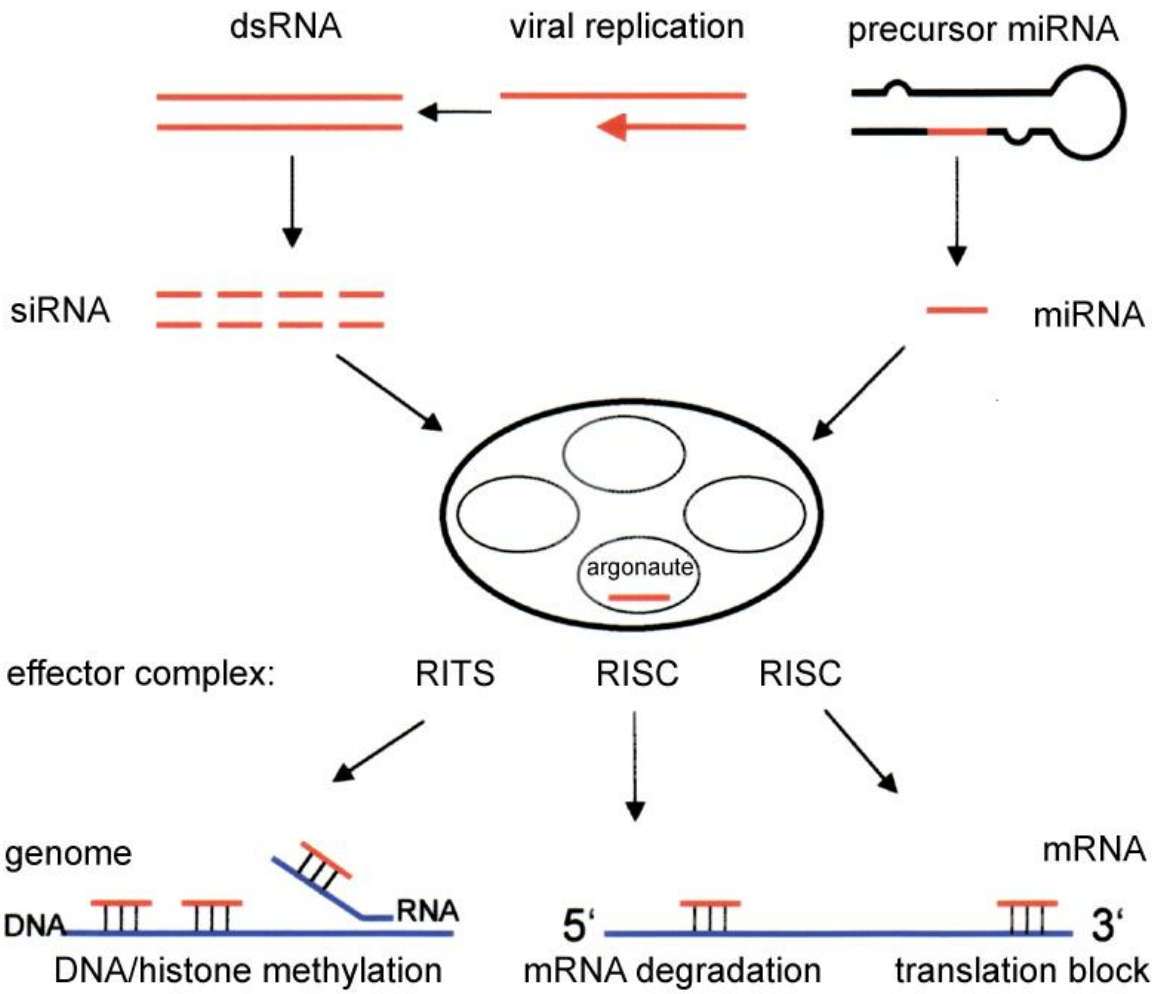
dsRNA formation



- RdRP = RNA-dependent RNA Polymerase – synthesis of compl. RNA strand templates:
  - transcripts cleaved by RISC
  - impaired mRNAs (without polyA or cap)
  - transcripts of RNA polymerase IV

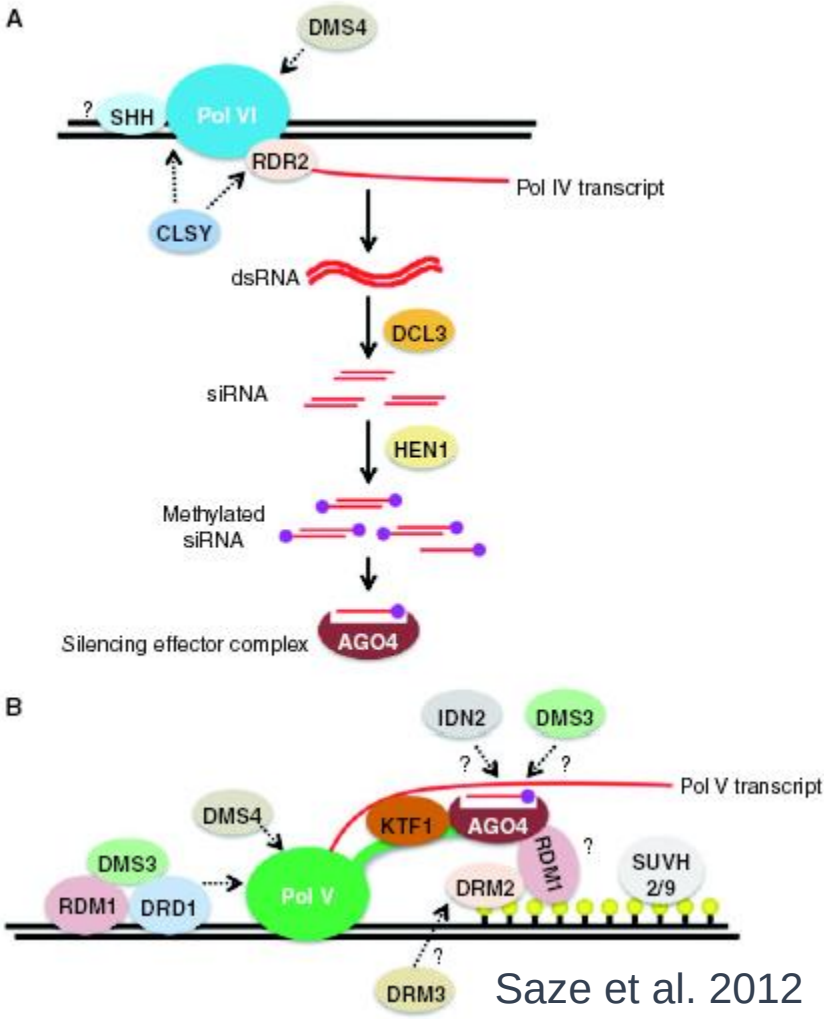
Overview of RNA interference

Overview of RNA interference. The dicer enzymes produce siRNA from double-stranded RNA and mature miRNA from precursor miRNA. miRNA or siRNA is bound to an **argonaute enzyme** and an effector complex is formed, either a **RISC (RNA-induced silencing complex)** or **RITS (RNA-induced transcriptional silencing**) complex. RITS affects the rate of transcription by histone and DNA methylation, whereas RISC degrades mRNA to prevent it from being translated.



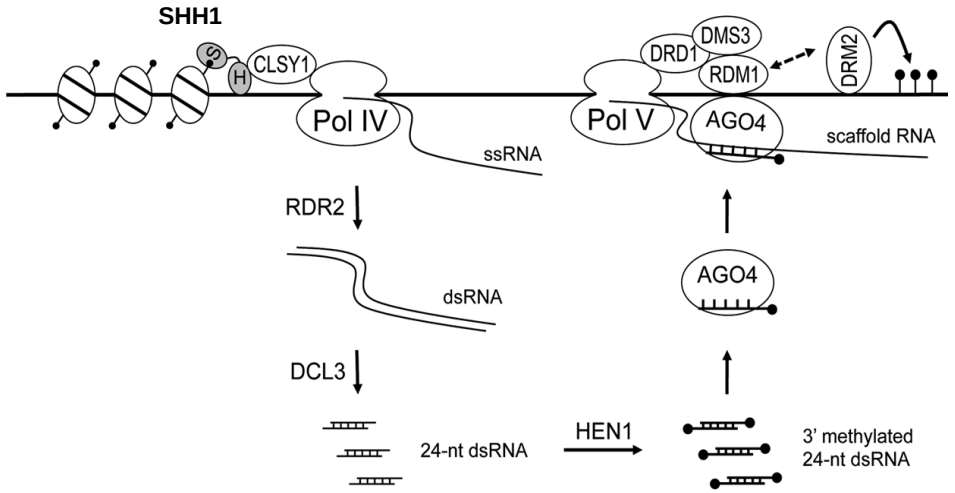
Matzke MA, Matzke AJM – This figure is adapted from one by Matzke MA, Matzke AJM (2004) Planting the Seeds of a New Paradigm. PLoS Biol 2(5): e133 doi:10.1371/journal.pbio.0020133.

RNA-directed DNA methylation (in detail)



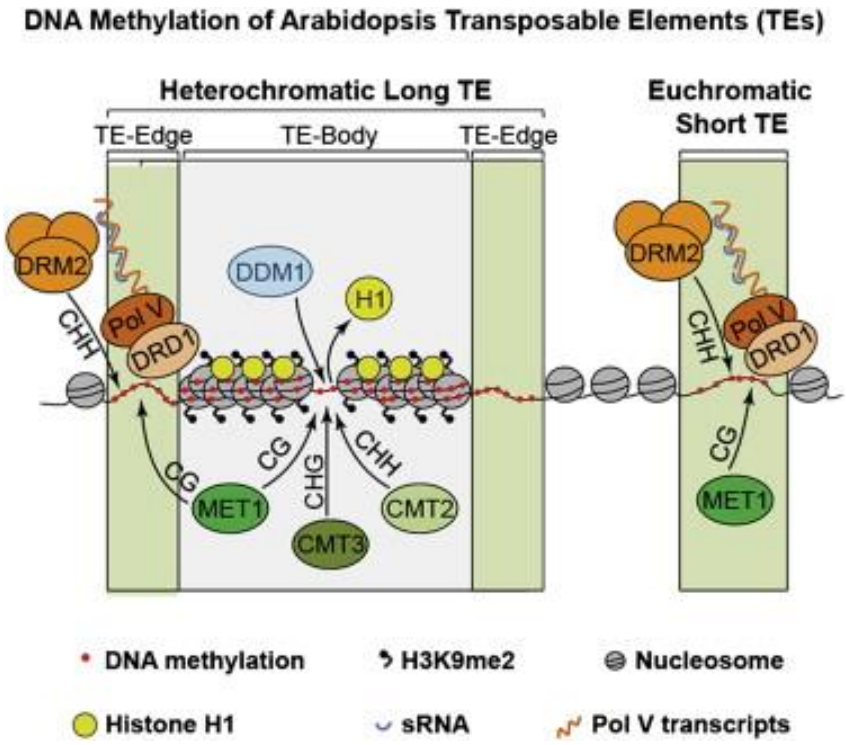
Saze et al. 2012

Pol IV a V – RNA polymerases
RDR2 - RNA dep.RNA polymerase
DCL3 – dicer-like protein
AGO4 – ARGONAUTE
DRM2 – *de novo* metyltransferase
DRD1 – chromatin remodelling protein
SHH1 – dual histon-code reading
(H3K9me2, H3K4)

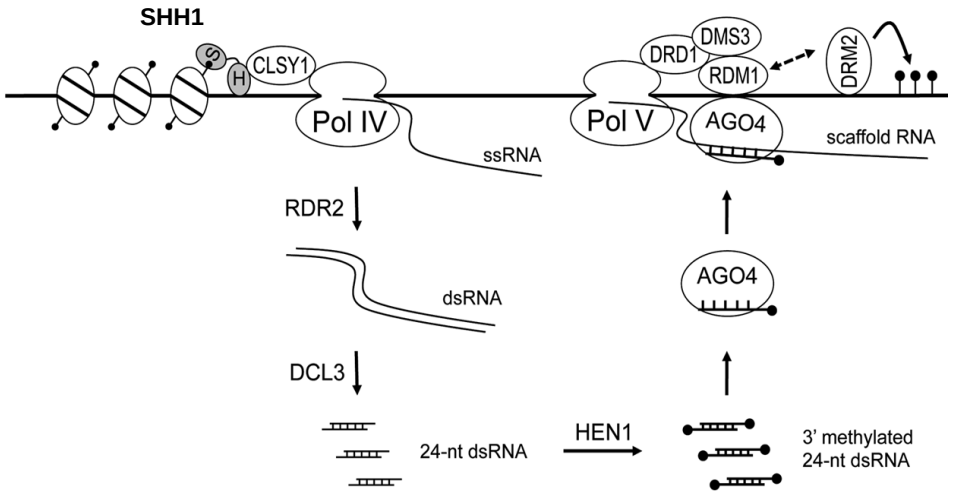


Zhang et al. 2013

RNA-directed DNA methylation
– why so complicated and energy consuming?



Zemach et al. 2013



Zhang et al. 2013

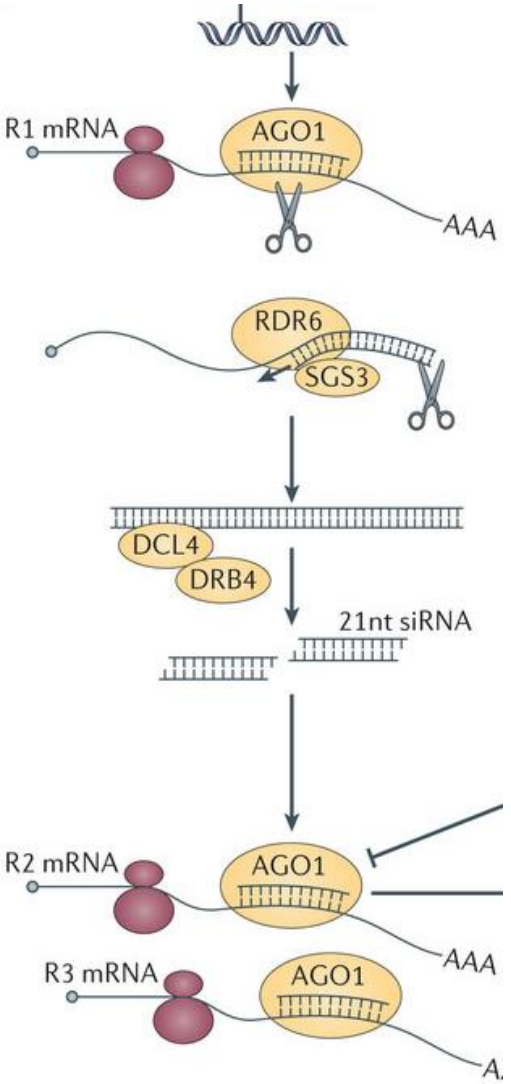
Secondary siRNA formation

- target RNA (mRNA, TAS transcripts)
- cleaved by Ago + primary sRNA
(miRNA or siRNA)

- RDR6 – complementary strand synthesis:
dsRNA → DCL2(4) → secondary siRNA

Function of secondary siRNA
- signal amplification
- formation of siRNA from neighbor seq.
(transitivity – new targets)

ta-siRNAs (miRNA na TAS)
(trans-acting siRNA – widening of miRNA targets)



Cosupression in Petunia

- overexpression of pigment gene (enzyme for pigment synthesis) caused loss of pigmentation in flower sectors

- occurrence of aberrant transcripts due to overexpression
- formation of dsRNA from aberrant transcripts by RdRP (RDR6)
- formation of siRNAs that silence both transgene and internal gene

Interfering RNA

The phenomenon of RNA interference collides with the concept of transcription factors

Although transcription factors start their own transcription, but interfering RNAi decide which transcripts will be used

How was the mechanism of RNAi created?

Primitive immune system

↓

Protection of cells against viral infection

↓

dsRNA? → that's a virus!

↓

If a cell detects dsRNA, considers it as a virus - which must be destroyed!

This was followed by adaptation of RNA interference

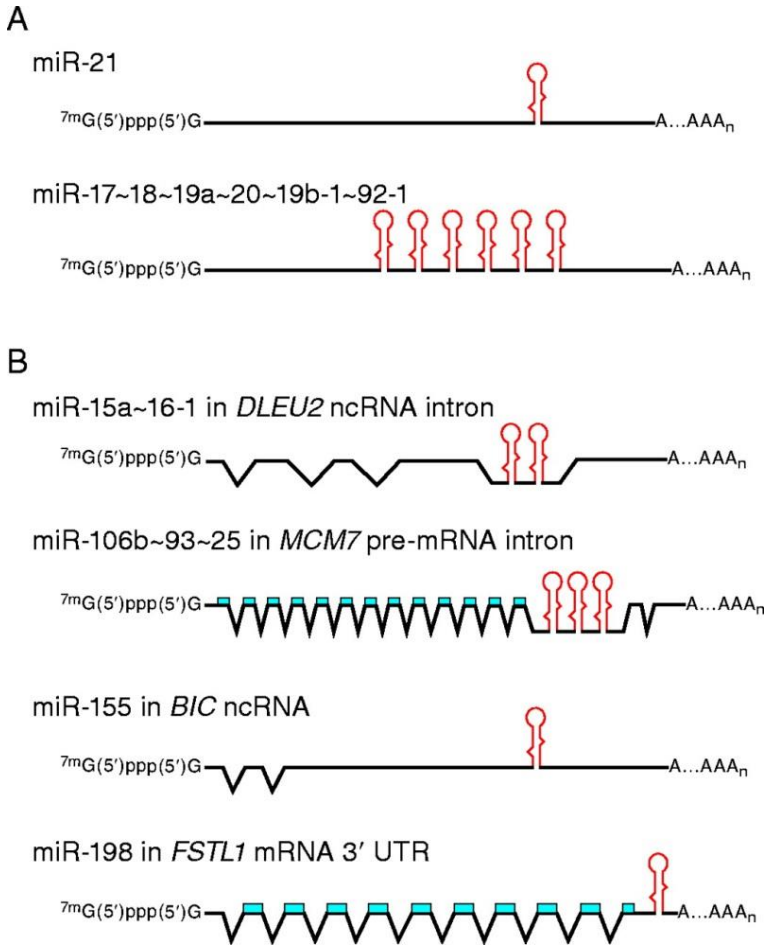
The resulting mechanism of defense against viruses was subsequently adapted for eukaryotic cells, where it serves as a regulatory mechanism for the rapid locking of translation at the current "unnecessary" transcripts

This allows ontogeny

Has RNAi been an evolutionary phenomenon leading to multicellular?

How are miRNAs formed? - I

Discovered only in plants, animals and fungi



pri-miRNA
(hundreds to thousand bp)

- mostly transcription from areas where there are not structural genes
- But even within introns and exons
- coordinated transcription hnRNA

How are miRNAs formed? - II

RNA polymerase II

pri-miRNA (100-1000 bp)

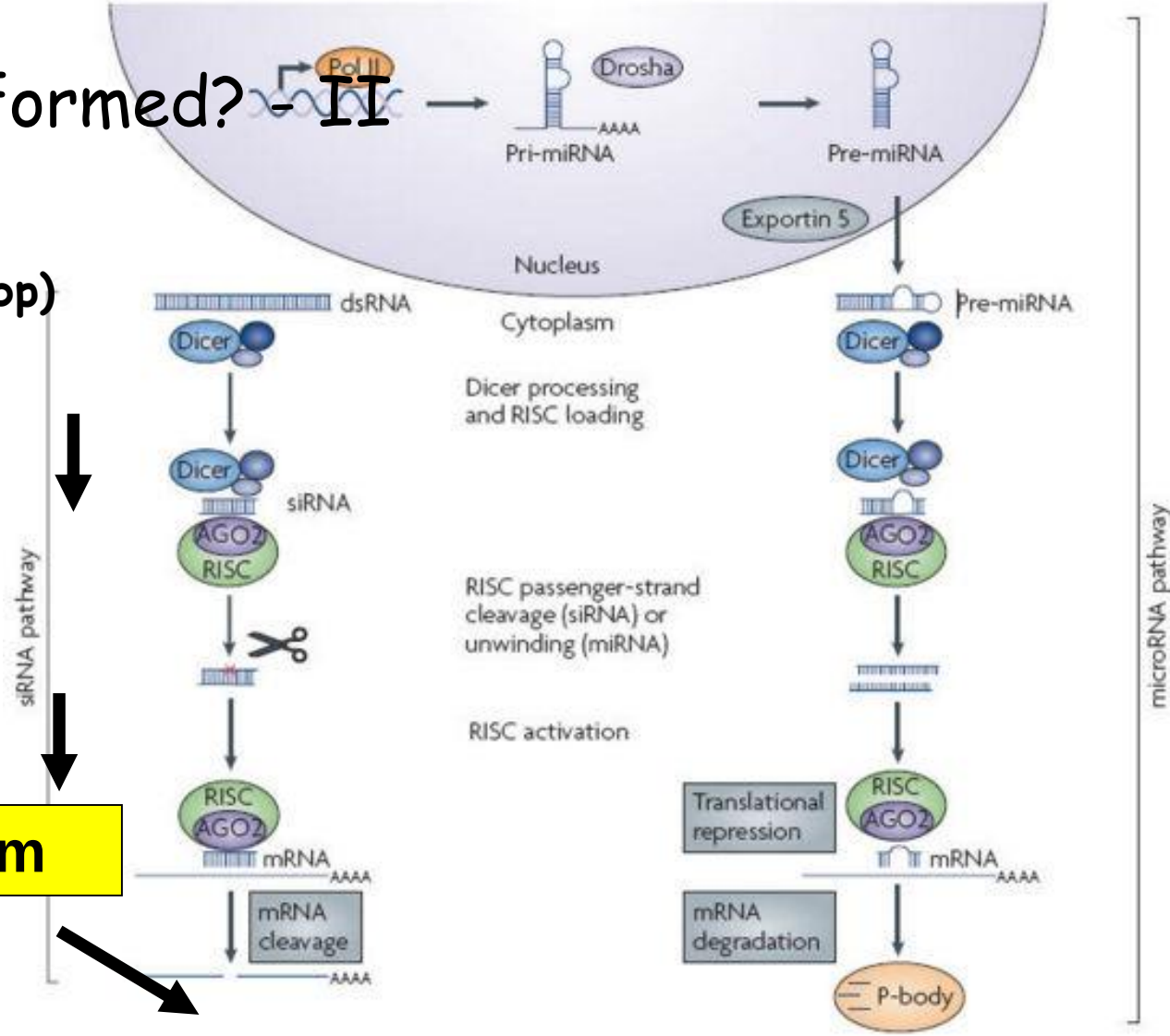
RNase III - DROSHA

cleavage of edges and formation of

pre-miRNA (70 bp, hairpin)

export to cytoplasm

RNase III - DICER

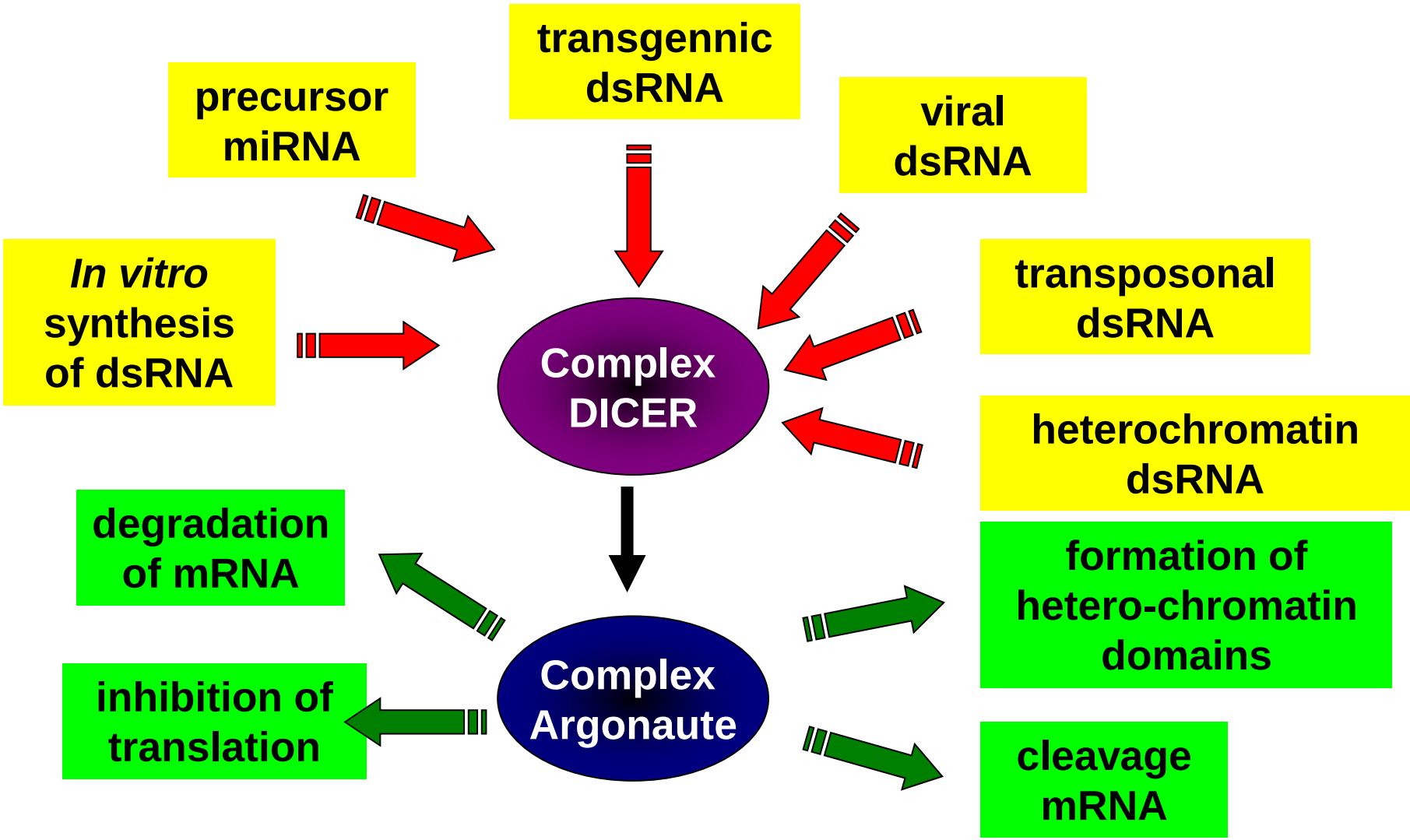


adjustment to dsRNA = miRNA/miRNA* (21 bp)

degradation of miRNA*

action miRNA

Path of RNAi from signal to action



Denli AM and Hannon GJ, *Trends in Biochemical Sciences*, 2003

Site of action of interfering RNA

Transcription

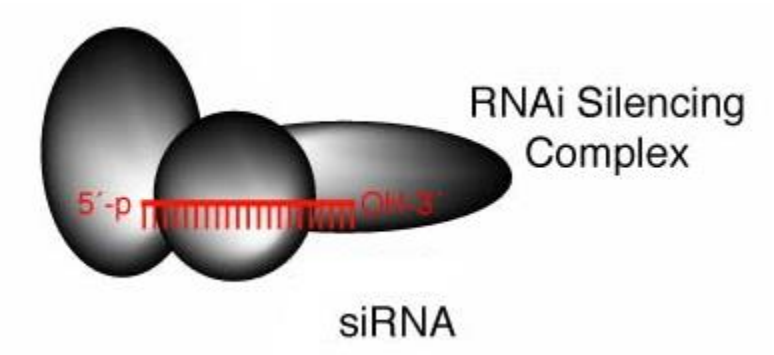
- siRNA
- metylation of sequences in promoter

Posttranscriptional processes

- siRNA, miRNA, piRNA
- degradation mRNA
- inhibition of adjustments of mRNA
- blocking of translation
- activation of interferon

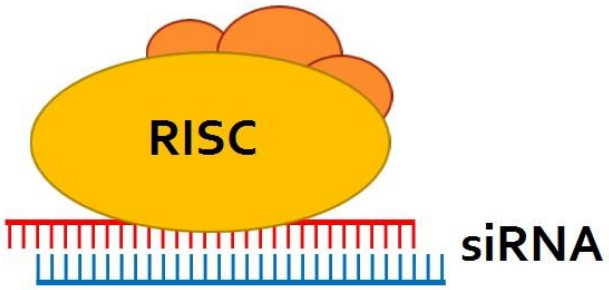
Mechanism of RNA interference - I

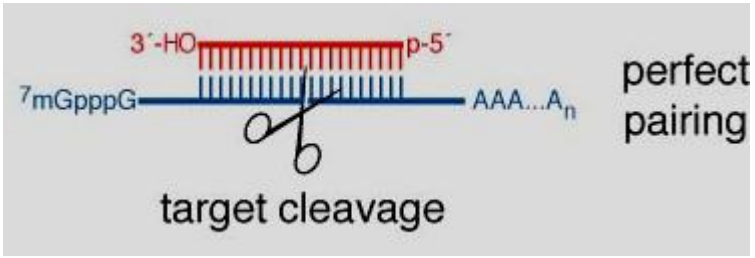
Resulting molecule of siRNA or miRNA is incorporated in „RNA-induced silencing complex" (RISC)



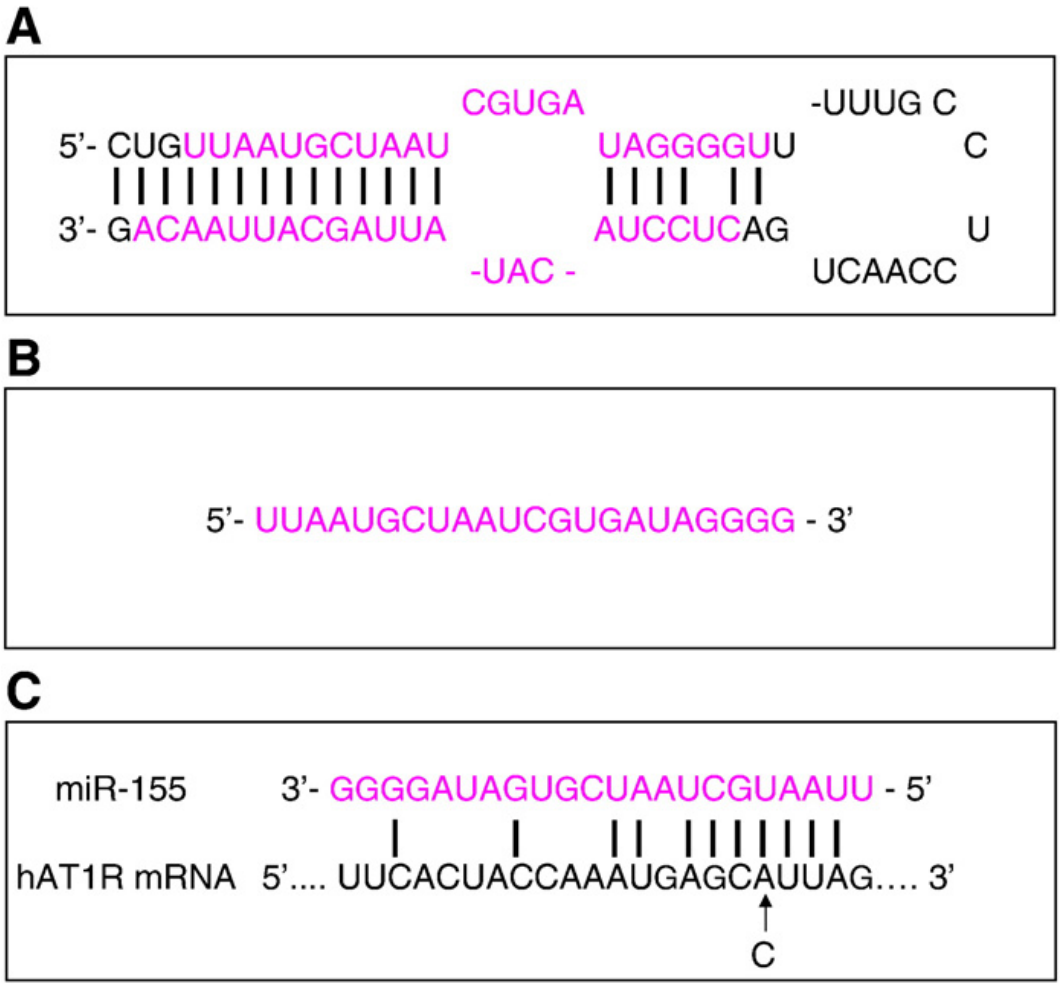
Mechanism of RNA interference - II

Based on homology of siRNA or miRNA for mRNA, RISC complex causes degradation of this mRNA





Structure of RNAi and its target site



gene for miRNA-155 (pre-miRNA-155)

mature miRNA-155

interaction of miRNA-155 with target sequence

Fundamental differences between the siRNA and miRNA

- Both types regulate expression

- **siRNA originated in dsRNA**
- **siRNA is often associated with a foreign RNA (usually viral), and is 100% complementary**
- **miRNA comes from molecules of ssRNA, which forms a dsRNA hairpin structures**
- **miRNAs regulate post-transcriptional gene expression**

Other differences between the siRNA and miRNA

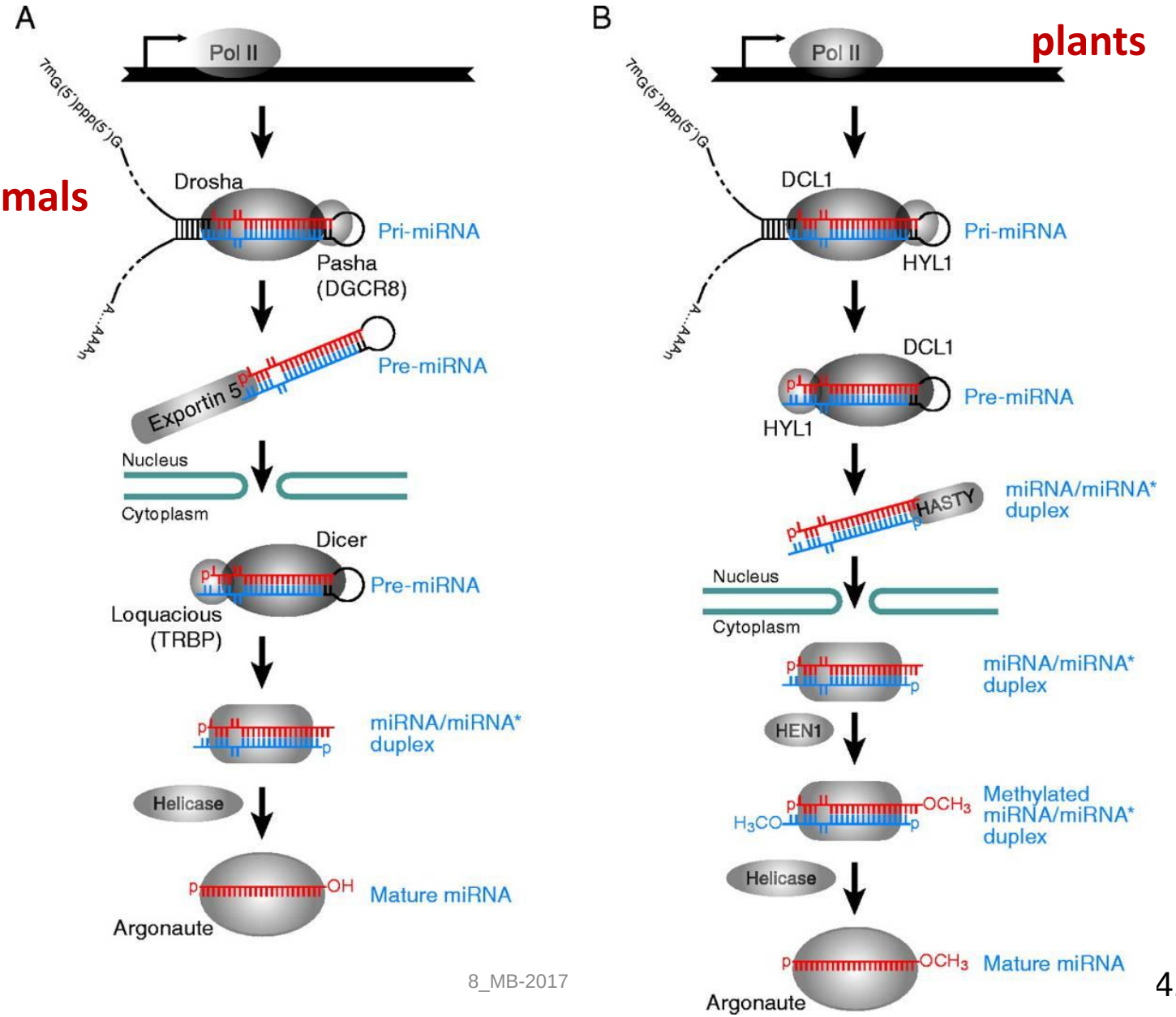
siRNA

- dsRNA
- protection against viruses and transposons
- protection against overproduction
- cause degradation of target molecules
- Absolute complementarity with the target sequence

miRNA

- ssRNA (hairpins)
- regulation of ontogeny and development processes
- do not cause degradation, just translation blockade
- complementarity to the target sequence is not absolute
- formed by activity of RNA polymerase II

Differences in miRNA biogenesis



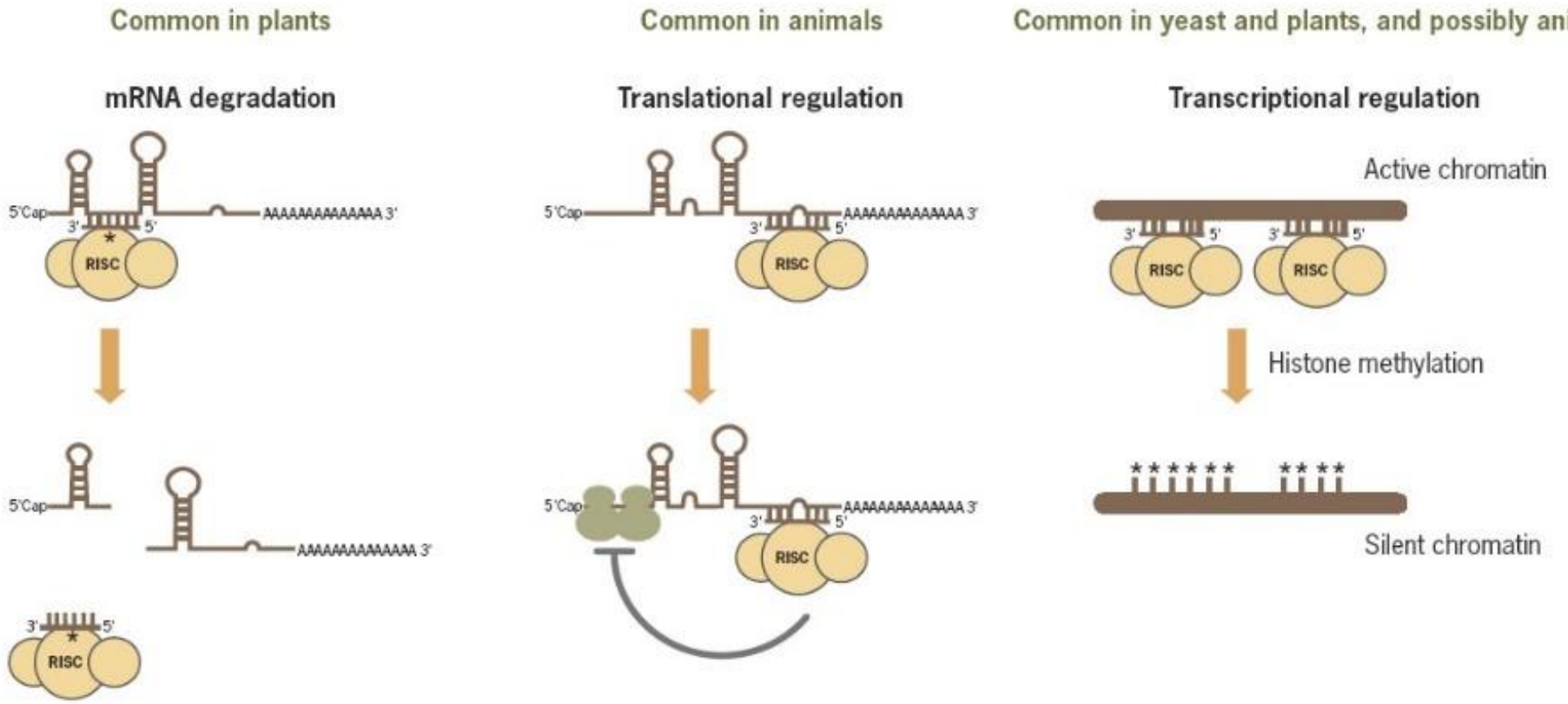
TEST

Differences between plant and animal miRNA

	Plants	Animals
source	intergenic regions	intergenic regions, introns
miRNA clusters	rare	common
mechanism	mRNA cleavage	translation repression
target site on mRNA	ORF	3´-terminus

TEST

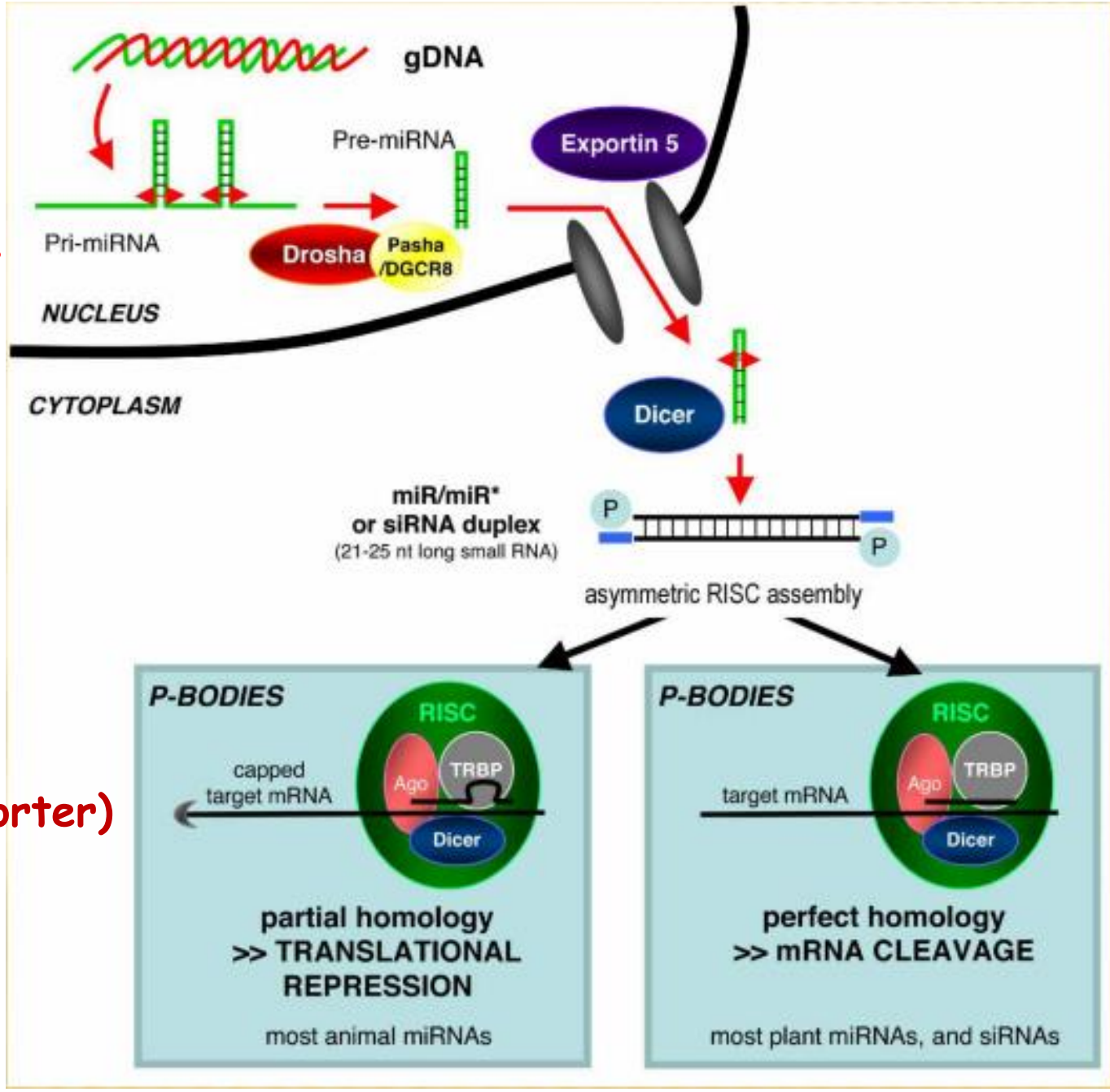
RNA interference overview



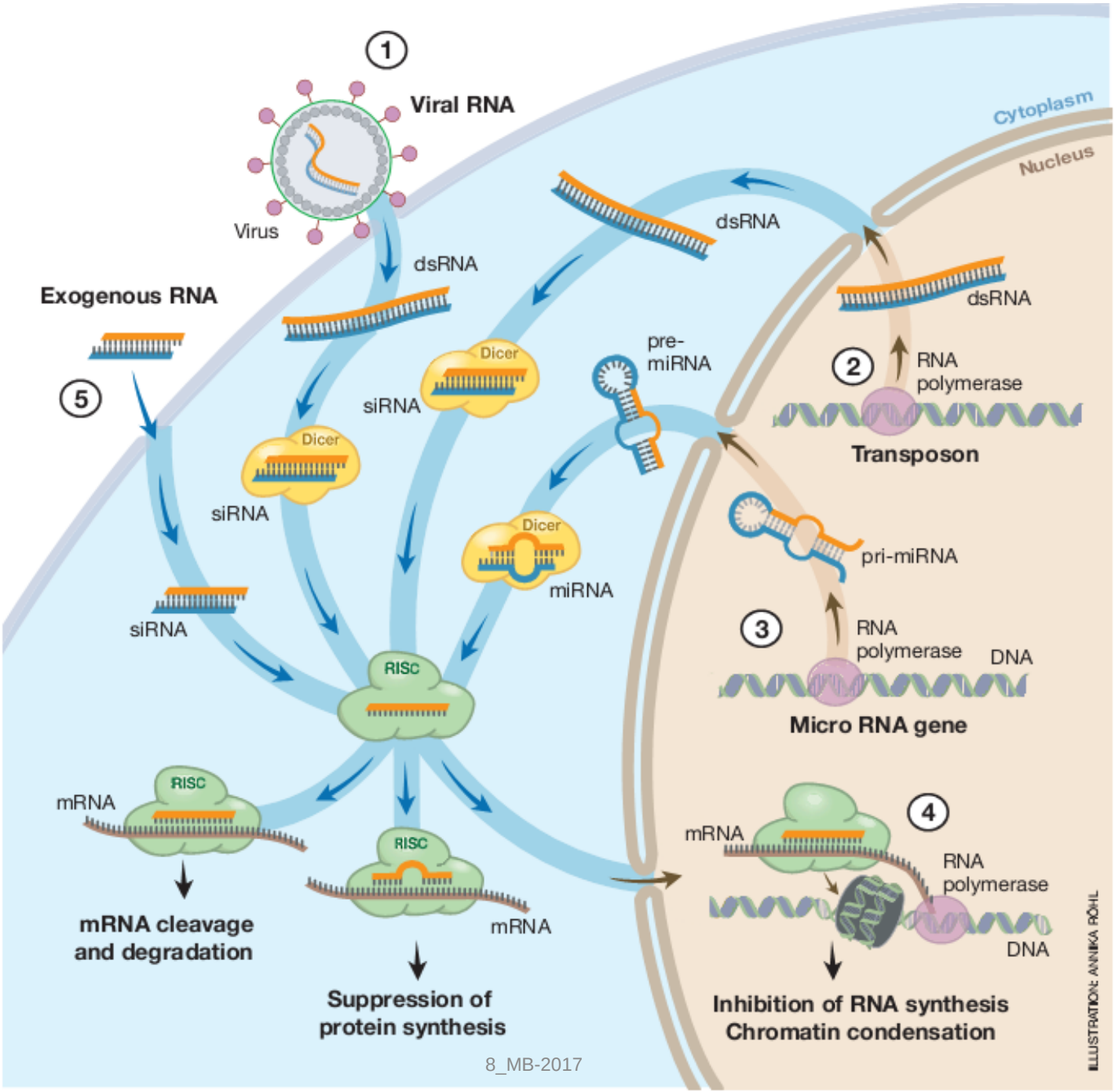
RNA interference

based on enzyme degradation or translation inhibition of specific mRNA

Drosha (RnasaIII)
Pasha (protein)
Exportin 5 (transporter)
Dicer (RNasaIII)
RISC (multiprotein complex)



TEST


1
Viral RNA
Virus
Cytoplasm
Nucleus
dsRNA
Exogenous RNA
5
dsRNA
Dicer
siRNA
pre-
miRNA
dsRNA
2
RNA
polymerase
Transposon
Dicer
siRNA
Dicer
miRNA
pri-miRNA
siRNA
3
RNA
polymerase
DNA
Micro RNA gene
RISC
RISC
mRNA
RISC
mRNA
4
mRNA
RNA
polymerase
DNA
mRNA cleavage
and degradation
Suppression of
protein synthesis
Inhibition of RNA synthesis
Chromatin condensation
ILLUSTRATION: ANNIKA RÖHL

siRNA and miRNA utilisation:

1) gene analysis
2) gene therapies
3) anti-viral vaccines
4) transgenic organisms that have transiently inhibited selected genes

- iRNA usage does not fall under GMO
- Yet usage of cassettes producing iRNA does!

RNAi therapeutic applications - I

1) **RNAi as antivirotics, should block expression of viral genes and viral genome replication. Most anticipated RNAi therapy target is HIV.**

2) **RNAi induced epigenetic changes on local chromatin structure could specifically control gene expression.**

3) **It is assumed that 35% to 70% of human genes are transcribed into hnRNA transcripts that later undergo alternative splicing. Defects in alternative splicing can lead to severe diseases. RNAi could be used to block these defective alternatively spliced molecules.**

RNAi therapeutic applications - II

4) **RNAi can target genes involved in metabolic diseases. For example the central role in insuline resistance in diabetes mellitus II is due to defects in gene expression.**

5) **Gene „knock-outs" in pathogen genomes can be used as a research tool to gain a better understanding over pathogenic modes of action and therefore aid in developing effective countermeasures.**

piRNA = Piwi-interacting RNA

piRNA properties

- described in animals
- form complexes with Piwi proteins (piwi proteins - regulatory proteins responsible for maintaining incomplete differentiation in stem cells and maintaining the stability of cell division rates in germ line cells)
- affect ontogenesis (sperm is not produced without piRNA transcription)
- do they transport miRNA to target sequences?

- **26 to 31 bp**
- **in silico analysis described 52 934 possible piRNA molecules in mice, 52 099 in human and 47 024 for rats**
- **originate from only a handful of intergenic clusters as ssRNA**

Piwi-interacting RNA (piRNA) is the largest class of small non-coding RNA molecules expressed in animal cells. piRNAs form RNA-protein complexes through interactions with piwi proteins.

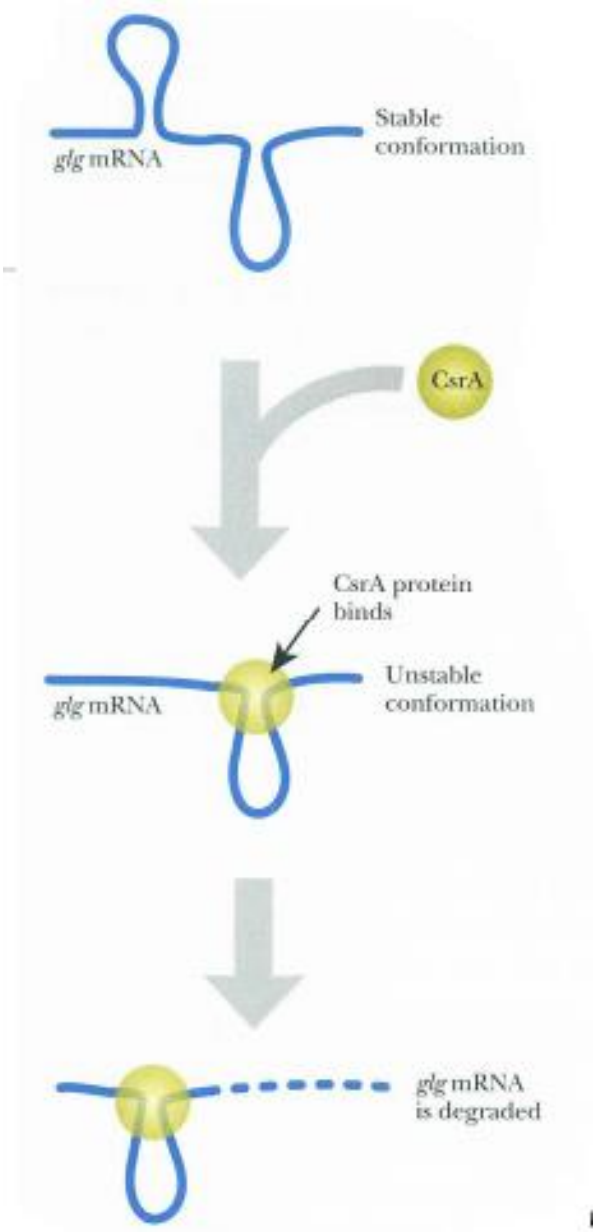
These piRNA complexes have been linked to both epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly those in spermatogenesis.

They are distinct from microRNA (miRNA) in size (26–31 nt rather than 21–24 nt), lack of sequence conservation, and increased complexity.
It remains unclear how piRNAs are generated, but potential methods have been suggested, and it is certain their biogenesis pathway is distinct from miRNA and siRNA, while rasiRNAs (repeat associated small interfering) are a piRNA subspecies.

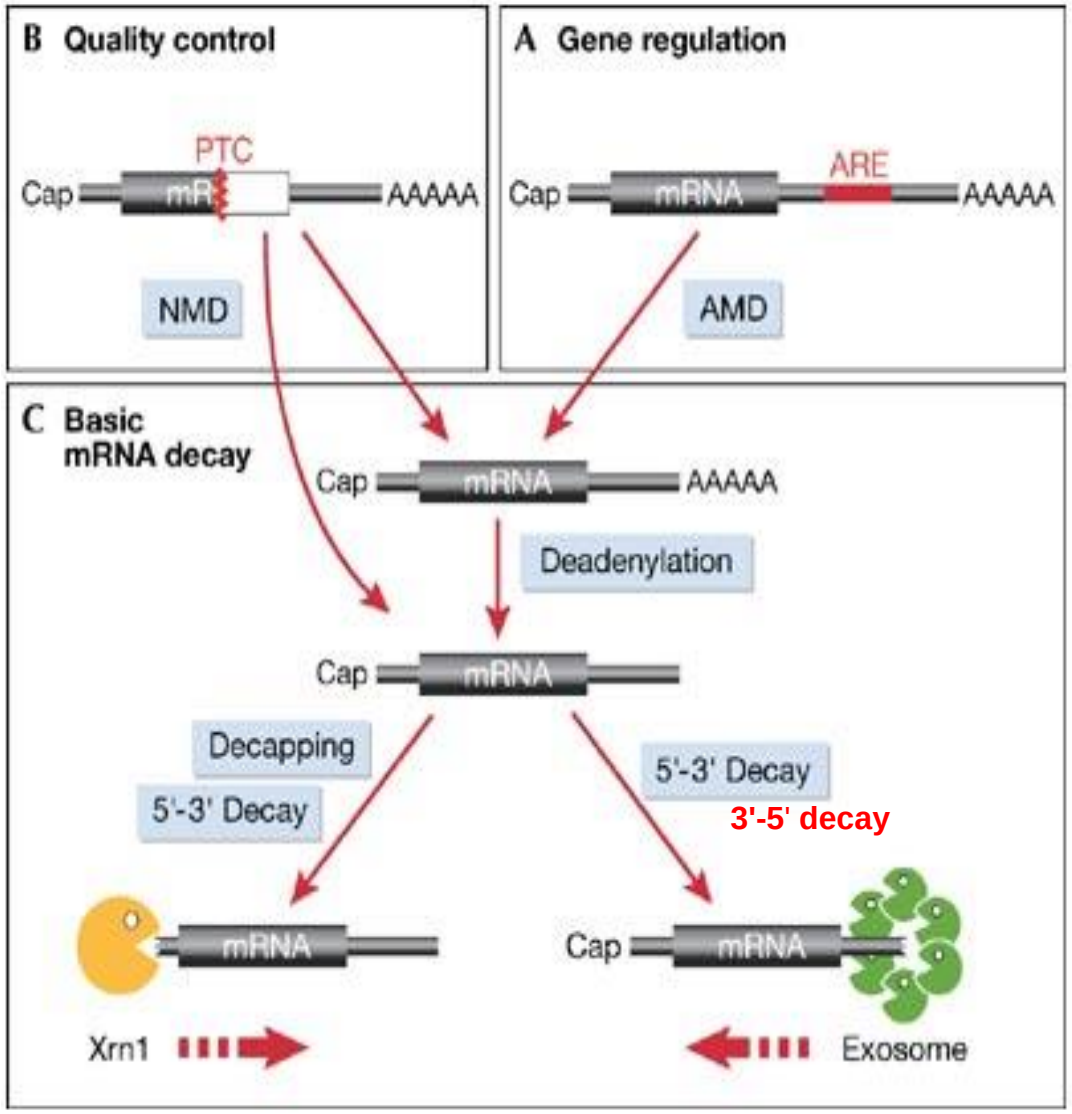
RNA stability

- mRNA has a short half-life, it is readily degraded by ribonucleases
- mRNA secondary structure is a key component in RNAse sensitivity

- mRNA secondary structure can be altered by protein binding – regulation signals



General scheme of messenger RNA decay pathways.



(A) mRNAs containing an AU-rich element (ARE) in their 3' UTR undergo rapid ARE-mediated mRNA decay (AMD) in resting cells. **Concealing ARE sequence from AMD induces gene expression. (B)** Quality control mechanisms. mRNAs that contain a premature termination codon (PTC) are recognized and specifically degraded by the nonsense-mediated mRNA decay (NMD) pathway. **(C)** The basic mRNA decay machinery in the cytoplasm initially removes the poly(A) tail through the activity of deadenylating enzymes. Subsequently, the mRNA can be further degraded from the 3' end by a complex of 3'–5' exonucleases known as the exosome. Alternatively, the mRNA is decapped at the 5' end, and the 5'–3' exonuclease Xrn1 proceeds to degrade the body of the mRNA.

RNAa (RNA activation) molecules activate genes!

- described in 2006
- RNAa mode of action – protect ARE sequences
- RNAi shutdown genes for 5 to 7 days, RNAa activates genes for 13 days

- The molecular mechanism of RNAa is not fully understood.
- Similar to RNAi, it has been shown that mammalian RNAa requires members of the Ago clade of Argonaute proteins, particularly Ago2, but possesses kinetics distinct from RNAi.
- In contrast to RNAi, promoter-targeted agRNAs induce prolonged activation of gene expression associated with epigenetic changes

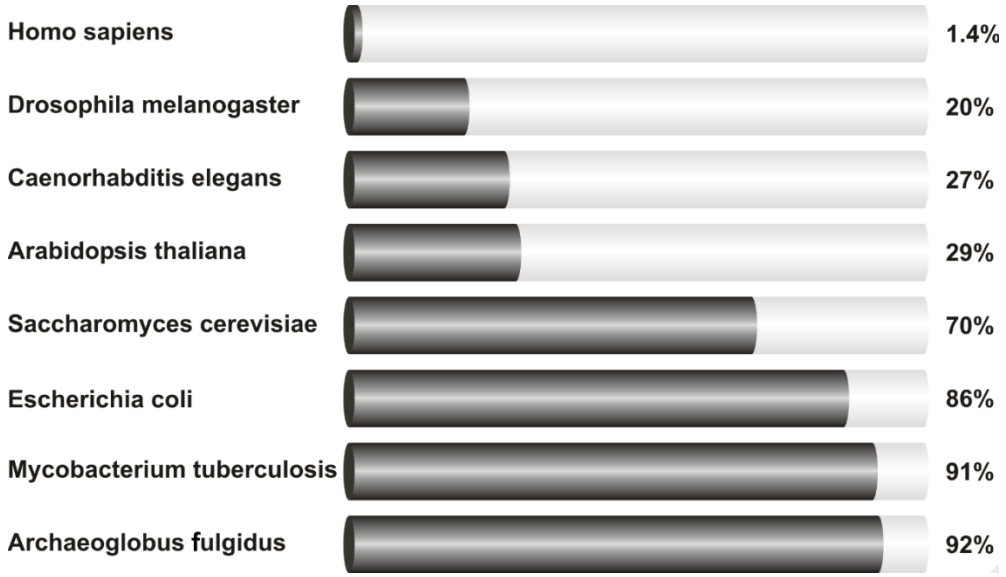
RNAa therapies?

1) RNAa can be used on its own

2) We still have to consider that the treatment can deliver complex effects. For example small interfering RNAi can act not only as a negative but at the same time as a positive regulator → inhibiting one gene can indirectly activate another. Such side effects are to be expected also with RNAa.

World of noncoding RNAs

encoding genes represent less than 2% of the total genome sequence
vs.
at least 90% of the human genome is actively transcribed
the more complex organism, the more it comprises non-coding RNAs



Recent evidence suggests that the non-coding RNAs (ncRNAs) may play major biological roles in cellular development, physiology and pathologies. NcRNAs could be grouped into two major classes based on the transcript size: small ncRNAs and long ncRNAs.

Sana *et al. Journal of Translational Medicine* 2012, **10**:103
http://www.translational-medicine.com/content/10/1/103

JOURNAL OF TRANSLATIONAL MEDICINE

REVIEW Open Access

Novel classes of non-coding RNAs and cancer

Jiri Sana[1,2], Petra Faltejskova[1,2], Marek Svoboda[1] and Ondrej Slaby[1,2,3*]

A new classes of non-coding RNAs

Table 1: Types of recently discovered human non-coding RNAs.

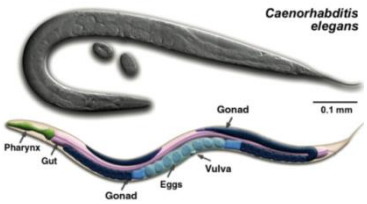
	Class	Symbol	Characteristic	Disease / biological function associations
Small non-coding RNAs	MicroRNAs	miRNAs	18-25 nt; account 1-2% of the human genome; control the 50% of protein-coding genes; guide suppression of translation; Drosha and Dicer dependent small ncRNAs	initiation of various disorders including many, if not all, cancers / regulation of proliferation, differentiation, and apoptosis; involved in human development
	all interfering RNAs	siRNAs	19-23 nt; made by Dicer processing; guide sequence specific degradation of target mRNA;	great potential in diseases treatment / posttranscriptional gene silencing mainly through RISC degradation mechanism; defence against pathogenic nucleic acids
	wi-interacting RNAs	piRNAs	26-30 nt; bind Piwi proteins; Dicer independent; exist in genome clusters; principally restricted to the germline and somatic cells bordering the germline	relationship between piRNAs and diseases has not yet been discovered / involved in germ cell development, stem self-renewal, and retrotransposon silencing
	all nucleolar RNAs	snoRNAs	60-300 nt; enriched in the nucleolus; in vertebrate are excised from pre-mRNA introns; bind snoRNP proteins	association with development of some cancers / important function in the maturation of other non-coding RNAs, above all, rRNAs and snRNAs; miRNA-like snoRNAs regulate mRNAs
	oter-associated small RNAs	PASRs	20-200 nt; modified 5' (capped) ends; coincide with the transcriptional start sites of protein- and non-coding genes; made from transcription of short capped transcripts	relationship with diseases has not yet been discovered / involved in the regulation of the transcription of protein-coding genes by targeting epigenetic silencing complexes
	Transcription initiation RNAs	tiRNAs	~ 18 nt ; have the highest density just downstream of transcriptional start sites; show patterns of positional conservation; preferentially located in GC-rich promoters	
	Centromere repeat associated small interacting RNAs	crasiRNAs	34-42 nt; processed from long dsRNAs;	relationship between crasiRNAs and diseases has not yet been discovered / involved in the recruitment of heterochromatin and/or centromeric proteins
	Telomere-specific small RNAs	tel-sRNAs	~ 24 nt; Dicer independent; 2'-O-methylated at the 3' terminus; evolutionarily conserved from protozoa to mammals; have not been described in human up to now	relationship between tel-sRNAs and diseases has not yet been discovered / epigenetic regulation
	Pyknons		subset of patterns of variable length; form mosaics in untranslated and protein-coding regions; more frequently in 3' UTR	expected association with cancer biology / possible link with posttranscriptional silencing of genes, mainly involved in cell communication, regulation of transcription, signaling, transport, etc.
Long non-coding RNAs	Long intergenic nonoding RNAs	lincRNAs	ranging from several hundreds to tens of thousands nts; lie within the genomic intervals between two genes; transcriptional cis-regulation of neighbouring genes	involved in tumorigenesis and cancer metastasis / involved in diverse biological processes such as dosage compensation and/or imprinting
	Long intronic nonoding RNAs		lie within the introns; evolutionary conserved; tissue and subcellular expression specified	aberrantly expressed in human cancers / possible link with posttranscriptional gene silencing
	Telomere-associated noRNAs	TERRAs	100 bp - >9 kb; conserved among eukaryotes; synthesized from C-rich strand; polyadenylated; form inter-molecular G-quadruplex structure with single-stranded telomeric DNA	possible impact on telomere-associated diseases including many cancers / negative regulation of telomere length and activity through inhibition of telomerase
	Long noRNAs with dual functions		both protein-coding and functionally regulatory RNA capacity	deregulation has been described in breast and ovarian tumors / modulate gene expression through diverse mechanisms
	Pseudogene RNAs		code for a protein; potential to regulate their protein-coding cousin; made through retrotrans-position; tissue specific	and cancer progression / regulation of tumor suppressors and oncogenes by acting as microRNA decoys
	Transcribed-ultraconserved regions	T-UCRs	longer than 200 bp; absolutely conserved between orthologous regions of human, rat, and mouse; located in both intra- and intergenic regions	expression is often altered in some cancers; possible involvement in tumorigenesis / antisense inhibitors for protein-coding genes or other ncRNAs

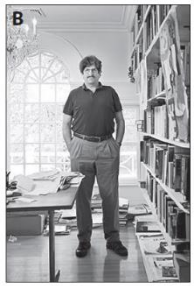
Sana et al, J Transl Med, 2012

HISTORY

1998 Fire, Mello – RNA interference

1993 Ambros, Ruvkun – discovery of miRNA lin-4





1999 Tuschl, Zamore, Bartel, Sharp
-RNAi 21-23 fragments

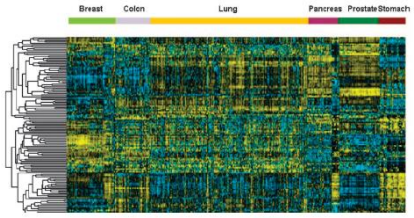

2006 Nobel Prize winners Andrew Fire and Craig Mello for their discovery of the RNAi mechanism.

2000-2001 Hannon
-Ago2, Dicer

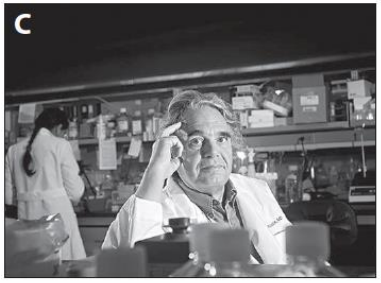
Cell, 1993

Nature, 1998

2002 Zamore
RNAi and miRNA effector share its orbit

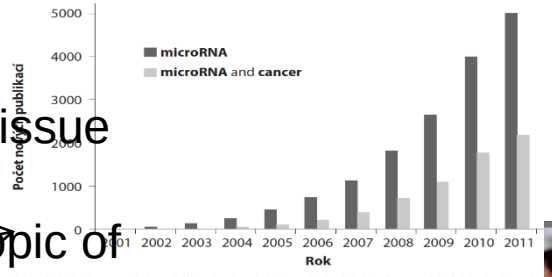


PNAS, 2004



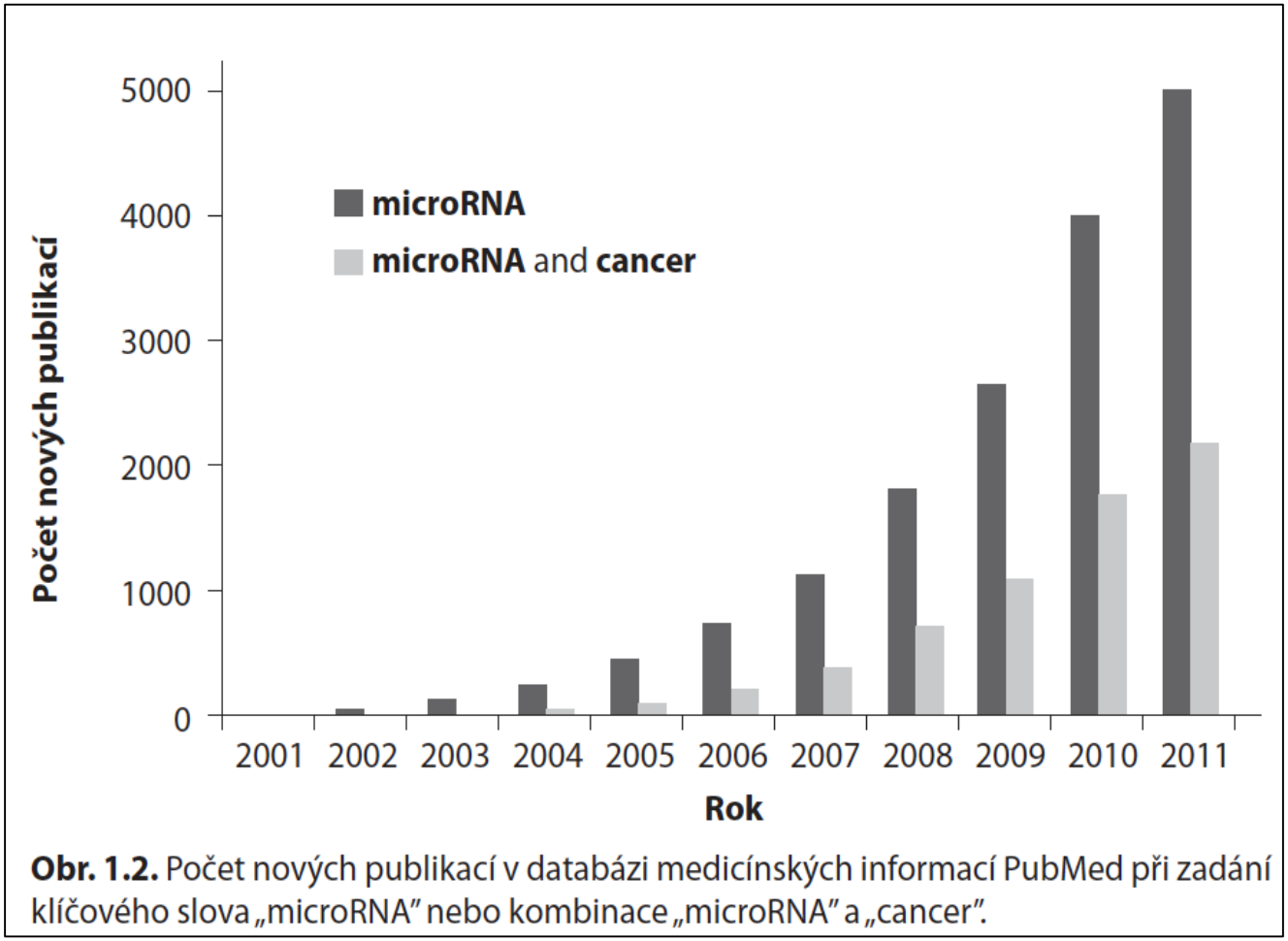
2002 Croce, Calin miR-15,miR-16 in CLL
2004 Croce
50% miRNA genes on chromosome fragile sites

2006 Croce
Deregulation of miRNAs in tumor tissue
…
2007 The first original work on a topic of miRNAs in oncology in the Czech Republic ☺



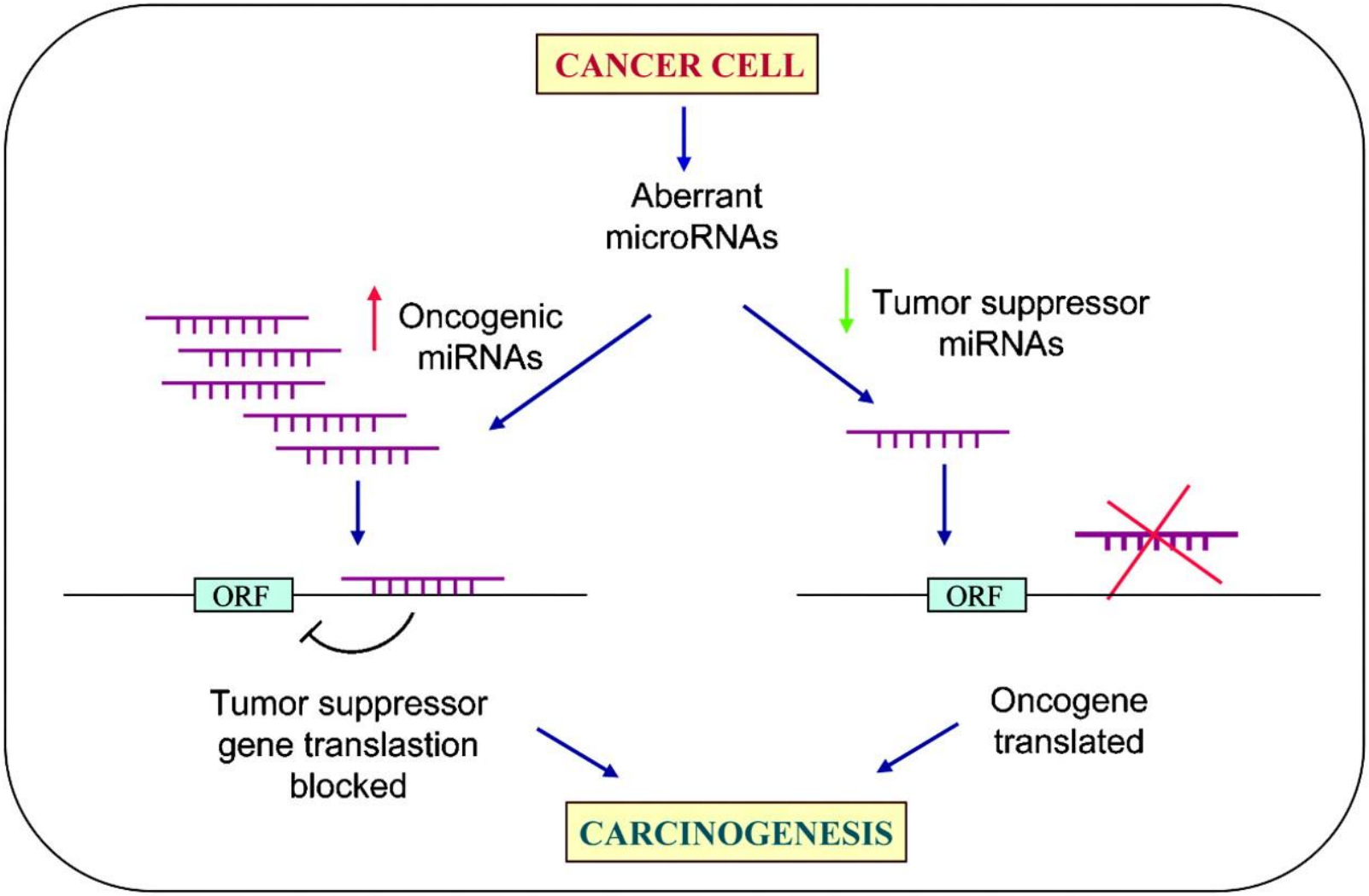
Obr. 1.2. Počet nových publikací v databázi medicínských informací PubMed při zadání dotazu obsahujícího nejen kombinace „microRNA" a „cancer".

microRNA = 43832
microRNA and cancer = 19433
19.10.2015 23.29

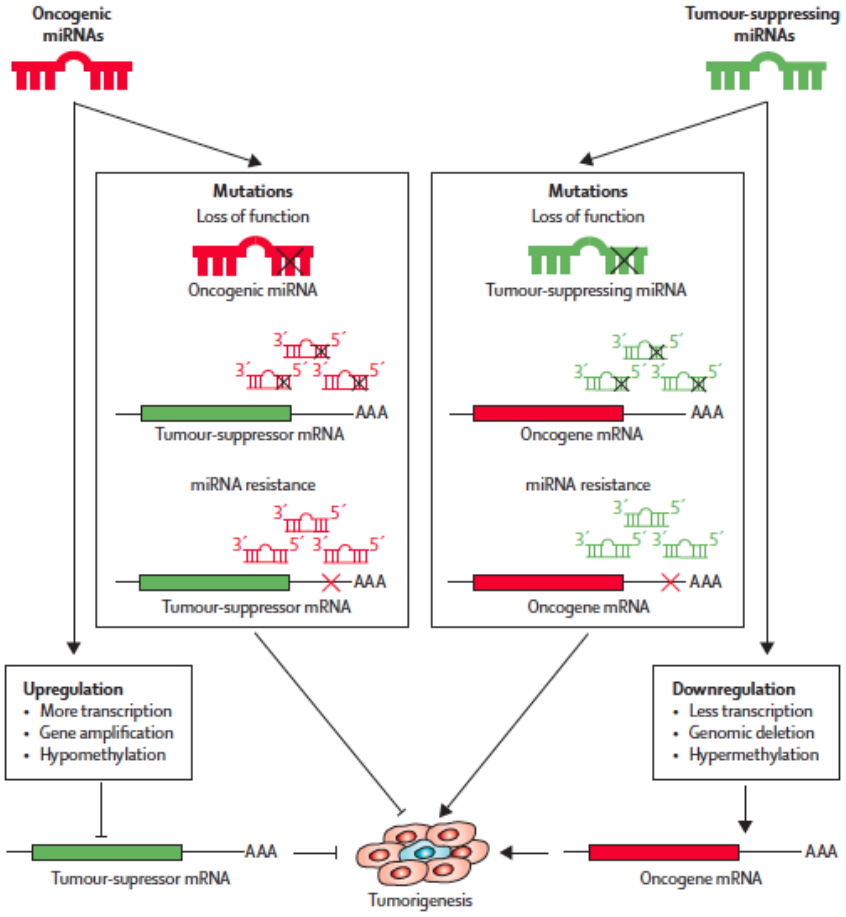


Obr. 1.2. Počet nových publikací v databázi medicínských informací PubMed při zadání klíčového slova „microRNA" nebo kombinace „microRNA" a „cancer".

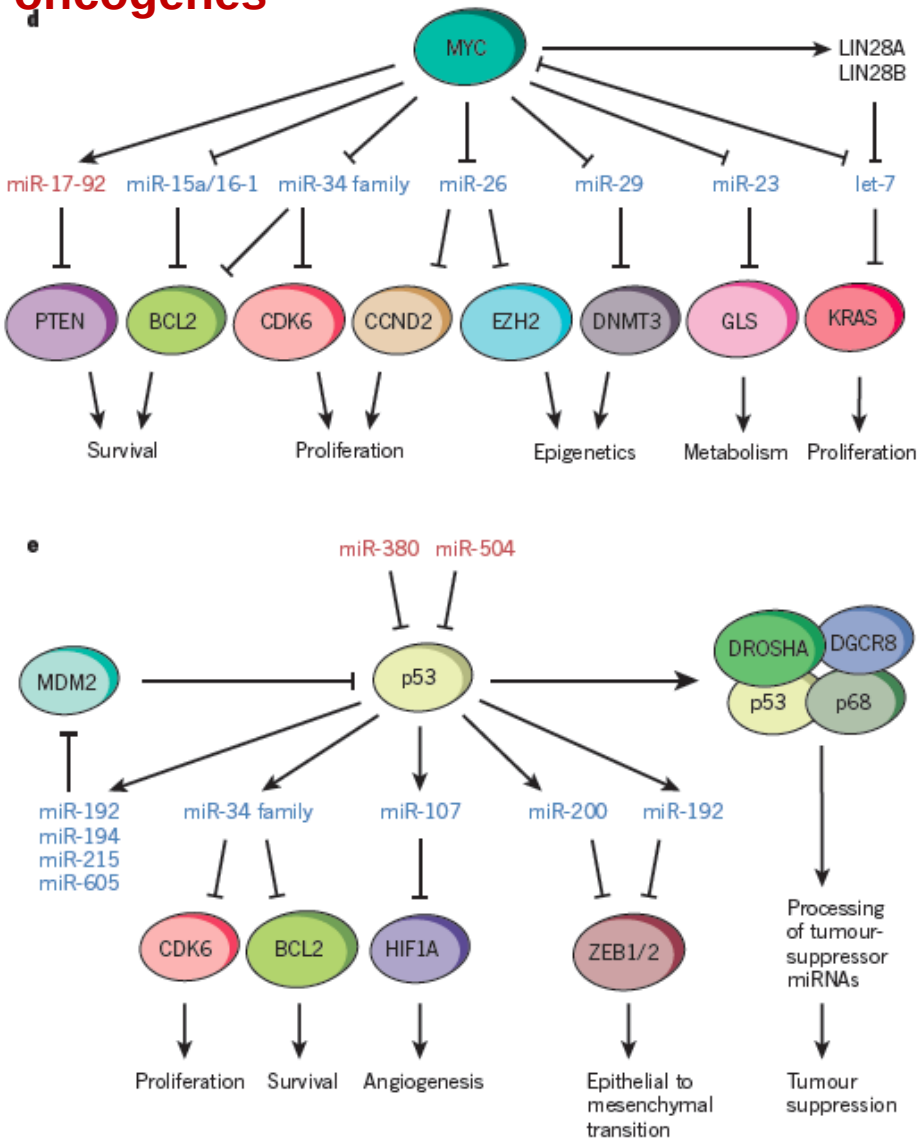
MicroRNAs as tumor suppressors or oncogenes



MicroRNAs as tumor suppressors or oncogenes

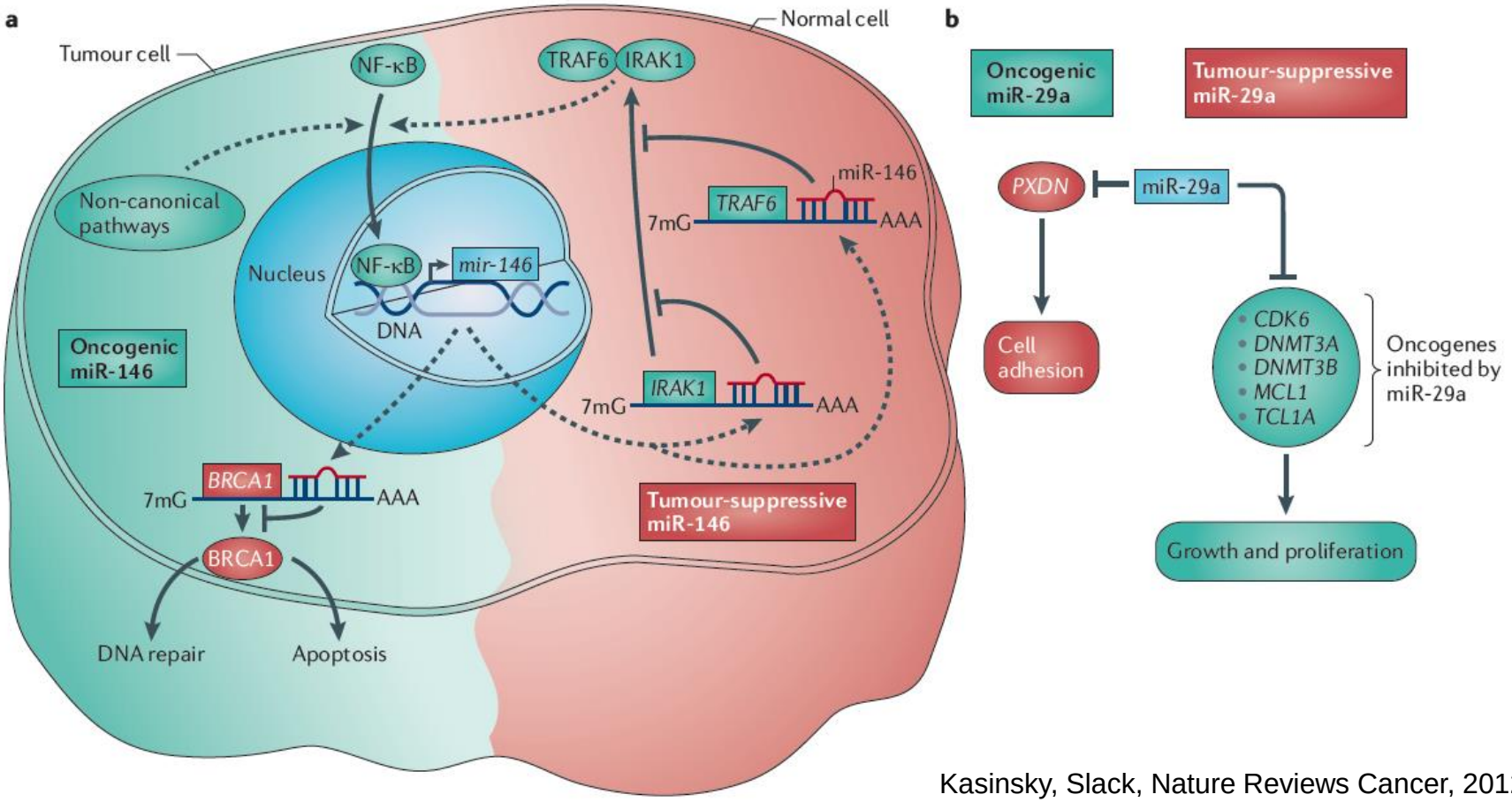


Kong et al, Lancet Oncology 2012



Lujambio, Nature 2012

MicroRNAs as oncogenes or TS depending on the context



Kasinsky, Slack, Nature Reviews Cancer, 2012

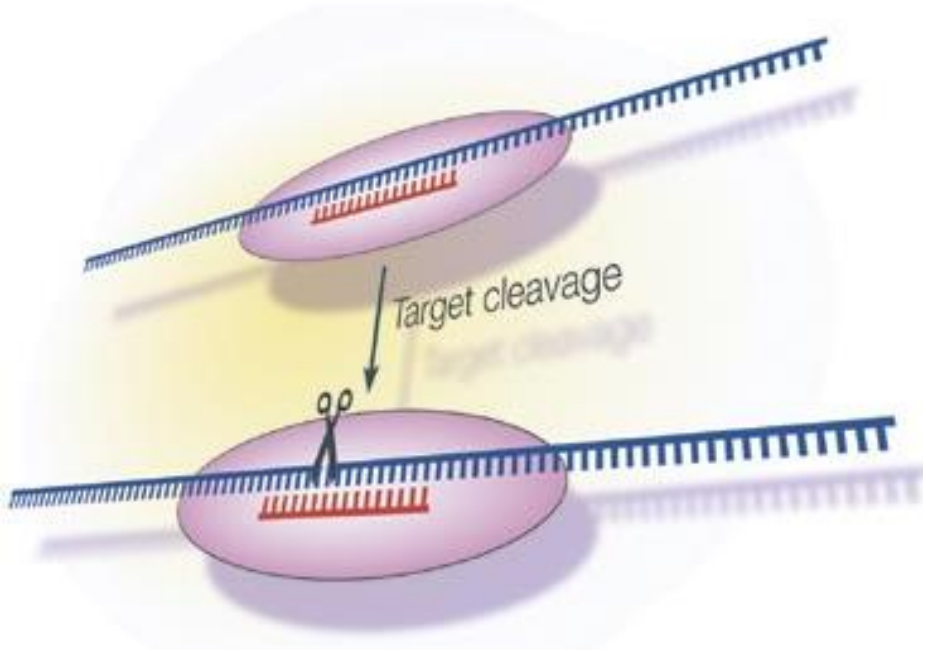
- https://www.youtube.com/watch?v=Vh3-NHdjnyQ

- microRNA siRNA
- https://www.youtube.com/watch?v=5YsTW5i0Xro

- https://www.youtube.com/watch?v=cK-OGB1_ELE

RNA interference in bacteria and archae?

- **First mentioned in 2007**



Sheilagh Molloy (2007): First evidence of prokaryotic RNAi?
Nature Reviews Microbiology 5, 329.

CRISPR system

In 2008, it was described RNAi analogous system designed to the degradation of viral NA

- **It uses internal "virus" sequences inserted in the inverted repeats (CRISPR)**
- **CRISPR = clusters of regularly interspaced short palindromic repeats**
- After transcription of this sequence leads to their progressive cleavage by **Cas proteins**
- **The resulting products interfere with the nucleic acid of the entering virus**

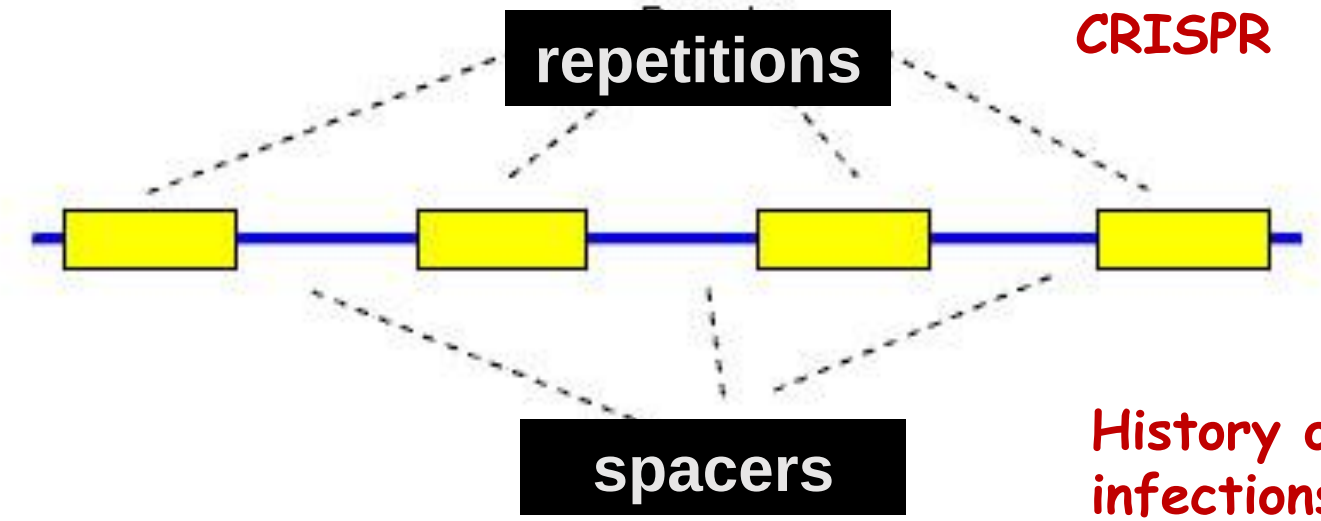
- Each of repeats followed by short segments called **Spacer DNA**, obtained during previous meetings with relevant bacterial viruses or plasmids.

Brouns et al. (2008): Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes, Science 321, 960-964

Structure of CRISPR

Towards the end of 2008, the CRISPR reported in about 40% of early the sequenced bacteria and archaea

All repeats contain a short length of 24-48 nucleotides and space of approximately the same length



Marraffini a Sontheimer (2008): Science 322, 1843 – 1845

Edgar (2007): BMC Bioinformatics 8:18

CRISPR / Cas system is a prokaryotic immune system, providing resistance against foreign genetic elements such as plasmids or phages, [3], [4], and therefore constitutes a form of acquired immunity. Spacer DNA of these exogenous genetic elements detected and deactivated in a manner analogous to the mechanism of RNA interference in eukaryotic organisms. [5] CRISPR loci have been found in about 40% bacteria and archaea in 90% [6]. CRISPR interference technology has enormous potential for the application, including altering the human germ line, animals (and other organisms) or modification genes food crops. Delivering a protein and appropriate guidance Cas9 RNA into the cell genome of the target organism can be cut open at any desired point. [7] [8] [9] **CRISPRy in connection with specific endonucleases, intended for editing the genome or targeted regulation of genes have already been tested in a variety of organisms** [10]. From an ethical point of view seems to be especially worrisome possibility to edit the human germ line. [11]

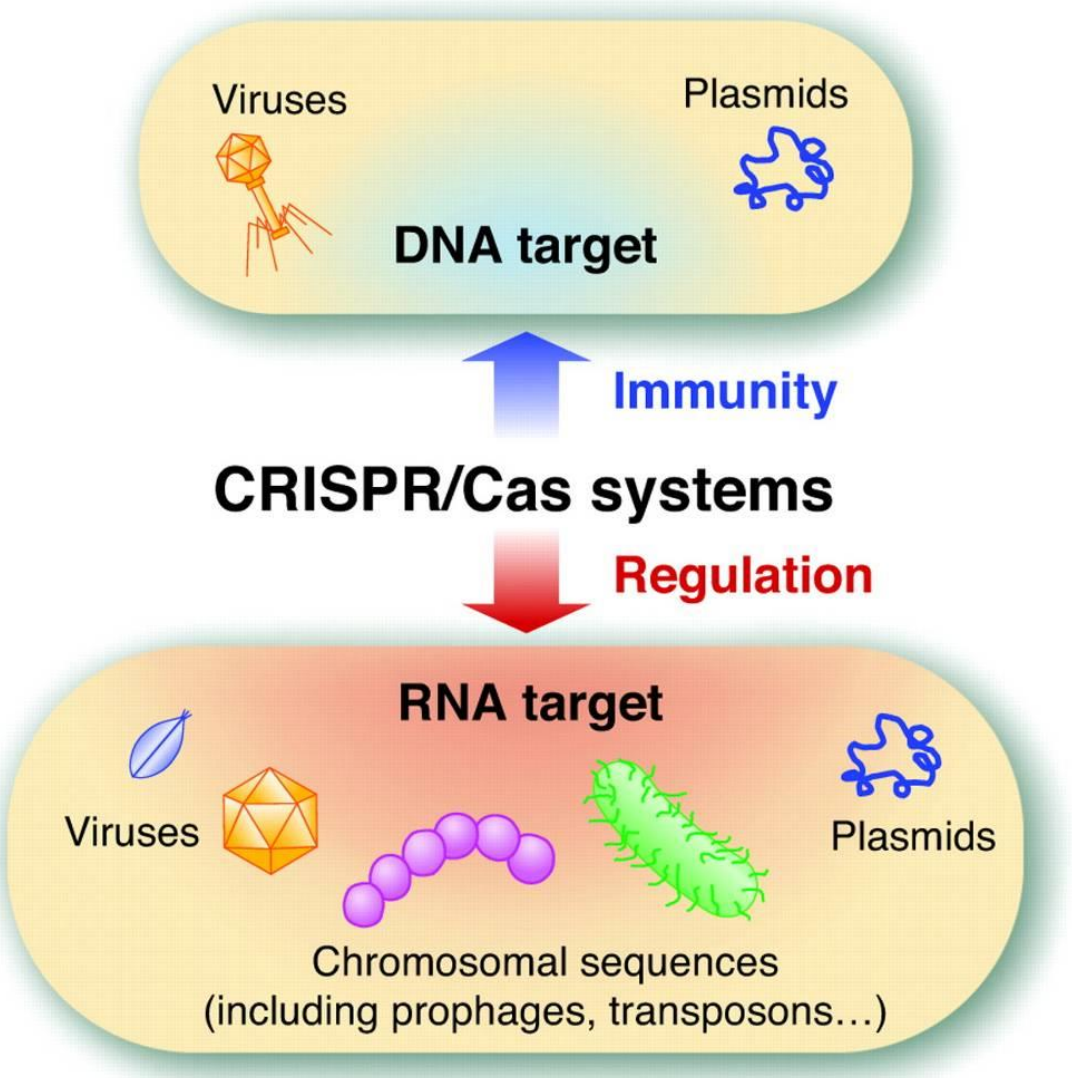
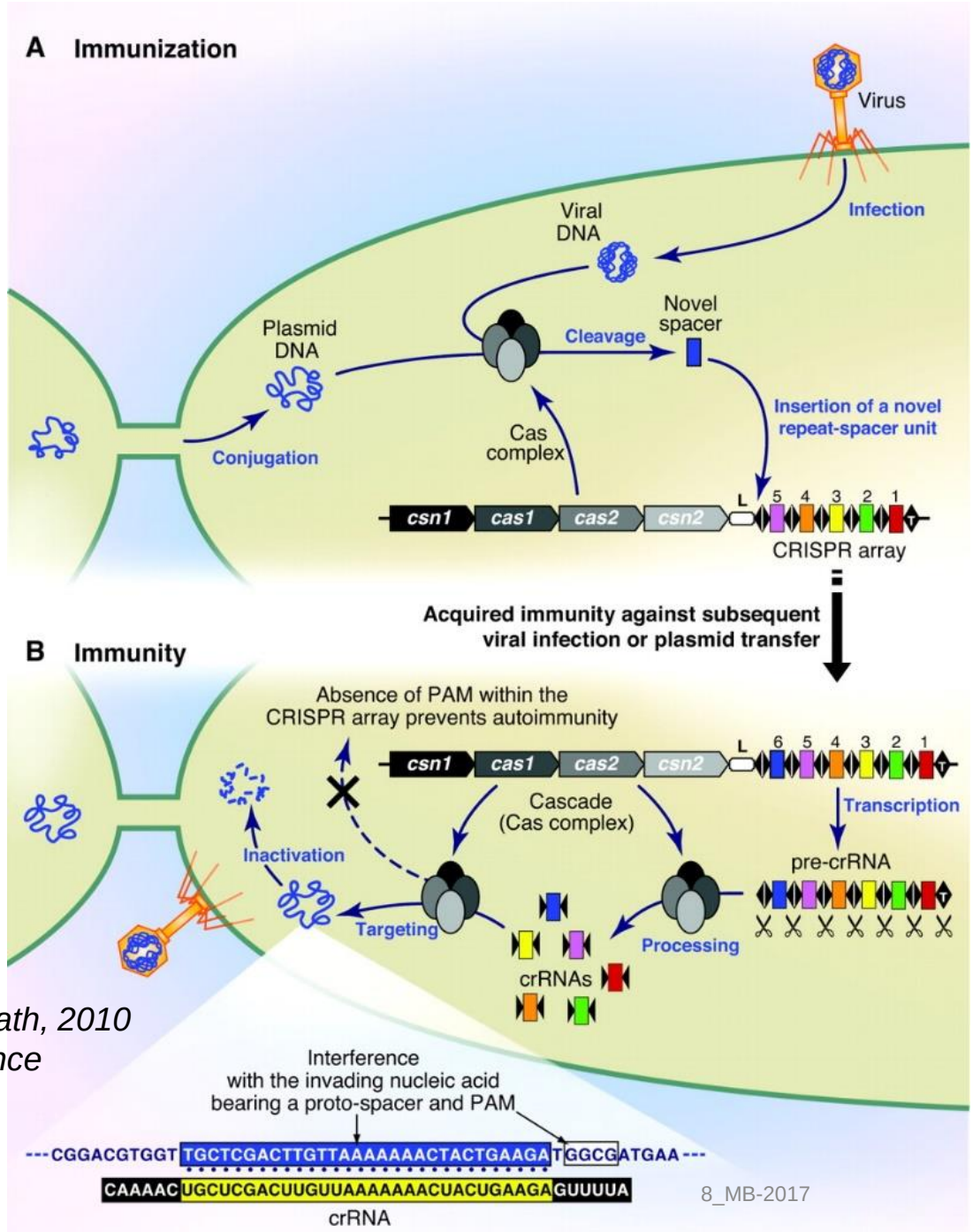


Fig. 3. CRISPR interference. The CRISPR/Cas systems may target either DNA or RNA to interfere with viruses, plasmids, prophages, or other chromosomally encoded sequences.

P. Horvath et al., Science 327, 167-170 (2010)



Horvath, 2010 Science

Overview of the CRISPR/Cas mechanism of action. (**A**) Immunization process: After insertion of exogenous DNA from viruses or plasmids, a Cas complex recognizes foreign DNA and integrates a novel repeat-spacer unit at the leader end of the CRISPR locus. (**B**) Immunity process: The CRISPR repeat-spacer array is transcribed into a pre-crRNA that is processed into mature crRNAs, which are subsequently used as a guide by a Cas complex to interfere with the corresponding invading nucleic acid. Repeats are represented as diamonds, spacers as rectangles, and the CRISPR leader is labeled L.

CRISPR restricts horizontal transfer of DNA from Staphylococcus

- Spacer in CRISPR encodes crRNA
- CrRNA sequence is homologous to the gene nickase that occurs in almost all conjugative plasmids in Staphylococcus
- Binding of crRNA to nickase preventing conjugation and plasmid transformation
- Interference occurs at the level crRNA-DNA rather than mRNA-crRNA
- CRISPR prevents the spread of antibiotic resistance

Marraffini a Sontheimer (2008): Science 322, 1843 - 1845

- **CRISPR/Cas9**

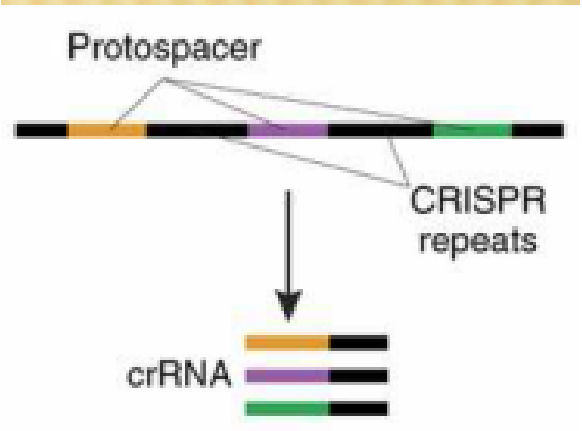
- **CRISPR-associated protein 9 –** nuclease from Streptococcus pyogenes

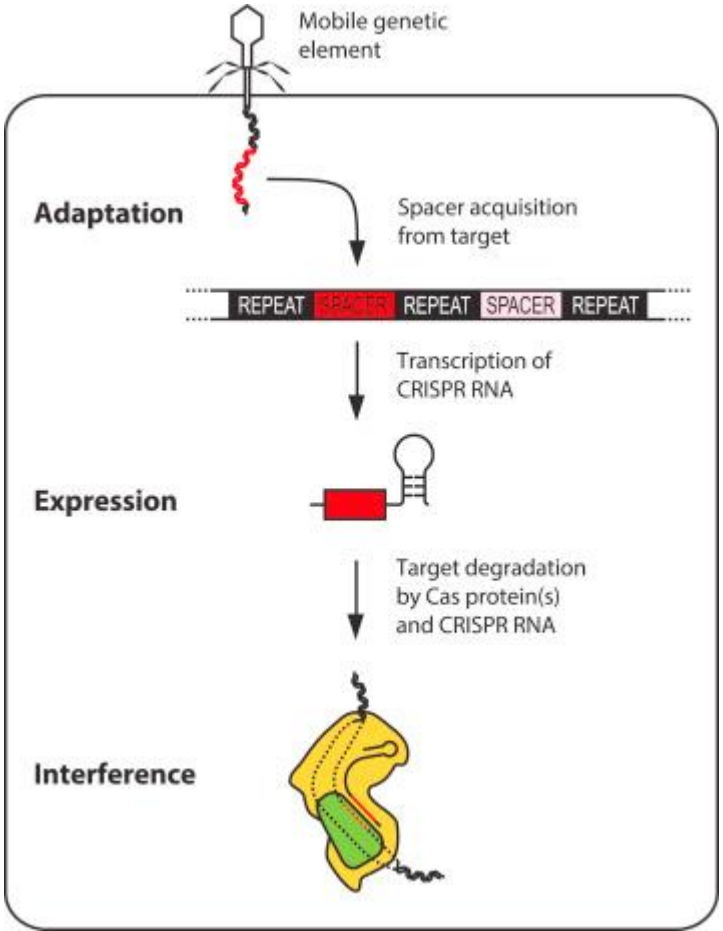
- adaptive immunity against bacterial viruses (generally foreign DNA)

- RGN – RNA-guided nuclease

- **sequence specificity is determined by the interactions of DNA-RNA**

- Bacteria – incorporation of foreign DNA into the CRISPR repeats in the genome
- These subsequently transcribed into RNA (crRNA)
- **crRNA – protospacer** –fragment of foreign DNA repeat - CRISPR

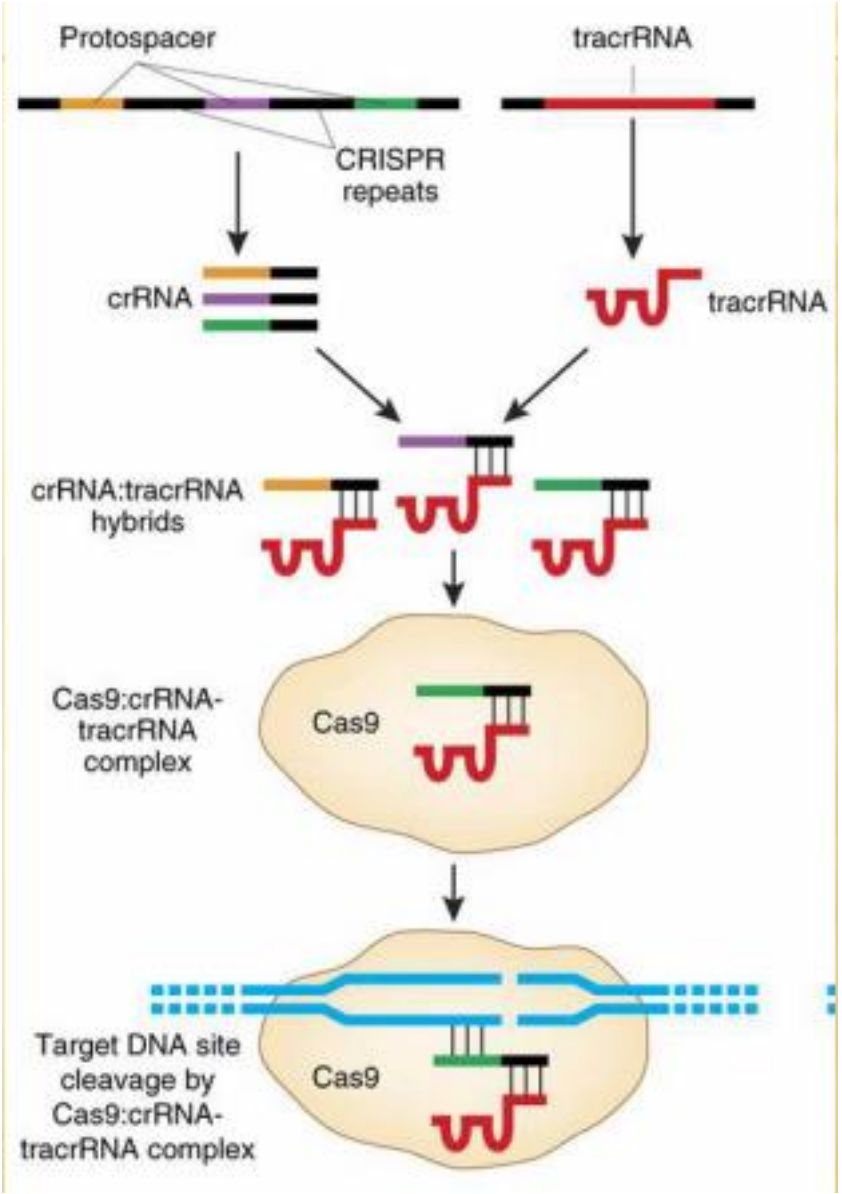




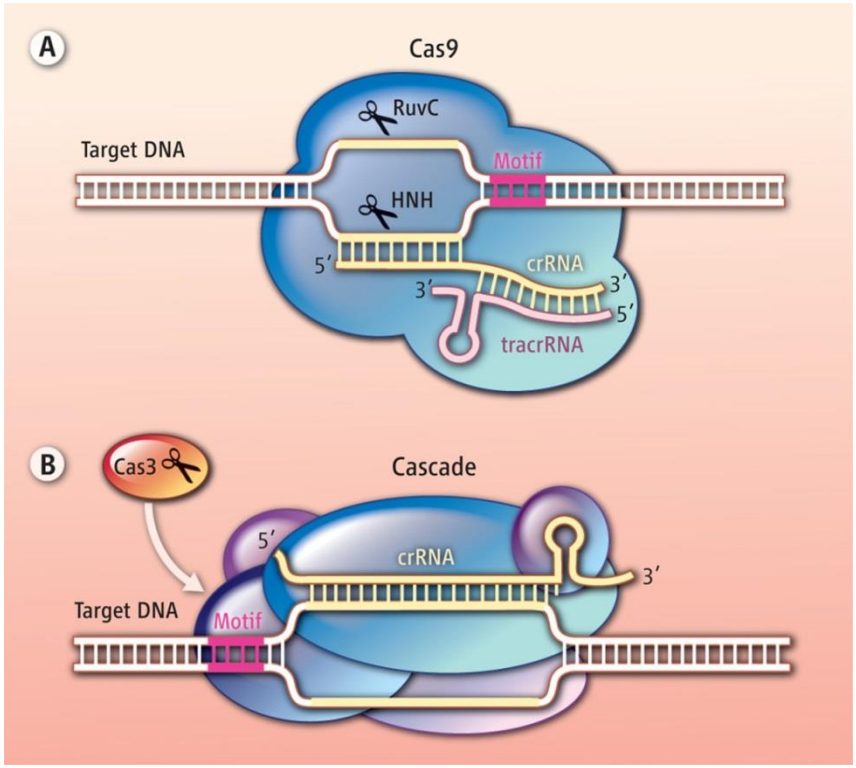
https://www.youtube.com/watch?v=MnYppmstxIs

What is CRISPR?

- crRNA then hybridized with CRISPR transactivating RNA (tracrRNA)
- -This complex interacts with the RNA **nuclease Cas9**
- - protoscpacer RNA directs the entire complex to the foreign DNA (sequence complementarity)
- - resulting ribonucleoprotein complex cleaves the foreign complementary DNA



Mechanism of action



A) Nuclease Cas9 recognizes a target sequence through crRNA and tracrRNA

B) The fission process to participate in a total of five Cas proteins; Cas3 is a nuclease and helicase

Brouns, SJJ (2012): Science 337: 808-809

Genome Editing Glossary

Cas = CRISPR-associated genes

Cas9, Csn1 = a CRISPR-associated protein containing two nuclease domains, that is programmed by small RNAs to cleave DNA

crRNA = CRISPR RNA

dCAS9 = nuclease-deficient Cas9

DSB = Double-Stranded Break

gRNA = guide RNA

HDR = Homology-Directed Repair

HNH = an endonuclease domain named for characteristic histidine and asparagine residues

Indel = insertion and/or deletion

NHEJ = Non-Homologous End Joining

PAM = Protospacer-Adjacent Motif

RuvC = an endonuclease domain named for an *E. coli* protein involved in DNA repair

sgRNA = single guide RNA

tracrRNA, trRNA = trans-activating crRNA

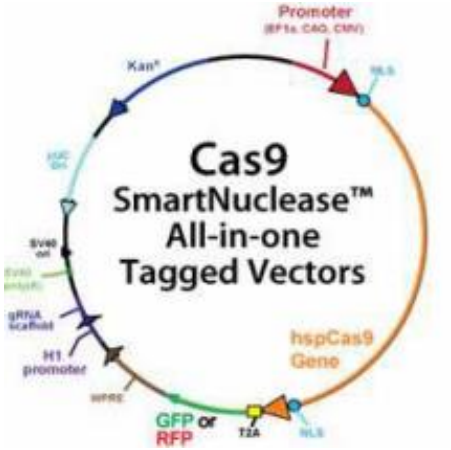
TALEN = Transcription-Activator Like Effector Nuclease

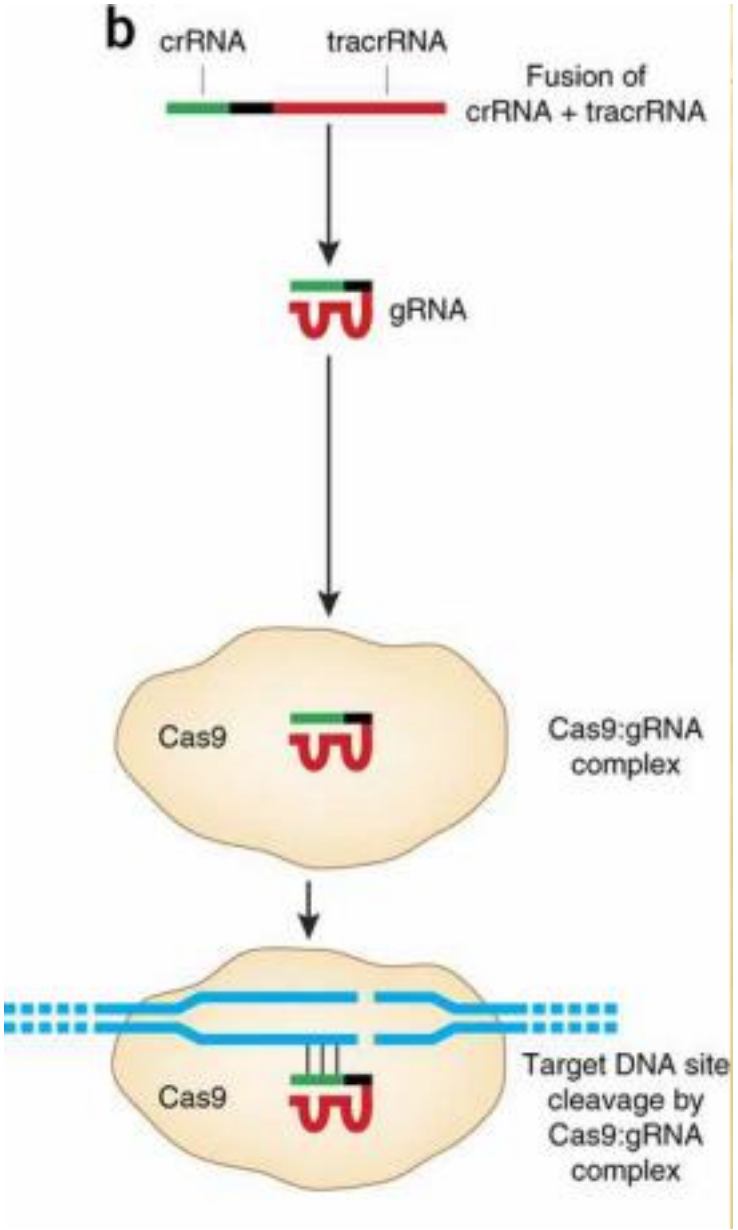
ZFN = Zinc-Finger Nuclease

Year 2013 - enzymes involved in CRISPR mechanism are used as the latest achievement in the preparation of GMO in vitro mutagenesis

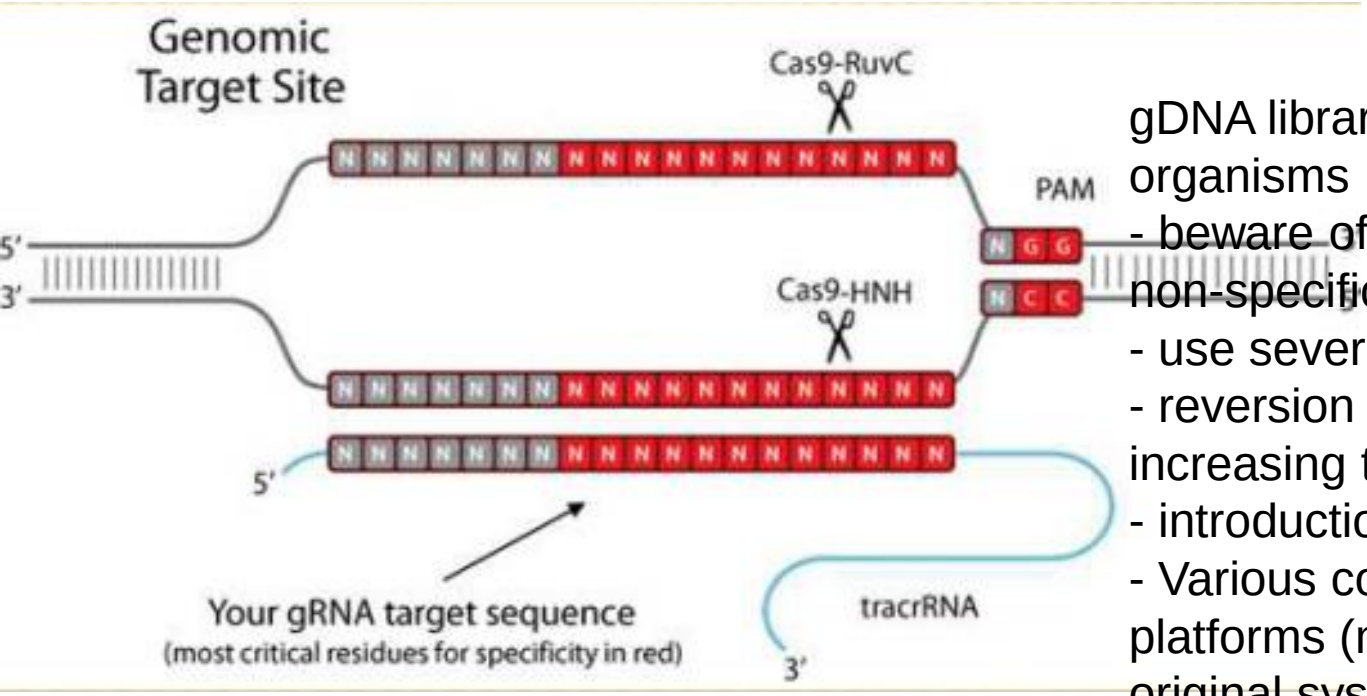
CRISPR/Cas9

- the whole system is modified for targeted mutagenesis
- – vector - gRNA = crRNA tracr + RNA
-
- part gRNA and 20nt complementary section to the target site in the genomic DNA
- - + Coexpression of Cas9 nuclease (even the same vector)

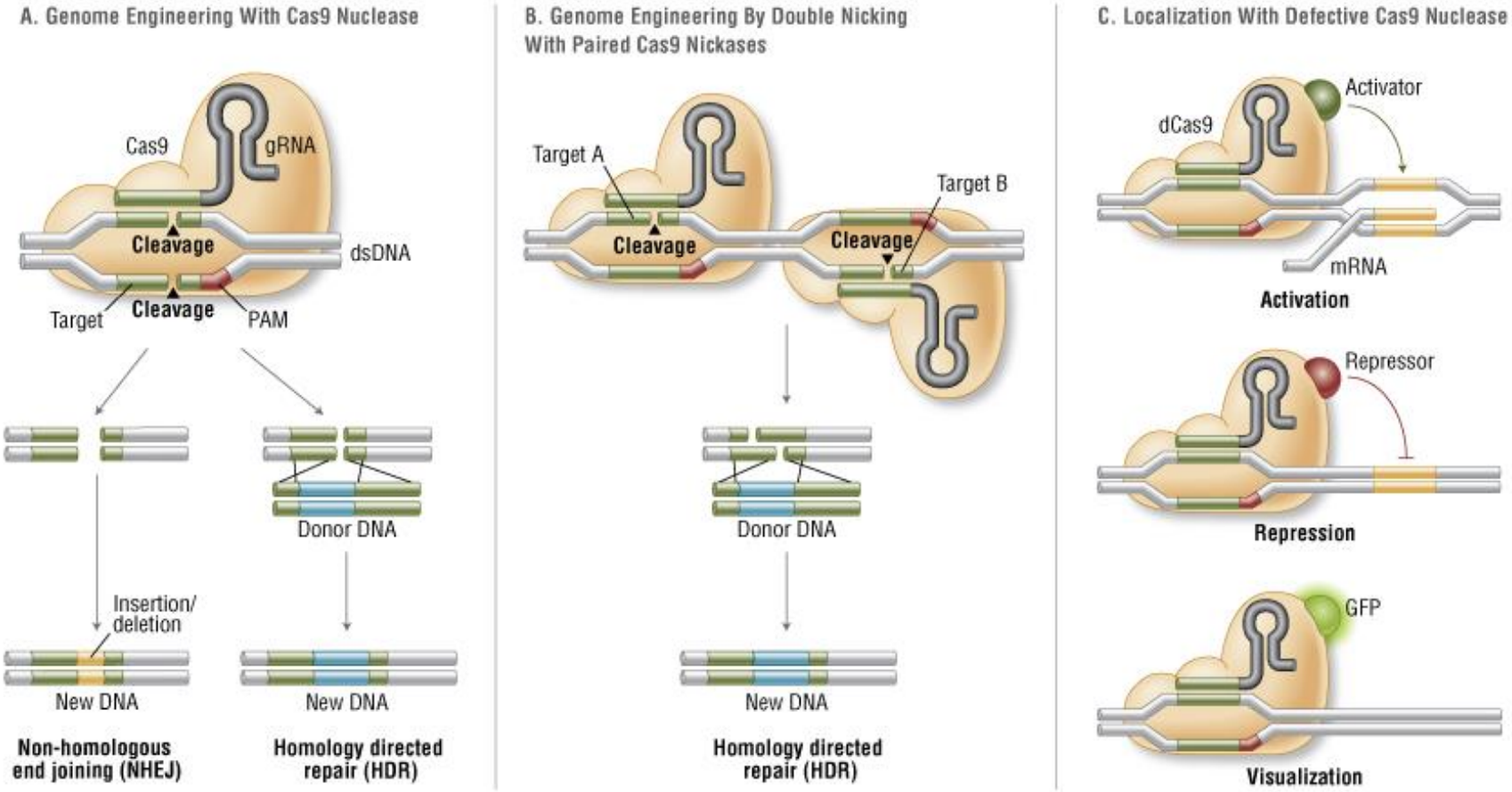




- PAM – protospacer adjacent motif
- sequence in the vicinity of gDNA
- required for efficient cleavage by Cas9 nuclease
- the original system "NGG" (but the development of systems with other sequences)
- according to the system target sequence must be in the N 20 -GG



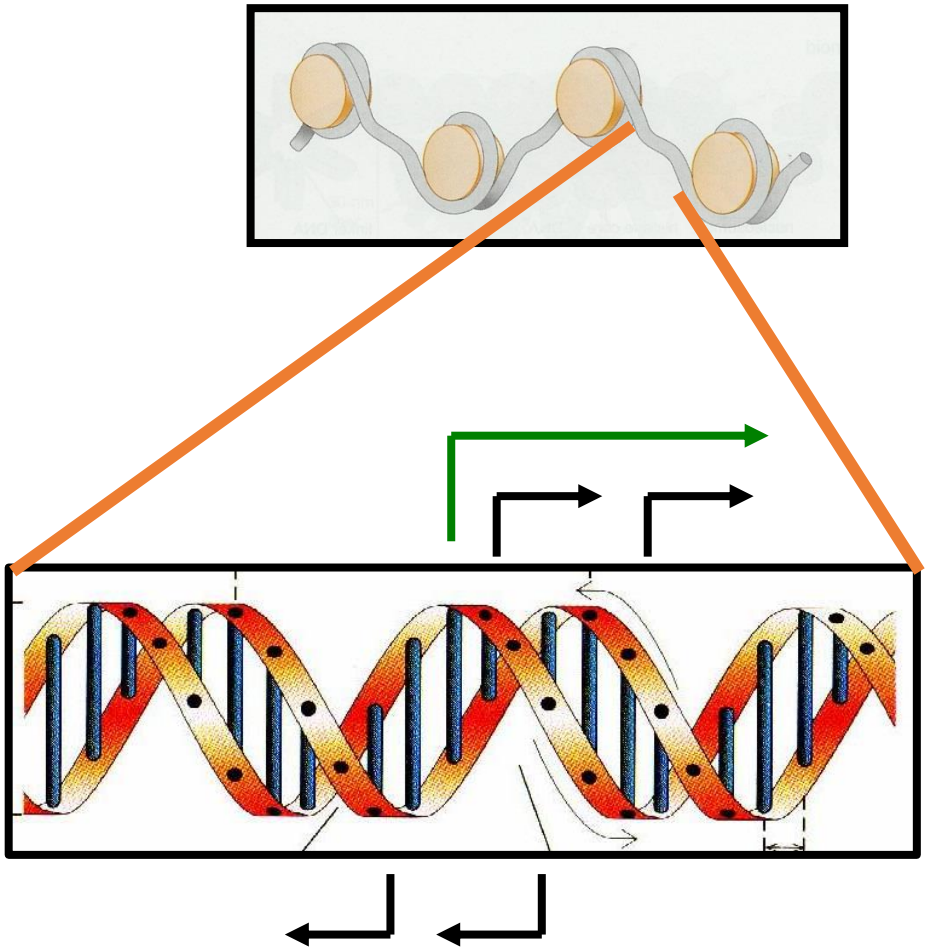
gDNA libraries available for various organisms
- beware of non-specific binding - non-specific cleavage
- use several different gDNA
- reversion of the phenotype by increasing the expression
- introduction of mutation
- Various companies - different platforms (modifications of the original system)
- available software can be used to design



A. Wild-type Cas9 nuclease site specifically cleaves double-stranded DNA activating double-strand break repair machinery. In the absence of a homologous repair template non-homologous end joining can result in indels disrupting the target sequence. Alternatively, precise mutations and knock-ins can be made by providing a homologous repair template and exploiting the homology directed repair pathway.
B. Mutated Cas9 makes a site specific single-strand nick. Two sgRNA can be used to introduce a staggered double-stranded break which can then undergo homology directed repair.
C. Nuclease-deficient Cas9 can be fused with various effector domains allowing specific localization. For example, transcriptional activators, repressors, and fluorescent proteins.

PASRs – 12/2008

PaRNA = promotor associated RNA



transcriptionally active region is between nucleosomes

- transcription of hnRNA

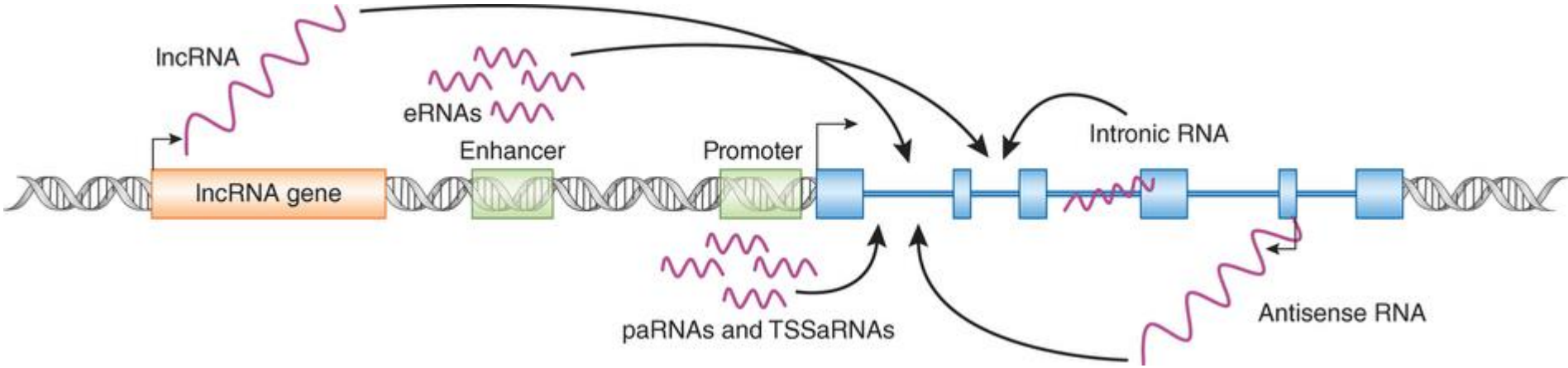
- short RNA generated by transcription in both directions

Buratowski (2008): Science 322, 1804-1805

paRNA

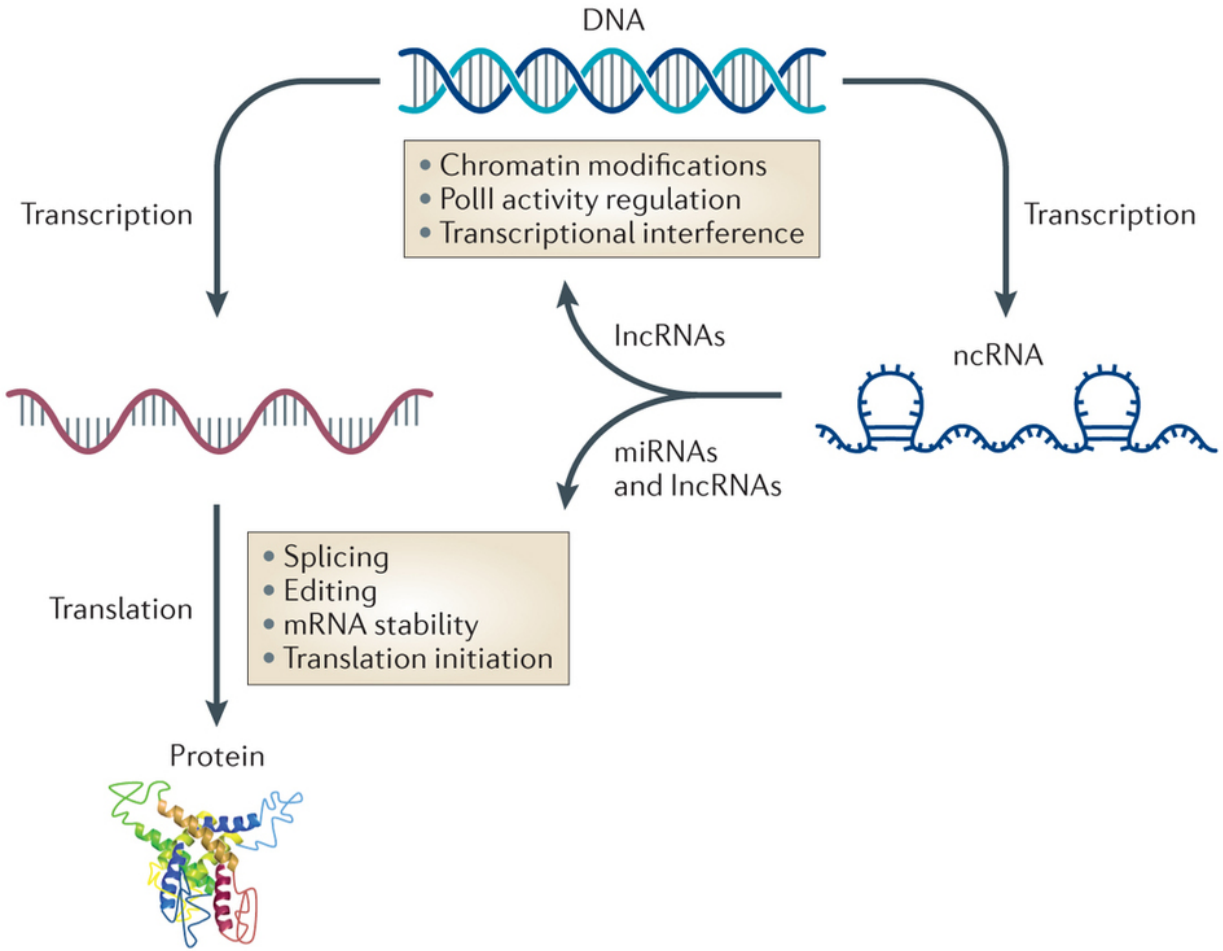
- paRNA is actually simple antisense RNA
- occurs concurrently with transcription of hnRNA

➢it is synthesized by RNA polymerase II



Their expression is often coordinated with that of neighboring protein-coding genes, and in many cases, related transcripts can influence each other at one step or another during their biogenesis.

lncRNA – long non-coding RNA

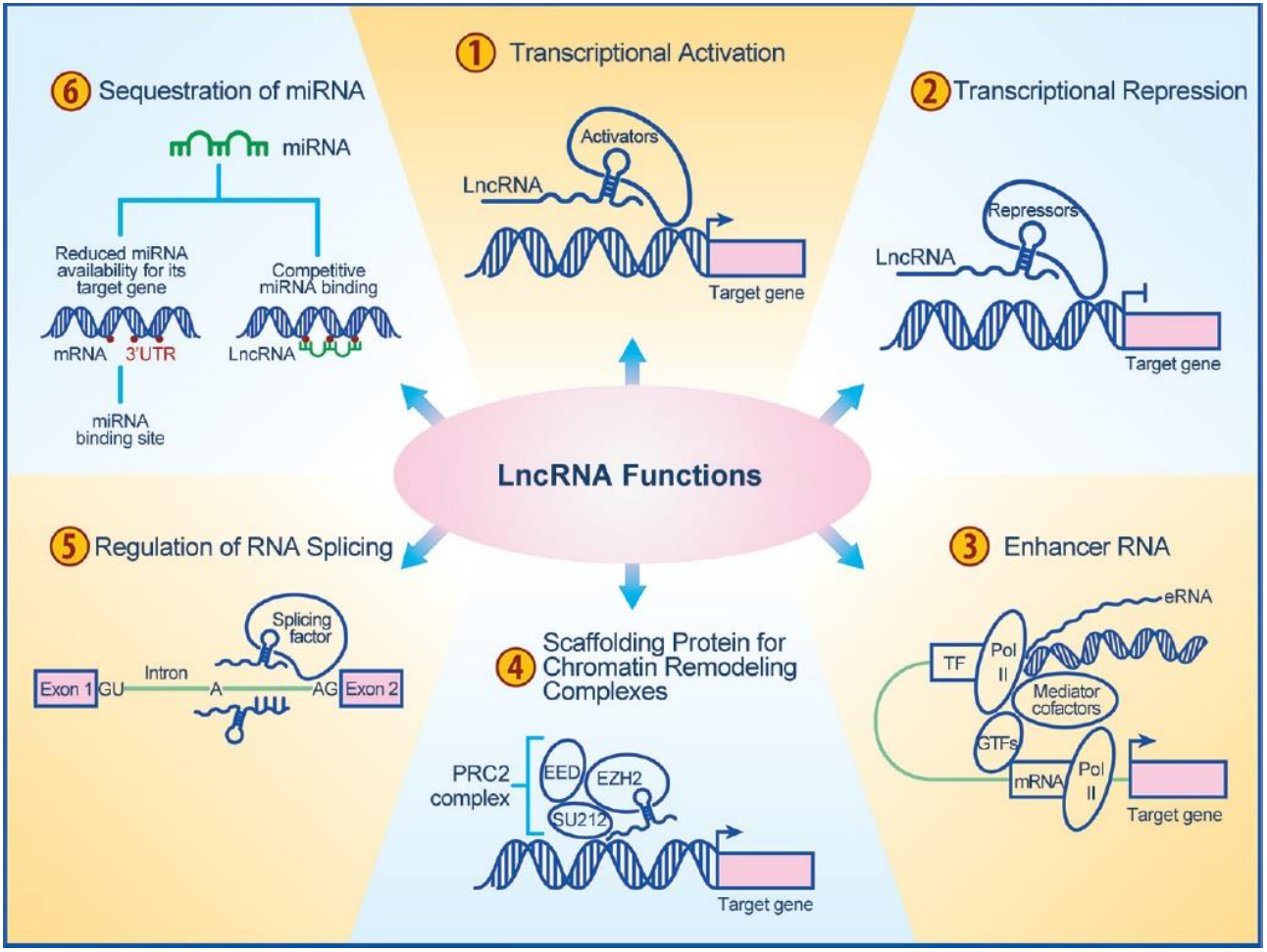


Nature Reviews | Drug Discovery

Long non-coding RNAs (long ncRNAs, lncRNA) are non-protein coding transcripts longer than 200 nucleotides.[1] This somewhat arbitrary limit distinguishes long ncRNAs from small regulatory RNAs such as microRNAs (miRNAs), short interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and other short RNAs.[2]

2009

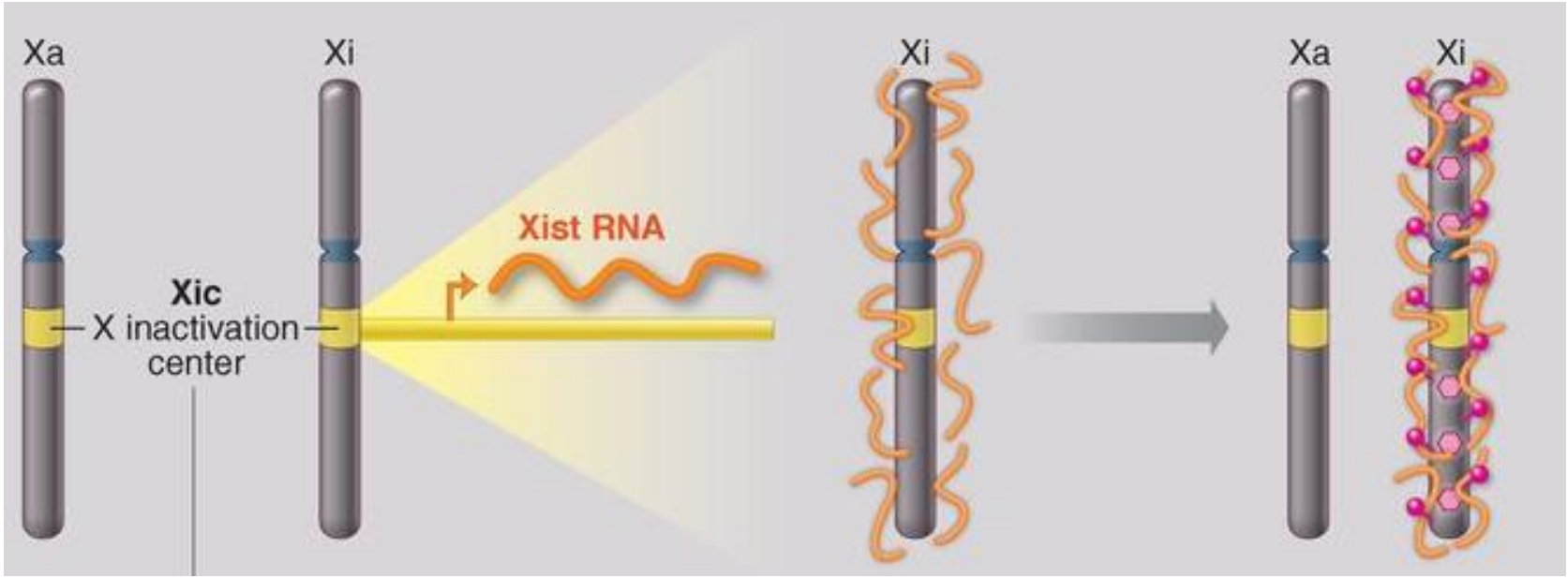
Long ncRNAs in the regulation of gene transcription



Long ncRNAs in gene-specific transcription

In eukaryotes, RNA transcription is a tightly regulated process. NcRNAs can target different aspects of this process, targeting transcriptional activators or repressors, different components of the transcription reaction including RNA polymerase (RNAP) II and even the DNA duplex to regulate gene transcription and expression (Goodrich 2006). In combination these ncRNAs may comprise a regulatory network that, including transcription factors, finely control gene expression in complex eukaryotes.

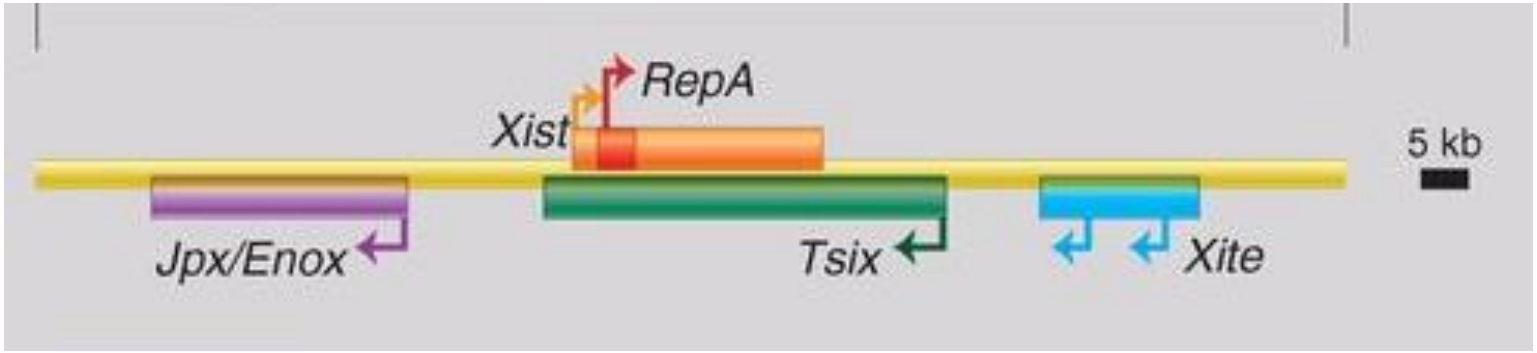
LncRNAs
in the inactivation of the X chromosome



- Xist lncRNA formed transcription inactivation center (Xic) inactive X chromosome (Xi)
- Xist RNA covers the entire chromosome expression and sleep patterns of modifications of histones and DNA

J T Lee (2012): Science 2012;338:1435-1439

The core area Xic and its lncRNA

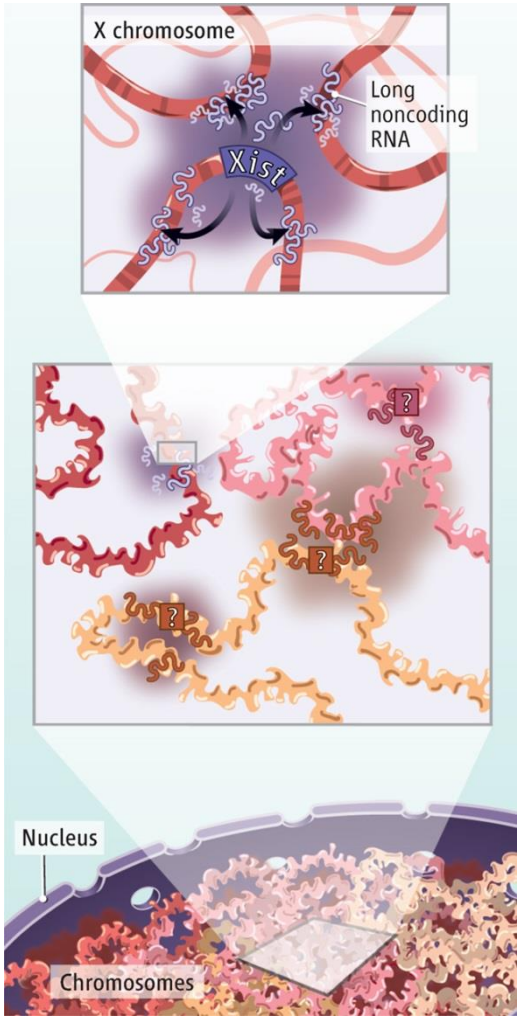


Tsix = antisense transcript= negative regulator of Xist

Jpx = positive regulator Xist

J T Lee (2012): Science 2012;338:1435-1439

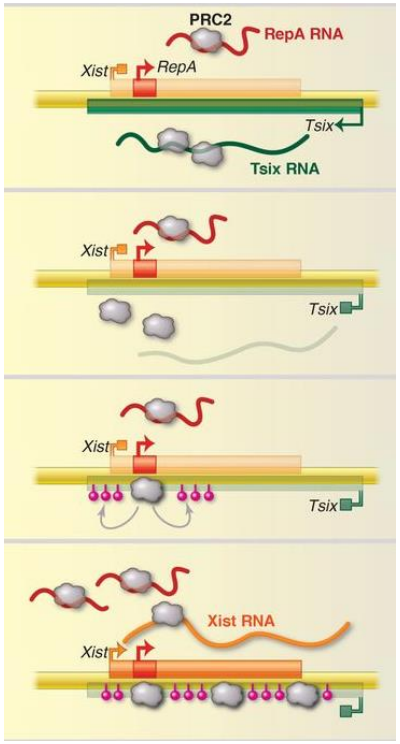
Targeting Xist requires three dimensions



- **Primary binding site for Xist located near the site of transcription locus Xist**
- **Xist does not bind to specific sequences (sequential dependency), but in those places where they happen to appear after transcription (spatial dependence)**
- **Only then effect of Xist spread over the genome further**

Dimond A a Fraser P (2013): Science 2013; 341:720-721

Interaction lncRNA-protein for initiation XCI

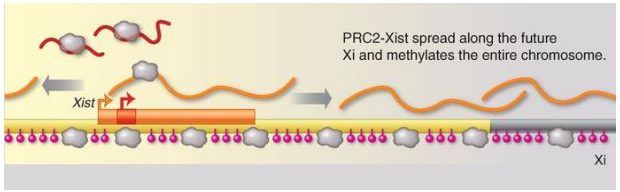


Tsix preventing connection PRC2-RepA to chromatin and prevents X chromosome inactivation

If Tsix does not form, complex PRC2-RepA binds to chromatin

PRC2 methylates future Xi

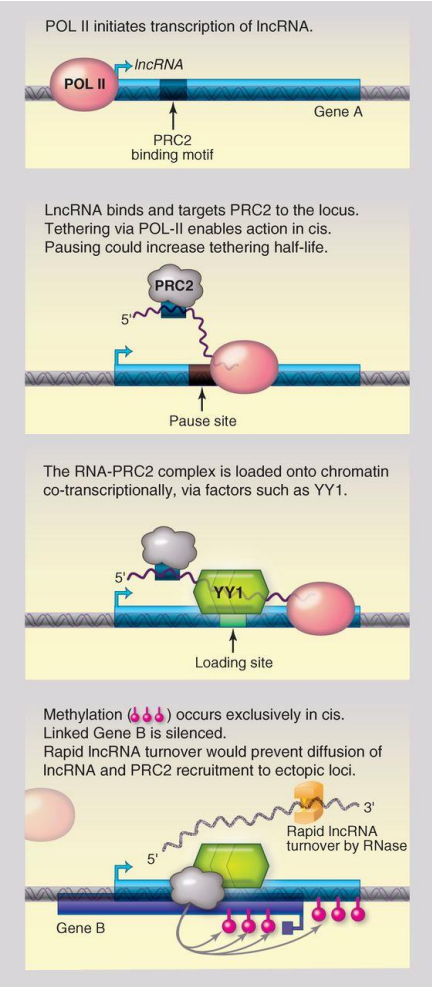
Expression of Xist RNA that binds to PRC2



PRC2-Xist extends along the future Xi and methylate an entire chromosome

J T Lee (2012): Science 2012;338:1435-1439

LncRNAs connects epigenetic complexes to chromatin …

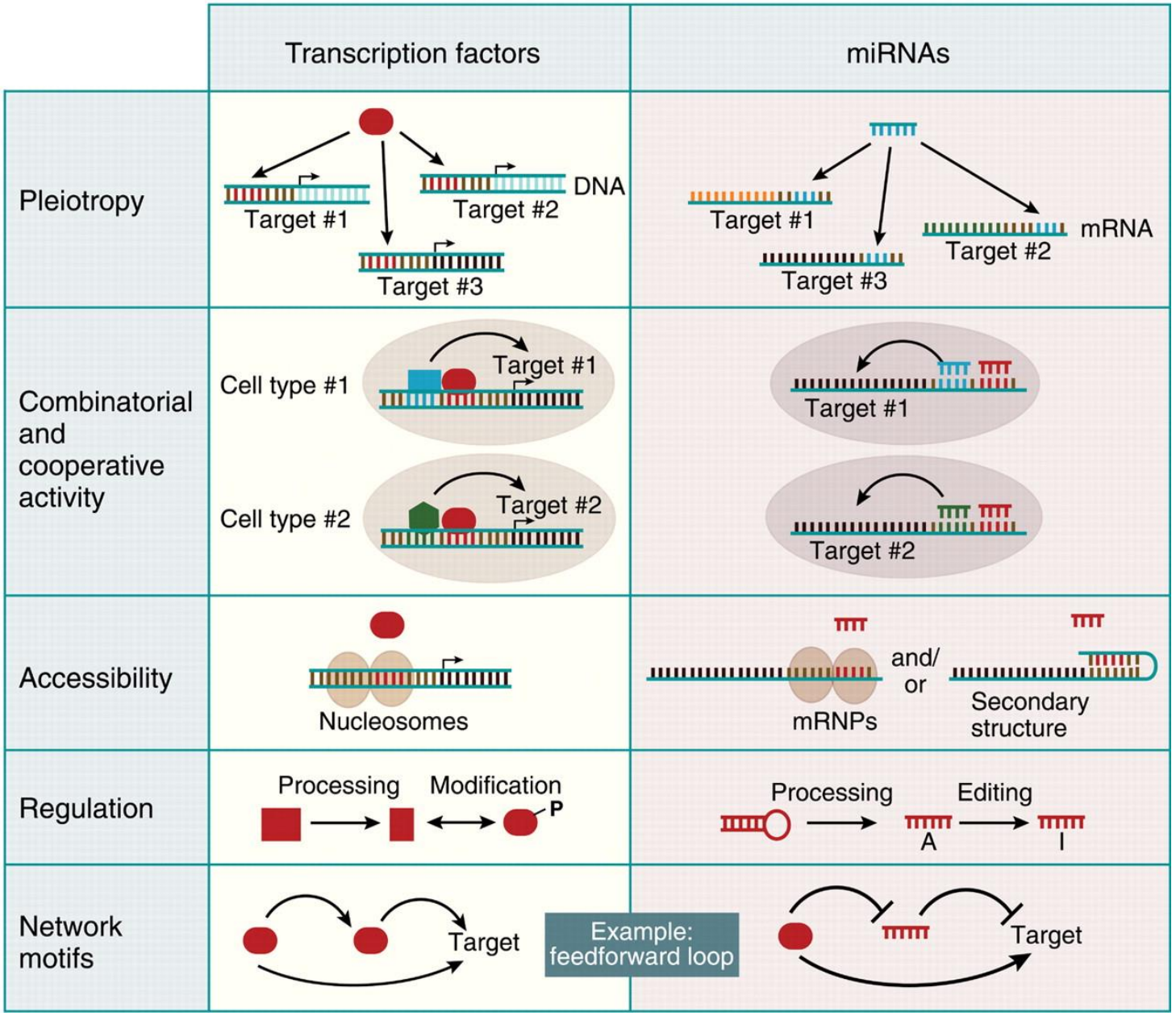


… allowing allele and locus-specific regulation

- Emerging lncRNA binds to the complex epigenetic (eg. PRC2)
- Along with him is bound to chromatin through DNA binding factors (e.g. YY1 for Xist RNA)
- Epigenetic modifications are put to sleep gene
- LncRNA rapid degradation prevents its diffusion to other loci

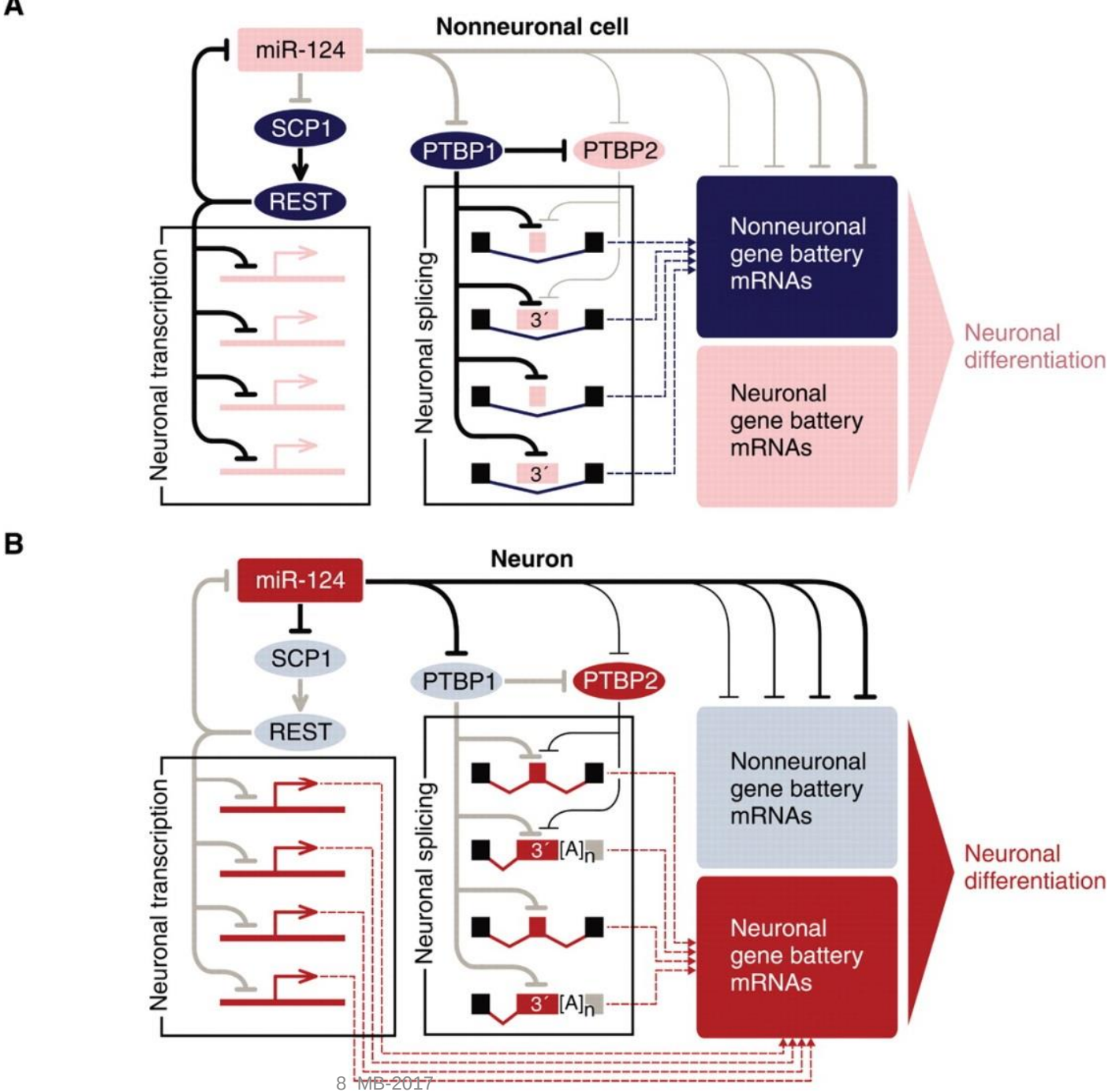
J T Lee (2012): Science 2012;338:1435-1439

Principles of regulation of miRNA and TF



O. Hobert Science 319, 1785 -1786 (2008)

Regulatory network of miR-124



E. V. Makeyev et al.. Science 319, 1789 -1790 (2008)

- https://www.youtube.com/watch?v=2pp17E4E-O8&t=4s