Small RNAs

RNA interference
Identification and function

Press Release - 2 October 2006

The Nobel Assembly at Karolinska Institutet decided to award The Nobel Prize in Physiology or Medicine for 2006 jointly to

Andrew Fire and Craig Mello

for their discovery of

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

This year's Nobel Laureates have discovered a fundamental mechanism for controlling the flow of genetic information. Our genome operates by sending instructions for the manufacture of proteins from DNA in the nucleus of the cell to the protein synthesizing machinery in the cytoplasm. These instructions are conveyed by messenger RNA (mRNA). In 1998, the American scientists Andrew Fire and Craig Mello published their discovery of a mechanism that can degrade mRNA from a specific gene. This mechanism, RNA interference, is activated when RNA molecules occur as double-stranded pairs in the cell. Double-stranded RNA activates biochemical machinery which degrades those mRNA molecules that carry a genetic code identical to that of the double-stranded RNA. When such mRNA molecules disappear, the corresponding gene is silenced and no protein of the encoded type is made.

RNA interference occurs in plants, animals, and humans. It is of great importance for the regulation of gene expression, participates in defense against viral infections, and keeps jumping genes under control. RNA interference is already being widely used in basic science as a method to study the function of genes and it may lead to novel therapies in the future.

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

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

Nature (1998) 391, 806 - 811

Nature **391**, 806-811 (19 February 1998)

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

Andrew Fire ……. and Craig C. Mello

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene. 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. 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.

Purified antisense and sense RNAs covering a 742-nucleotide segment of *unc-22* had only marginal interference activity, requiring a very high dose of injected RNA to produce any observable effect (Table 1). In contrast, a sense–antisense mixture produced highly effective interference with endogenous gene activity.

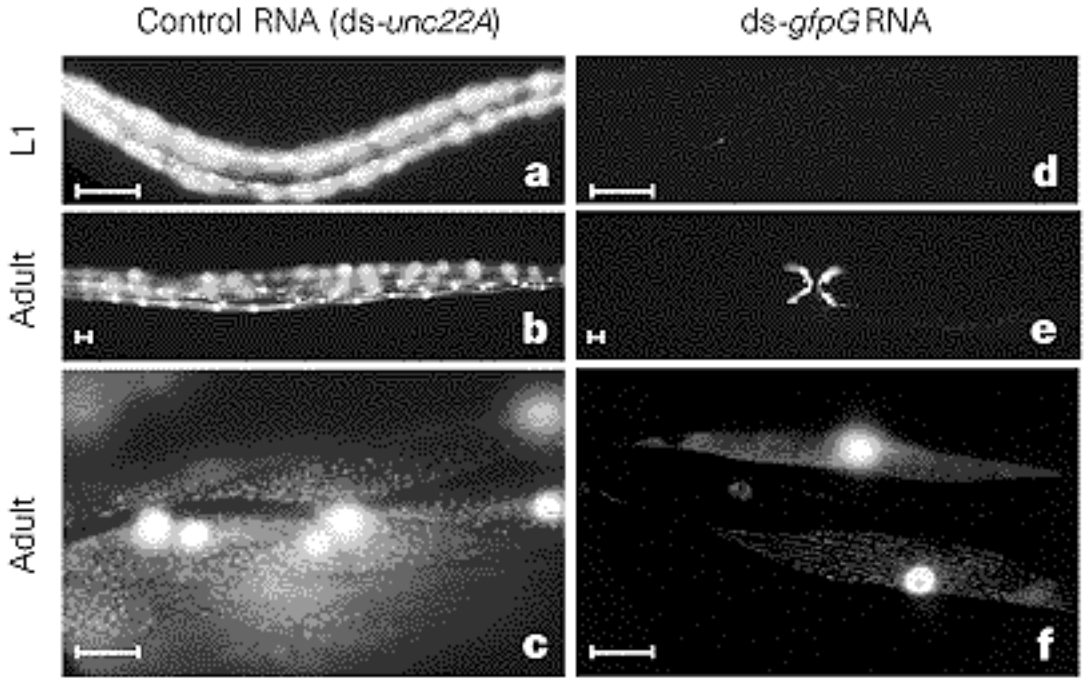
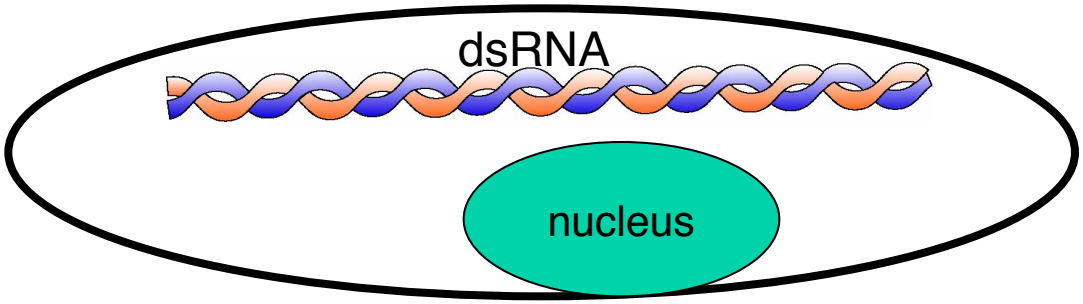


Figure 2 Analysis of RNA-interference effects in individual cells. Fluorescence micrographs show progeny of injected animals from GFP-reporter strain PD4251. **a–c, Progeny of animals injected with a control RNA (double-stranded (ds)-unc22A**). a, Young larva, b, adult, c, adult body wall at high magnification. These GFP patterns appear identical to patterns in the parent strain, with prominent fluorescence in nuclei (nuclear-localized GFP–LacZ) and mitochondria (mitochondrially targeted GFP). **d–f, Progeny of animals injected with ds-gfpG.** Only a single active cell is seen in the larva in d, whereas the entire vulval musculature expresses active GFP in the adult animal in e. f, Two rare GFP-positive cells in an adult: both cells express both nuclear-targeted GFP–LacZ and mitochondrial GFP.

At high concentrations of *gfp* dsRNA, we saw interference in virtually all striated body-wall muscles, with occasional lone escaping cells, including cells born during both embryonic and postembryonic development. The non-striated vulval muscles, which are born during late larval development, appeared to be resistant to interference at all tested concentrations of injected dsRNA.

What's RNA interference (RNAi)?

- RNAi it's a process of gene silencing triggered by a dsRNA



RNAi + PTGS

RNA interference (RNAi)

Post-transcriptional gene silencing (PTGS)

Virus-induced gene silencing (VIGS)

Homology-dependent silencing

Quelling

Cosuppression

Characteristics of PTGS

- is sequence specific
- duplication of coding sequences is required for silencing
- both endogenous genes and transgenes are silenced (co-suppression)
- acts at post-transcriptional level
- gene silencing correlates with the presence of transgenic tandem repeats
- the accumulation of unexpected transgenic chimeric transcripts correlates with gene silencing
- reverts *via* excision of transgenic copies
- acts *in trans* in heterokaryons (it is mediated by a diffusible molecule)

Cosuppression

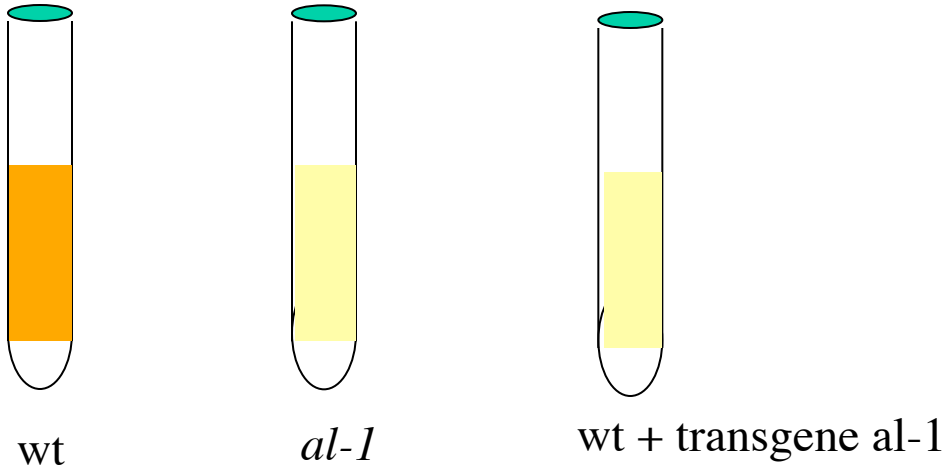
The overexpression of the CHS (Chalcone synthase) gene in petunia leads to lack of flower pigmentation instead of increasing it.

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

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

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



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

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

Initiation and Maintenance of Virus-Induced Gene Silencing

The Plant Cell, Vol. 10, 937–946, June 1998

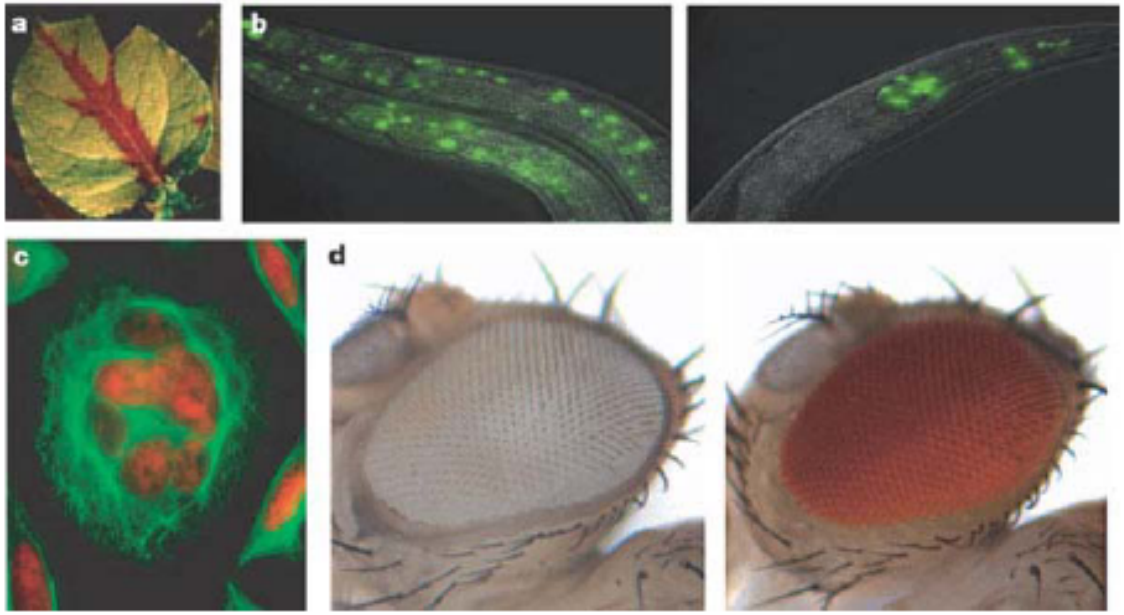
M. Teresa Ruiz, Olivier Voinnet, and David C. Baulcombe

The phytoene desaturase (PDS) gene of *Nicotiana benthamiana* was *silenced in plants infected with potato virus X (PVX) vectors carrying PDS inserts, and a green fluorescent protein (GFP) transgene was silenced in plants infected with PVX–GFP. This virus-induced gene silencing (VIGS) is post-transcriptional and cytoplasmic because it is targeted against exons rather than introns of PDS RNA and against viral RNAs.*

RNAi:

a tool for inhibiting gene expression *in vivo* in a sequence-specific way

Plants



C. elegans

HeLa cells

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

Drosophila

In plants, silencing can be triggered, for example, by engineered RNA viruses or by inverted repeat transgenes. In worms, silencing can be triggered by injection or feeding of dsRNA. In both of these systems, silencing is systemic and spreads throughout the organism. **a**, A silencing signal moves from the veins into leaf tissue. Green is green fluorescent protein (GFP) fluorescence and red is chlorophyll fluorescence that is seen upon silencing of the GFP transgene. **b**, *C. elegans* engineered to express GFP in nuclei. Animals on the right have been treated with a control dsRNA, whereas those on the left have been exposed to GFP dsRNA. Some neuronal nuclei remain florescent, correlating with low expression of a protein required for systemic RNAi59. **c**, HeLa cells treated with an ORC6 siRNA and stained for tubulin (green) and DNA (red). Depletion of ORC6 results in accumulation of multinucleated cells. Stable silencing can also be induced by expression of dsRNA as hairpins or snap-back RNAs. **d**, Adult *Drosophila* express a hairpin homologous to the white gene (left), which results in unpigmented eyes compared with wild type (right).

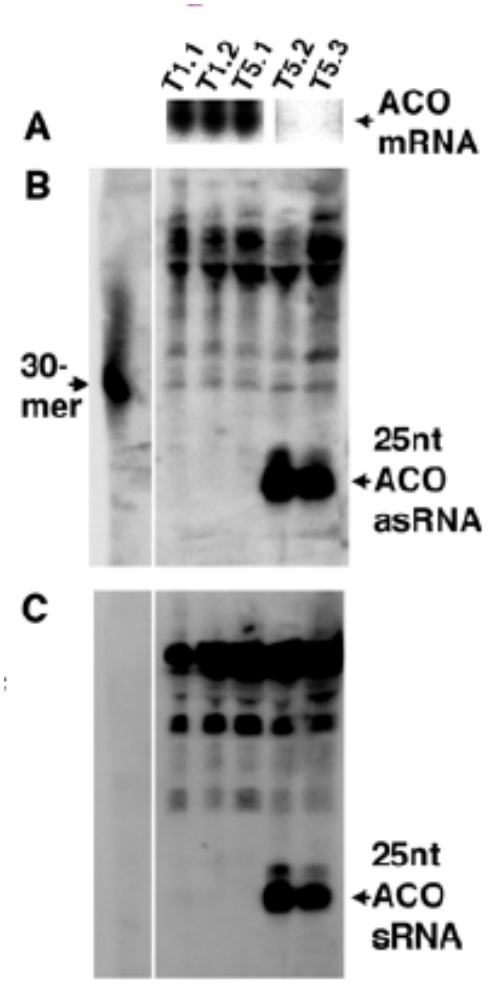
Cosuppression, PTGS, Quelling, VGS initially appeared different processes.

The identification in different organisms of mutants affecting these processes and the characterization of very short RNAs has allowed to produce a unifying theory.

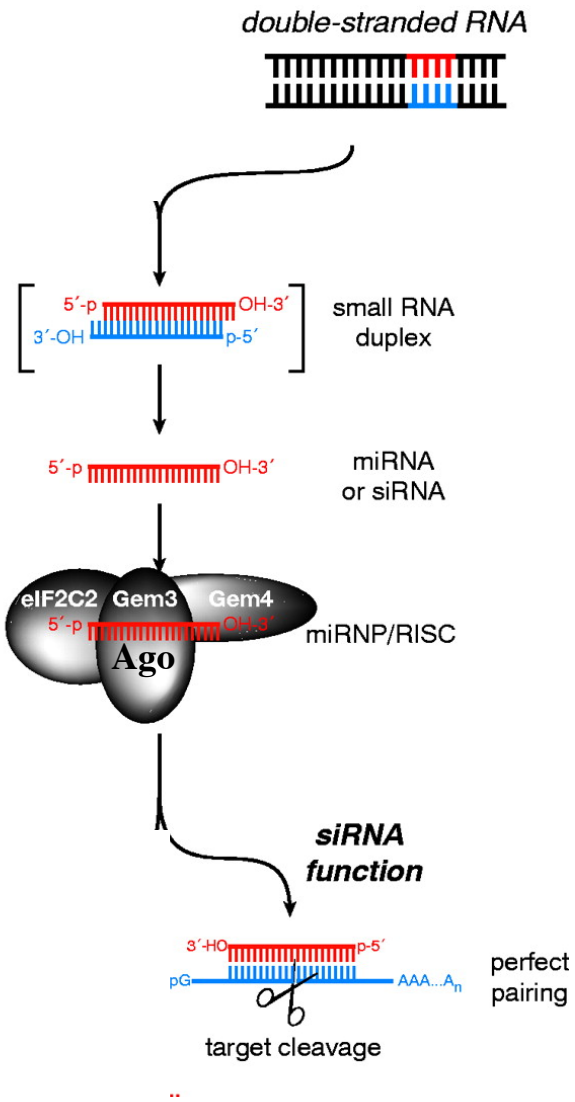
RNAi correlates with the production of small RNAs

- In plants, during PTGS, small RNA of 25 nt are found. They are absent in control plants.

- Such RNAs are complementary to both the sense and antisense sequences of the silenced gene

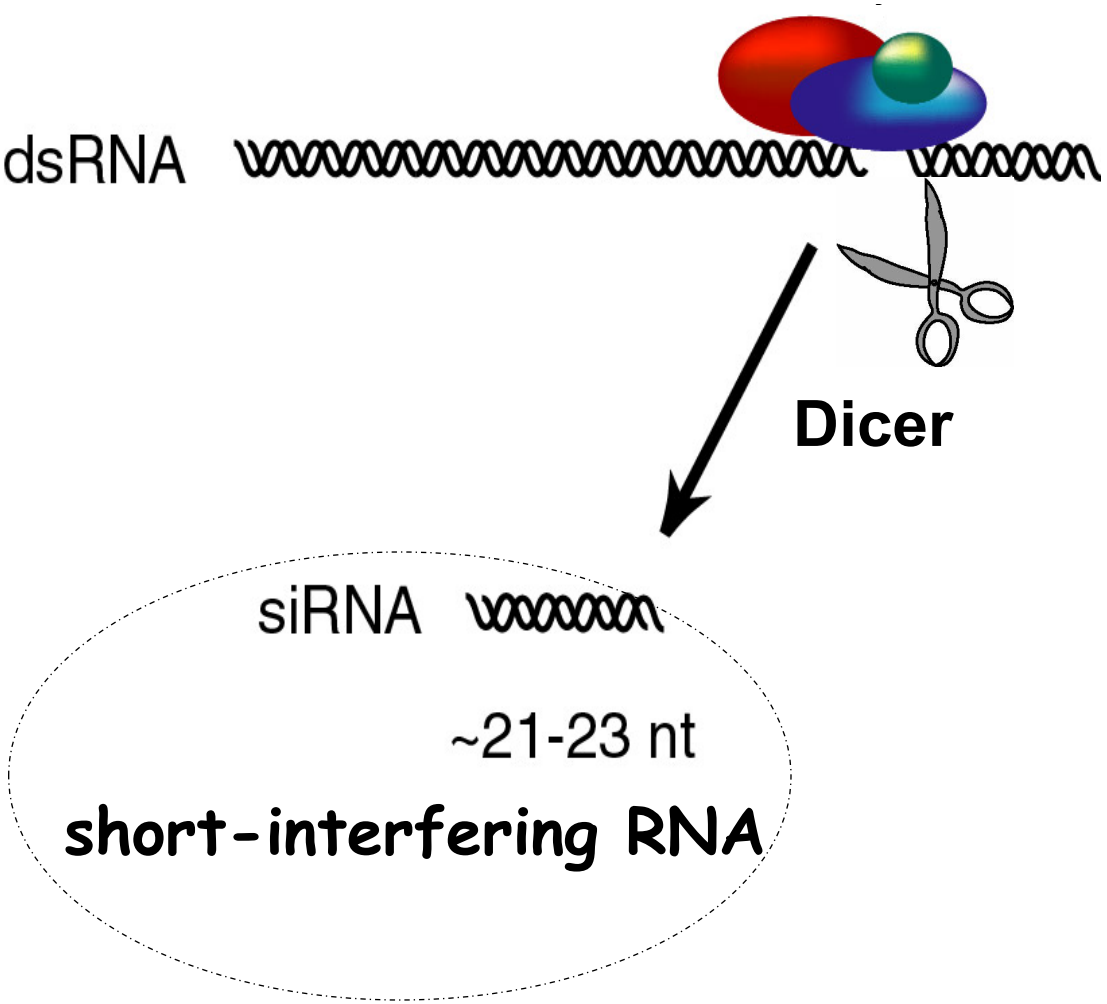


Hamilton e Baulcombe (Science,1999)

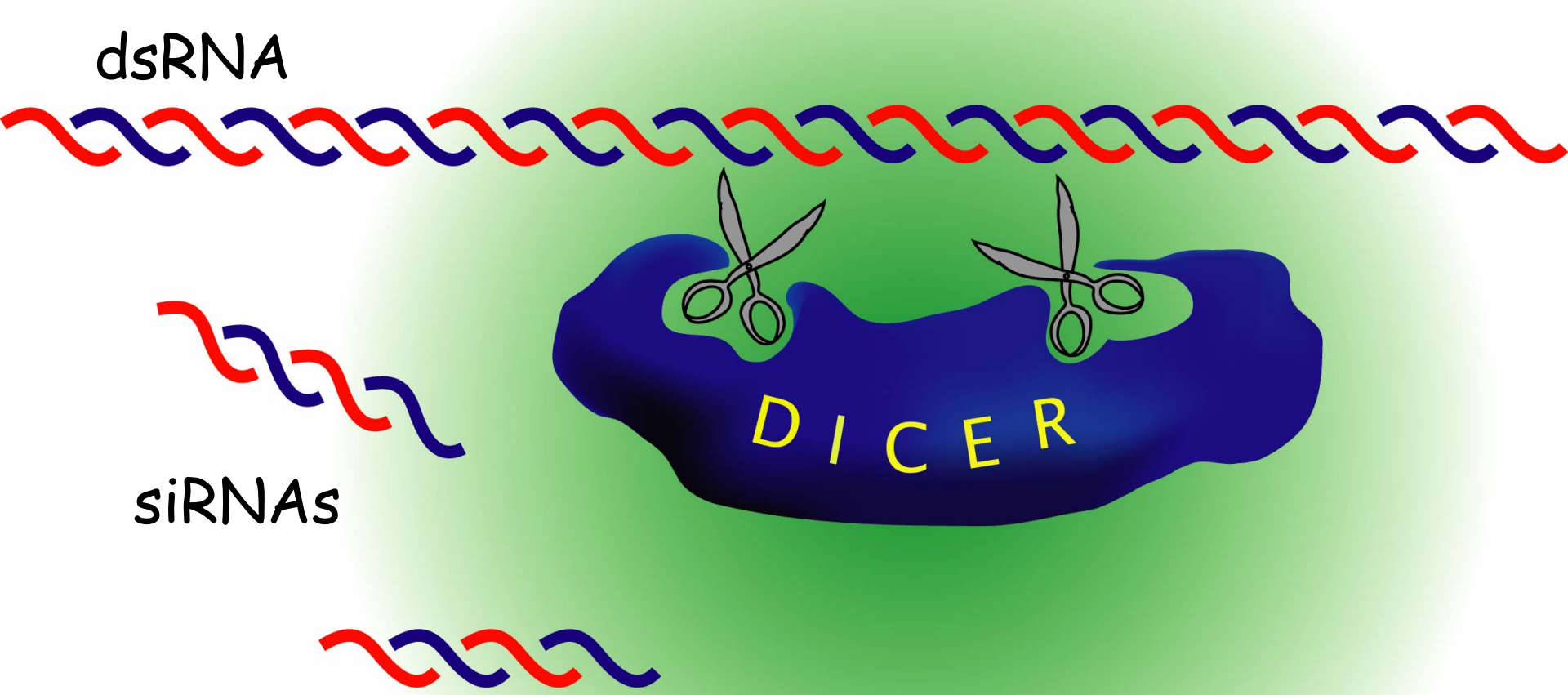

double-stranded RNA
5′-p
OH-3′
3′-OH
p-5′
small RNA duplex
5′-p
OH-3′
miRNA or siRNA
eIF2C2
Gem3
Gem4
5′-p
OH-3′
Ago
miRNP/RISC
siRNA function
3′-HO
p-5′
pG
AAA...An
perfect pairing
target cleavage

First step:

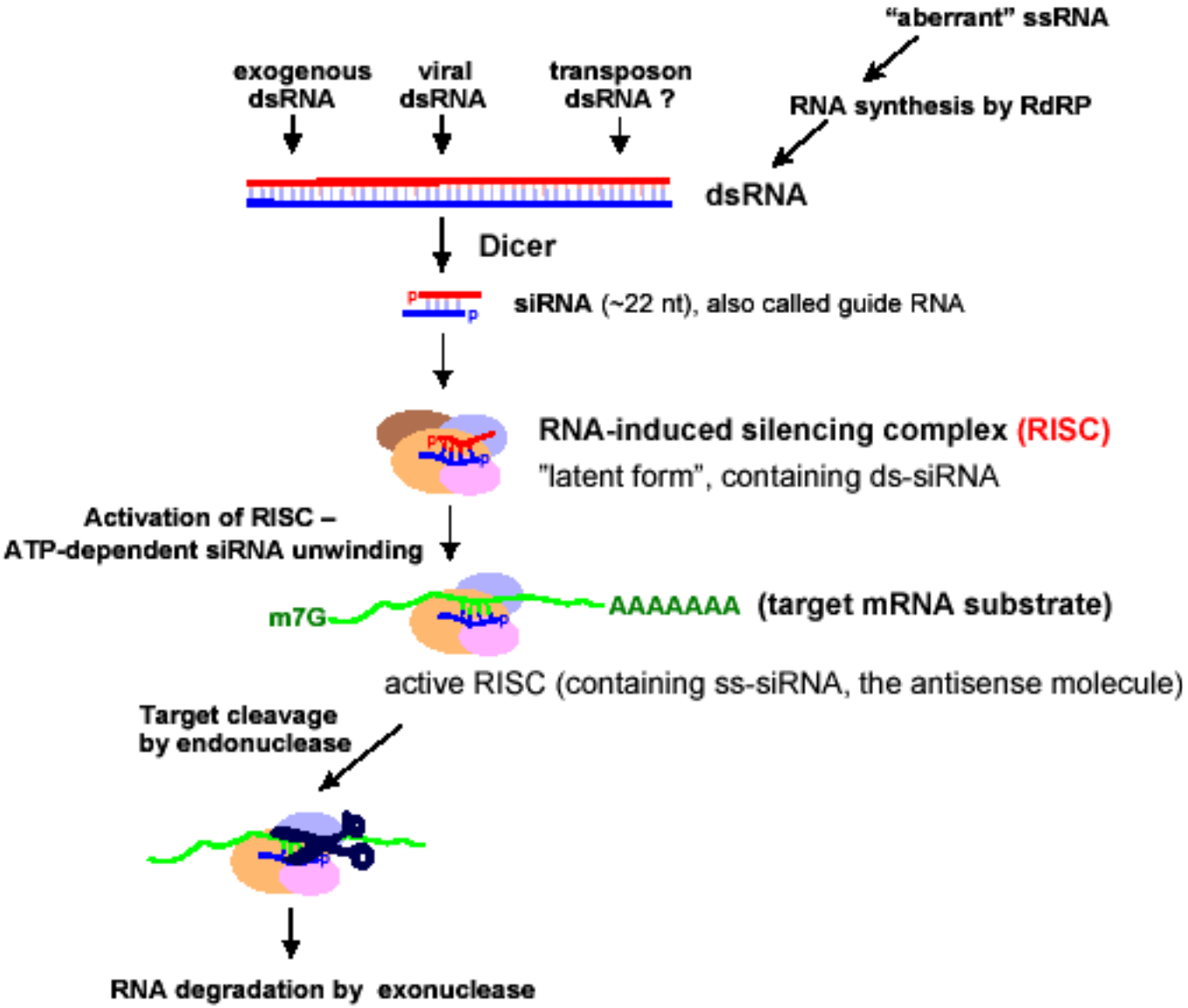
Processing of long dsRNA in 21-23 nt long pieces



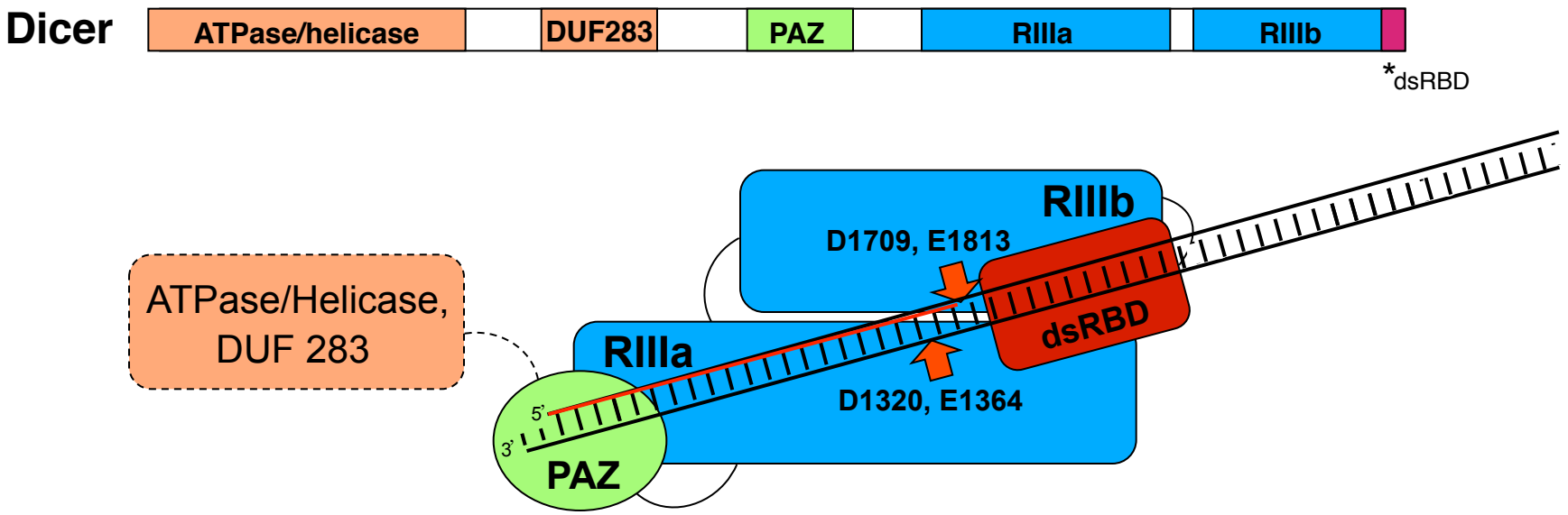
Dicer has two RNase III domains



RNAi mechanism

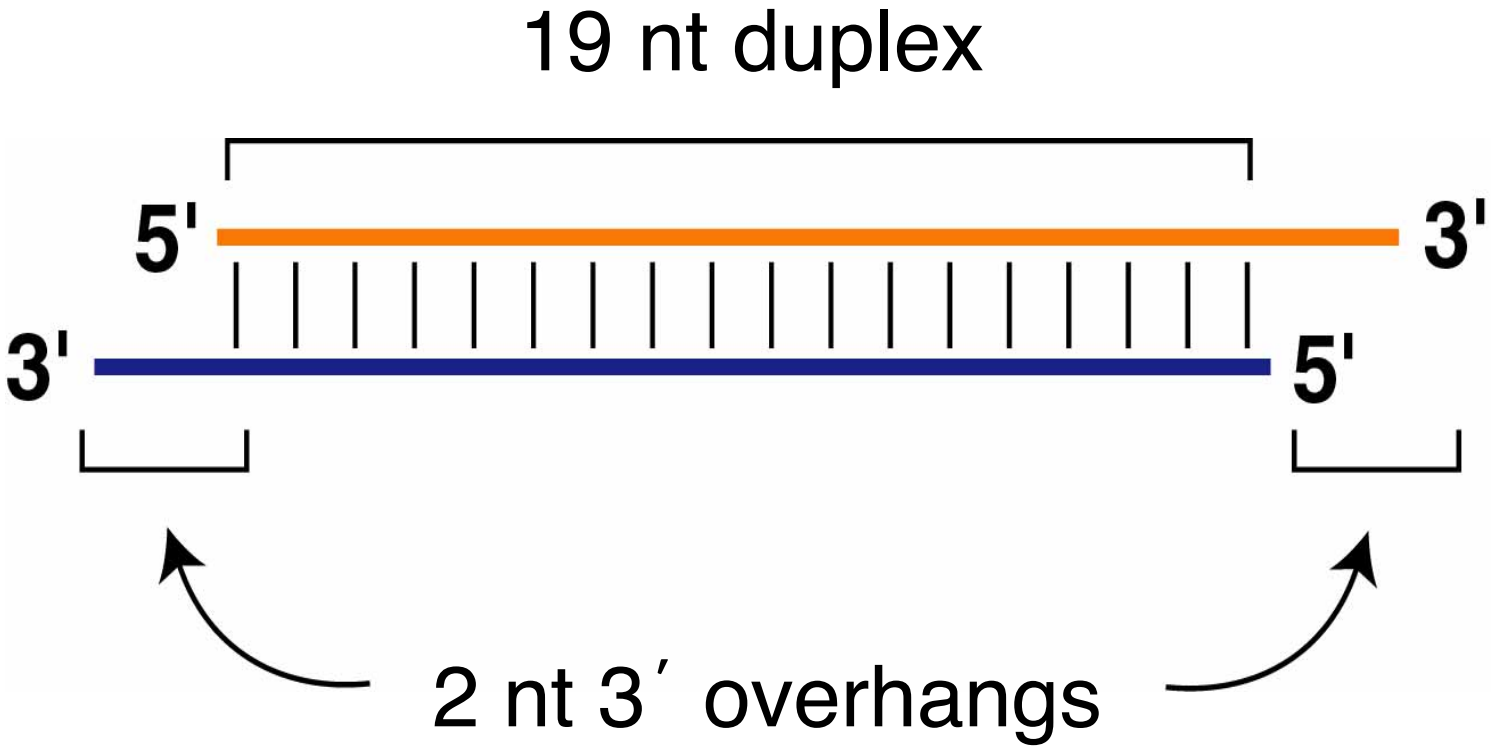


A model of dsRNA processing by Dicer



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

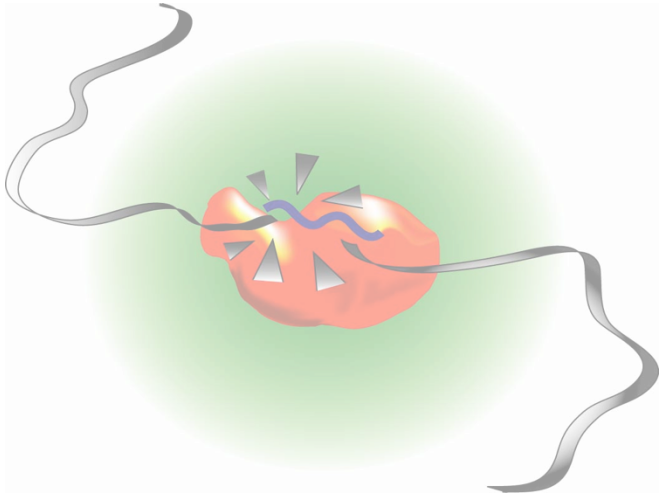
siRNAs have a well defined structure



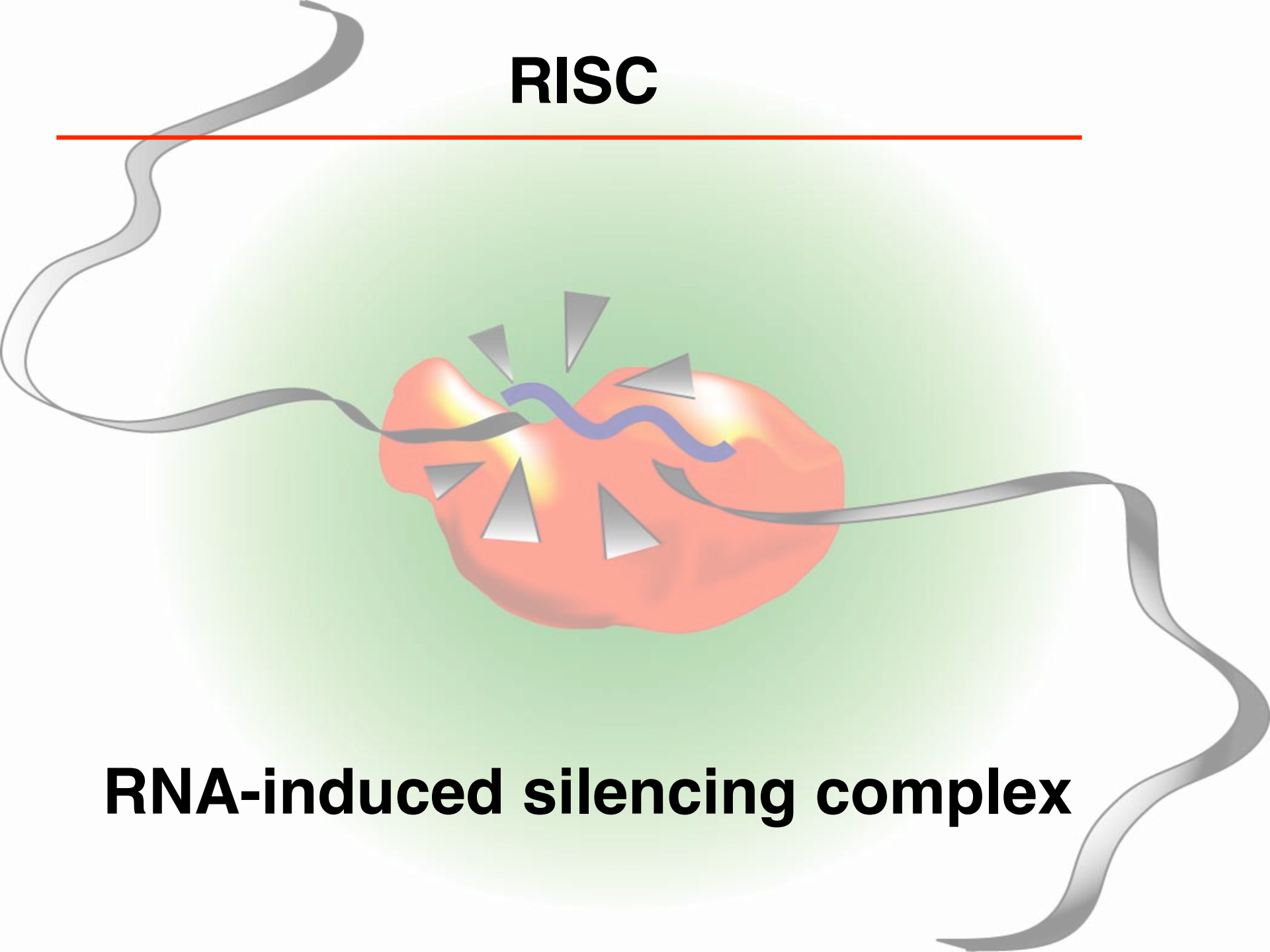
Second step:

The antisense siRNA is incorporated into the RISC complex and directs cleavage of the target RNA

- Dicer releases siRNAs to RISC through the interaction of its Paz domain with the one of AGO
- RISC is a multiprotein complex
- The activation of RISC is ATP-dependent and requires the unwinding of the duplex siRNA

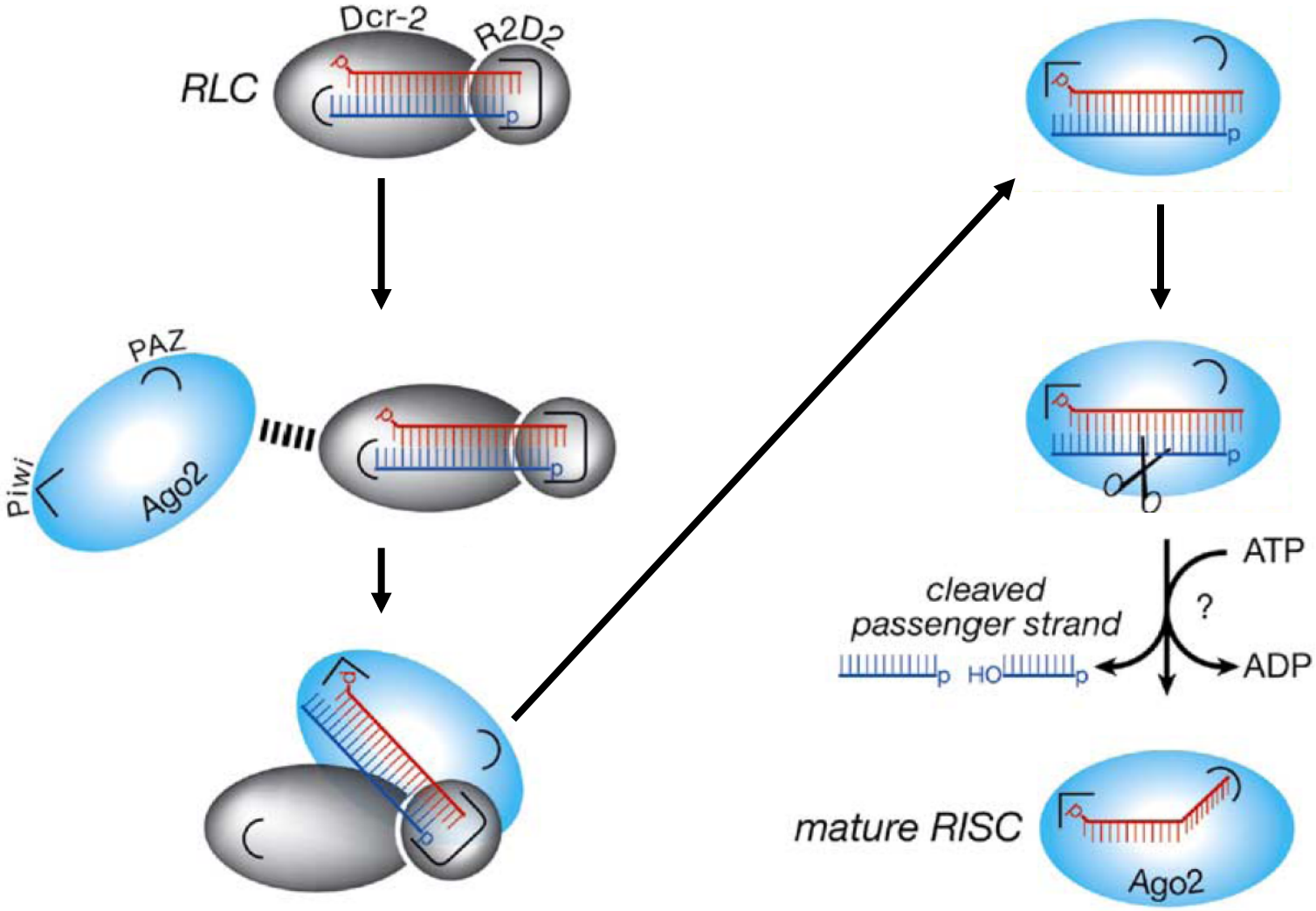


RISC



RNA-induced silencing complex

how this does it works in RLC?



Argonaute proteins

- characterized by PAZ and PIWI domains (PPD proteins)

- PAZ domains anchor the 3’ ends of small RNAs

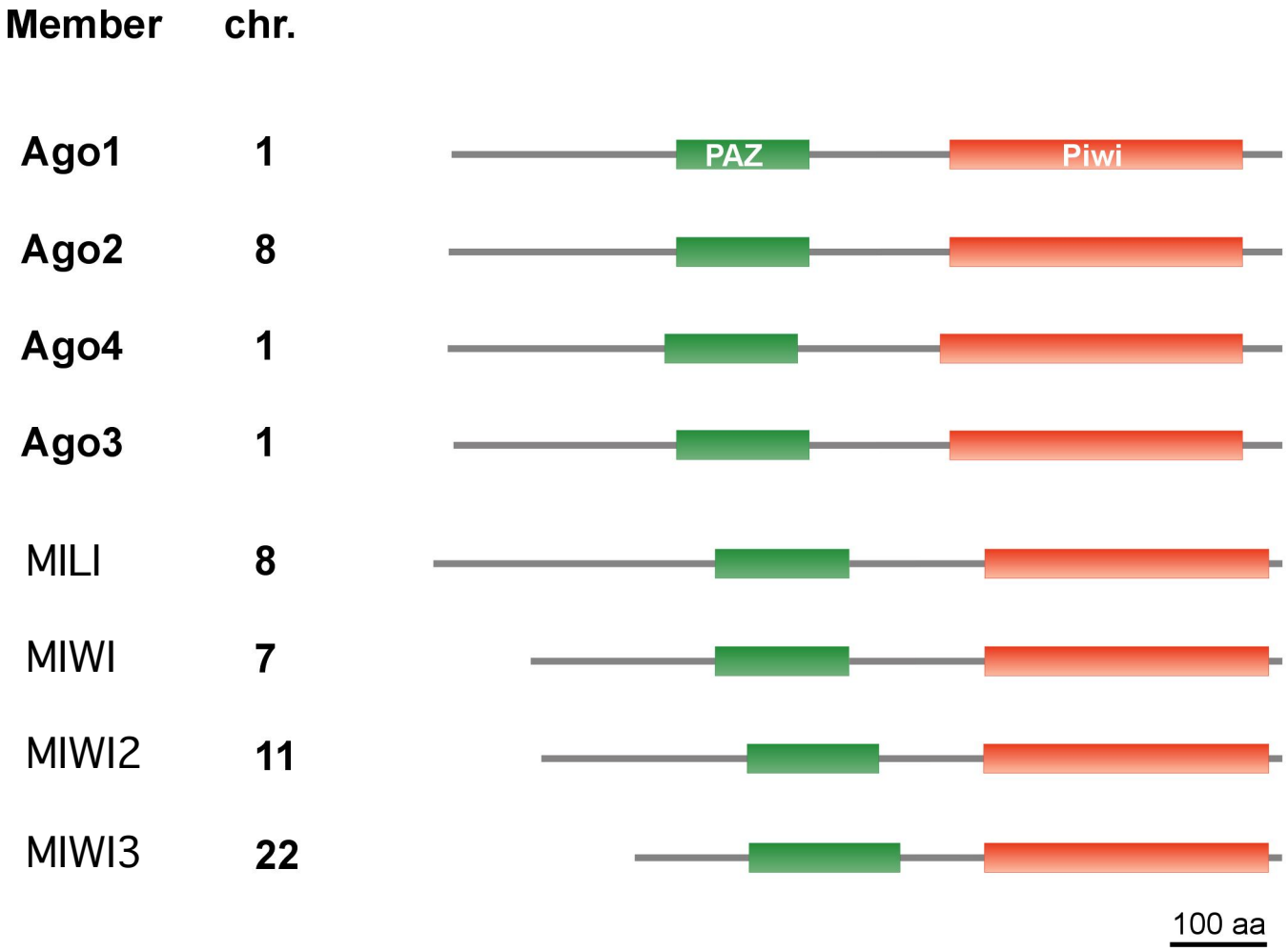
- PIWI domain is structurally similar to RNase H and can cleave complementary RNAs

- The MID domain anchors the 5’ end of the small RNA

- Ago proteins specifically bind small RNAs and are key-players in small RNA guided gene silencing

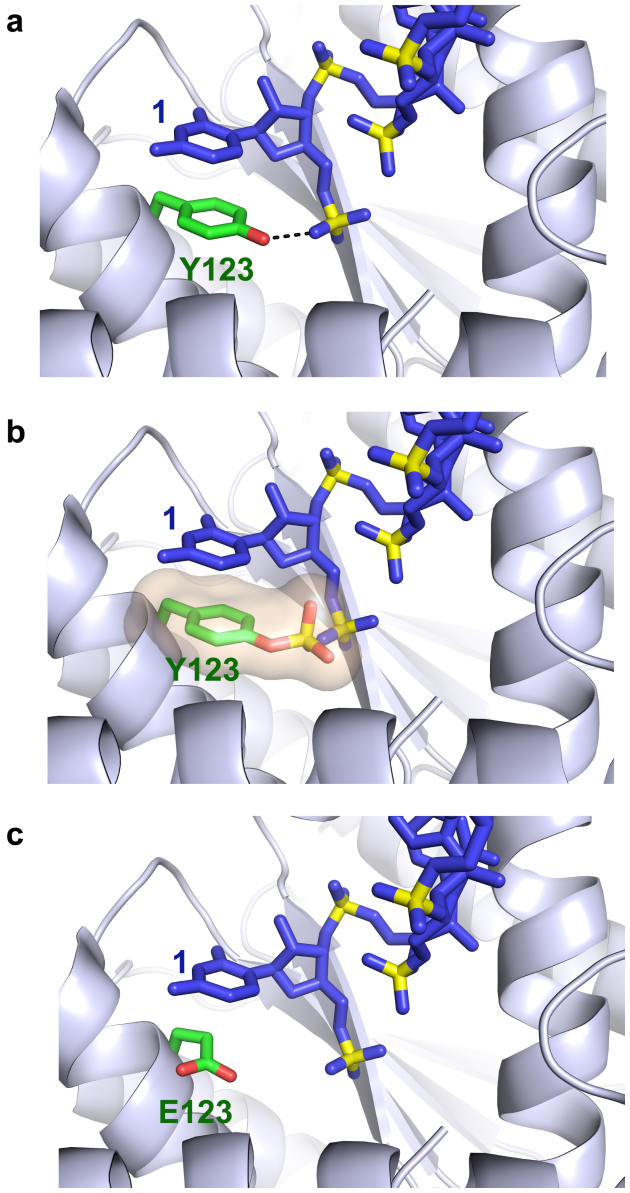
Ago proteins in different organisms	
S. pombe	1
D. melanogaster	5
C. Elegans	27
Mammals	4

The human Argonaute protein family



A type of small non-coding RNA known as piRNAs (**Piwi-interacting RNAs**) have recently been shown to have important and conserved function in germ cell development and maintenance. The Piwi proteins are highly conserved throughout evolution, belong to the Argonaute family and are close relatives of the Ago proteins that execute miRNA/siRNA function

Model of the *A.fulgidus* 5' phosphate binding pocket with Y123p



- Y529 is conserved between human Ago2 and *A.fulgidus* Ago (Y123)

- Y529F allows stacking interactions, Y529A destroys stacking interactions

- Y529E destroys stacking interactions and generates a negatively charged environment

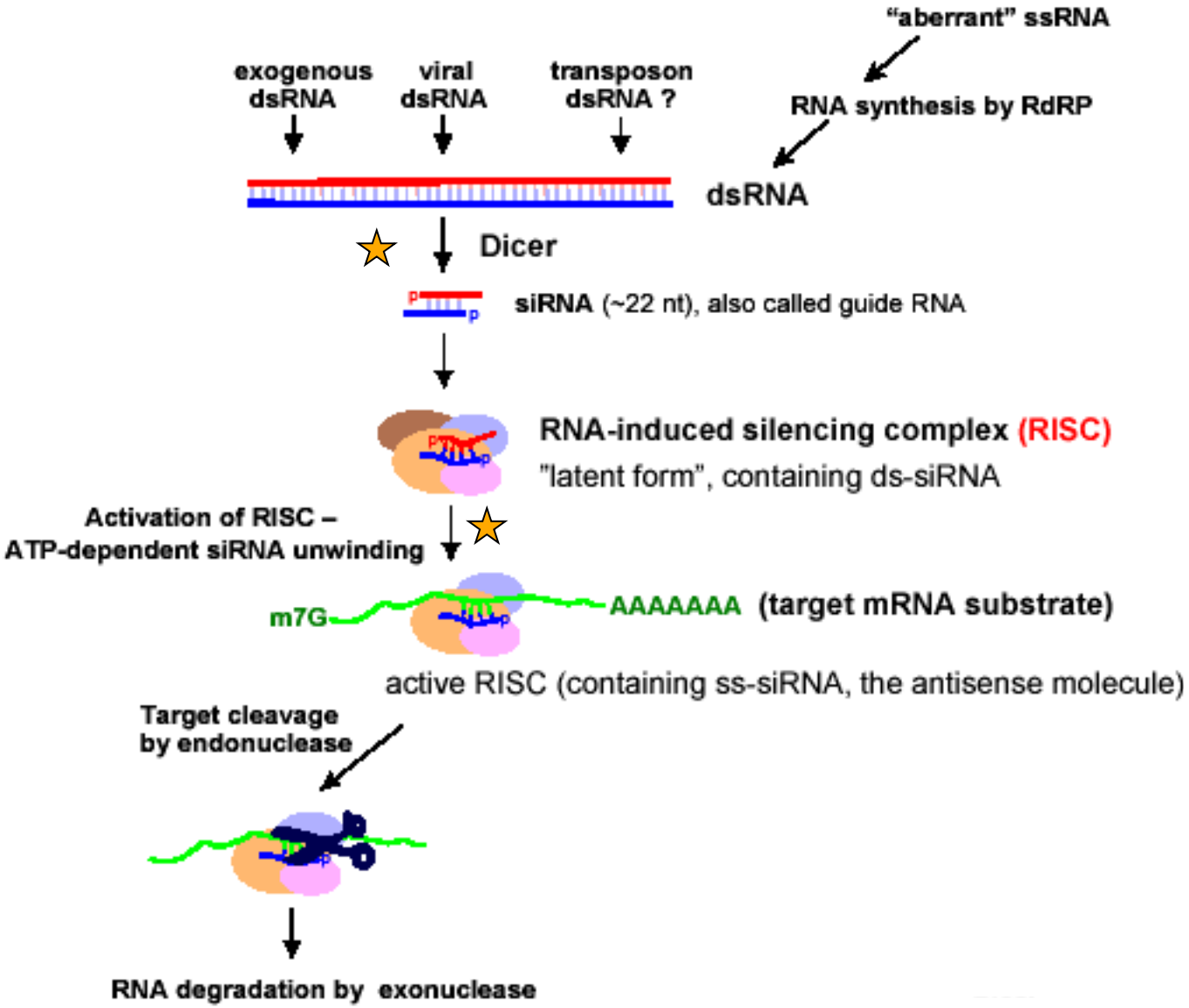
Y - Tyr
F - Phe
E - Glu

Wang Y., Patel D.

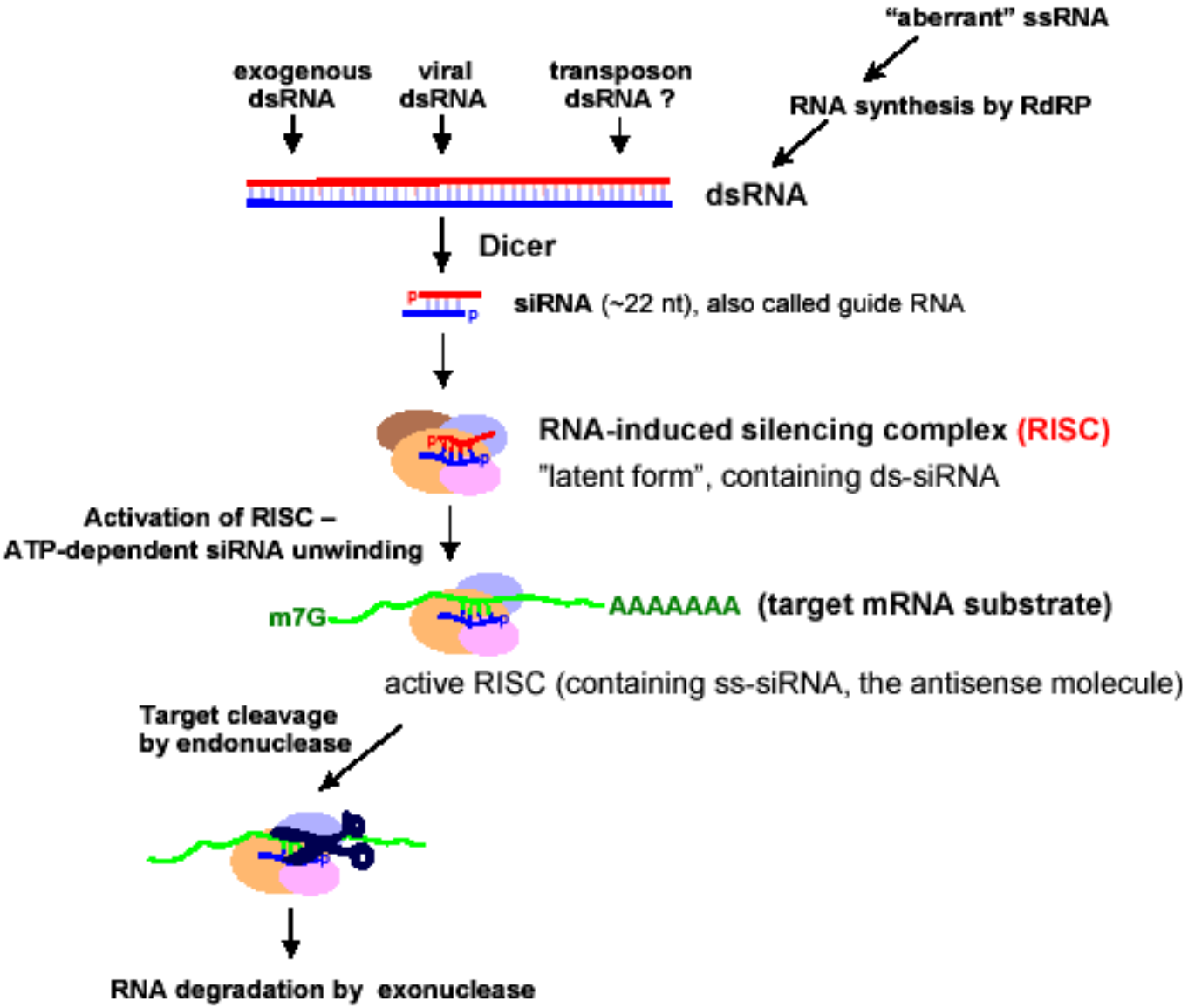
Summary II

- Ago proteins are phosphorylated at different sites
- Ago2 is phosphorylated at tyrosine 529 located in the 5'p binding pocket
- Ago2 Y529E generates a negatively charged environment in the 5'p binding pocket
- Ago2 Y529E interacts with Dicer and TNRC6B suggesting correct folding
- Ago2 Y529E has reduced cleavage activity and P-body localization
- due to reduced small RNA binding
- Ago2 Y529 phosphorylation could be a switch to inactivate Ago2 reversibly

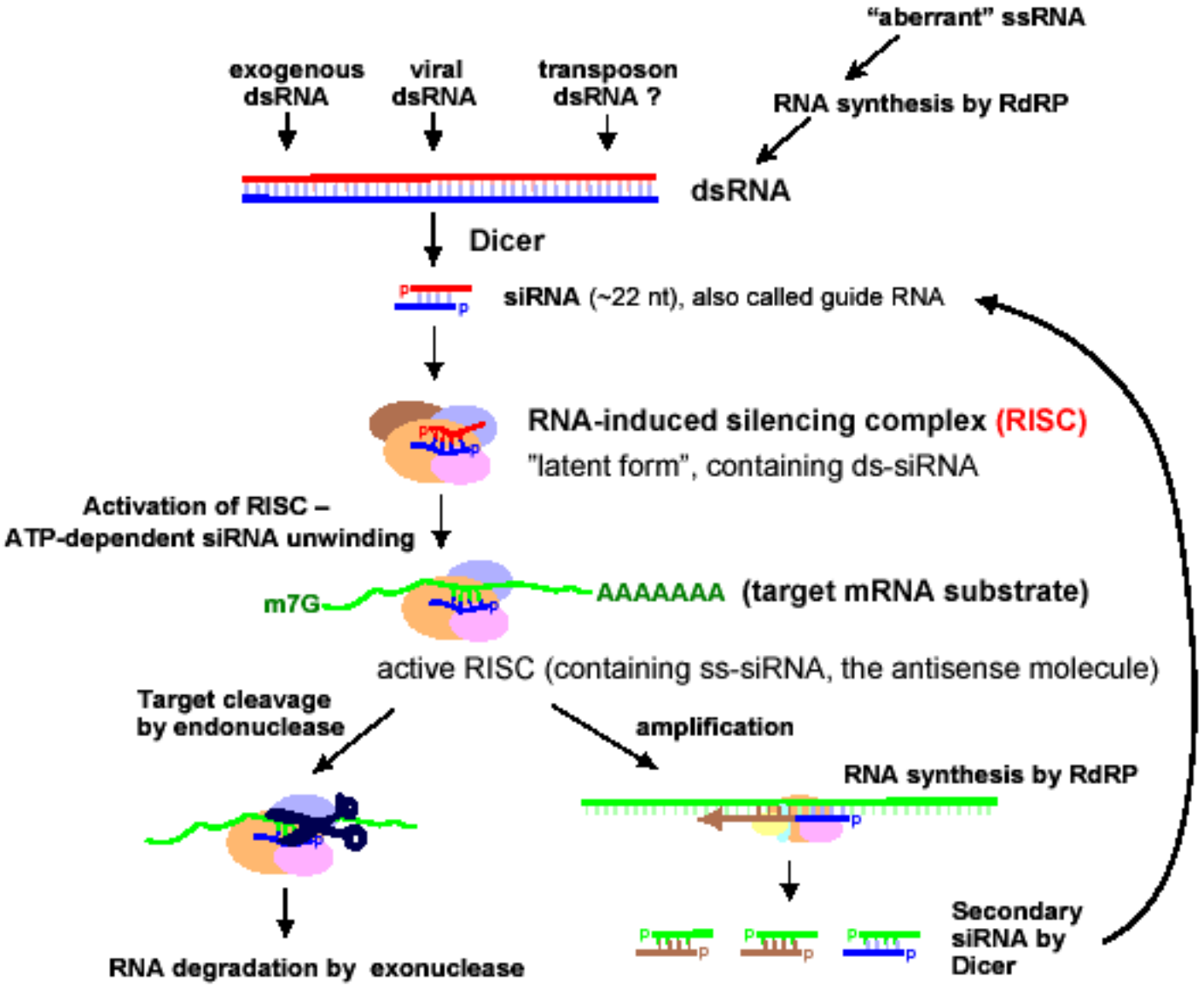
RNAi mechanism



RNAi mechanism



Meccanismo dell' RNAi



RNAi functions

- Control of exogenous parasites (viruses or genes)
- Control of endogenous parasites (transposons)
- Developmental regulation:
  control of translational - miRNAs
- Regulation of chromatin structure
- ?

miRNA

Identification and function

Lee RC, Feinbaum RL, Ambros V. *Cell. 1993 75:843-54.*

The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14

lin-4 is essential for the normal temporal control of diverse postembryonic developmental events in C. elegans. lin-4 acts by negatively regulating the level of LIN-14 protein, creating a temporal decrease in LIN-14 protein starting in the first larval stage (L1). We have cloned the C. elegans lin-4 locus by chromosomal walking and transformation rescue. We used the C. elegans clone to isolate the gene from three other Caenorhabditis species; all four Caenorhabditis clones functionally rescue the lin-4 null allele of C. elegans. Comparison of the lin-4 genomic sequence from these four species and site-directed mutagenesis of potential open reading frames indicated that lin-4 does not encode a protein. Two small lin-4 transcripts of approximately 22 and 61 nt were identified in C. elegans and found to contain sequences complementary to a repeated sequence element in the 3' untranslated region (UTR) of lin-14 mRNA, suggesting that lin-4 regulates lin-14 translation via an antisense RNA-RNA interaction.

Wightman B, Ha I, Ruvkun G. *Cell. 1993 75:855-62.*

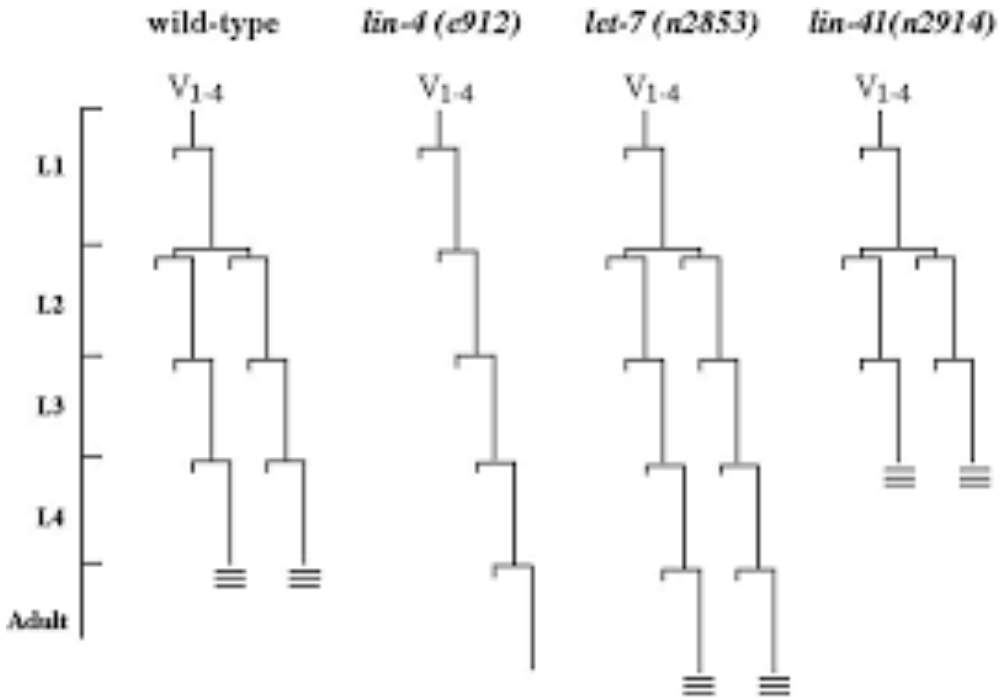
Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans.

During C. elegans development, the temporal pattern of many cell lineages is specified by graded activity of the heterochronic gene Lin-14. Here we demonstrate that a temporal gradient in Lin-14 protein is generated posttranscriptionally by multiple elements in the lin-14 3'UTR that are regulated by the heterochronic gene Lin-4. The lin-14 3'UTR is both necessary and sufficient to confer lin-4-mediated posttranscriptional temporal regulation. The function of the lin-14 3'UTR is conserved between C. elegans and C. briggsae. Among the conserved sequences are seven elements that are each complementary to the lin-4 RNAs. A reporter gene bearing three of these elements shows partial temporal gradient activity. These data suggest a molecular mechanism for Lin-14p temporal gradient formation: the lin-4 RNAs base pair to sites in the lin-14 3'UTR to form multiple RNA duplexes that down-regulate lin-14 translation.

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G.
Nature. 2000 403:901-6.

The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans.

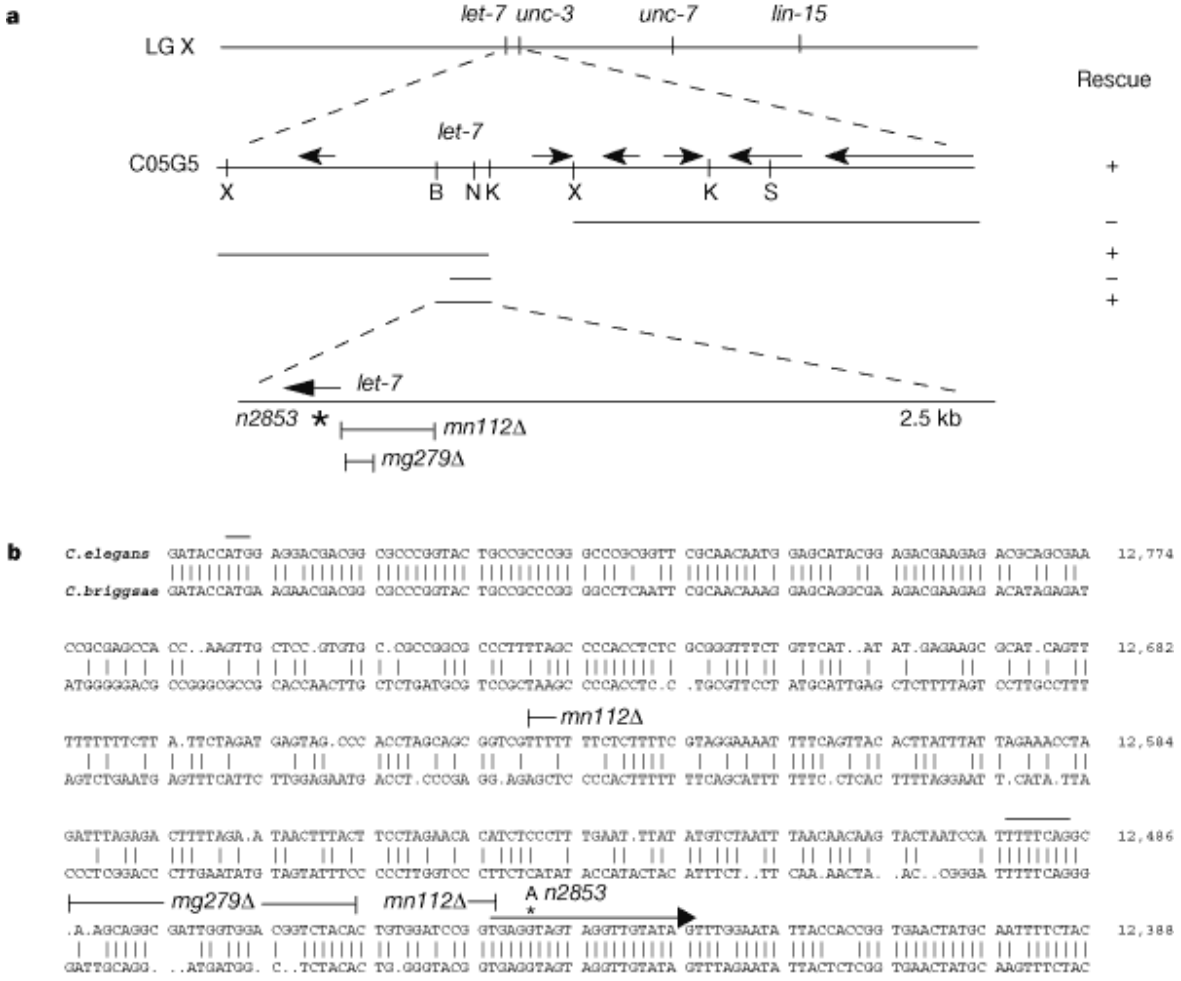
The C. elegans heterochronic gene pathway consists of a cascade of regulatory genes that are temporally controlled to specify the timing of developmental events. Mutations in heterochronic genes cause temporal transformations in cell fates in which stage-specific events are omitted or reiterated. Here we show that let-7 is a heterochronic switch gene. Loss of let-7 gene activity causes reiteration of larval cell fates during the adult stage, whereas increased let-7 gene dosage causes precocious expression of adult fates during larval stages. let-7 encodes a temporally regulated 21-nucleotide RNA that is complementary to elements in the 3' untranslated regions of the heterochronic genes lin-14, lin-28, lin-41, lin-42 and daf-12, indicating that expression of these genes may be directly controlled by let-7. A reporter gene bearing the lin-41 3' untranslated region is temporally regulated in a let-7-dependent manner. A second regulatory RNA, lin-4, negatively regulates lin-14 and lin-28 through RNA-RNA interactions with their 3' untranslated regions. We propose that the sequential stage-specific expression of the lin-4 and let-7 regulatory RNAs triggers transitions in the complement of heterochronic regulatory proteins to coordinate developmental timing.



Lin-4 reiteration of early fates at inappropriately late stages

lin-14 null mutations cause a phenotype opposite to that of *lin-4* and are completely epistatic to lin-4, which is consistent with lin-4 acting as a negative regulator of lin-14.
lin-14 mutants skip the expression of L1-specific events and precociously execute programs normally specific for L2, L3, L4 and adult stages.

let-7 gene activity causes reiteration of larval cell fates during the adult stage



The *let-7* gene sequence. a, Transgenic rescue. +, more than 90% rescue in multiple lines; -, no rescue. Arrows above C05G5 indicate predicted genes. b, Sequence comparison of *let-7* from *C. elegans* and *C. briggsae*. The 2.5-kb genomic fragment that fully rescues *let-7(mn112)* corresponds to nucleotide positions 14,208–11,749 of cosmid C05G5. The 21-nucleotide *let-7* transcript is indicated by an arrow. A truncation of the 2.5-kb fragment (to 12,446) deleting this transcript no longer rescues *let-7(mn112)* . The ATG at 12,857 and a 3' splice consensus TTTTCAG of a non-conserved open reading frame at 12,494 are indicated by bars.

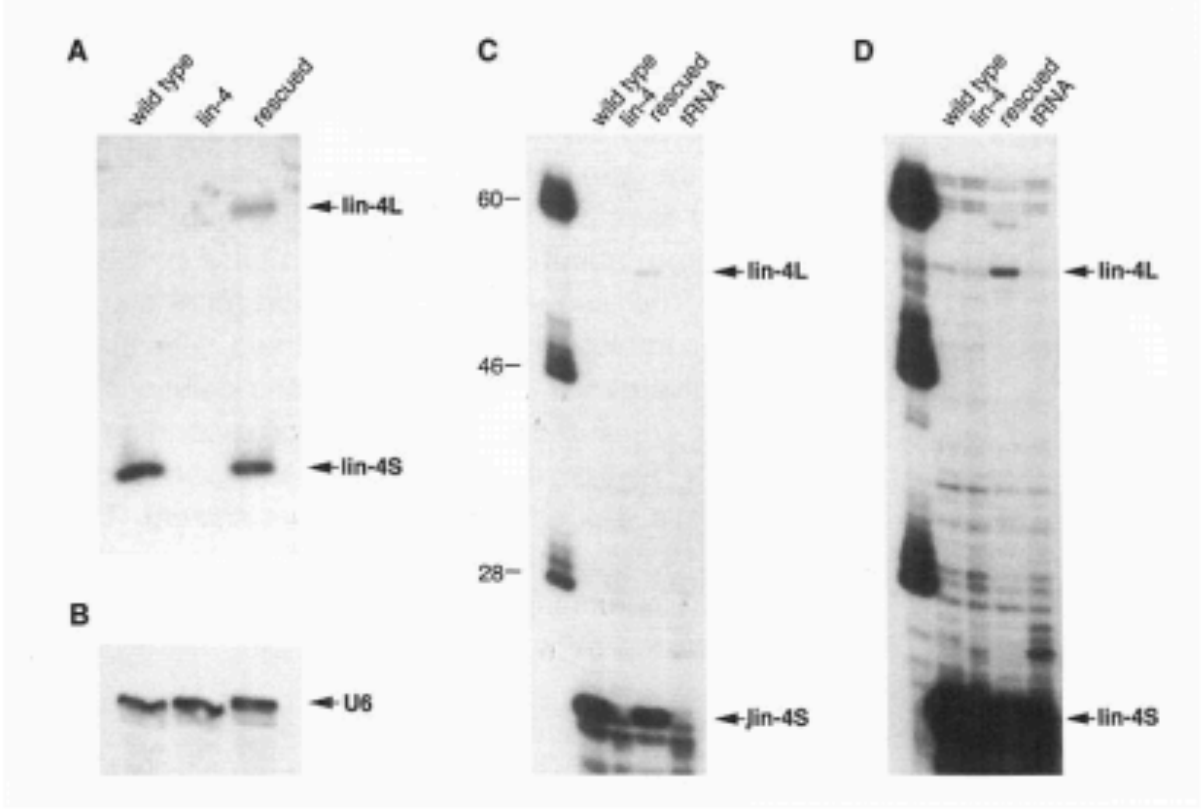


Figure 5. Identification of Two Small *lin-4* Transcripts

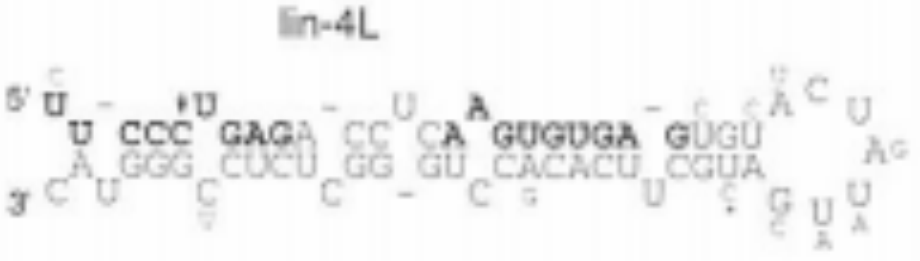
(A) Northern blot of total RNA from wild-type, *lin-4(e912)* (strain VT371), and *lin-4(e912)* rescued by transformation with pVTSal6 (strain VT510), probed with radiolabeled *lin-4* RNA probe. Although not shown here, similar northern blots have been done with DNA oligonucleotides loaded as size markers.

(B) Northern blot shown in (A), stripped and reprobed with an oligonucleotide complementary to U6 snRNA, to control for RNA loading.

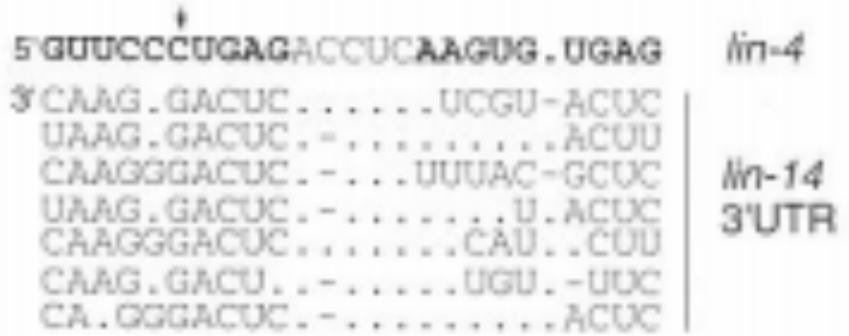
(C) RNAase protection analysis of total RNA from wild-type N2, *lin-4(e912)*, and a *lin-4* line rescued by pVTSal6 (strain VT510). The *lin-4* RNA probe used here and in (A) was generated as a runoff transcript from pMspI digested with EcoRI (T7RI, Figure 4). This *lin-4* probe covers nucleotides 506–568 and does not include the 3′ end of *lin-4*L; hence, *lin-4*L does not completely protect the probe in this assay. Probe fragments protected by *lin-4*S and *lin-4*L are indicated by the arrows. RNA size markers generated by runoff transcription of pBS are shown at the left. The gel was exposed to film for 16 hr with an intensifying screen.

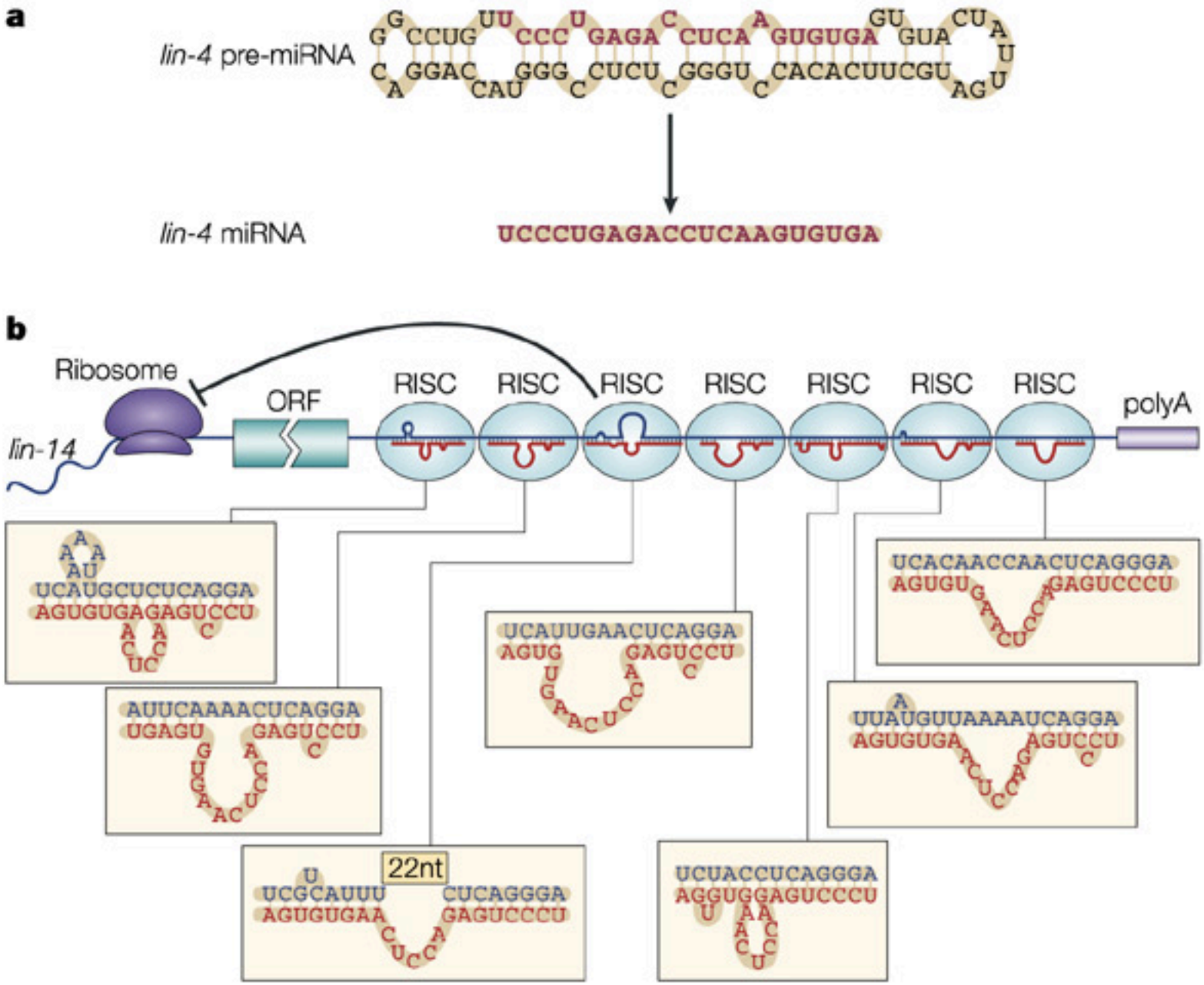
(D) Same gel shown in (C), exposed to film for 96 hr with intensifying screen to better visualize the product protected by *lin-4*L. For both the Northern blots and RNAase protection experiments, the estimated error in the lengths of RNA molecules is ±2 nt.

A.

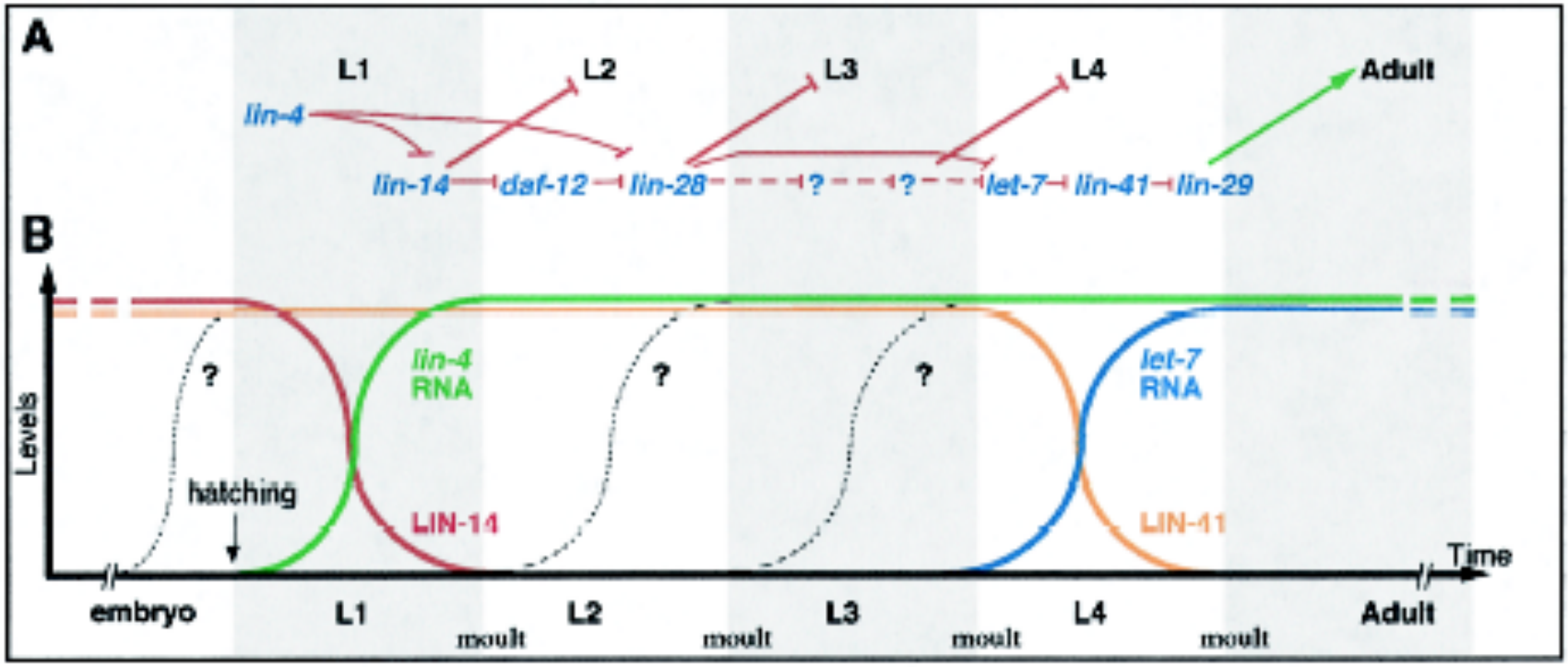

lin-4L
lin-4S
5'UUCCCUGAGACCUCAAGUGUGA3'

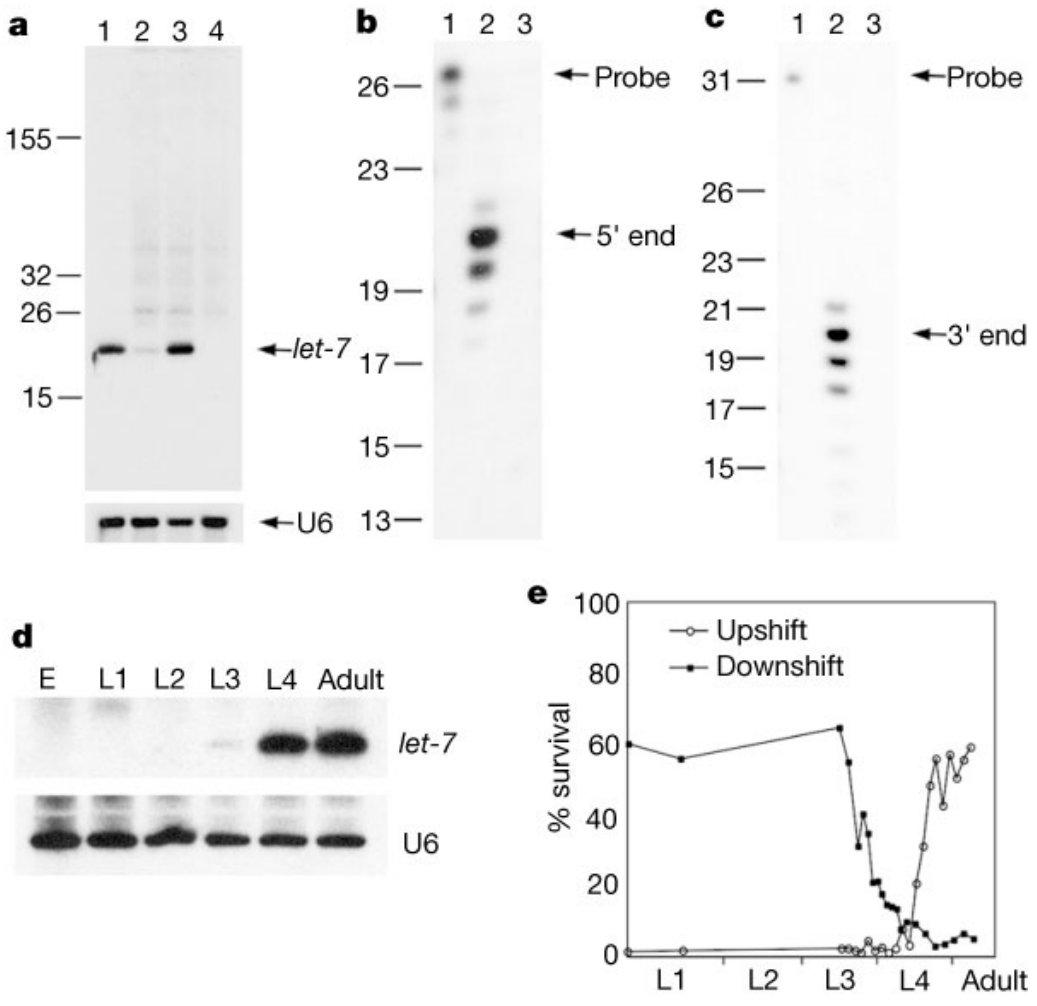
B.


5'GUUCCCUGAGACCUCAAGUG.UGAG
lin-4
3'CAAG.GACUC.....UCGU-ACUC
UAAG.GACUC.-.....ACUU
CAAGGGACUC.-...UUUAC-GCUC
UAAG.GACUC.-.....U.ACUC
CAAGGGACUC.....CAU..CUU
CAAG.GACU..-.....UGU.-UUC
CA.GGGACUC.-.....ACUC
lin-14
3'UTR


a
lin-4 pre-miRNA
lin-4 miRNA
UCCCUGAGACCUCAAGUGUGA
b
Ribosome
ORF
lin-14
RISC
RISC
RISC
RISC
RISC
RISC
RISC
polyA
22nt

The stRNAs *lin-4* and *let-7* control the developmental timing in C.elegans by repressing the translation of lin-14 and lin-41 mRNAs

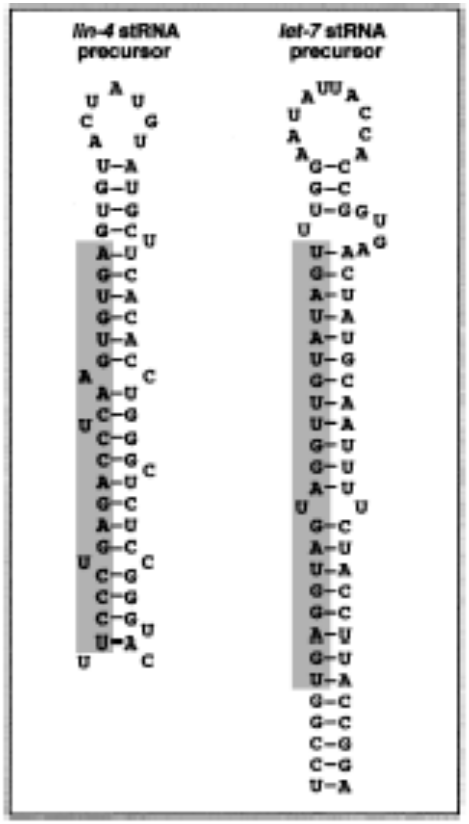




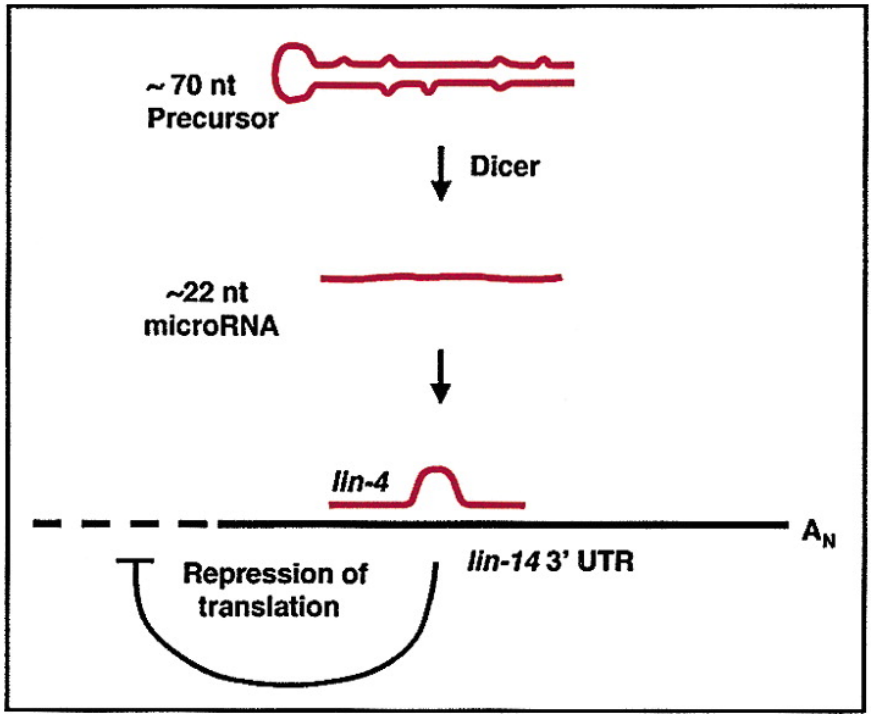
The 21-nucleotide *let-7* RNA. a, Northern blot of total RNA from mixed stage wild-type (lane 1), *let-7(n2853)* (lane 2), *lin-28(n719)* (lane 3) and *lin-28(n719); let-7(mn112) unc-3(e151)* animals (lane 4) probed with p249N. b, c, S1 nuclease transcript mapping. b, 5' probe p263 undigested (lane 1), and digested after hybridization to wild-type RNA (lane 2) or tRNA (lane 3). c, 3' probe p267 undigested (lane 1), and digested after hybridization to wild-type RNA (lane 2) or tRNA (lane 3). Sizing 1 nucleotide. d, Northern blot of wild-type RNA from the first 3 hours of each developmental stage. e, Temperature-sensitive period of *let-7(n2853)* viability.

Inhibitors of translation - miRNAs

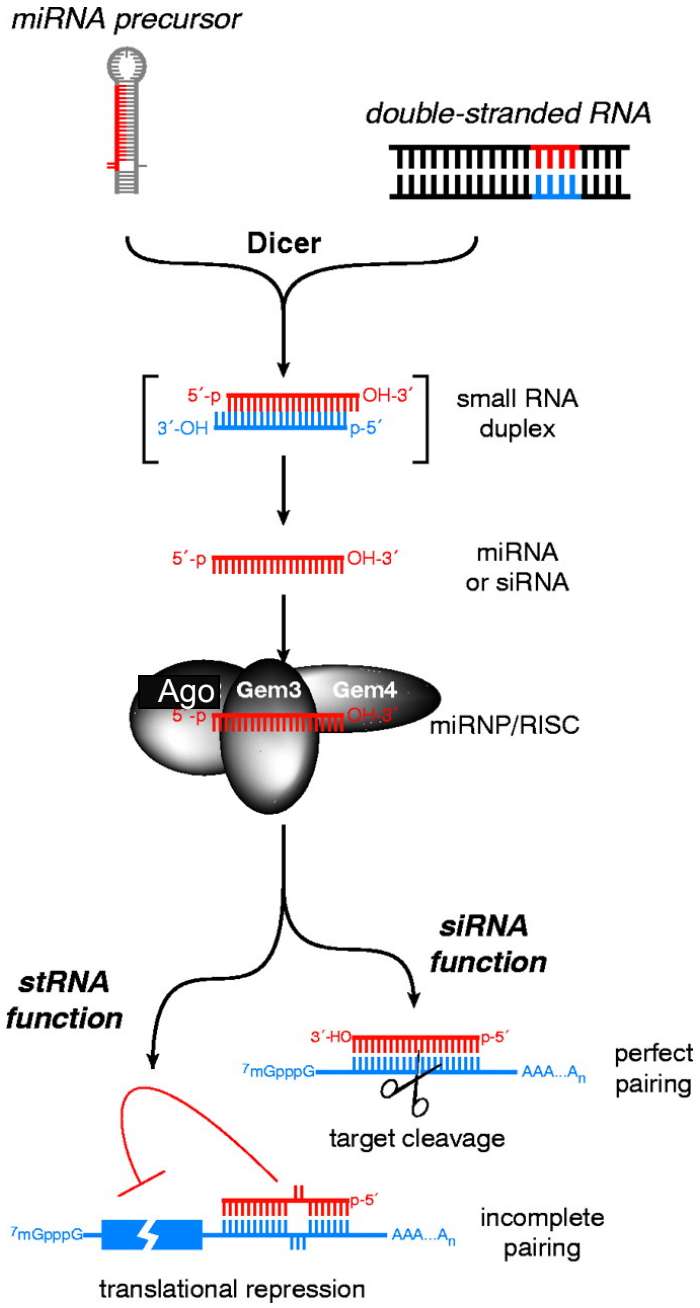
The genomes of *C. elegans*, *Drosophila*, human and plants encode for microRNAs (miRNAs) = small temporal RNAs (stRNAs)



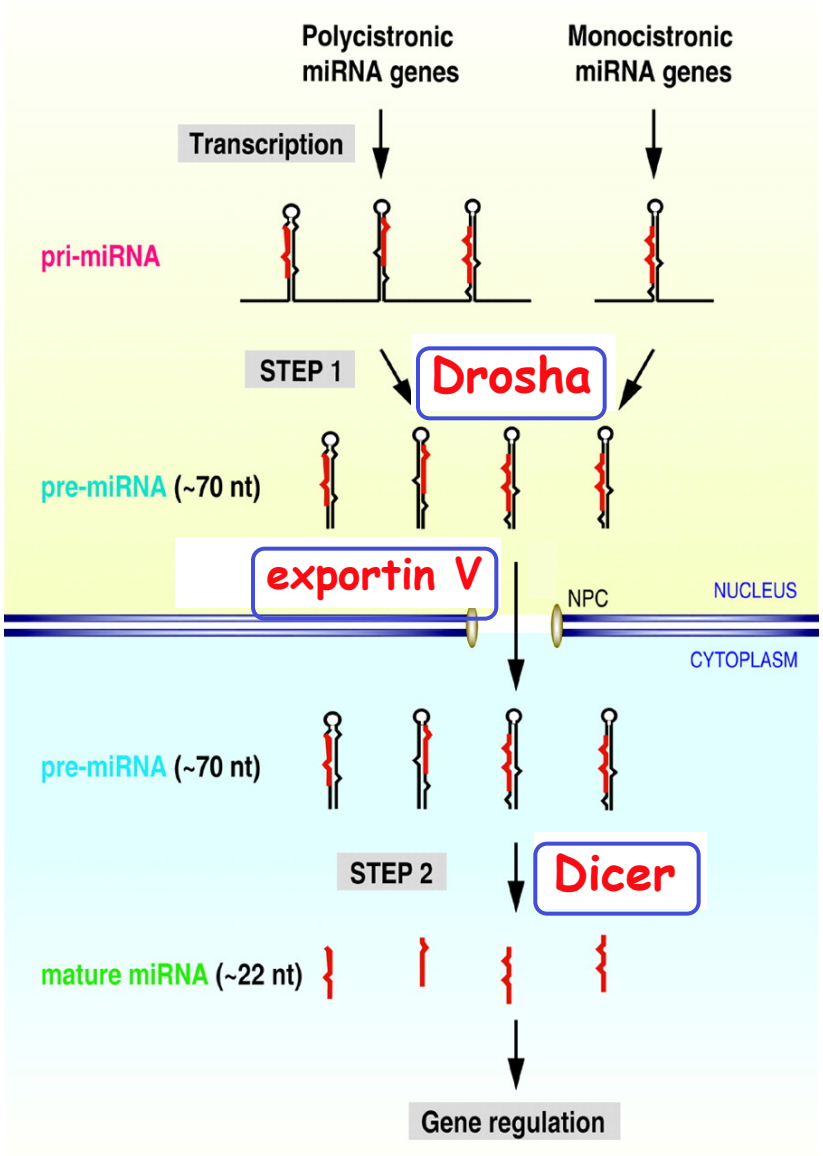
stRNAs


~70 nt
Precursor
Dicer
~22 nt
microRNA
lin-4
A_N
lin-14 3' UTR
Repression of
translation

Model for a common pathway in which miRNAs direct translational repression and siRNAs direct target RNA destruction (RNAi)



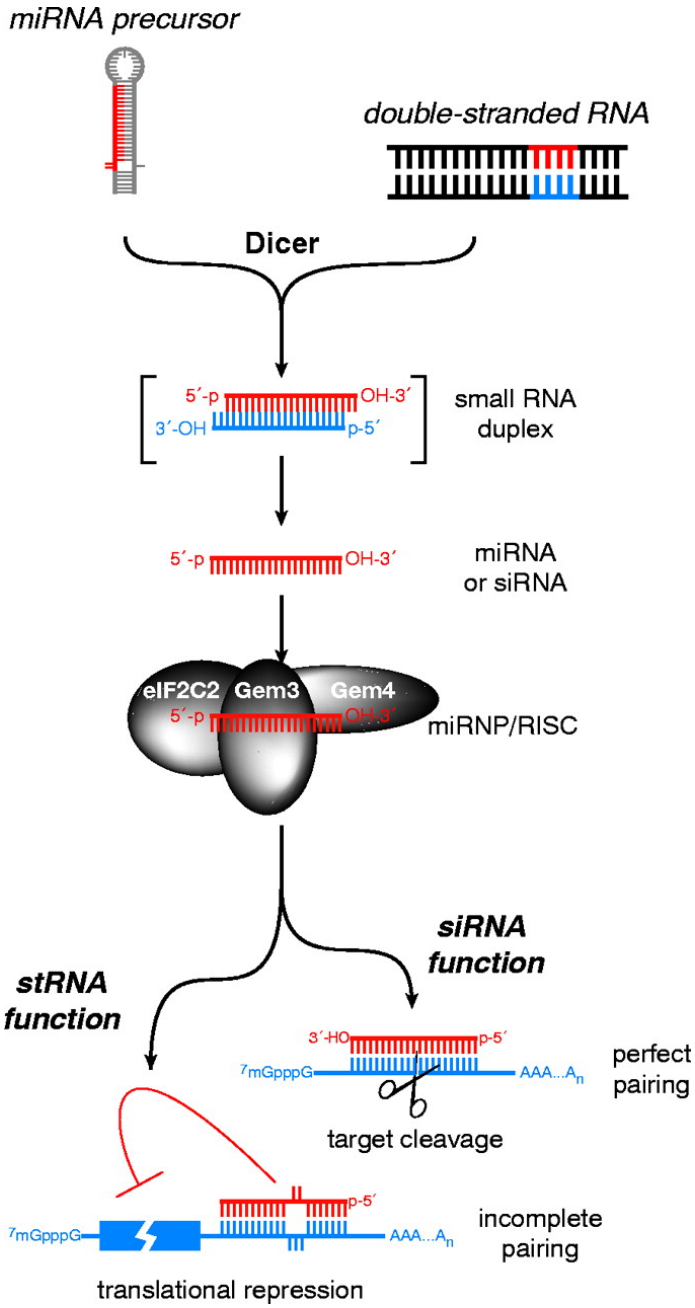
Biosynthesis of miRNAs



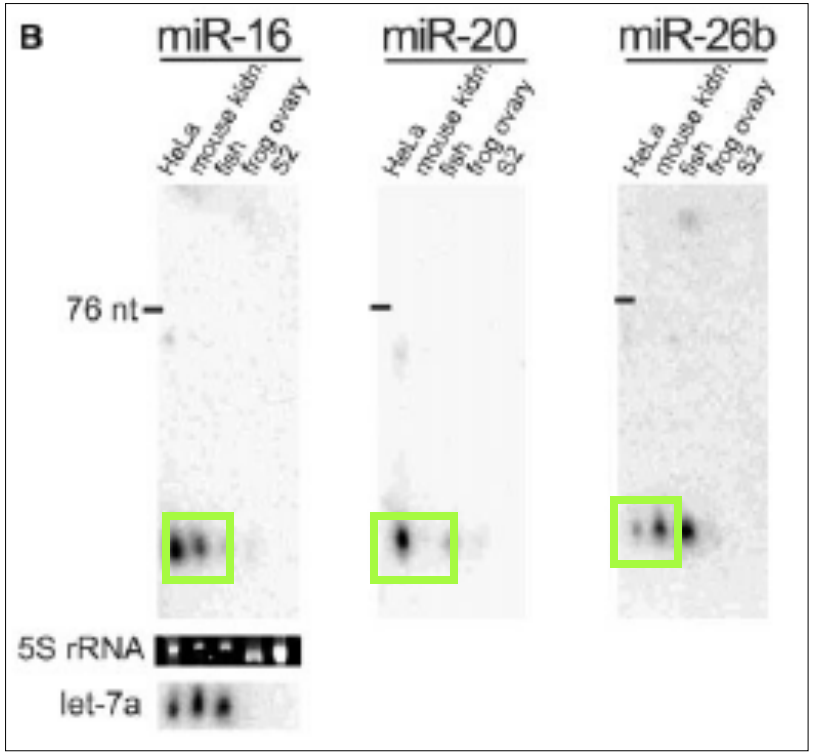
Model

for a common pathway
in which miRNAs direct
translational repression
and siRNAs direct target
RNA destruction (RNAi)
the difference depending
on the type of pairing
with the substrate

!! RISC will act stoichiometrically in repressing translation but catalytically in RNA destruction via RNAi



"Identification of novel genes coding for small expressed RNAs"
(Lagos-Quintana et al., 2001)



In questo lavoro,vennero individuati dei piccoli RNA non codificanti in cellule umane HeLa ed in topo, accertando così la presenza dei miRNA nei mammiferi

…2000…

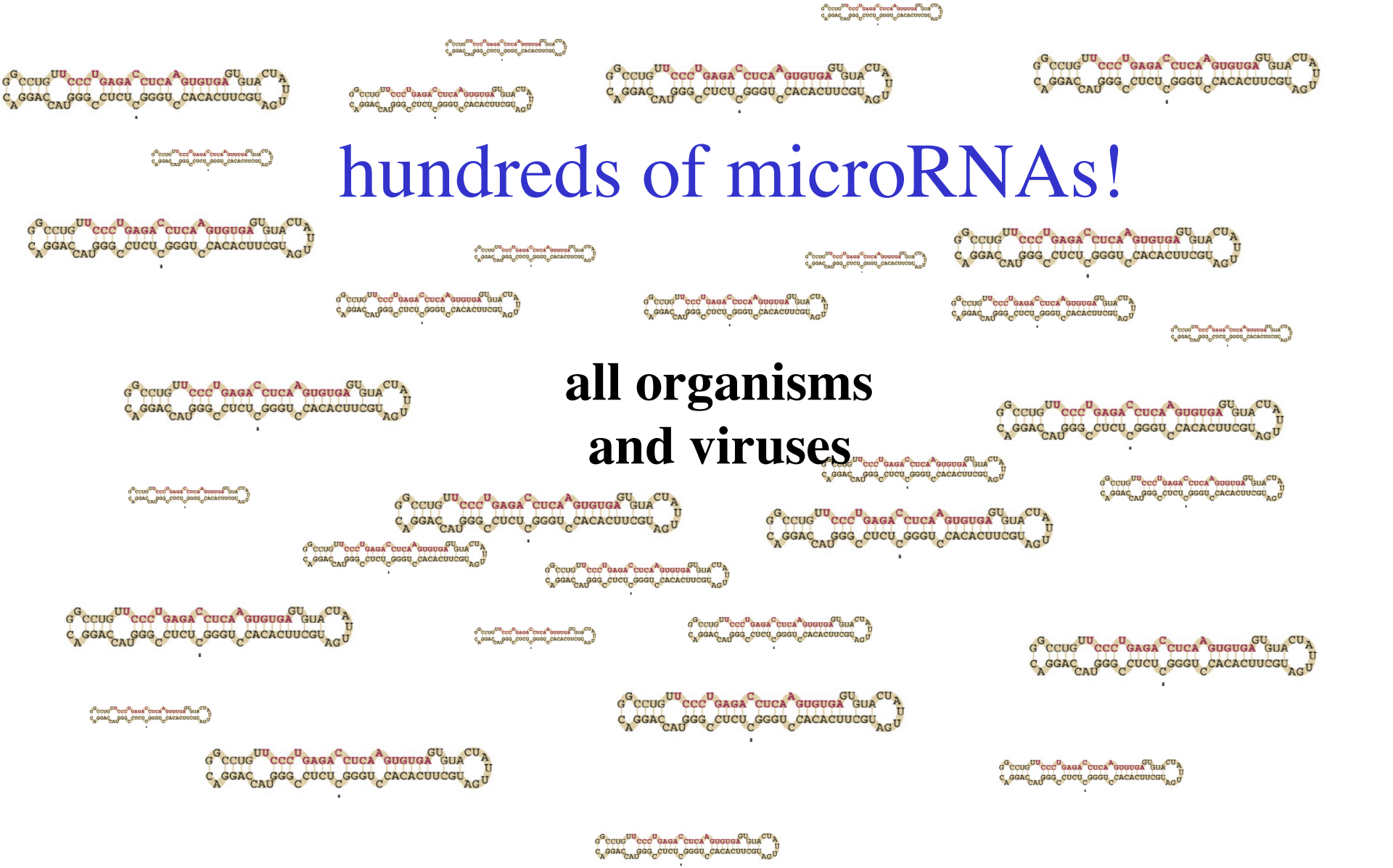
2 microRNAs

5’-ucccugagaccucaaguguga-3’ **lin-4** *C. elegans*

5’-ugagguaguagguuguauaguu-3’ **let-7** *C. elegans and vertebrates*

both involved in developmental regulation

…after 2000



hundreds of microRNAs!

all organisms and viruses

microRNAs

- **complex family of 21-23 nucl small RNAs**
(~1% of higher eukaryotes genes)

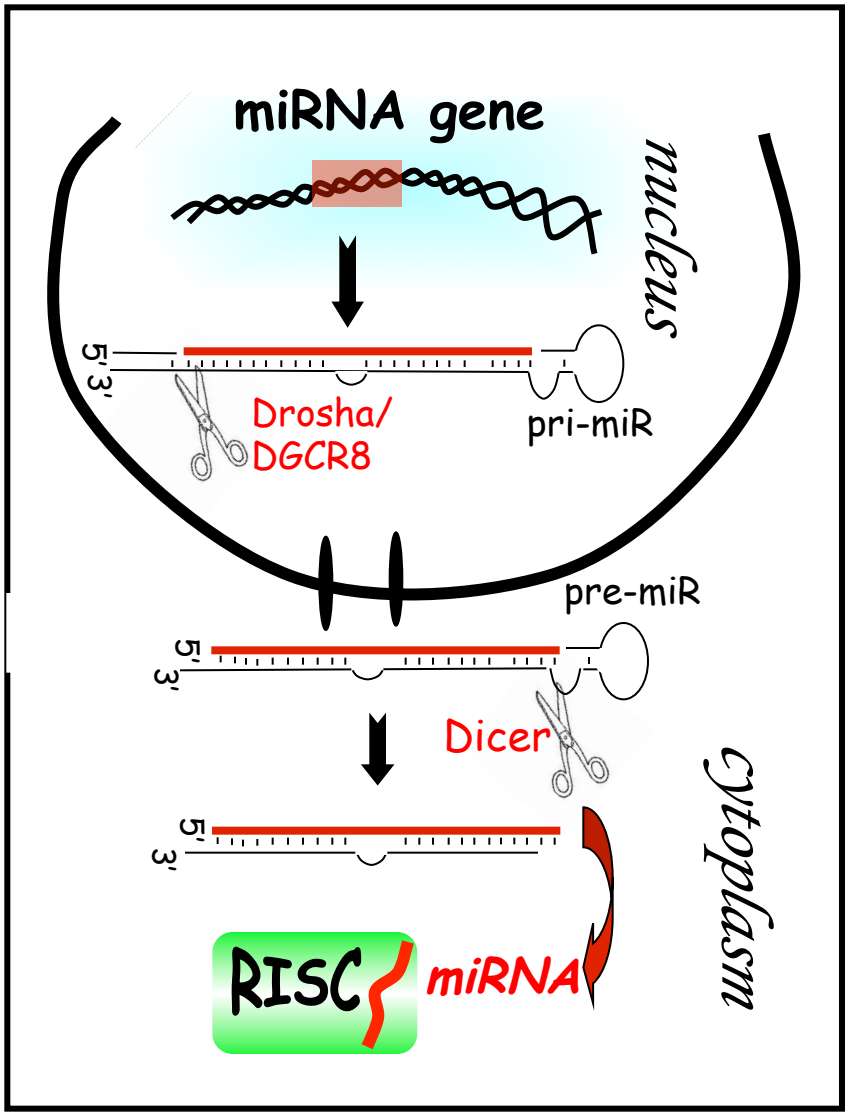
present in metazoa and plants

78	*D.melanogaster*
116	*C.elegans*
1000	*H.sapiens*
112	*A.thaliana*

and mammalian viruses

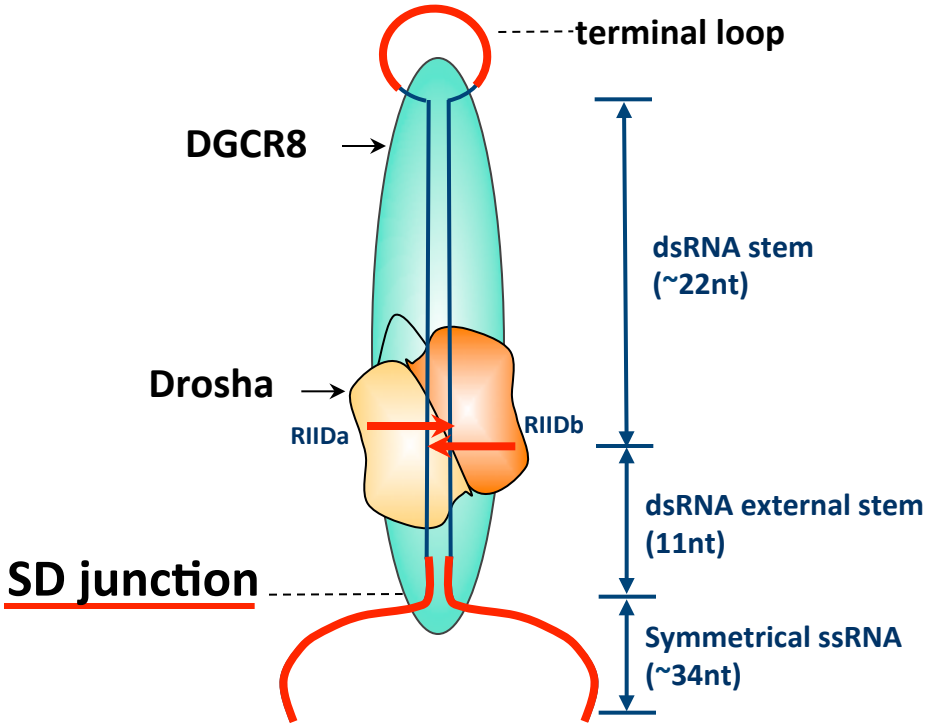
5-11 Herpesviruses , EBV

- they control cell growth and differentiation by regulating mRNA stability and translation

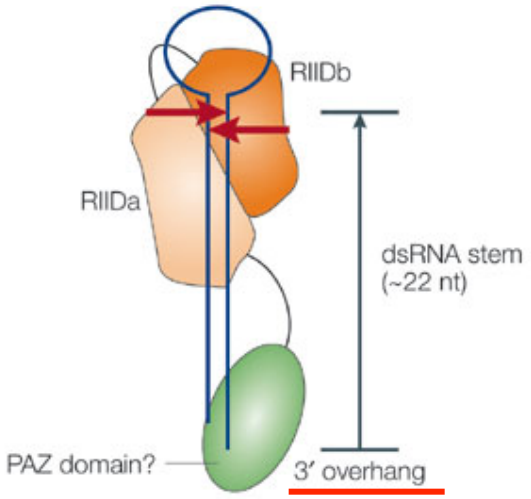


“Drosha” and “Dicer” RNases

Pri-miRNA processing by Drosha



Pre-miRNA processing by Dicer

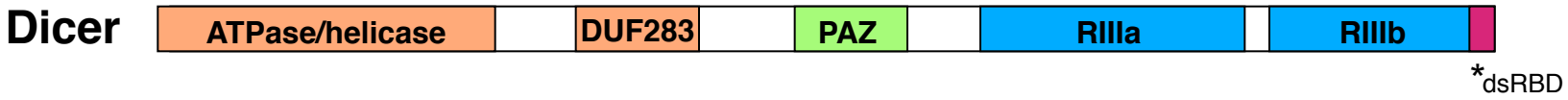


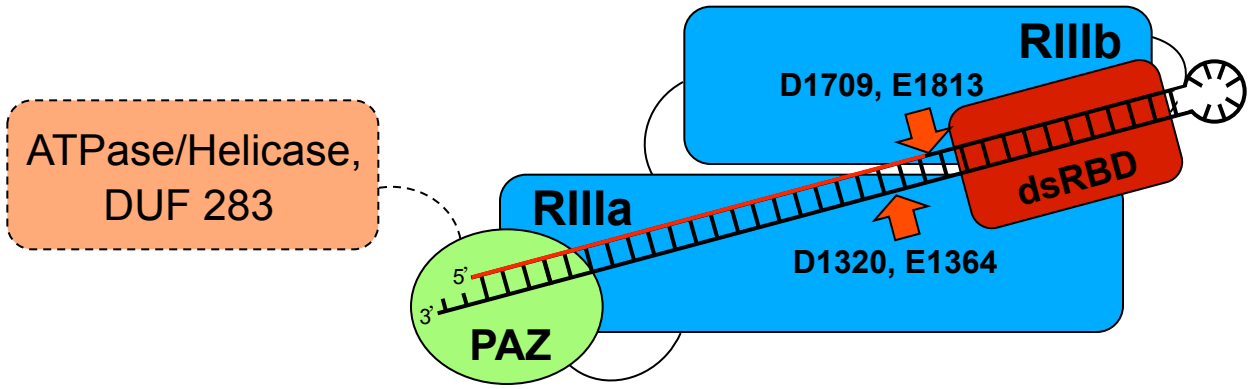
‘‘ssRNA-dsRNA Junction Anchoring’’ Model

Han *et al.,* Cell 2006

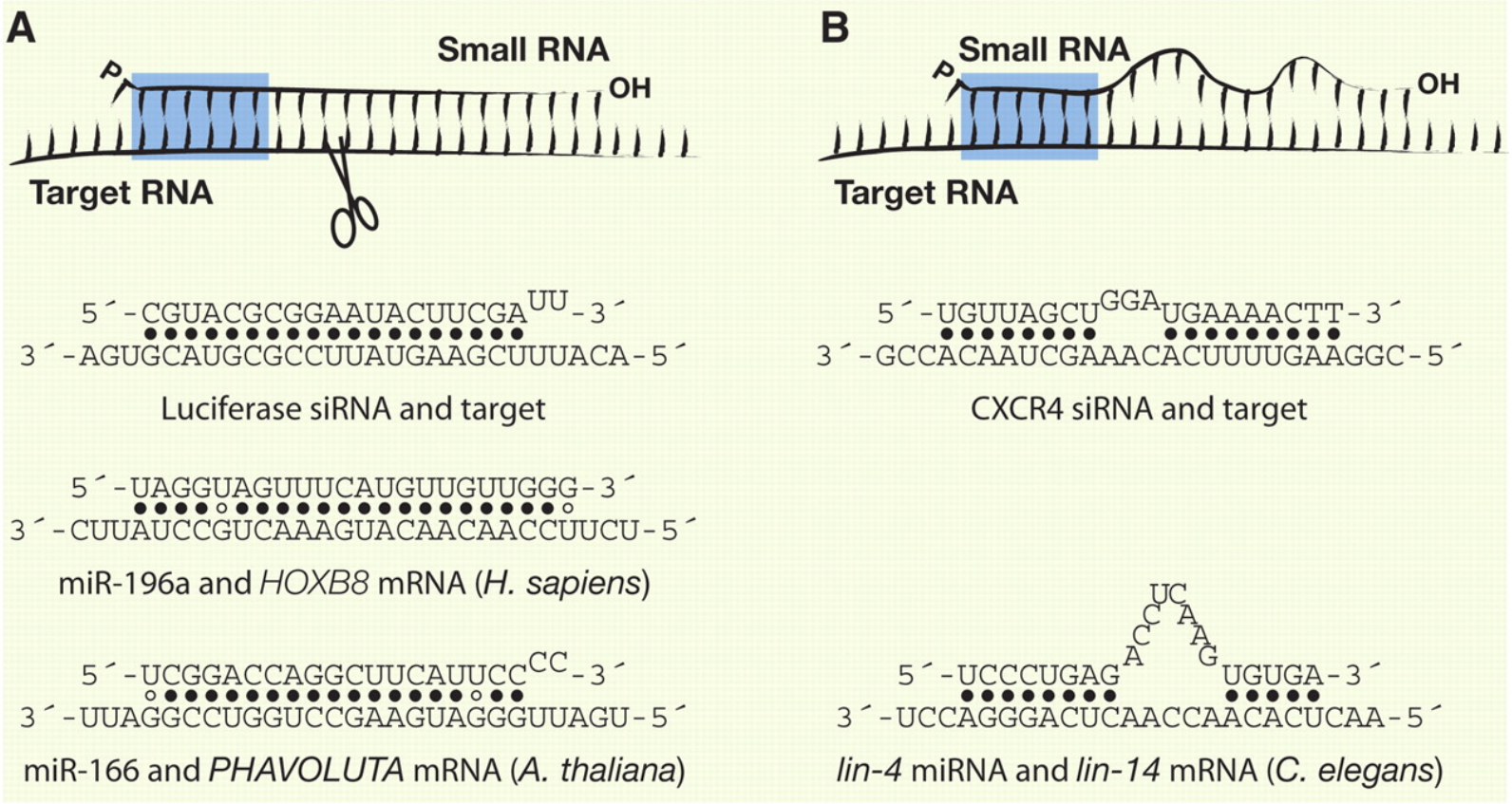
- **They generate 2-nt-long 3’ overhangs at the cleavage site.**

A model of dsRNA and miRNA processing by Dicer





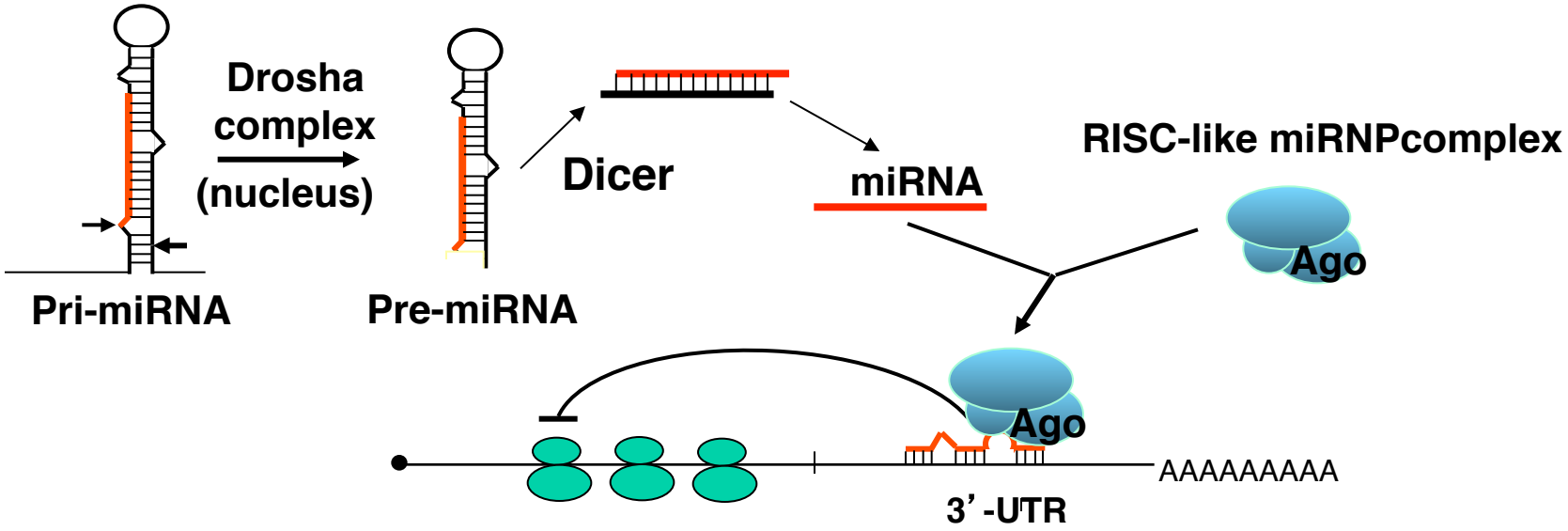
- Dicer functions as a monomer (i.e., intra-molecular y-dimer)
- PAZ domain recognizes the end
- Dicer has a single processing center, with two independent
- catalytic sites
- Each RNase III domain cuts one RNA strand in a polar way



Small RNA binding modes.

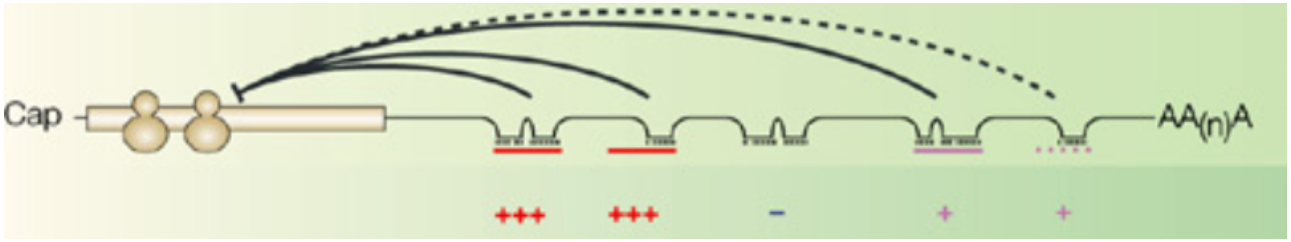
(A) Extensive pairing of a small RNA to an mRNA allows the Piwi domain of a catalytically active Argonaute protein (e.g., Ago2 in humans or flies) to cut a single phosphodiester bond in the mRNA, triggering its destruction. Synthetic siRNAs typically exploit this mechanism, but some mammalian miRNAs (such as miR-196a) and most, if not all, plant miRNAs direct an Argonaute protein to cut their mRNA targets. (B) Partial pairing between the target RNA and the small RNA, especially through the "seed" sequence—roughly nucleotides 2 to 7 of the small RNA—tethers an Argonaute protein to its mRNA target. Binding of the miRNA and Argonaute protein prevents translation of the mRNA into protein. siRNAs can be designed to trigger such "translational repression" by including central mismatches with their target mRNAs; animal miRNAs such as *lin-4*, the first miRNA discovered, typically act by this mode because they are only partially complementary to their mRNA targets. The seed sequence of the small RNA guide is highlighted in blue.

MicroRNAs: ~21-nt regulators of gene expression



- Conserved from plants to humans
- ~1000 miRNAs may operate in primates
- Tissue- and development-specific expression
- ~30% of all human genes are predicted to be miRNA targets
- Function as miRNPs, with Argonautes (Ago), FMRP, Gemins

miRNAs and their mRNA targets: emerging patterns



Bartel et al., 2004

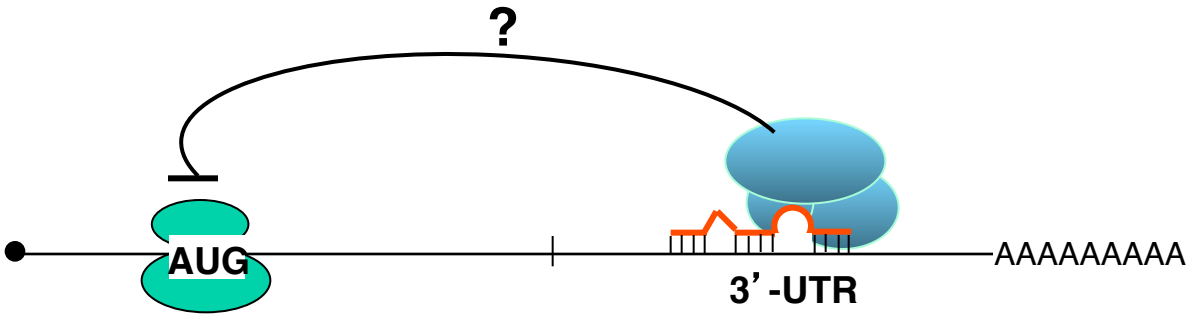
- Bind to mRNA 3' -UTRs; targets spread over kilobases of 3' -UTR
- One miRNA can control activity of many different mRNAs
- A single mRNA can be controlled by more than one miRNA

Major advantages of miRNA regulation?

- Networking and fine-tuning of gene expression
- Rapid (and possibly reversible) repression

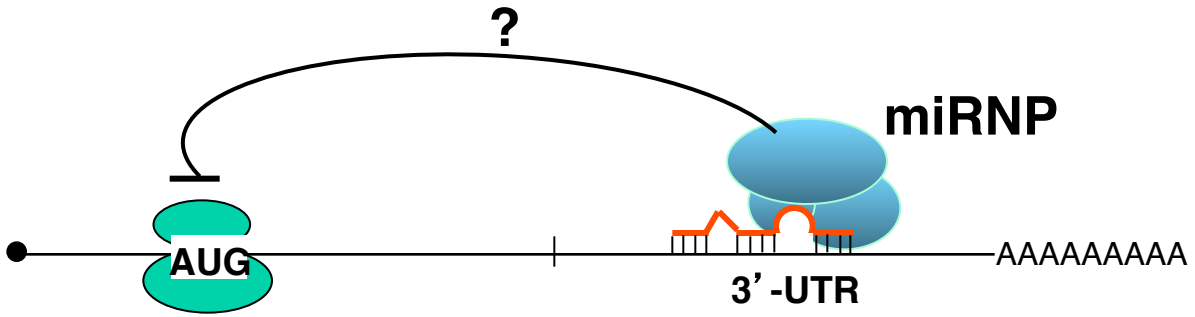
miRNAs: major challenges

- Identification of mRNA targets
- Mechanism of inhibition of protein accumulation



From W. Filipowicz

How do miRNAs inhibit productive translation?



Observations:

- Proteins do not accumulate (different metazoa)
- mRNA levels largely unaffected (*C. elegans*, *Drosophila*, mammals) but some recent papers (Lim et al., 2005; Pasquinelli et al., 2005) report considerable mRNA degradation
- mRNA associated with polysomes (*C. elegans*; Olson & Ambros, '99)

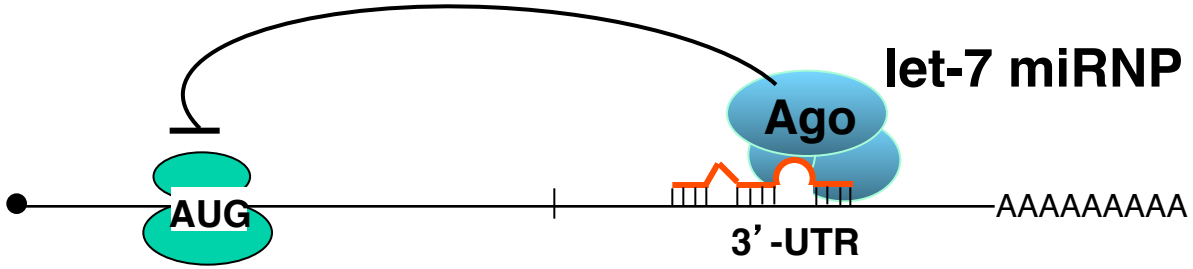
Questions:

- Is translation per se inhibited? Or proteins are made but undergo proteolysis?
- If translation is targeted, what is the mechanism?
- Contribution of mRNA degradation
- Do all miRNAs function in the same way?

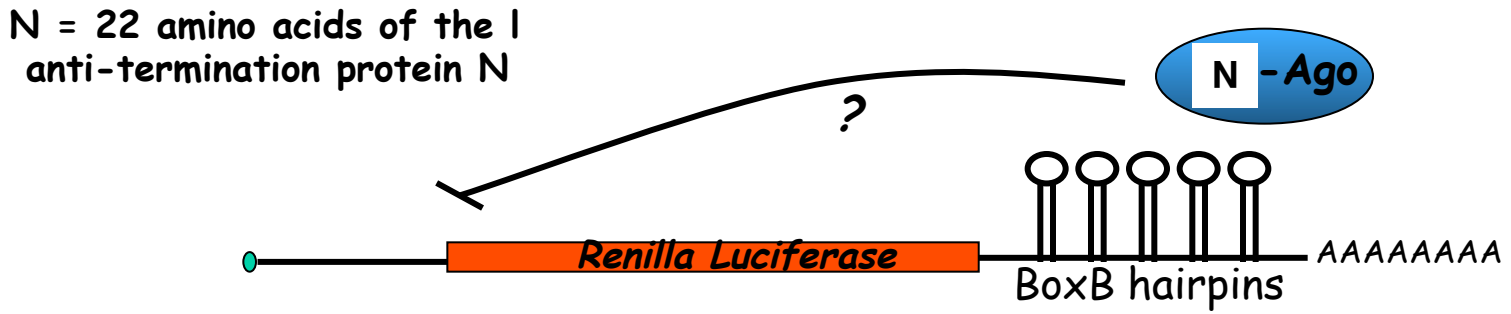
From W. Filipowicz

mRNA reporters to study miRNA-mediated repression

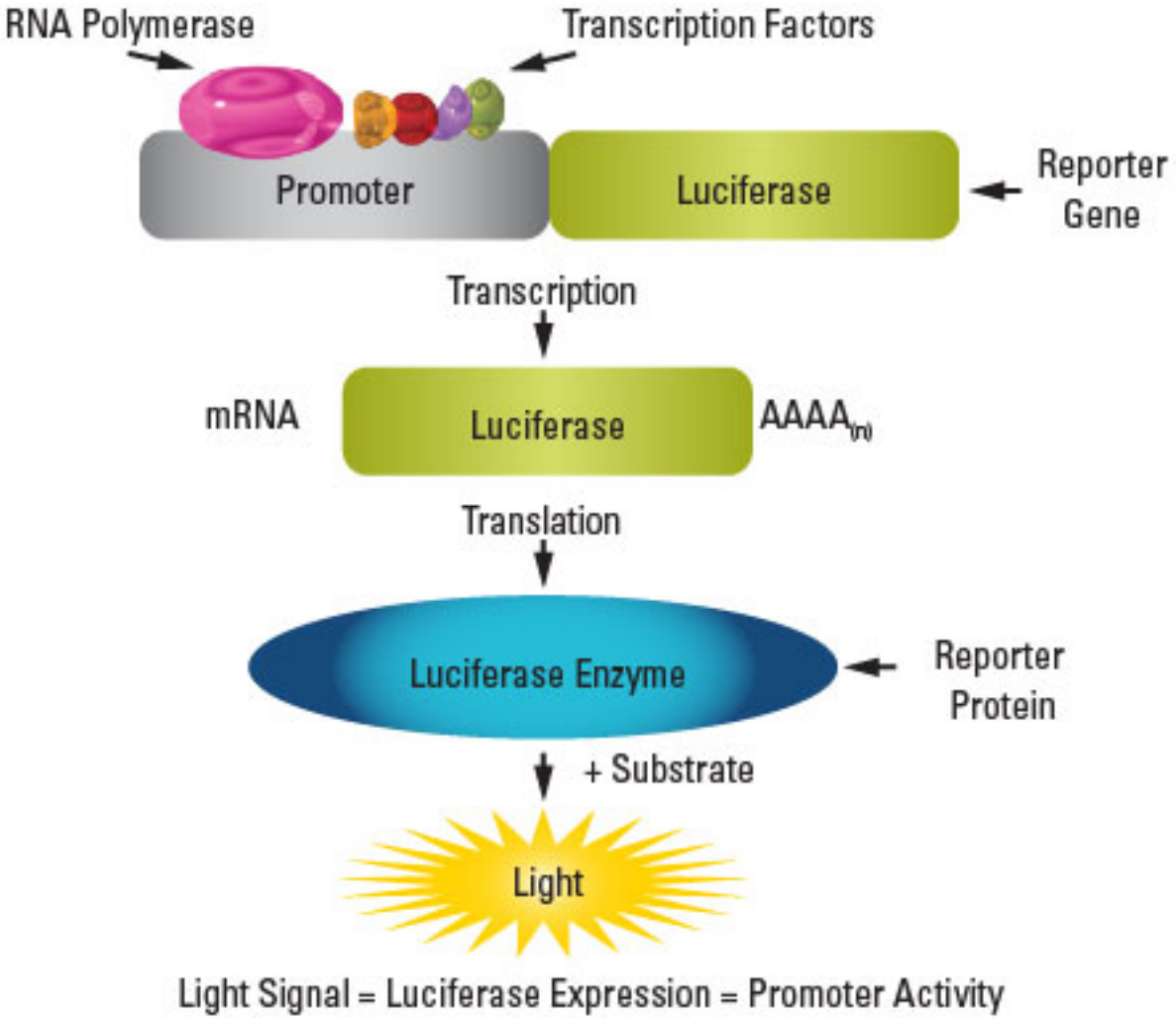
- Reporters responding to the endogenous let-7 miRNP

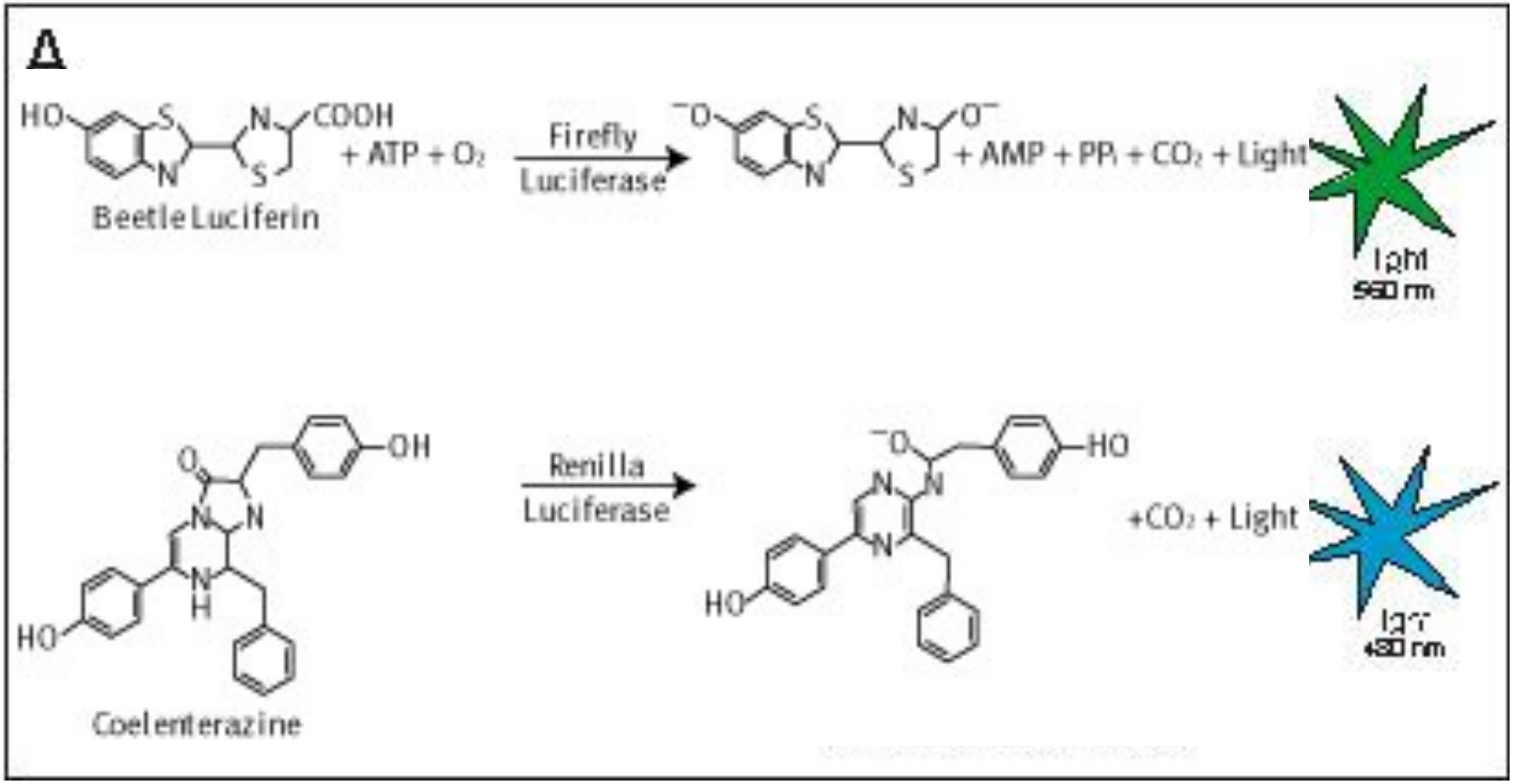


- Reporters responding to the miRNP protein tethering

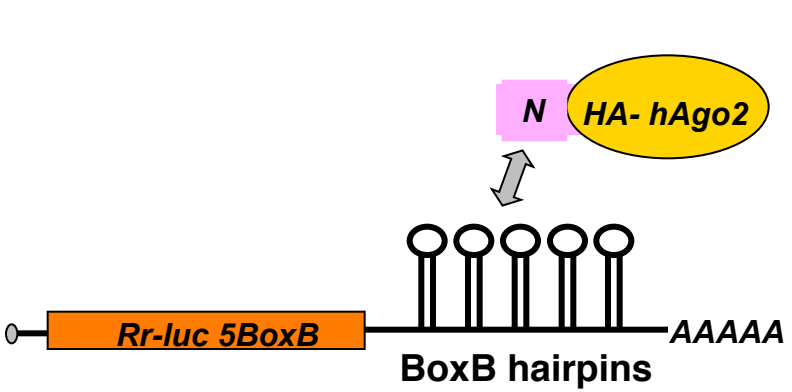


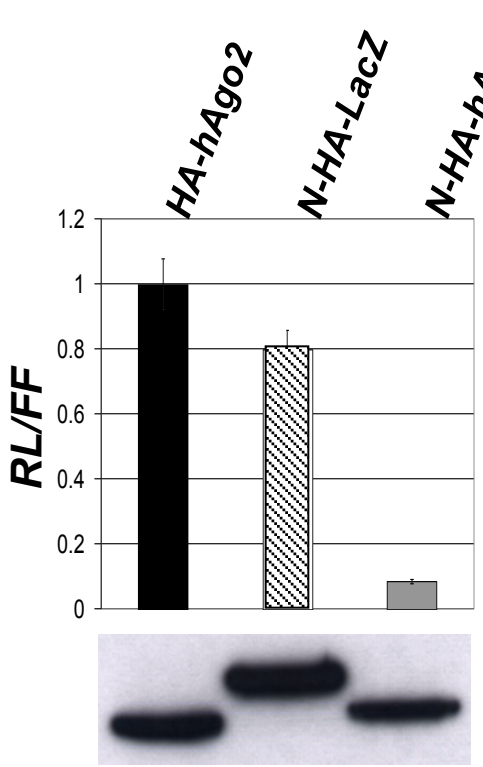
From W. Filipowicz


RNA Polymerase
Transcription Factors
Promoter
Luciferase
Reporter Gene
Transcription
mRNA
Luciferase
AAAA(n)
Translation
Luciferase Enzyme
Reporter Protein
+ Substrate
Light
Light Signal = Luciferase Expression = Promoter Activity


A
HO
S
N
COOH
N
S
Beetle Luciferin
+ ATP + O2
Firefly
Luciferase
−O
S
N
O−
N
S
+ AMP + PPi + CO2 + Light
Light
560 nm
O
OH
N
N
H
HO
Coelenterazine
Renilla
Luciferase
−O
HO
N
N
N
HO
+CO2 + Light
Light
480 nm

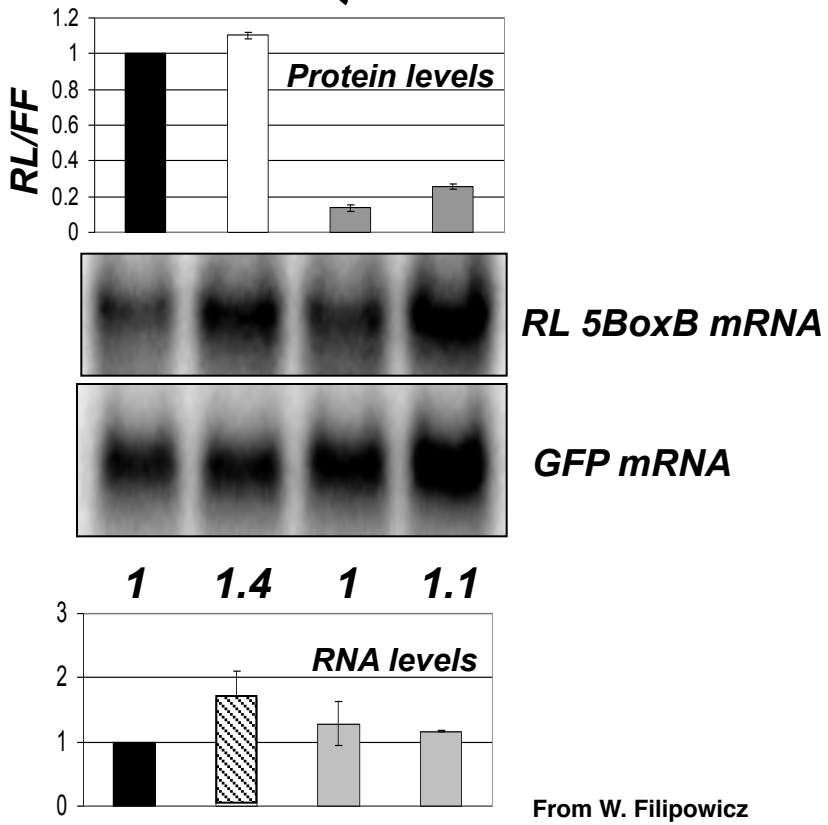
Tethering of human Argonautes to the reporter mRNA mimics the effect of miRNAs





Reporter activity

Western (α-HA Ab)

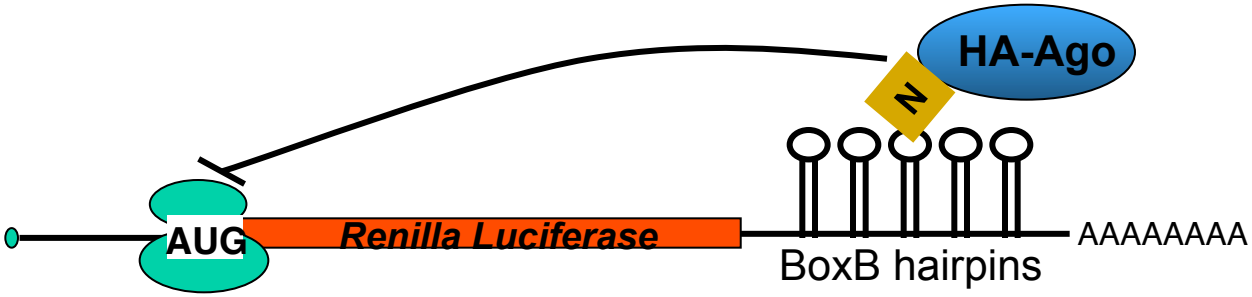


From W. Filipowicz

Summary of tethering experiments:

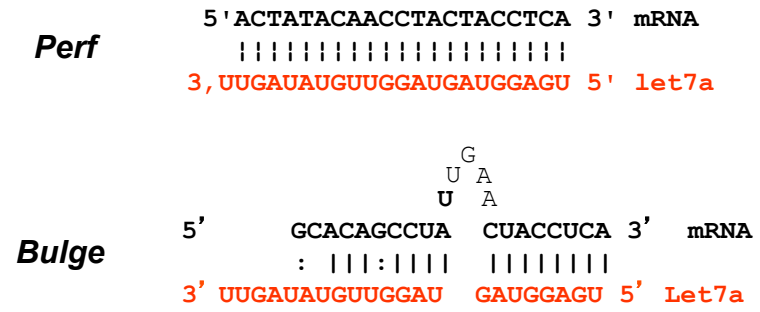
- All tethered hAgo proteins (hAgo2, 3 & 4) inhibit protein accumulation
- Inhibition occurs by miRNA-like mechanism (several BoxB required, no dependence on position in 3'UTR, etc)

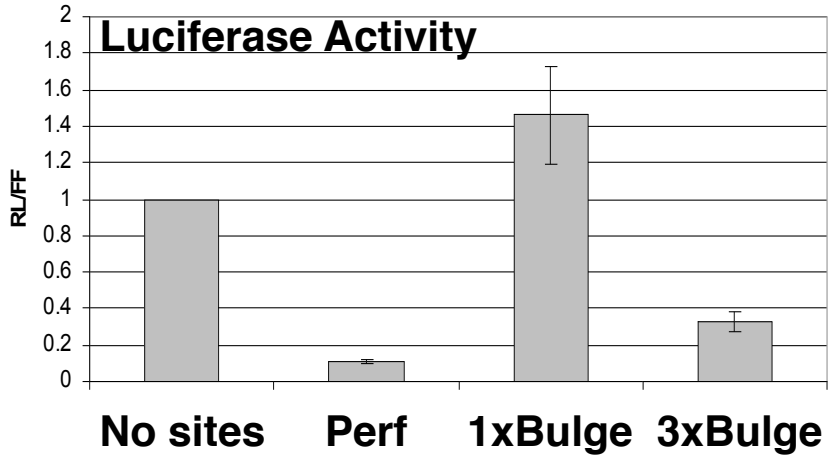
- Main function of miRNAs is to guide repressive proteins to the mRNA
- Integrity of hAgo2 is essential; Ago as a repressor?

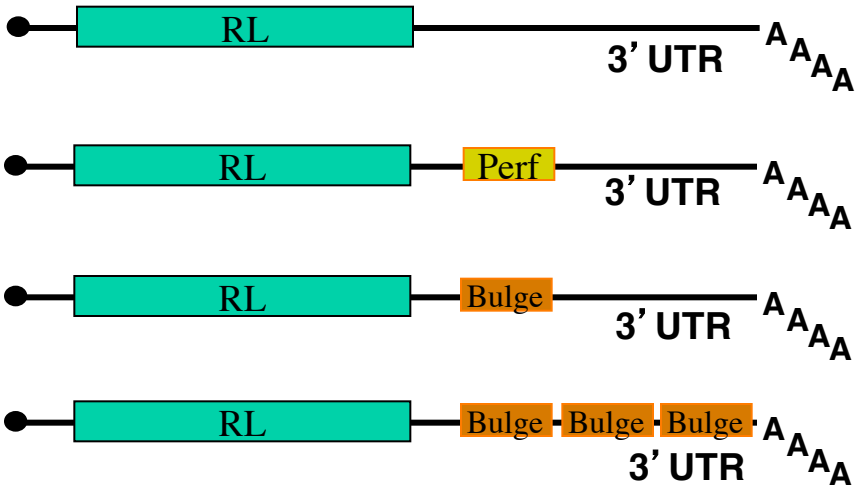


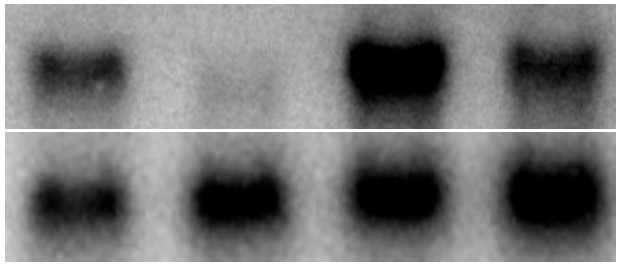
From W. Filipowicz

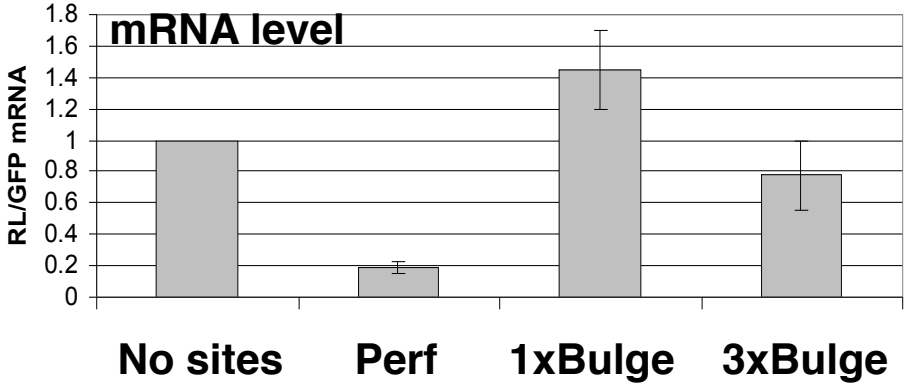
Inhibition of productive translation by endogenous let-7 miRNP in HeLa cells



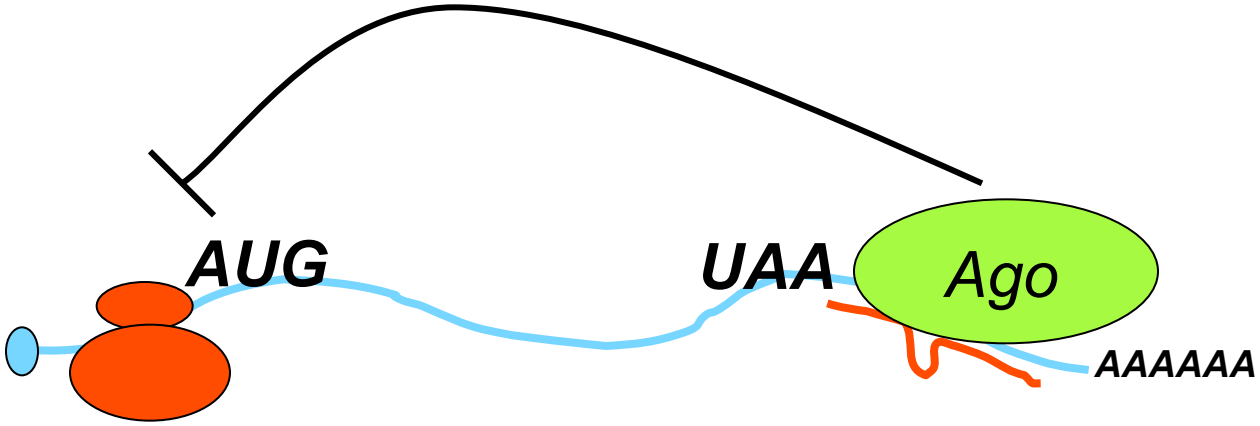




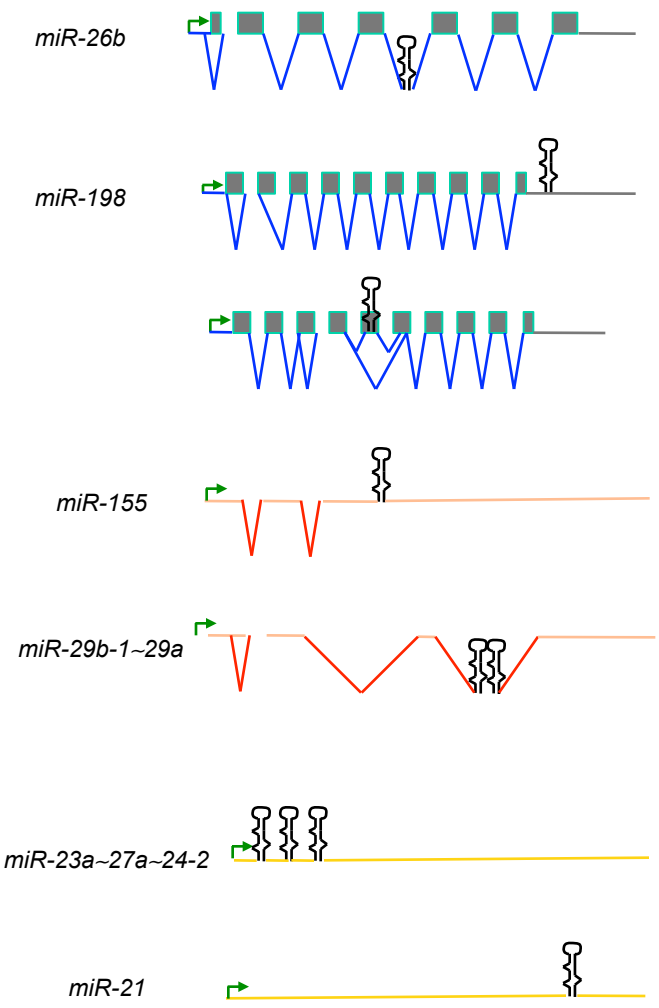


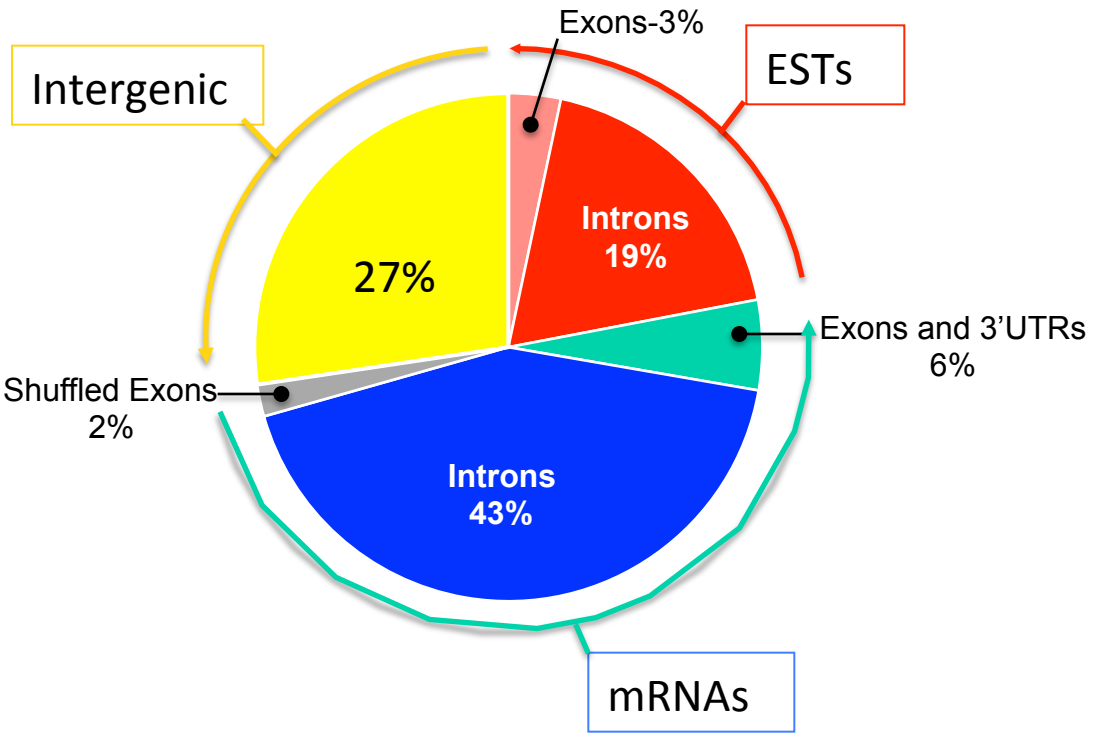


Translational initiation as a target of repression



Genomic organization of human microRNAs

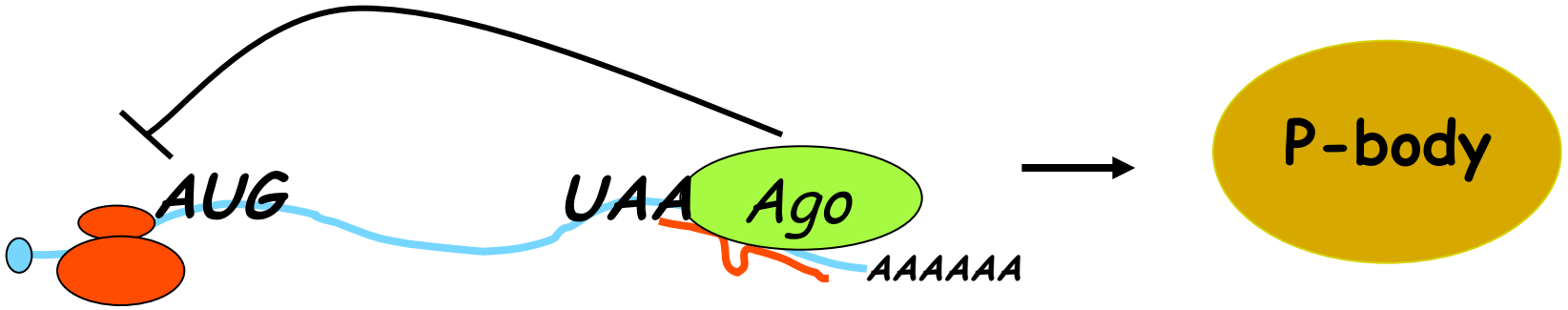




Morlando et al., **NSMB** (2008)

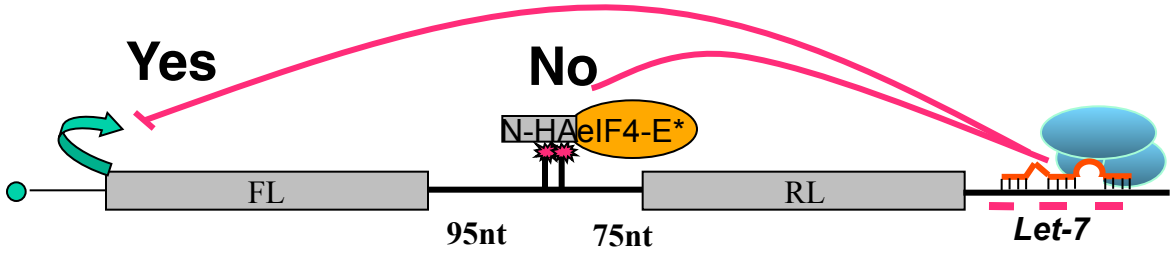
Repressed mRNAs in P-bodies:

- cause or consequence of translational inhibition?
- two-step versus one step model



Support for the two-step model

- PBs grow in size and accumulate mRNAs following inhibition of initiation
- Ribosomes and most IFs absent from PBs; ribosome clearance required (Chx)
- Bicistronic mRNA translation data
-

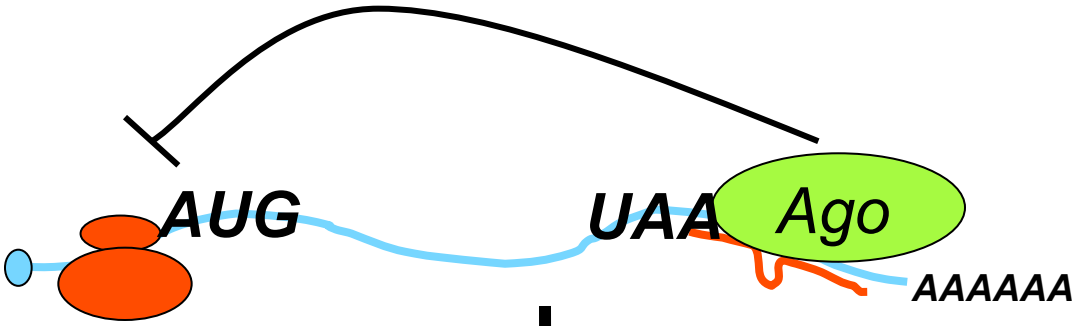


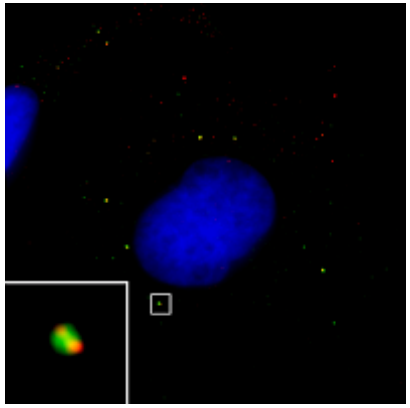
From W. Filipowicz

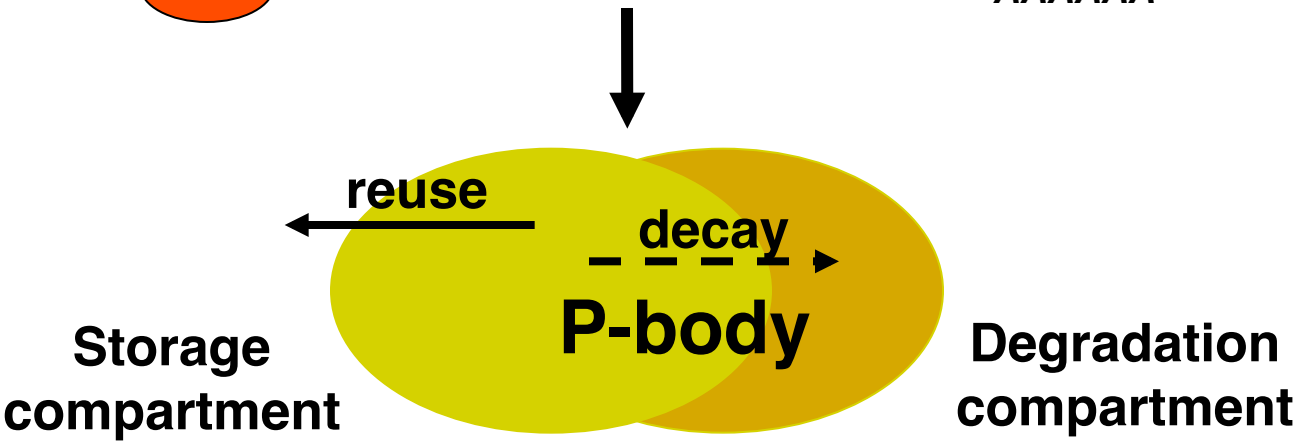
PBs as both mRNA storage and degradation sites

Support for the storage function:

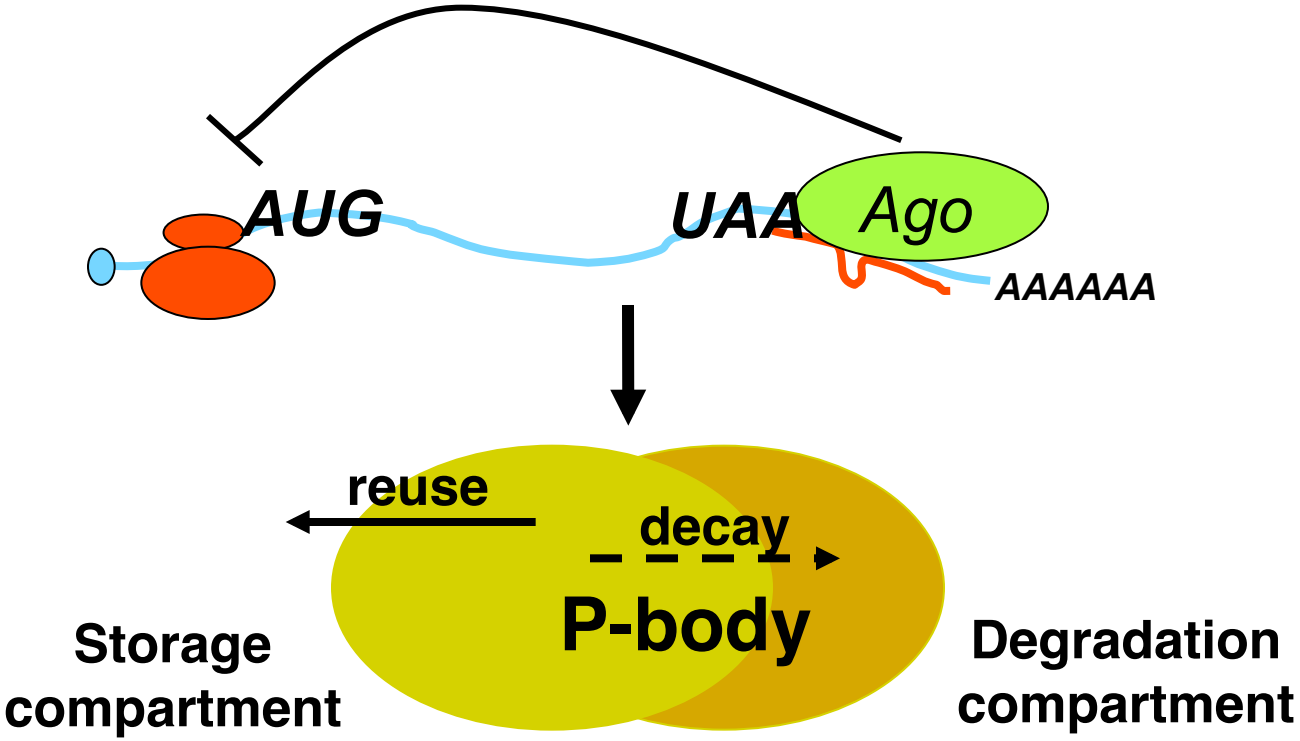
- Accumulation of mRNAs following inhibition of initiation in PBs
- mRNAs return to translation after release of inhibition (Parker et al., 2005)
- mRNAs undergoing repression but not those undergoing degradation can be visualized in PBs
- Two PB subcompartments?





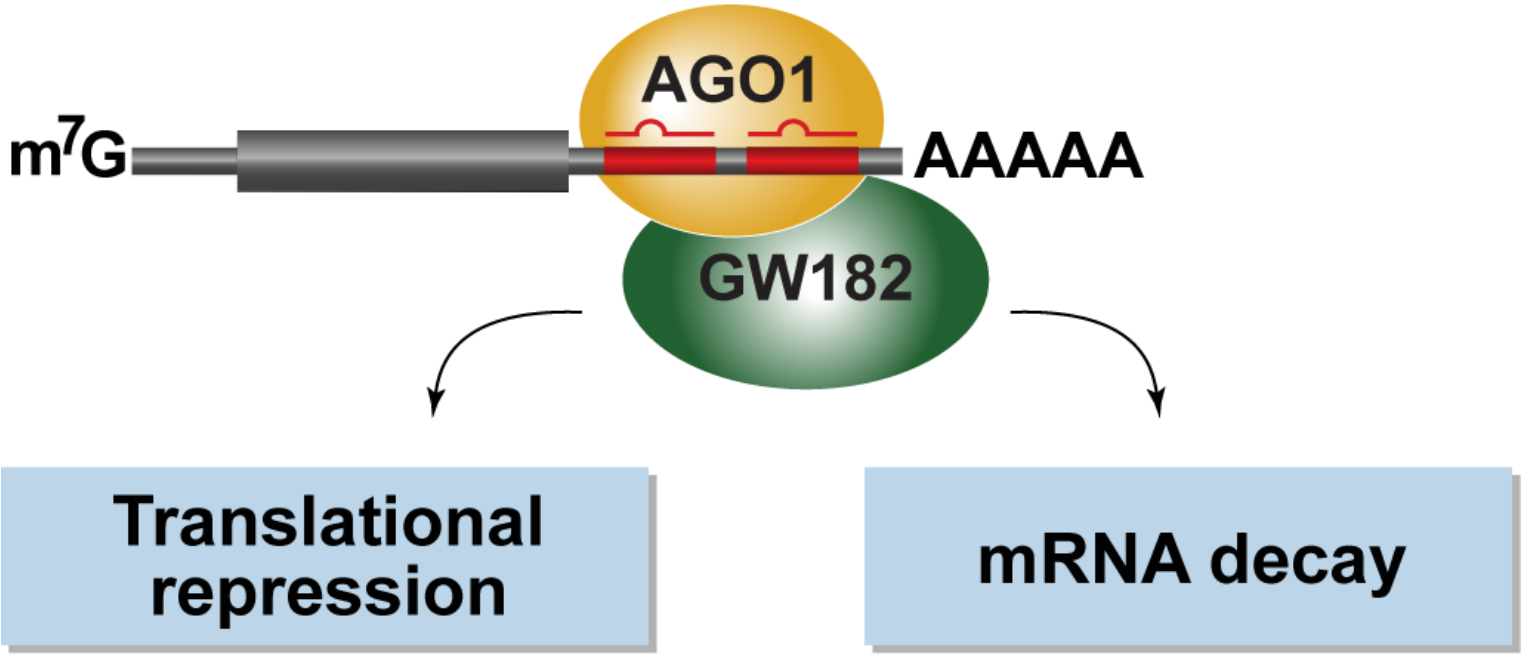


miRNAs may affect gene expression in different ways

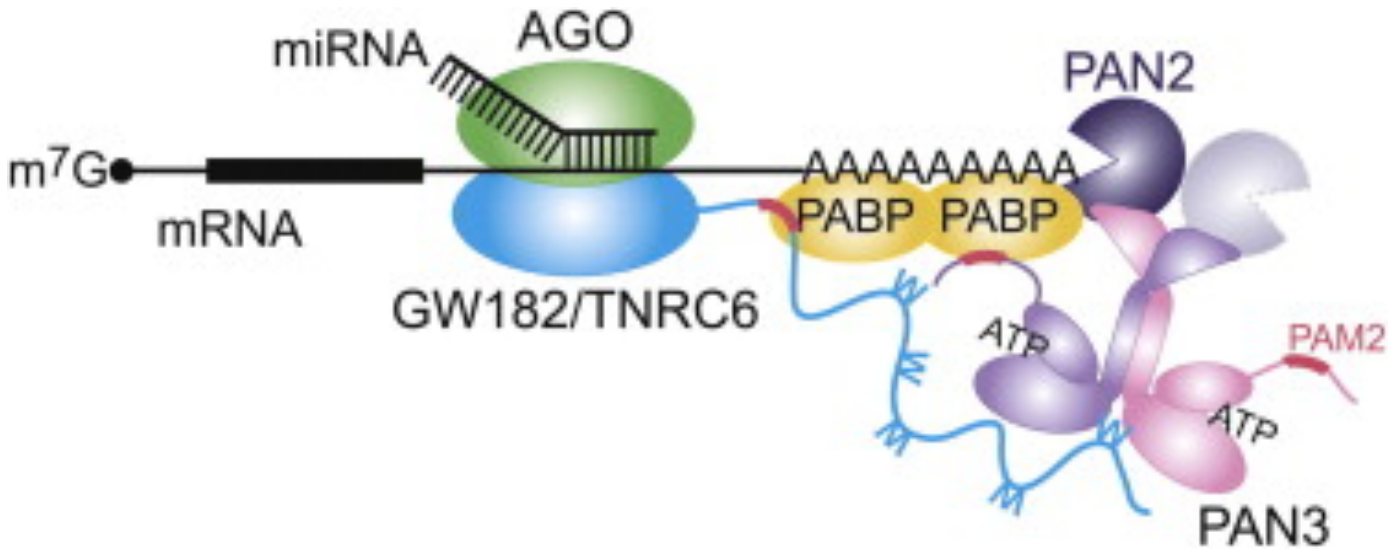


- Translational repression versus degradation: role of P-bodies
- Examples of miRNAs acting by alternative mechanisms
  - perfectly complementary miRNAs acting by the RNAi mechanism
  - miR-16 attracting mRNA degradative machinery to mRNA targets
  - stimulatory effect of miR-122 on HCV replication

GW182-AGO1 interaction is essential for silencing



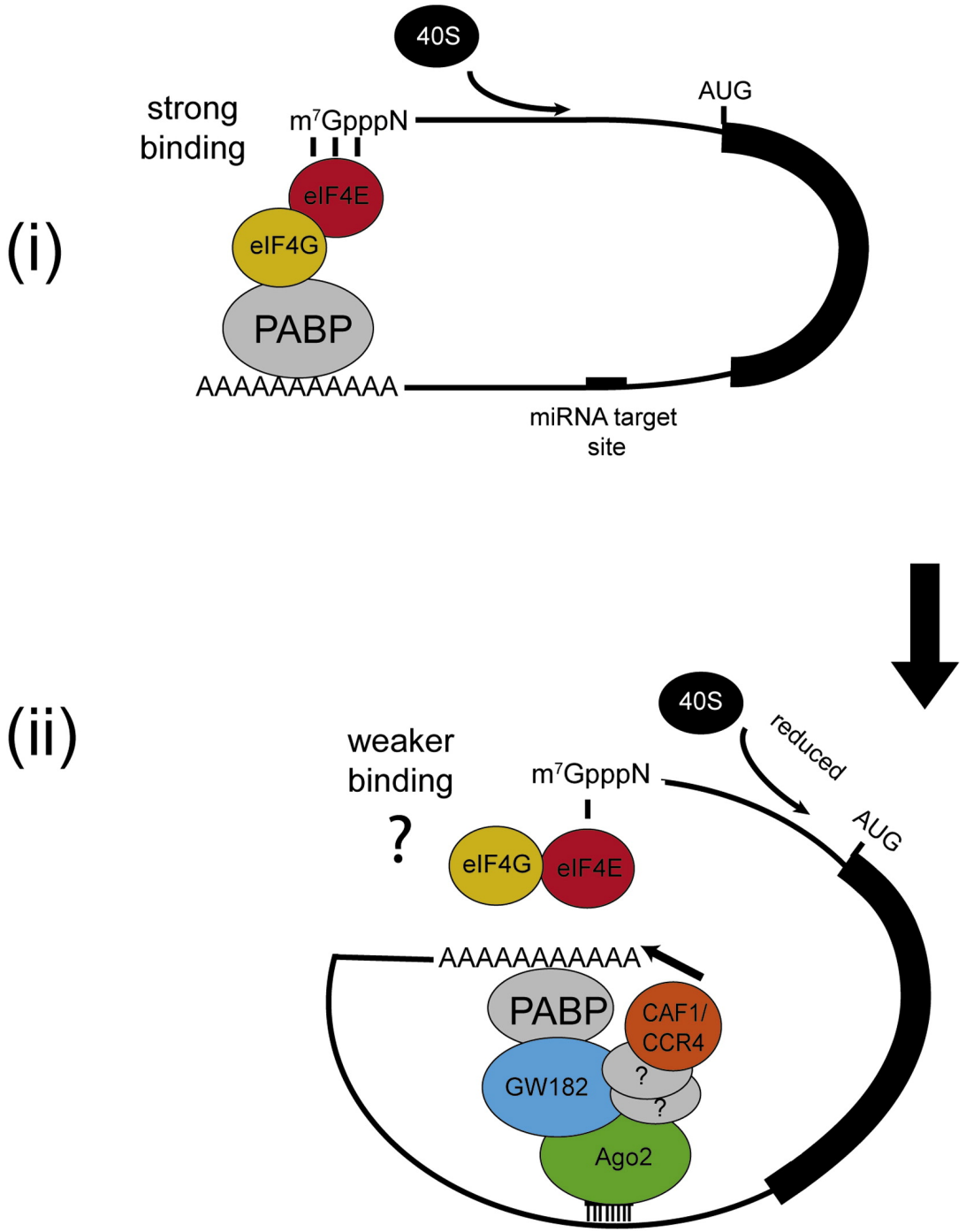
Behm-Ansmant et al. 2006. Genes & Dev. 20:1885



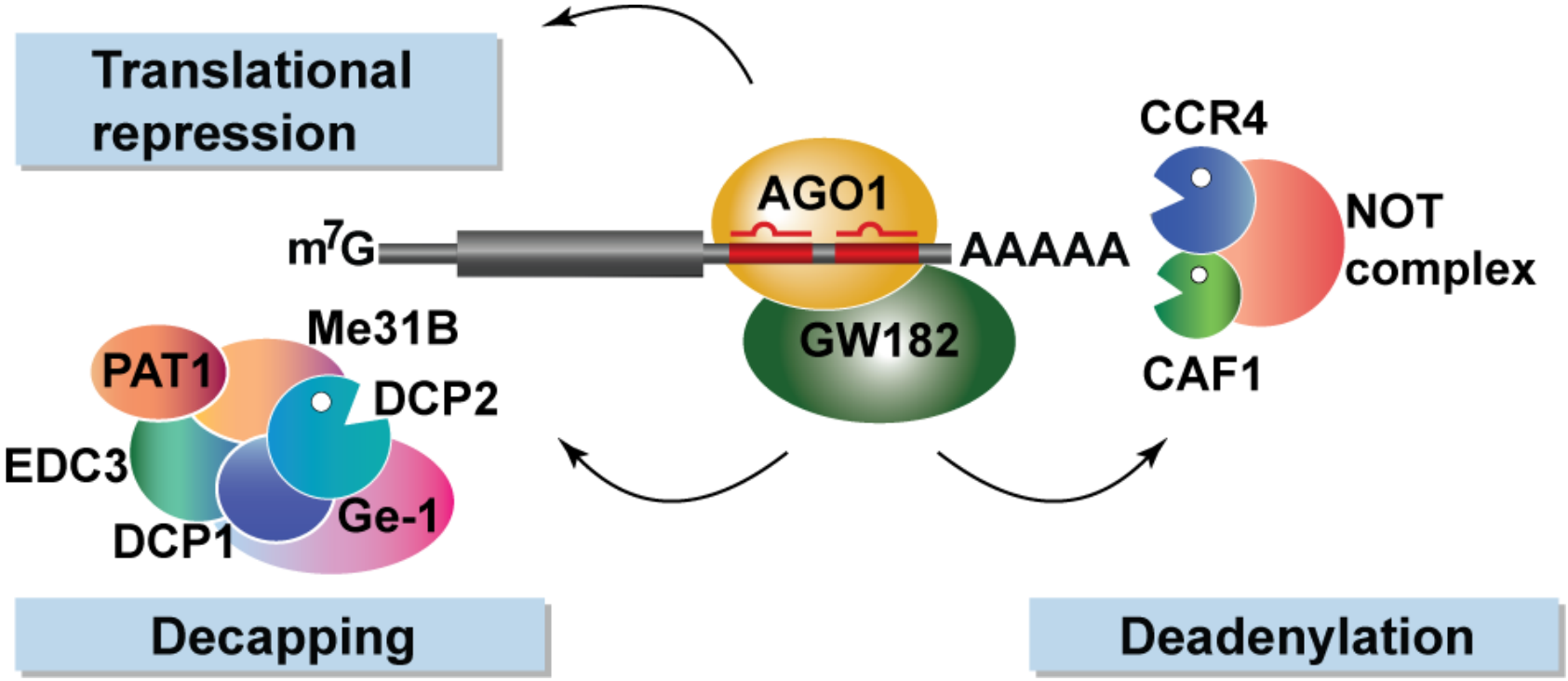
Ago (and PAN3) harbours tandem W-binding pockets (if deleted no silencing)

The distance between these pockets is similar to the distance between the W domains

- Redundancy in the way these proteins interact
- Flexible scaffolds that accomodate W residues in hydrophobic pockets
- Flexible tails catching the binding partner


(i)
40S
strong
binding
m7GpppN
AUG
eIF4E
eIF4G
PABP
AAAAAAAAAAA
miRNA target
site
(ii)
weaker
binding
?
40S
reduced
m7GpppN
AUG
eIF4G
eIF4E
AAAAAAAAAAA
PABP
CAF1/
CCR4
GW182
?
?
Ago2

Model for miRNA-mediated gene silencing

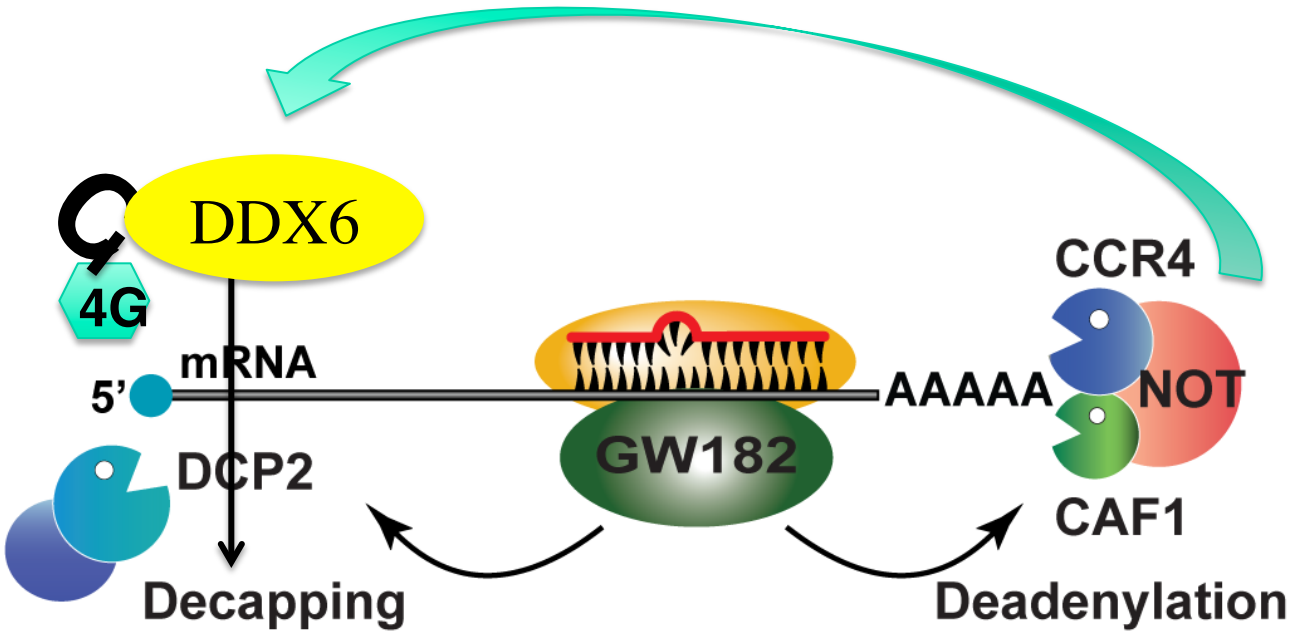


Behm-Ansmant et al. 2006. Genes & Dev. 20:1885

Eulalio et al. 2007. Genes & Dev. 21:2558

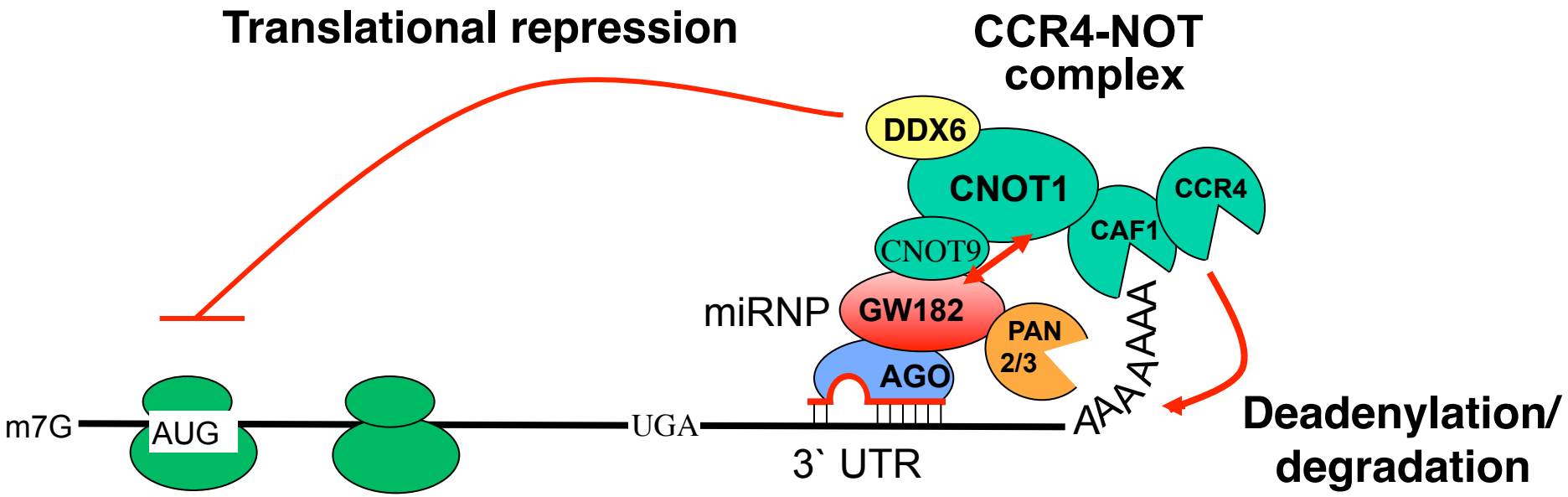
Witold Filipowicz – Basel

miRNA repress translation



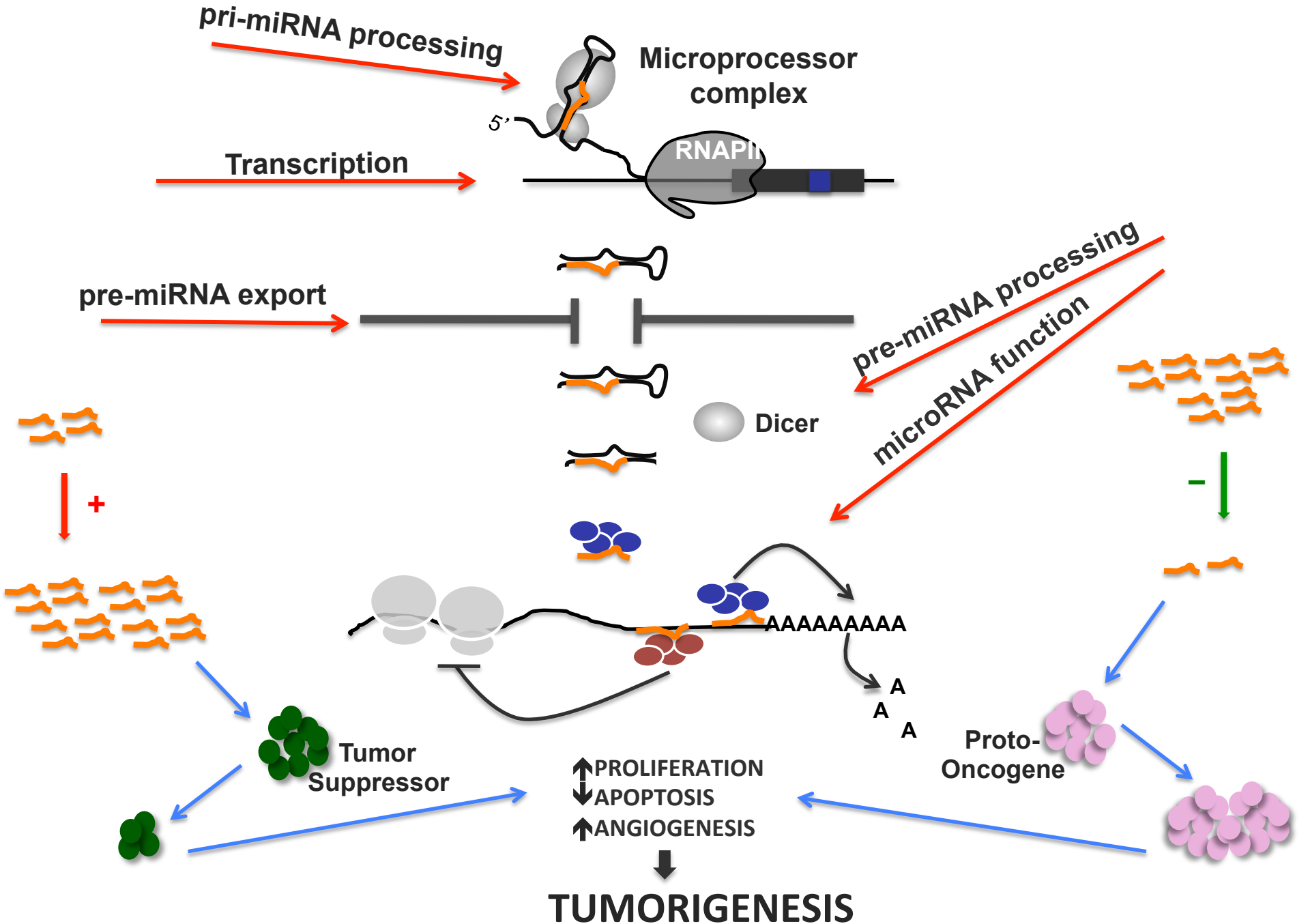
GW182 recruits NOT1 through WG/S/T domains
NOT1 is involved in repression of translation, it recruits DDX6
eiF4G interacts with the DEAD box helicase DDX6
DDX6 represses translation
DDX6 is a decapping activator

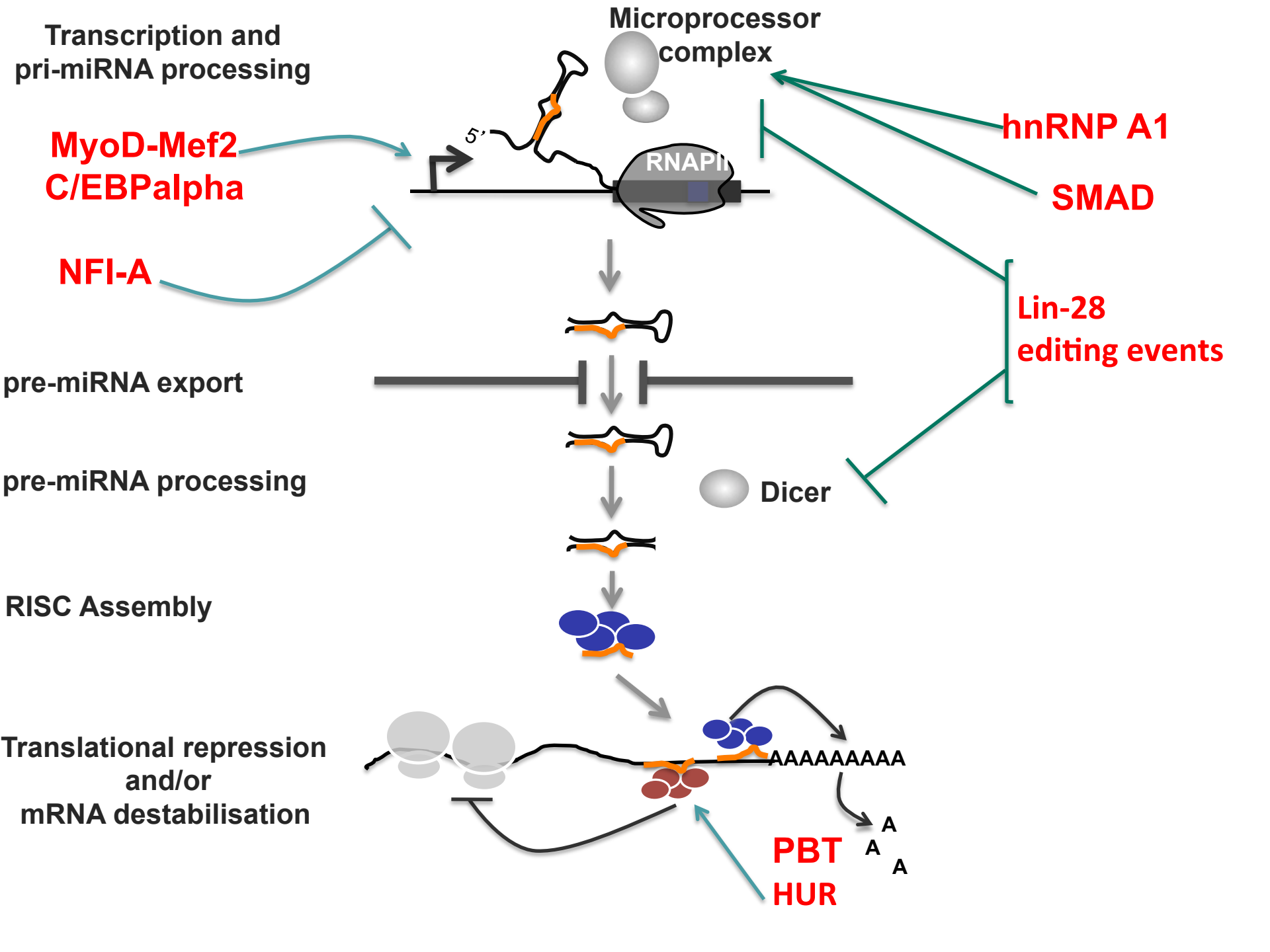
Central role of the CCR4-NOT in miRNA repression



- **Layers of protein effectors in miRNA repression:**
  an opportunity for sophisticated regulation

microRNAs and cancer




Transcription and
pri-miRNA processing
Microprocessor
complex
MyoD-Mef2
C/EBPalpha
NFI-A
5'
RNAPII
hnRNP A1
SMAD
Lin-28
editing events
pre-miRNA export
pre-miRNA processing
Dicer
RISC Assembly
Translational repression
and/or
mRNA destabilisation
AAAAAAAAA
A
A
A
PBT
HUR

Therapeutic use of miRNAs and anti-miRNAs

micro		
miR-122	RG-101 for Hepatitis C Virus Infection	REGULUS THERAPEUTICS
miR-21	RG-012 for Kidney Fibrosis Hepatocelluar Carcinoma	SANOFI
miR-103/107	Metabolic	AstraZeneca
miR-19	Oncology	AstraZeneca
miR-10b	Glioblastoma	REGULUS THERAPEUTICS
miR-221	Hepatocelluar Carcinoma	SANOFI

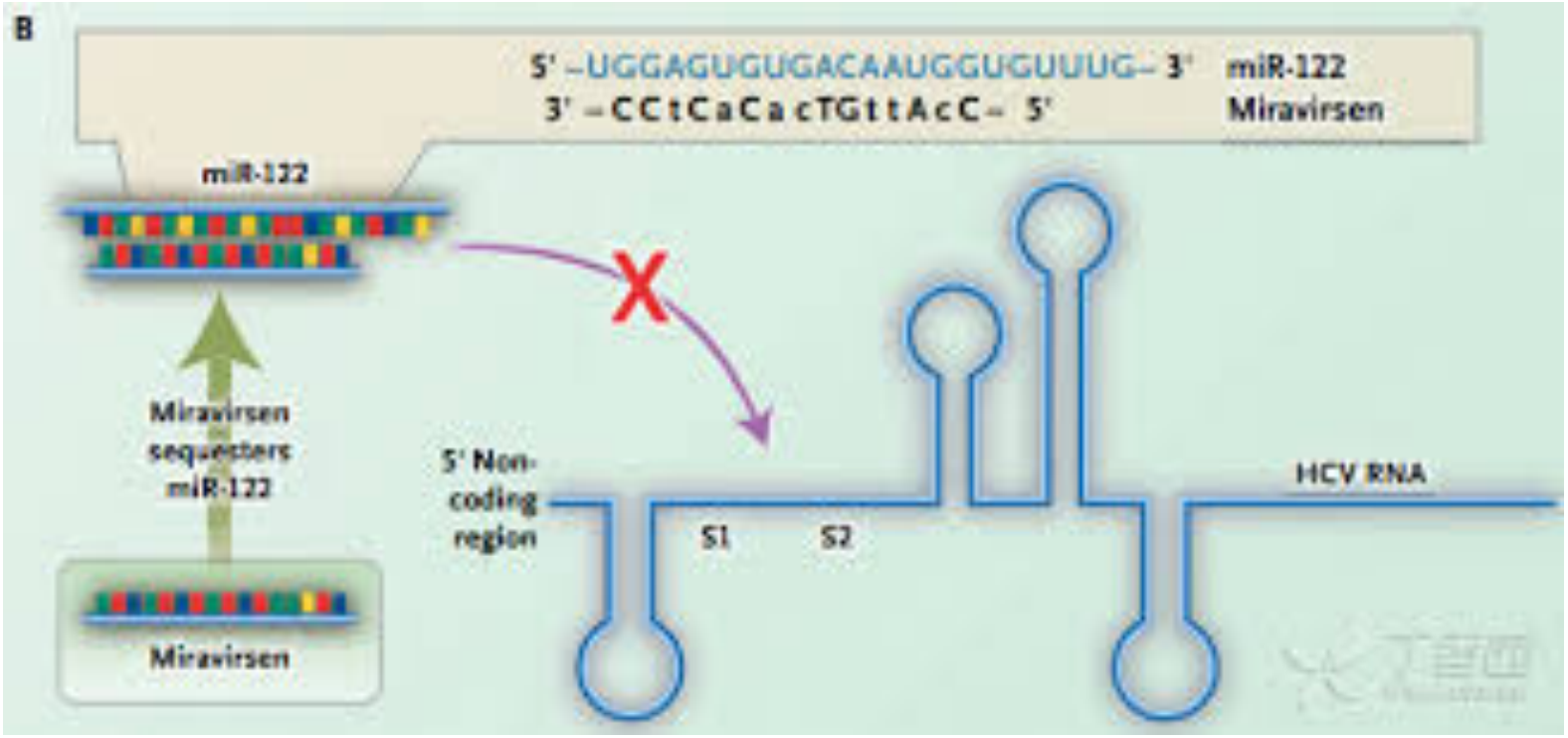
santaris pharma a/s

RNA Medicines for the 21st Century

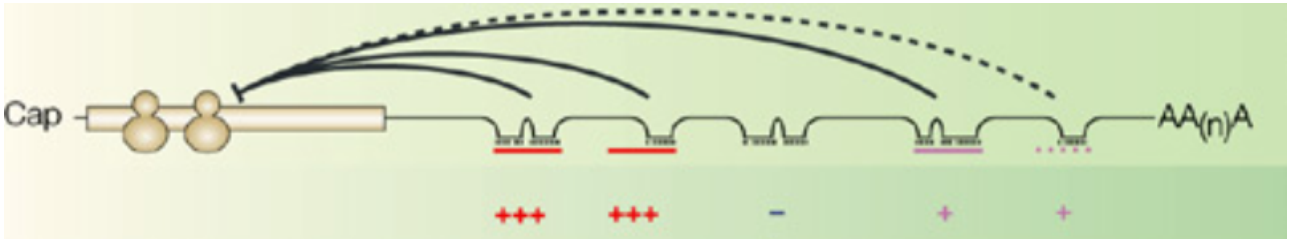
Miravirsen

is a LNA anti-sense oligonucleodite targeting and blocking miR-122

phase 2 trial



miRNAs and their mRNA targets: emerging patterns



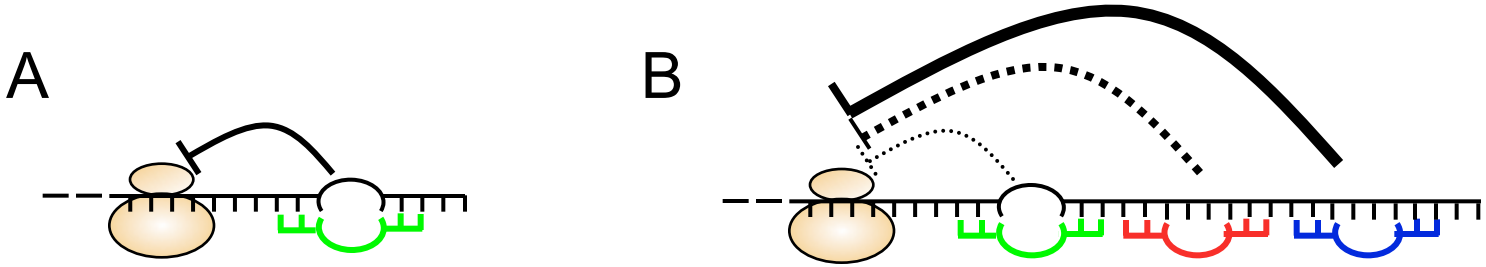
Bartel et al., 2004

- Bind to mRNA 3’-UTRs; targets spread over kilobases of 3’-UTR
- One miRNA can control activity of many different mRNAs
- A single mRNA can be controlled by more than one miRNA

Major advantages of miRNA regulation?

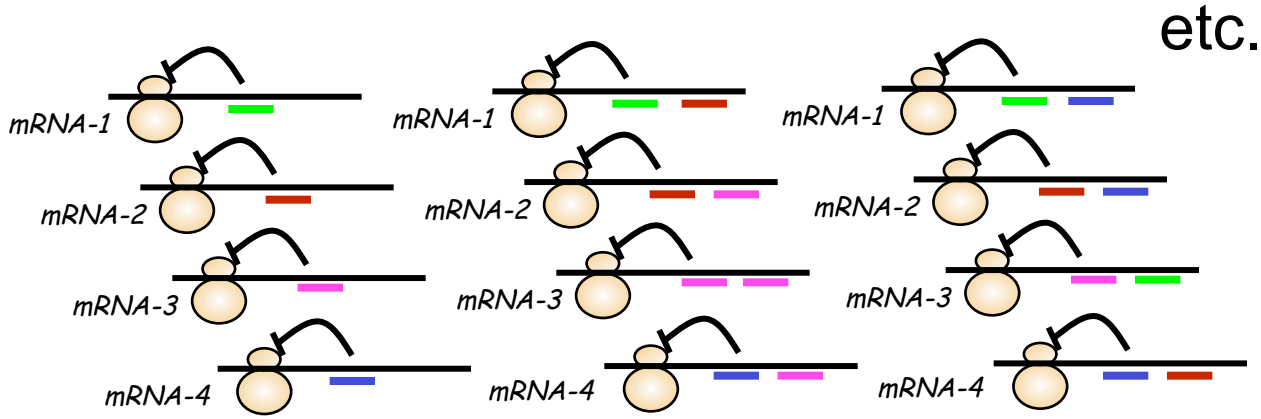
- Networking and fine-tuning of gene expression
- Rapid (and possibly reversible) repression

microRNAs and their pleiotropic activity provide a means for producing complex regulatory circuits



MULTIPLE TARGETS/ EFFECTORS Network of miRNAs

- The same mRNA can be regulated by different miRNAs
- Different combinations of miRNAs can regulate different sets of mRNAs
- cooperativity among miRNAs

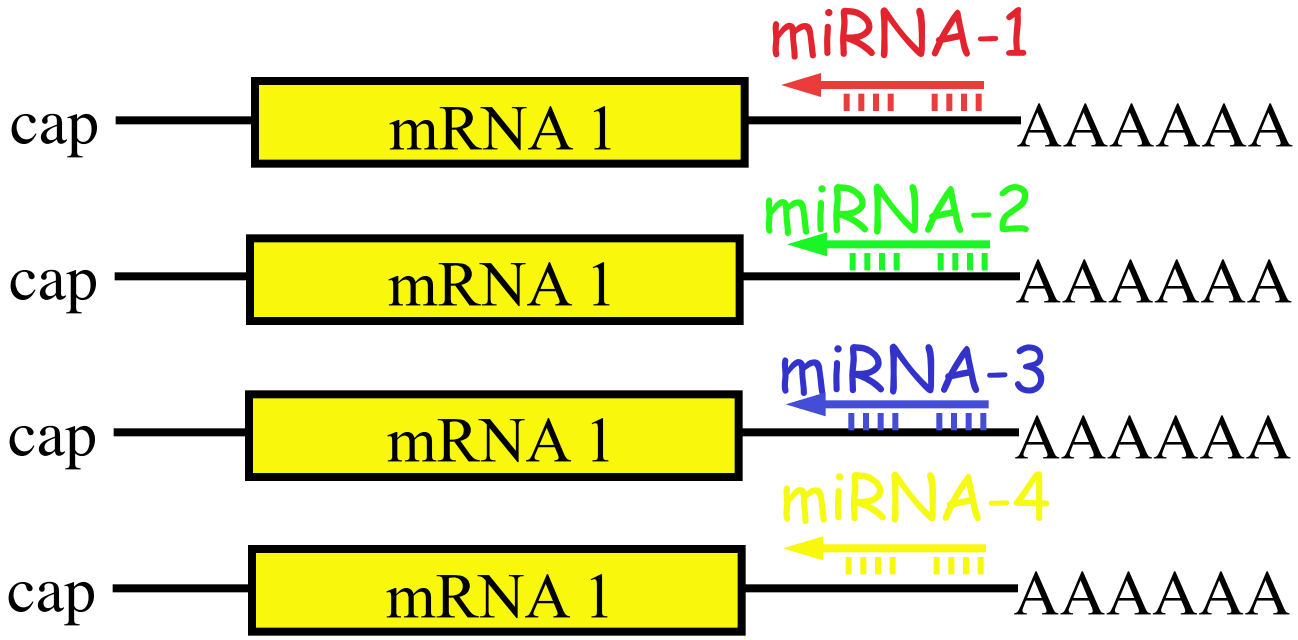


new conceptual implications derived from
the function of miRNAs

1)

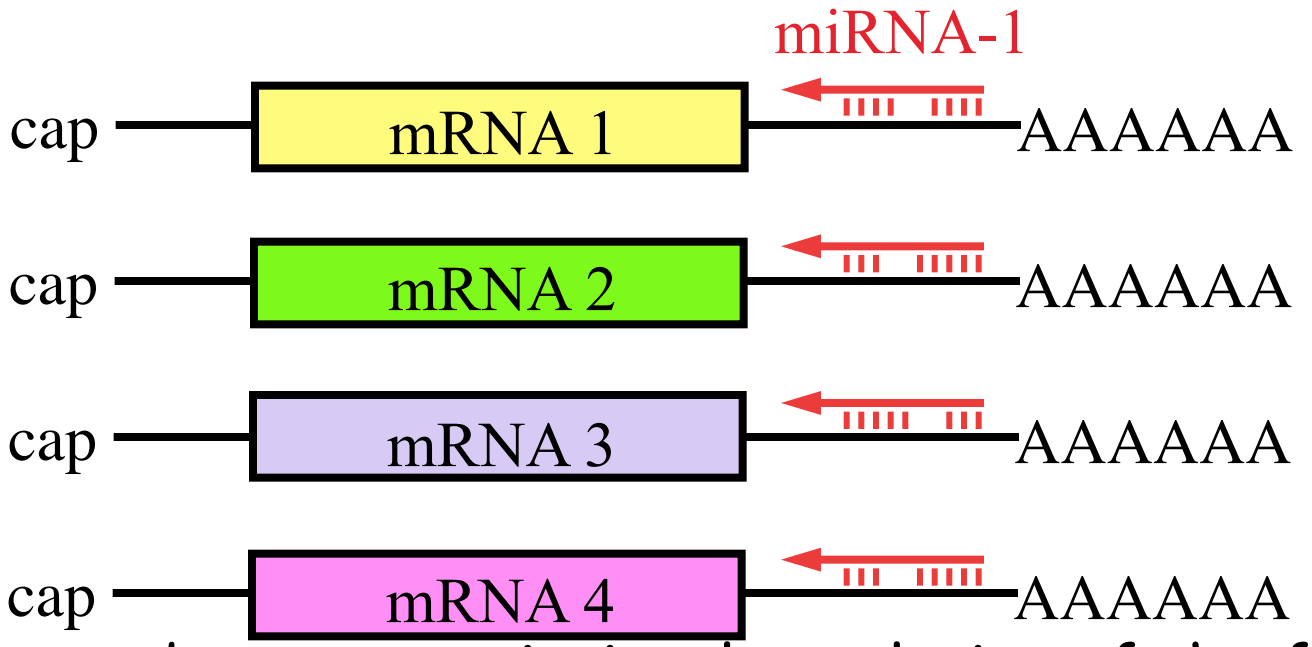
miRNA mode of action helps to explain how
the increase in complexity of regulatory circuits can be obtained
without changing the number of structural genes

Contemporary evolutionary advantages of microRNAs



A specific mRNA can be finely tuned in response to different stimuli in a cell type-specific way. Instead of providing the gene with different promoter regions it is the mRNA which is under the control of different miRNAs in different tissues

Contemporary evolutionary advantages of microRNAs



Independent transcriptional regulation of the four mRNAs

can be preserved while all four mRNAs are coregulated post-transcriptionally as a eukaryotic "regulon".

In specific tissues there will be master regulatory miRNAs

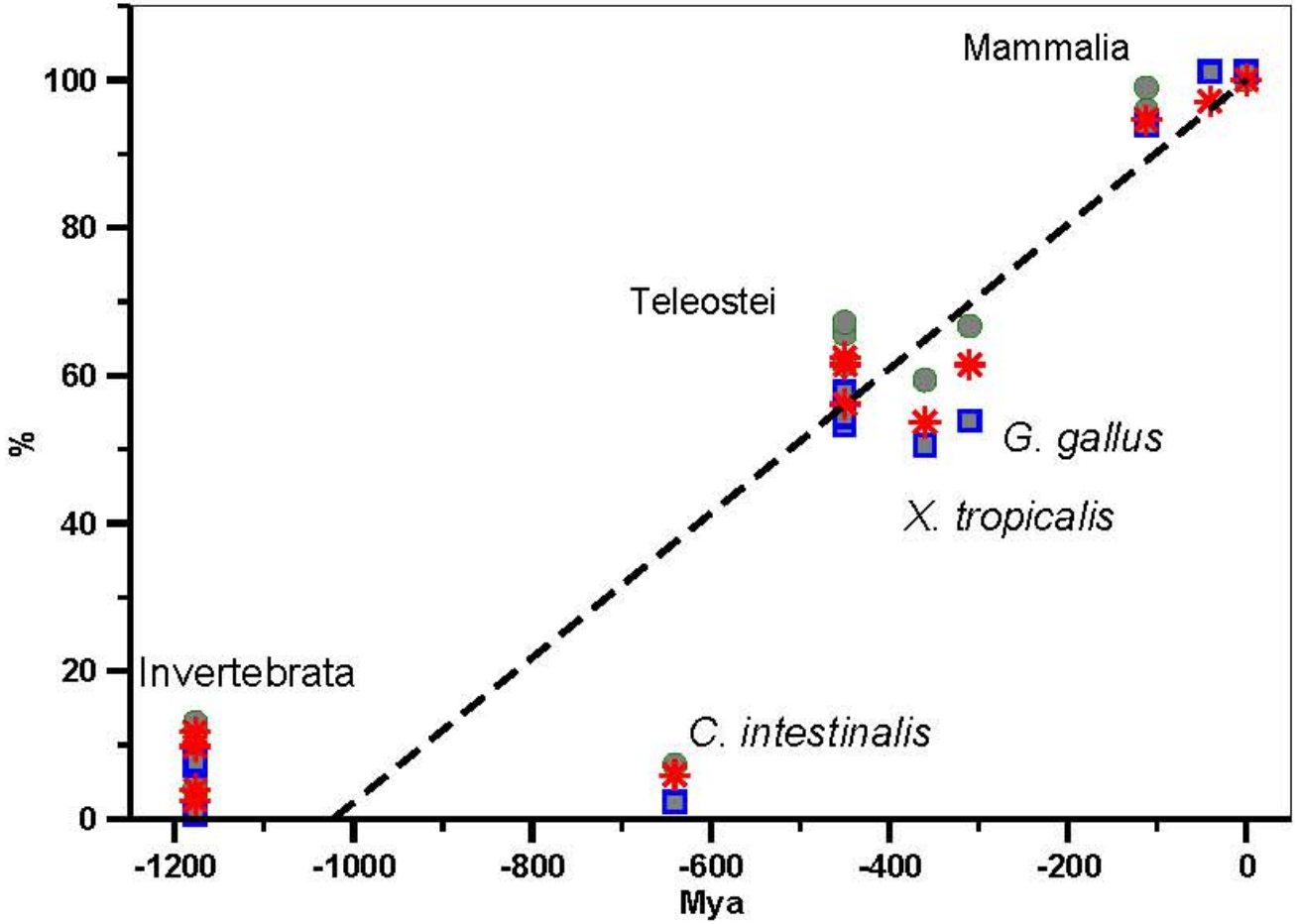


Fig. 8. Non-linear increase of miRNAs in Evolution. The human (green circles), rat (blue squares) and mouse (red stars) miRNAs listed in the `Rfam miRNA registry` v4.0 were blasted (cut off 10e-4) against the genomes of Invertebrata (*D. melanogaster*, *A. gambiae*, *C. elegans*, *C. briggsae*), *Ciona intestinalis*, Teleost fishes (*D. rerio*, *T. rubripes*, *T. nigroviridis*), *X. tropicalis*, *G. gallus* and Mammalia (*H. sapiens*, *M. musculus*, *R. norvegicus*). The percentage of mammalian miRNAs recovered are blotted against the evolutionary distance of those species.

Cell. 2005 Dec 16;123(6):1133-46.Click here to read Links

Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution.

Stark A,Brennecke J, Bushati N, Russell RB,Cohen SM.

MicroRNAs are small noncoding RNAs that serve as posttranscriptional regulators of gene expression in higher eukaryotes. Their widespread and important role in animals is highlighted by recent estimates that 20%-30% of all genes are microRNA targets. Here, we report that a large set of genes involved in basic cellular processes avoid microRNA regulation due to short 3'UTRs that are specifically depleted of microRNA binding sites. For individual microRNAs, we find that coexpressed genes avoid microRNA sites, whereas target genes and microRNAs are preferentially expressed in neighboring tissues. This mutually exclusive expression argues that microRNAs confer accuracy to developmental gene-expression programs, thus ensuring tissue identity and supporting cell-lineage decisions.

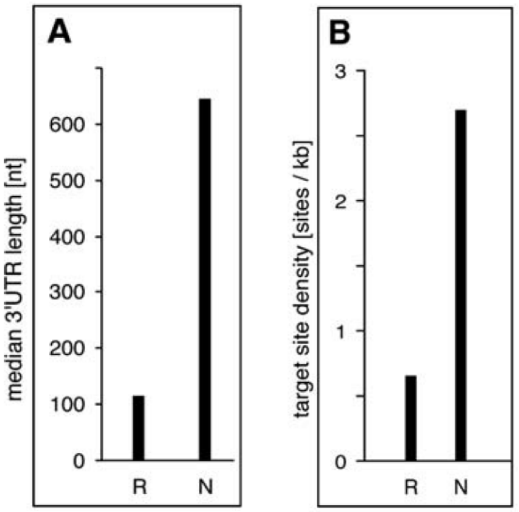
Improve 3'UTR targeting of microRNAs among species could raise organism complexity

Animal MicroRNAs Confer Robustness to Gene Expression and Have a Significant Impact on 3'UTR Evolution

D.melanogaster

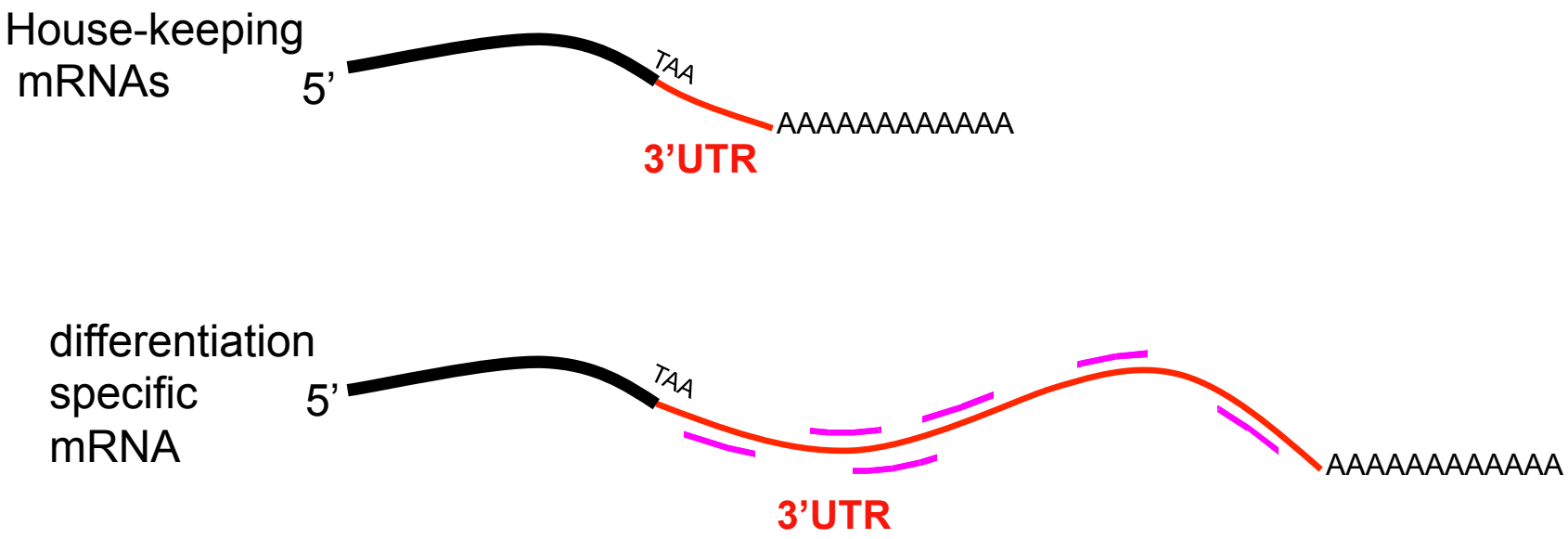
Table 1. miRNA Target and Antitarget Categories

Category	Description	# Genes	p(over) in Targets	p(under) in Antitargets
GO:0009887	Organogenesis	646	2.1E-34	7.3E-26
GO:0007399	Neurogenesis	364	2.2E-23	5.4E-19
GO:0007165	Signal transduction	791	2.7E-19	2.5E-14
GO:0030154	Cell differentiation	213	2.0E-11	5.4E-09
GO:0009790	Embryonic development	228	5.4E-11	1.4E-08
GO:0045165	Cell fate commitment	146	1.2E-10	3.8E-09
GO:0045449	Regulation of transcription	448	1.4E-09	2.8E-06
GO:0002009	Morphogenesis of an epithelium	104	1.0E-08	3.0E-08
GO:0007422	Peripheral nervous system development	95	4.5E-08	3.9E-07
GO:0009795	Embryonic morphogenesis	101	1.1E-07	5.2E-07
GO:0007498	Mesoderm development	135	3.5E-07	2.0E-04
Category	Description	# Genes	p(over) in Antitargets	p(under) in Targets
GO:0030529	Ribonucleoprotein complex	200	3.7E-06	1.3E-11
GO:0005840	Ribosome	128	2.4E-05	1.1E-11
GO:0006412	Protein biosynthesis	289	4.1E-03	3.8E-04
GO:0016070	RNA metabolism	190	7.4E-03	7.7E-04
GO:0016591	DNA-directed RNA polymerase II, holoenzyme	62	7.7E-03	5.6E-05
GO:0006119	Oxidative phosphorylation	61	1.8E-02	2.3E-04
GO:0006281	DNA repair	70	2.2E-02	4.7E-04
GO:0000502	Proteasome complex (sensu Eukarya)	37	2.6E-02	4.1E-04
GO:0006259	DNA metabolism	203	2.8E-02	3.9E-03
GO:0008380	RNA splicing	78	3.9E-02	1.4E-02



Stark et al. – Cell – 2005

D.melanogaster - While house-keeping genes have not increased the complexity of their 3'UTR, tissue-specific genes have drastically increased the length of their 3'UTR and the number of target sites for miRNAs



3'UTRs have been under selection to acquire or eliminate miRNA target sites

Increase in complexity of the 3' UTRs along the evolutionary scale:

increase in length and number of miRNA target sequences

the case of Homer 2

dendritic protein locally translated at synapses

Remodelling of the actin cytoskeleton - promotes the formation and maintenance of synaptic connections

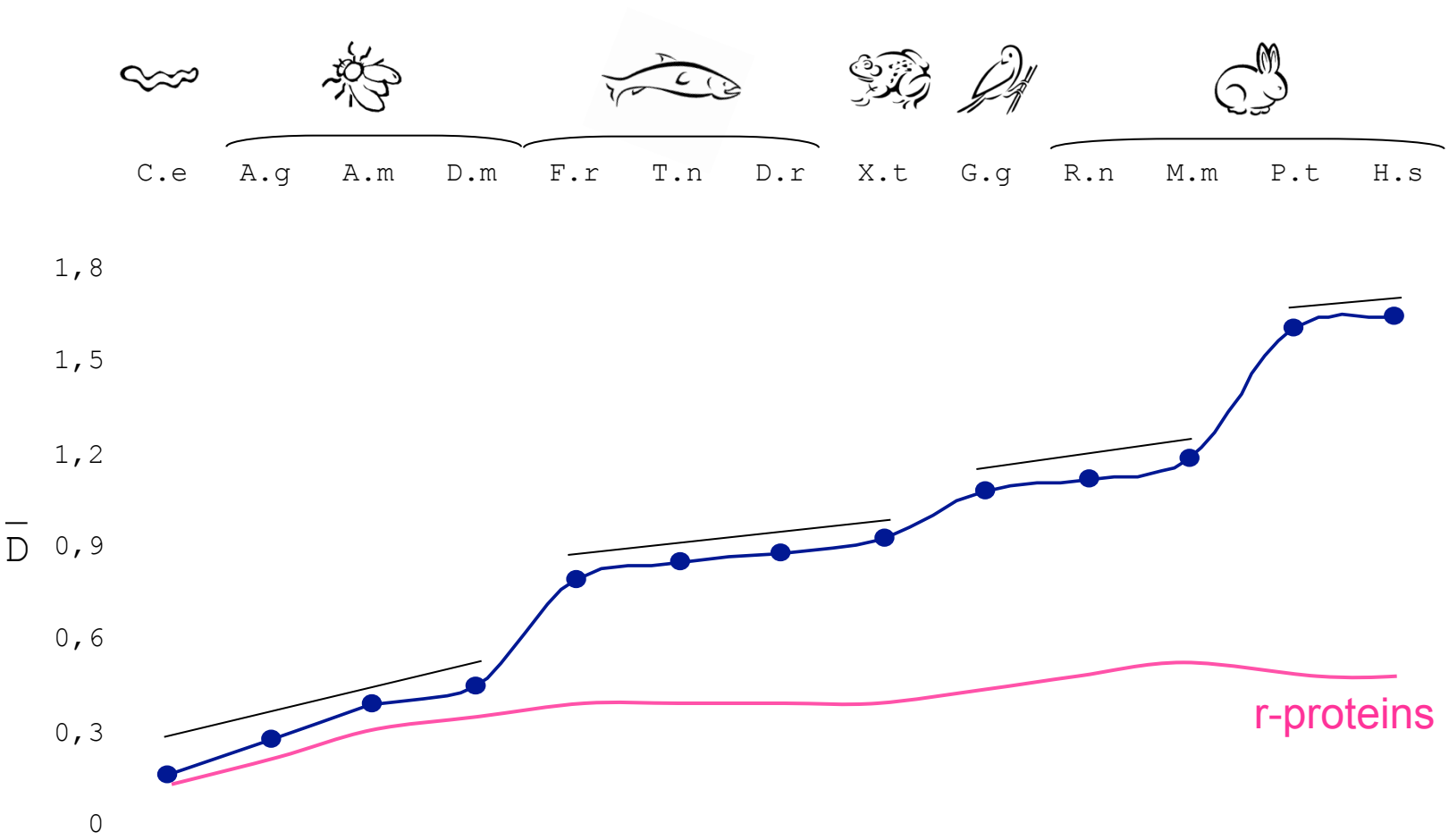
Local regulation of mRNA translation plays an important role in axon guidance, synaptic development and neuronal plasticity

BDNF activates translation of synaptic mRNAs and miRNAs counteract its action (miR 134 and LimK1)

+ other 14 conserved neuronal proteins

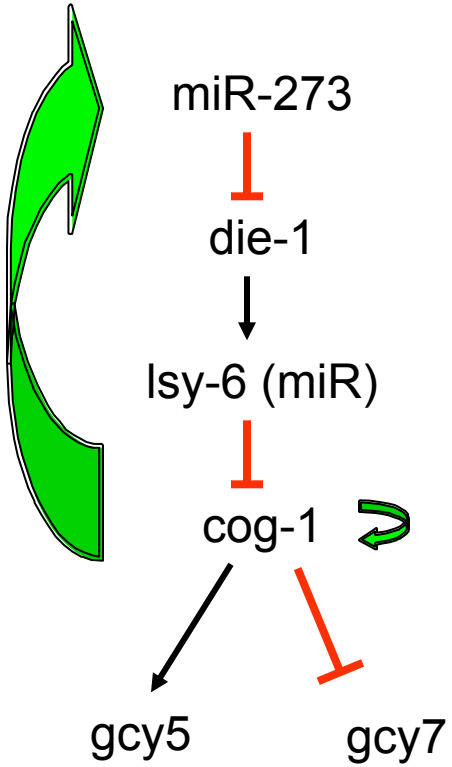
Genes conserved in evolution

For each species: number of miRNA targets for each 3'UTR
normalization for 3'UTR length
average of >50 genes



The mode of action of miRNAs helps to explain how differentiation commitments can be achieved

miRNAs allow **threshold overcomings**: in fact, very often they repress factors that positively feedback their own synthesis thus allowing to convert slight variations in factors concentration into strong and permanent commitments.



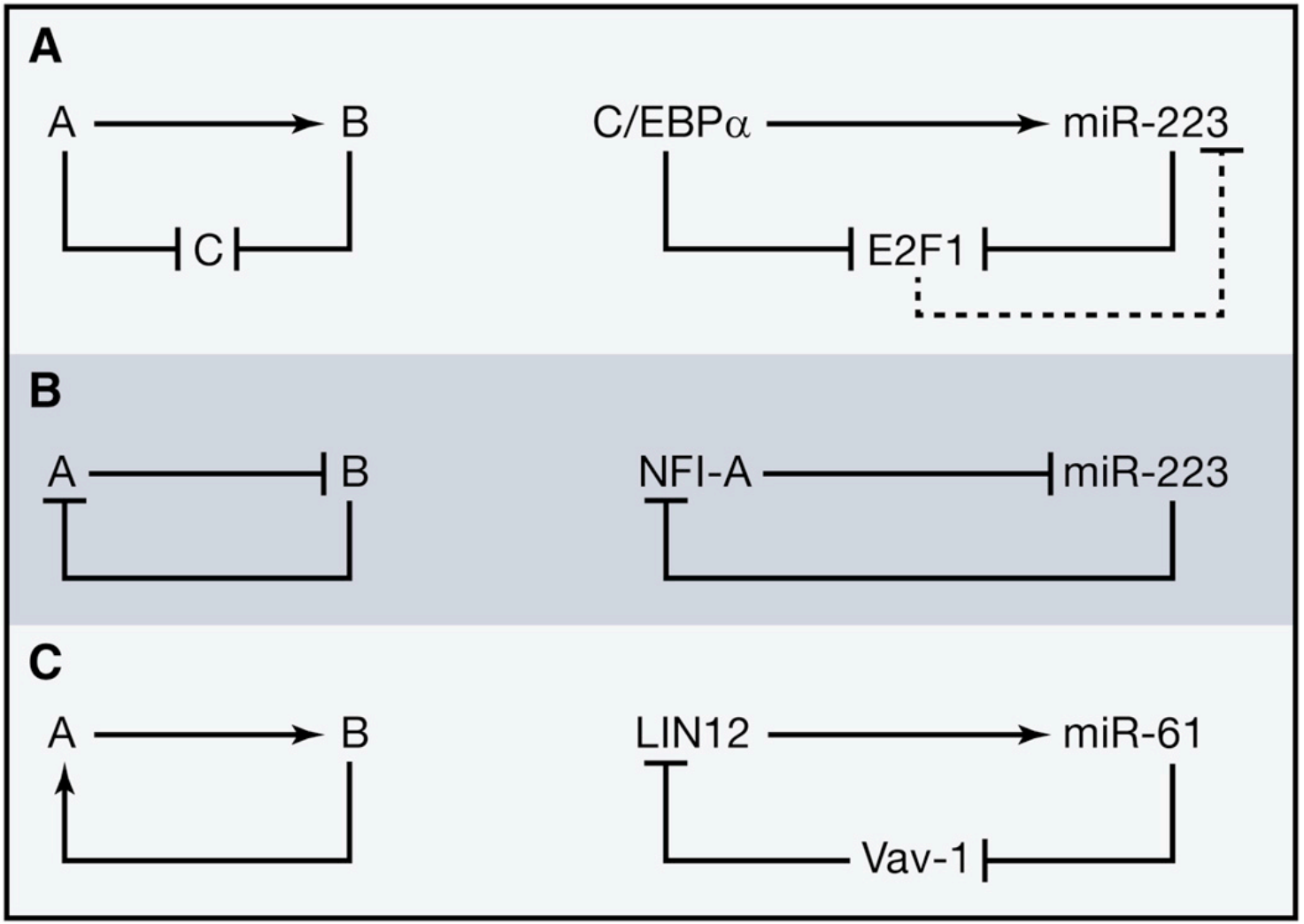
c.elegans ASE neurons (gustatory) respond to different stimuli - L/R gcy5 and gcy7

autoregulates its own promoter

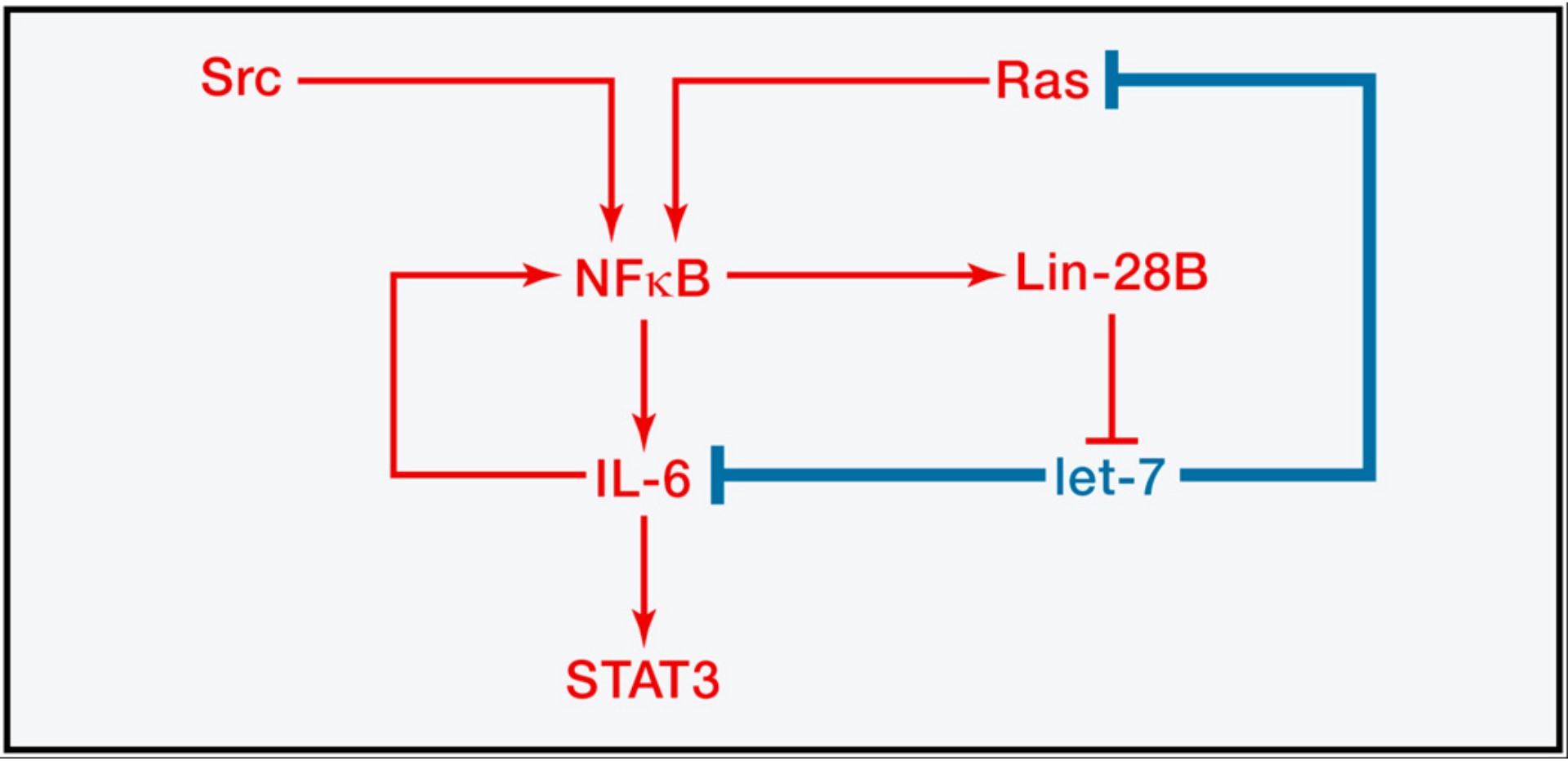
MicroRNAs acting in a double-negative feedback loop to control a neuronal cell fate decision.
Johnston RJ Jr, Proc Natl Acad Sci U S A. 2005 Aug 30; 102(35):12449-54.

chemoreceptors of the guanylyl cyclase (gcy) gene family

Roles for microRNAs in conferring robustness to biological processes
Margaret S. Ebert and Phillip A. Sharp – Cell, 2012, 149:515

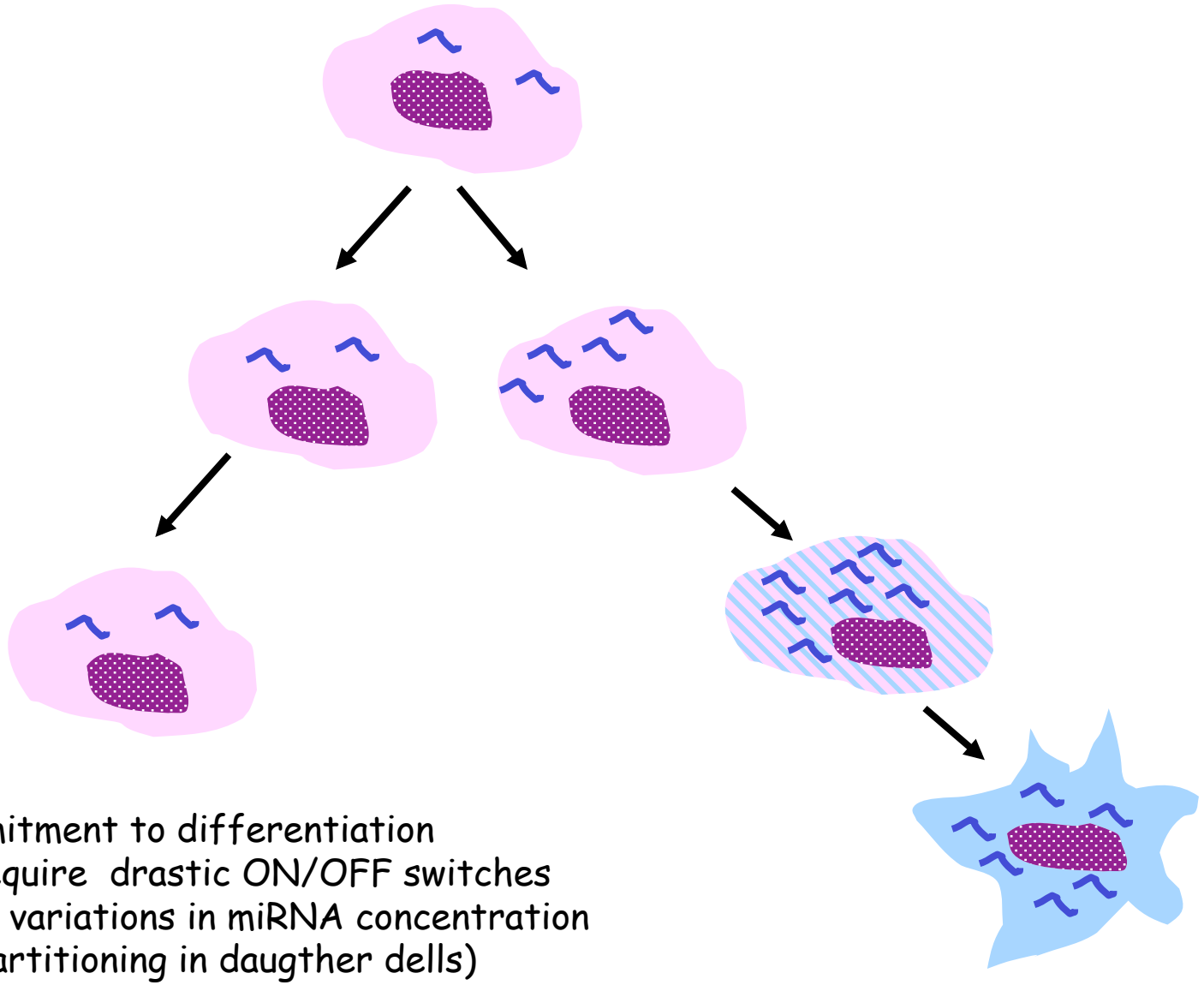


Network motifs for cell fate switches.



Positive feedback can amplify small changes. A transient inflammatory cue induces stable malignant transformation through an NF-κB/IL6 positive feedback network that is normally kept in check by let-7. Diagram adapted from (Iliopoulos et al., ...

Subtle changes in miRNA levels can lead to drastic and rapid changes in gene expression programs



Commitment to differentiation
would not require drastic ON/OFF switches
but only slight variations in miRNA concentration
(diff. partitioning in daugther dells)