RNA Interference and the World of Small RNAs

O, I die, Horatio;
The potent poison quite o'er-crows my spirit:
I cannot live to hear the news from England;
But I do prophesy the election lights On Fortinbras: he has my dying voice;
So tell him, with the occurrents, more and less, Which have solicited.

The rest is silence.

Hamlet, Act V, Scene II

From a review by Berstein et al. (2002)
Originally from a fellow named Shakespeare

PTGS (post transcriptional gene silencing) (1990)

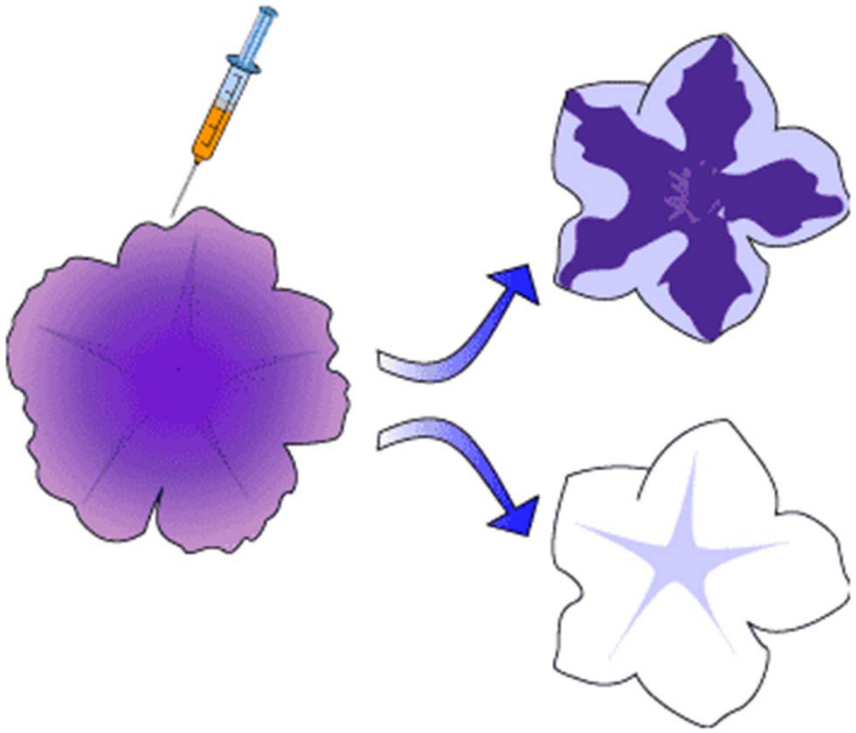
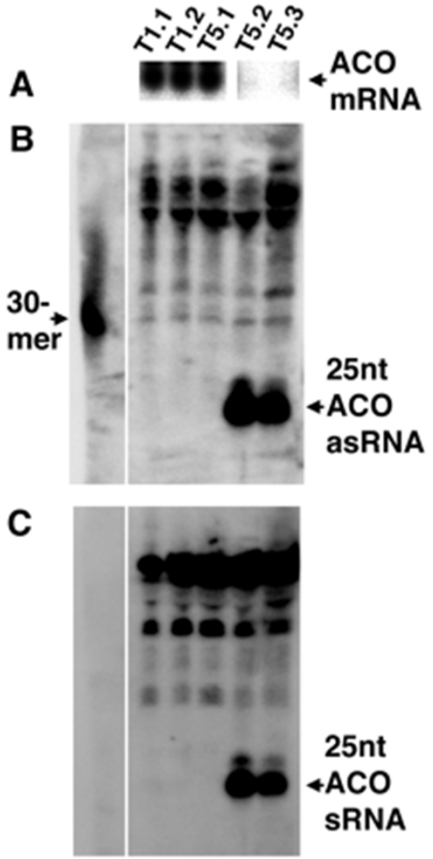


Figure 2. *A variegated petunia. Upon injection of the gene responsible for purple colouring in petunias, the flowers became variegated or white rather than deeper purple as was expected.*

David Baulcombe

Discovered 25 nt RNAs (1999)



Guo and Kemphues 1995 used antisense RNA to knock down expressionof a gene – It worked but the sense control also worked!

"Surprisingly, injection of in vitro synthesized sense RNA from the cDNA ZC22 also induced par-7 phenotypes at a high frequency among the progeny of injected worms. It is not clear what accounts for this effect."

Table 1. Scoring for Embryonic Lethality in RNA Injections

Molecules Injected	Number of Worms Injected	Percent Embryonic Lethality
ZC22 (*par-1*) antisense	16	52
ZC22 (*par-1*) sense	12	54
Clone *TS* antisense	8	0
Clone pZ1 antisense	8	0
DEPC–dH_2O	4	0

ZC22 is the nearly full-length cDNA clone (4 kb) for *par-1*; *TS* is *twinstar*, a Drosophila gene encoding cofilin (K. Gunsalus and M. Goldberg, personal communication); clone pZ1 is a zygotic cDNA clone identified by J. Watts (personal communication). The number of hatched versus unhatched embryos was counted for the first 2 days following the injections (a minimum of 100 embryos from each injected worm were counted).

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. The mixture was at least two orders of magnitude more effective than either single strand alone in producing genetic interference.

Andy Fire and Craig Mello (and a certain dynamite salesman)

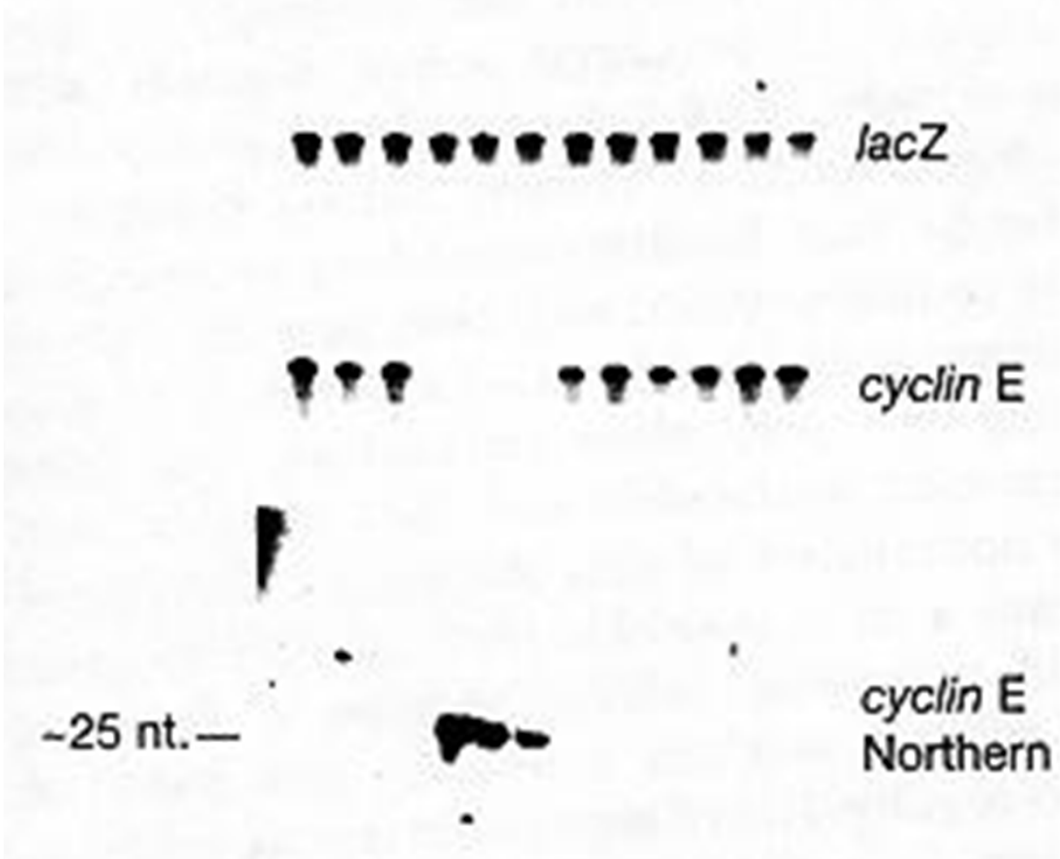
1997

Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F_1 phenotype
unc-22				*unc-22*-null mutants: strong twitchers[7,8]
*unc22A**	Exon 21–22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22B	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22C	Exon 21–22†	785	Sense + antisense	Strong twitchers (100%)
fem-1				*fem-1*-null mutants: femal (no sperm)[13]
fem1A	Exon 10‡	531	Sense	Hermaphrodite (98%)
			Antisense	Hermaphrodite (>98%)
			Sense + antisense	Female (72%)
fem1B	Intron 8	556	Sense + antisense	Hermaphrodite (>98%)
unc-54				*unc-54*-null mutants: paralysed[7,11]
unc54A	Exon 6	576	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
unc54B	Exon 6	651	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
unc54C	Exon 1–5	1,015	Sense + antisense	Arrested embryos and larvae (100%)
unc54D	Promoter	567	Sense + antisense	Wild type (100%)
unc54E	Intron 1	369	Sense + antisense	Wild type (100%)
unc54F	Intron 3	386	Sense + antisense	Wild type (100%)

Fractionation of editing activity

Drosophila cells were transfected with dsRNAs – got "loss-of-function phenotype.
A 540 nt cyclin fragment caused a G1 arrest, whereas transfection with the lacZ dsRNA had no effect. Size was important – 20-300 nt fragments had less effect than 400-500 nt fragments.
Cell extracts from tranfected cells showed specific degradation of exogenous mRNA targeted by the dsRNA.
Fractionated the in vitro activity by anion-exchange chromatography. The active fractions contained a 25 nt RNA homologous to the cyclic target.



In vitro RNAi extract

Tuschl and Zamore used a Drosophila embryo extract. Luciferin mRNA was added to get translation of luciferase. Pre-incubation of the extract for 10 min with a dsRNA specific for luciferin mRNA caused specific degradation of added luciferin mRNA. ATP was required and 21-25 nt ds RNA fragments were produced. The ds RNA determined the cleavage boundries along the mRNA sequence.

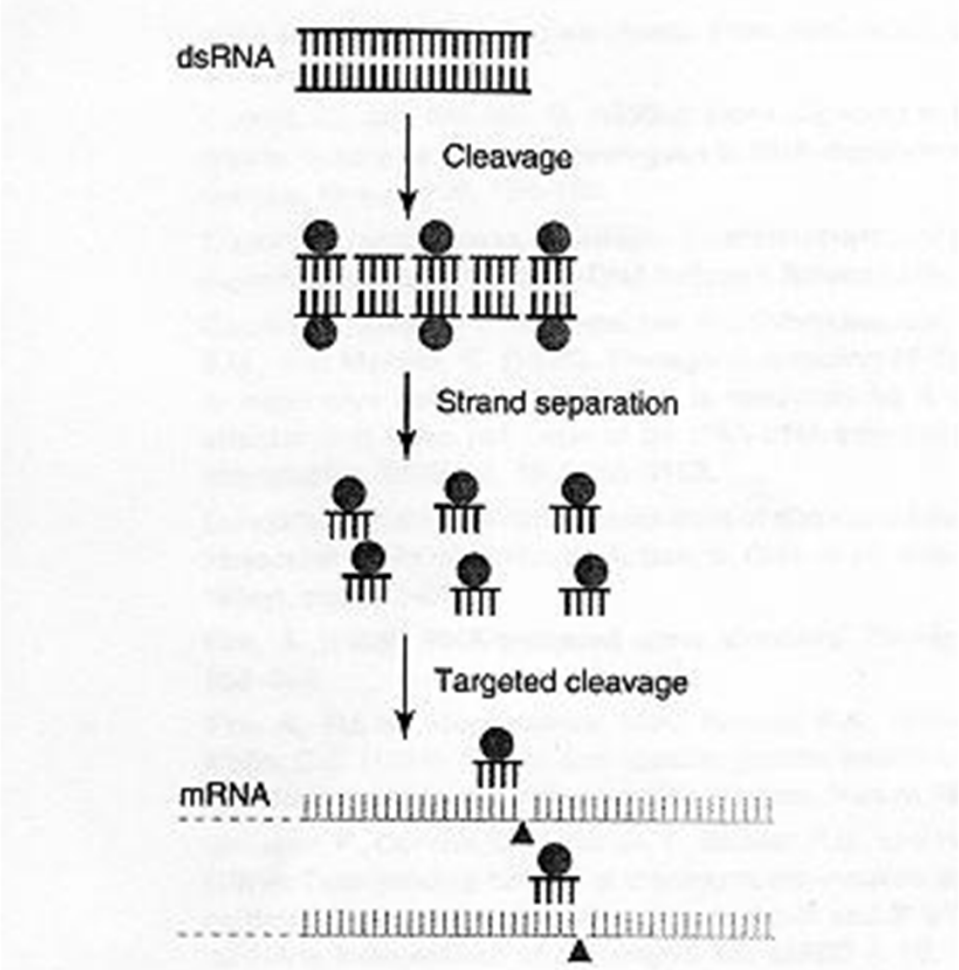
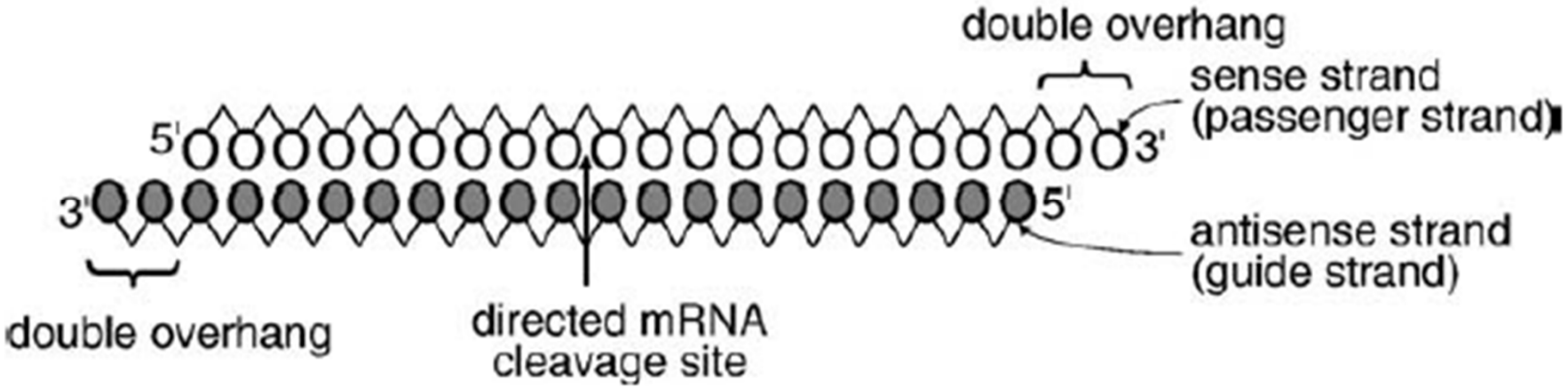


Figure 7. Proposed Model for RNAi

RNAi is envisioned to begin with cleavage of the dsRNA to 21–23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP-dependent helicase, possibly a component of the initial complex, to 21–23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (ochre circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

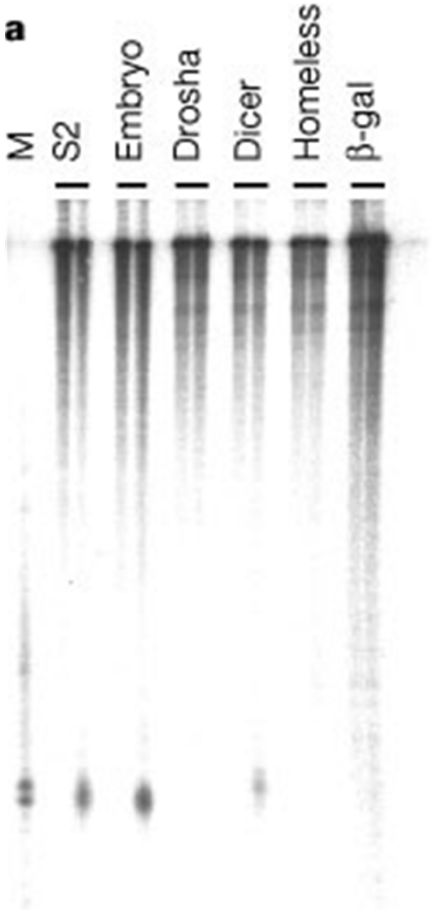
Structure of siRNAs



Identification of Dicer as the enzyme responsible for siRNAs

Genomic search for RNase III enzymes (double strand cutters)

IP of these enzymes. Only Dicer works

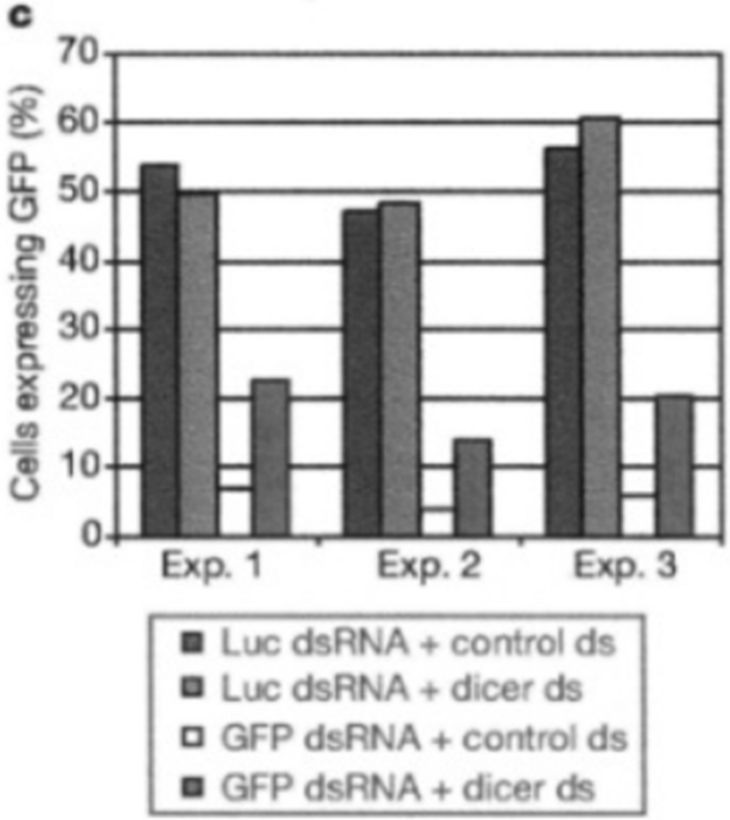


Dicer cleavage is ATP-dependent

The 23 nt product comigrates with the RISC product

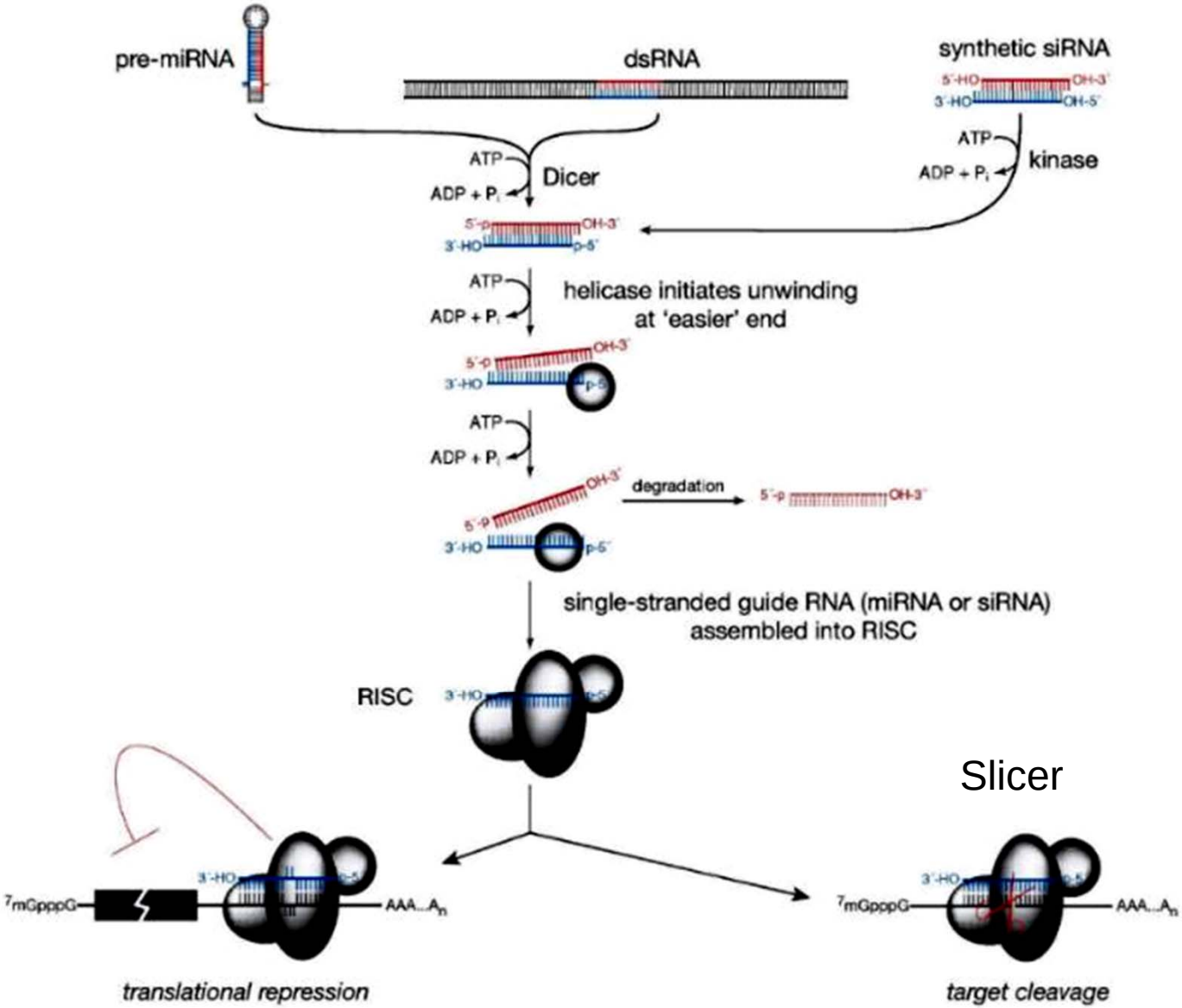
To prove definitely that Dicer was the enzyme involved in RNAi, they depleted Dicer activity from S2 cells by using RNAi itself. This resulted in the loss of the ability to silence an endogenous GFP transgene by RNAi.

cells (treated as above). Dicer dsRNA reduces the activity of Dicer-1 6.2-fold. **c**, GFP expression of co-transfected cells. Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plasmids plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase-9) or Dicer dsRNAs.



Cells co-transfected with GFP-plasmid and dsRNA

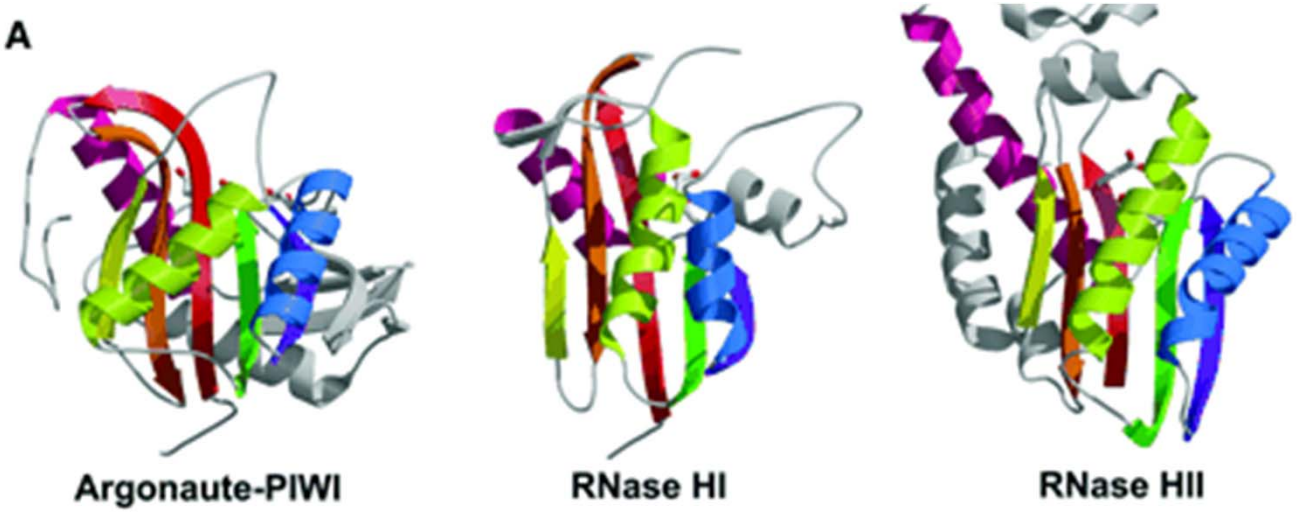
Only one strand is incorporated into the RISC complex



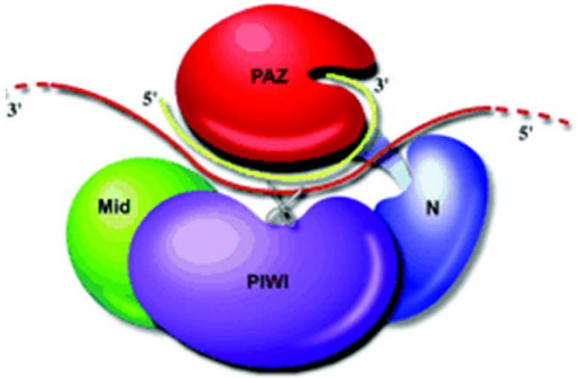
Identification of the "Slicer" Nuclease Activity in the RISC Complex

Argonaute is a protein found in the RISC complex and required for RNAi.

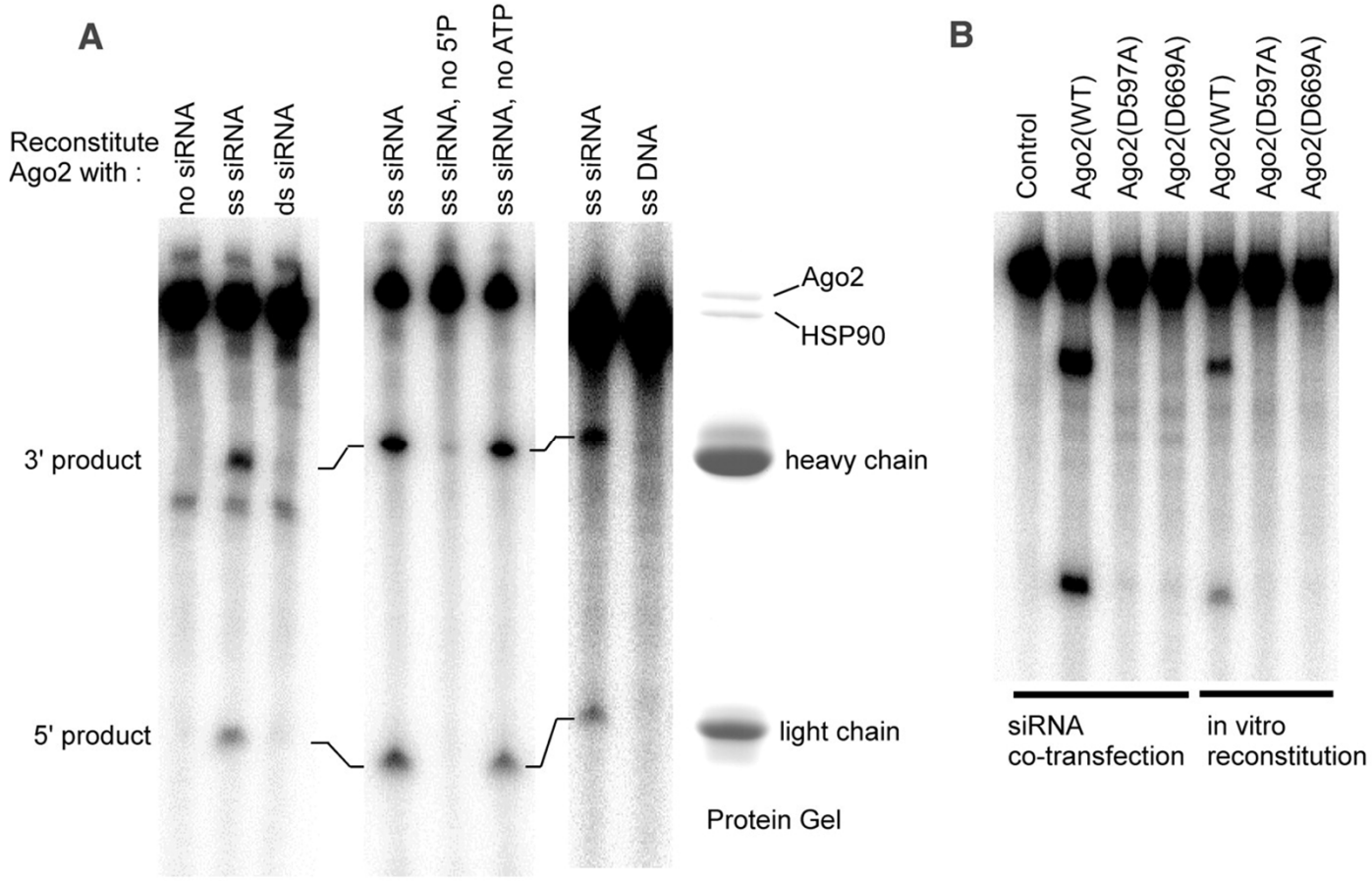
The PIWI domain is similar to the RNase H domain



This structure led to a model for siRNA-guided mRNA cleavage:



Reconstitution of Slicer activity with purified Argonaut-2 Protein



Ago2 protein was immunoaffinity purified from transiently transfected 293T cells. Immunoprecipitates were mixed in vitro with single- or double-stranded siRNAs or with a 21-nt DNA having the same sequence as the siRNA. Reconstituted RISC was tested for cleavage activity with a uniformly labeled synthetic mRNA. In B, mutants in Argo2 which were known to destroy activity were tested for activity in reconstituted material.

Micro RNAs

Several genes were discovered which were involved in developmental timing. They made RNA but no protein product!

Lin-4

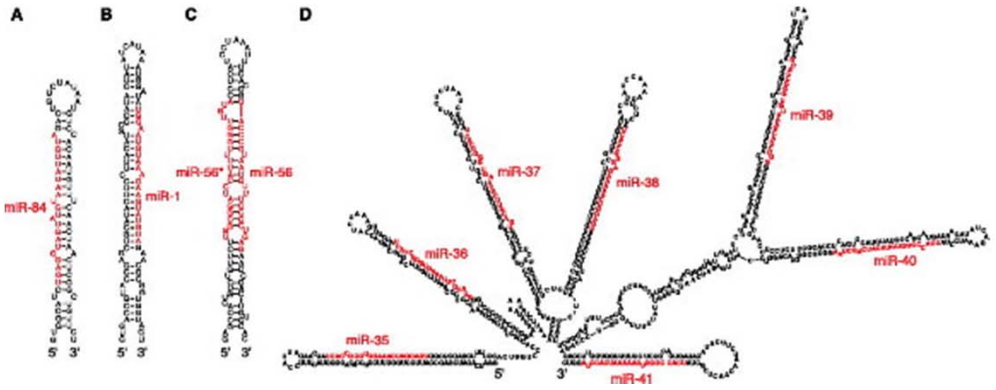
Complementary to 3' untranslated sequences of lin-14 mRNA and acts to developmentally repress the accumulation of lin-14 protein.

It also targets lin-28 and reduces protein expression.

Table 1. *D. melanogaster* miRNAs. The sequences given represent the most abundant, and typically longest, miRNA sequence identified by cloning. miRNAs frequently vary in length by one or two nucleotides at their 3′ termini. From 222 short RNAs sequenced, 69 (31%) corresponded to miRNAs, 103 (46%) to already characterized functional RNAs (tRNA, 7SL RNA, and rRNA), 30 (14%) to transposon RNA fragments, and 20 (10%) sequences had no database entry. The frequency for cloning a particular miRNA as a percentage relative to all identified miRNAs is indicated. Results of Northern blotting of total RNA isolated from staged populations of *D. melanogaster* are summarized. E, embryo; L, larval stage; P, pupa; A, adult; S2, Schneider-2 cells. The strength of the signal within each blot is represented from strongest (+++) to undetected (−). *let-7* stRNA was probed as the control. GenBank accession numbers and homologs of miRNAs identified by database searching in other species are provided in (21).

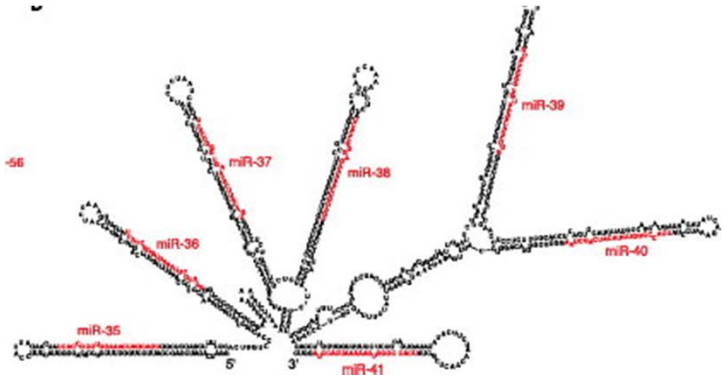
stRNA	Sequence (5′ to 3′)	Freq. (%)	E 0 to 3 hours	E 0 to 6 hours	L1 + L2	L3	P	A	S2
miR-1	UGGAAUGUAAAGAAGUAUGUAG	32	+	+	+++	+++	++	+++	-
miR-2a*	UAUCACAGCCAGCUUUGAUGAGC	3							
miR-2b*	UAUCACAGCCAGCUUUGAGGAGC	3	++	++	++	+++	++	+	+++
miR-3	UCACUGGGCAAAGUGUGUCUCA	9	+++	+++	-	-	-	-	-
miR-4	AUAAAGCUAGACAACCAUUGA	6	+++	+++	-	-	-	-	-
miR-5	AAAGGAACGAUCGUUGUGAUAUG	1	+++	+++	+/-	+/-	-	-	-
miR-6	UAUCACAGUGGCUGUUCUUUUU	13	+++	+++	+/-	+/-	-	-	-
miR-7	UGGAAGACUAGUGAUUUUGUUGU	4	+++	++	+/-	+/-	+/-	+/-	+/-
miR-8	UAAUACUGUCAGGUAAAGAUGUC	3	+/-	+/-	+++	+++	+	+++	-
miR-9	UCUUUGGUUAUCUAGCUGUAUGA	7	+++	++	+++	+++	+++	+/-	-
miR-10	ACCCUGUAGAUCCGAAUUUGU	1	+	+	++	+++	+/-	+	-
miR-11	CAUCACAGUCCGAGUUCUUGC	7	+++	+++	+++	+++	+++	+	-
miR-12	UGAGUAUUACAUCAGGUACUGGU	7	+	+	++	++	+	+++	+/-
miR-13a*	UAUCACAGCCAUUUUGACGAGU	1	+++	+++	+++	+++	+	+++	+++
miR-13b*	UAUCACAGCCAUUUUGAUGAGU	0							
miR-14	UCAGUCUUUUUCUCUCUCCUA	1	-	-	-	-	-	-	-
let-7	UGAGGUAGUAGGUUGUAUAGUU	0	-	-	-	-	+++	+++	-

* Similar miRNA sequences are difficult to distinguished by Northern blotting because of potential cross-hybridization of probes.



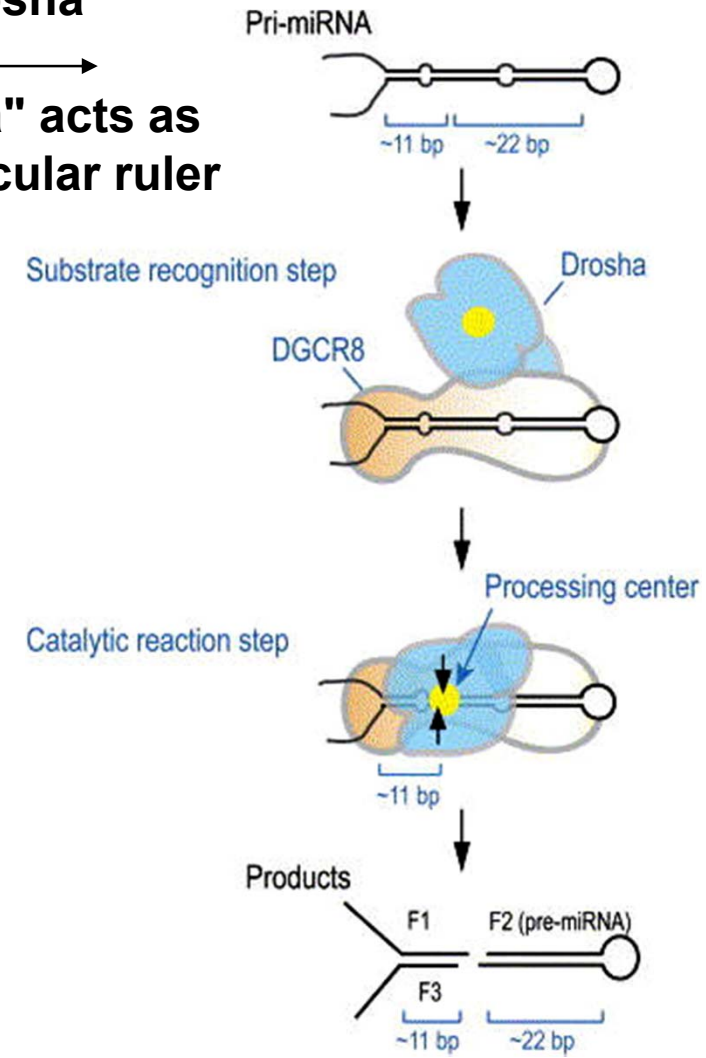
Dave Bartel
MIT

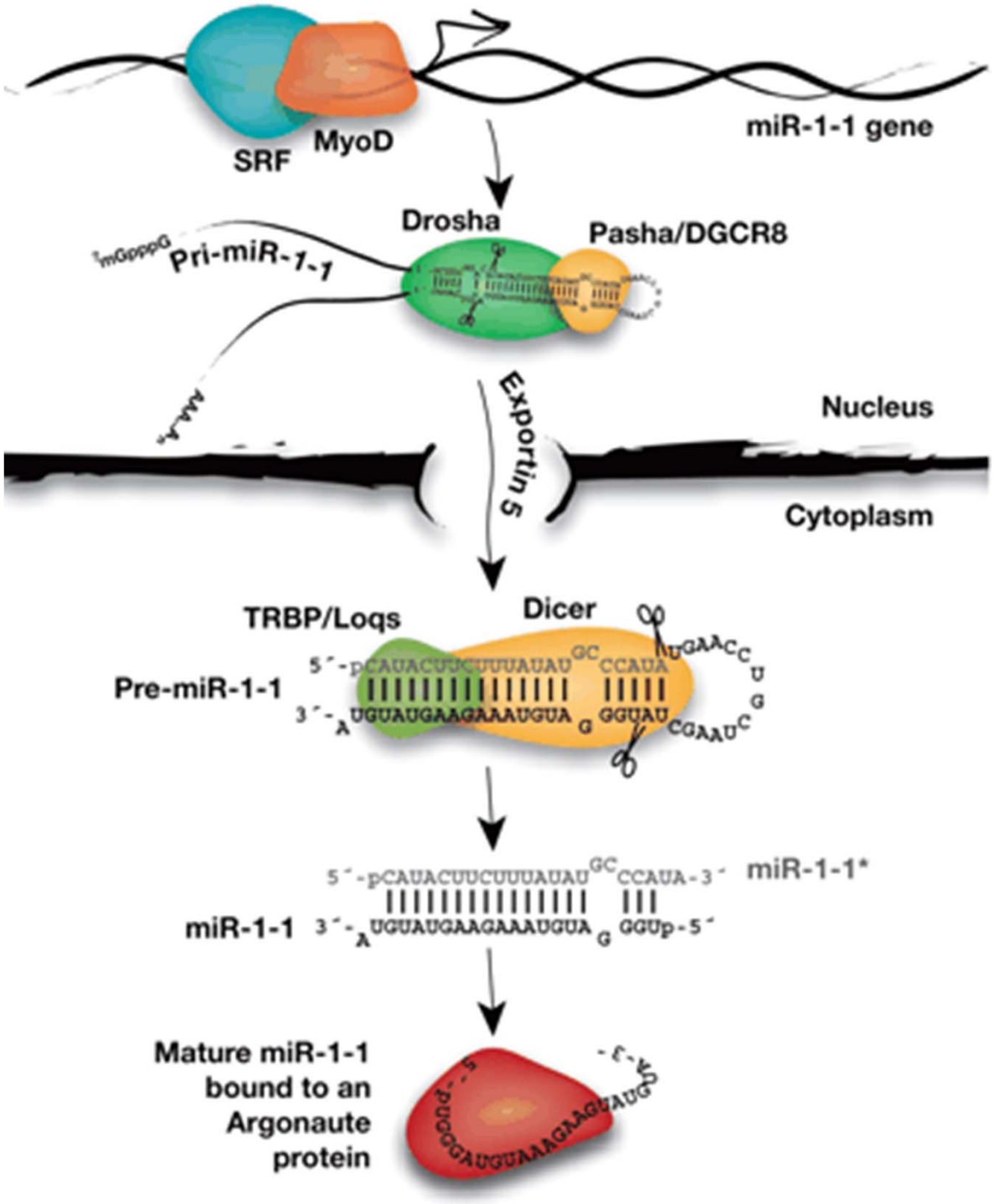
Processing of pri- MicroRNAs

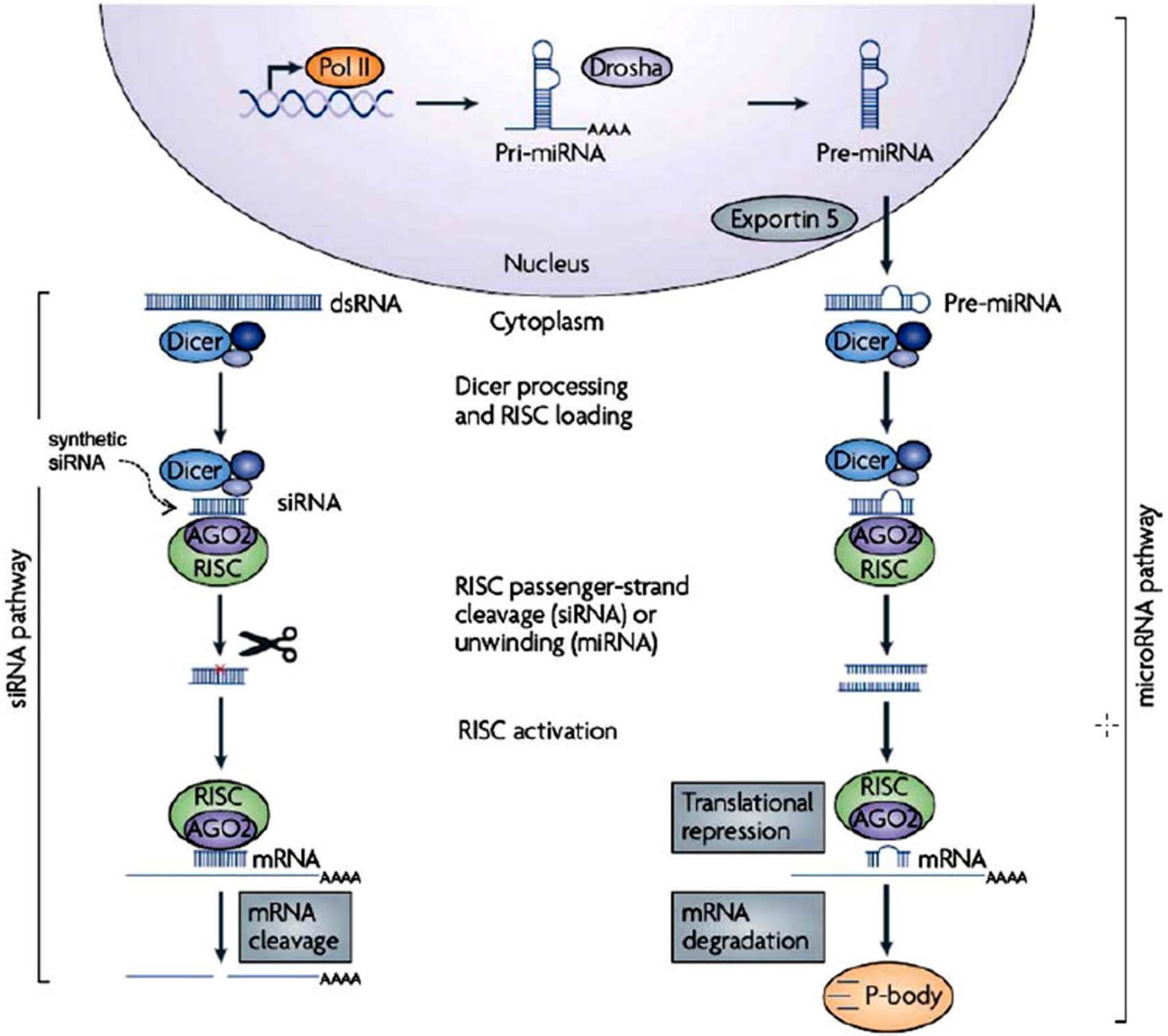


Drosha

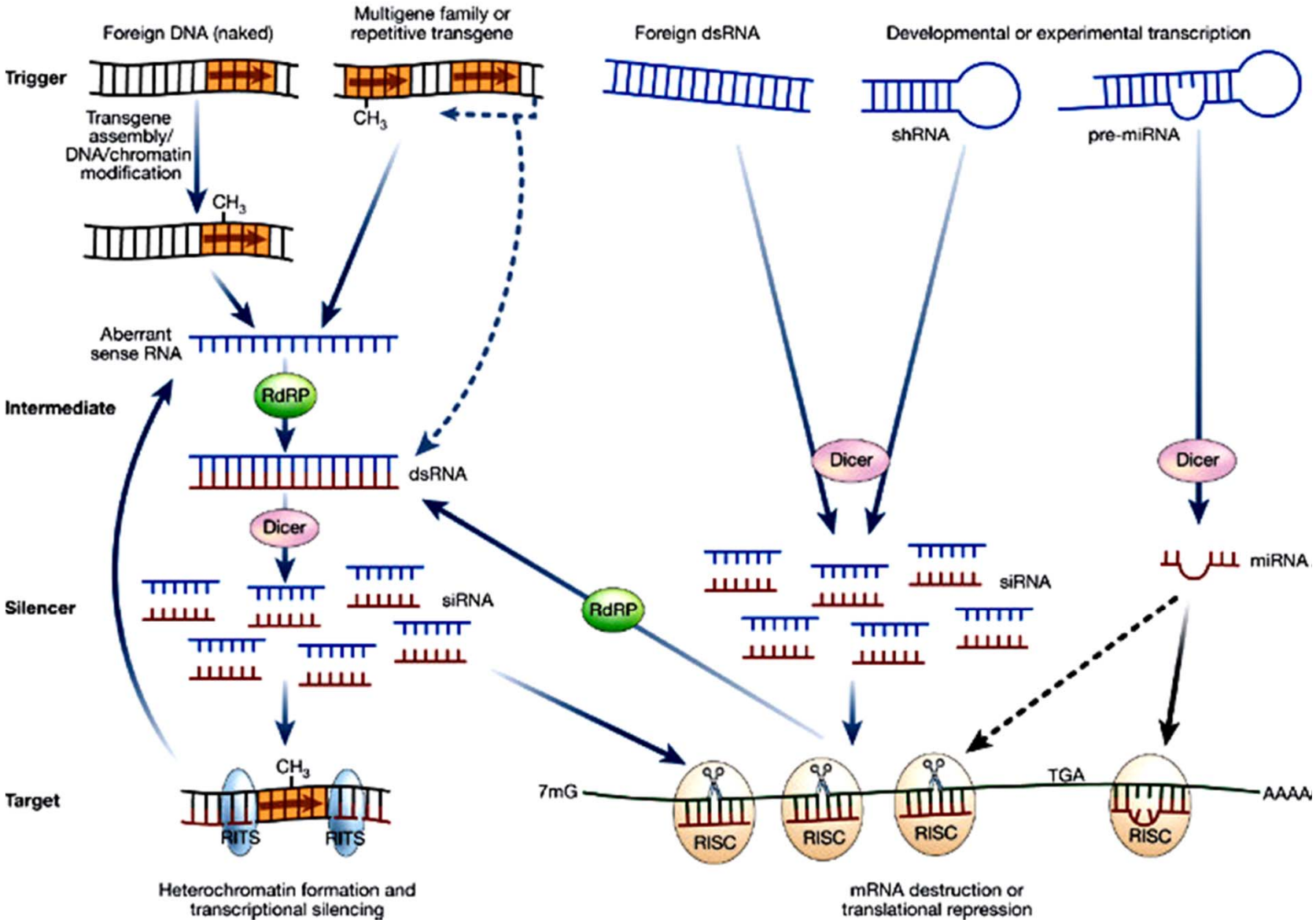
"Pasha" acts as a molecular ruler




SRF
MyoD
miR-1-1 gene
Drosha
Pasha/DGCR8
Pri-miR-1-1
Exportin 5
Nucleus
Cytoplasm
TRBP/Loqs
Dicer
Pre-miR-1-1
miR-1-1
miR-1-1*
Mature miR-1-1
bound to an
Argonaute
protein


Pol II
Drosha
Pri-miRNA
AAAA
Pre-miRNA
Exportin 5
Nucleus
Cytoplasm
dsRNA
Dicer
synthetic siRNA
siRNA
AGO2
RISC
Dicer processing and RISC loading
RISC passenger-strand cleavage (siRNA) or unwinding (miRNA)
RISC activation
mRNA
mRNA cleavage
Translational repression
mRNA degradation
P-body
siRNA pathway
microRNA pathway

Multiple roles of miRNAs



A chronology of some of the major discoveries and events in RNA silencing.

October 2000
Double-stranded RNA shown to direct DNA methylation

January 2001
Dicer shown to make siRNAs

May 2001
RNAi discovered in human cells

July 2001
Dicer found to make microRNAs (miRNAs)

October 2001
miRNAs are established as a large class of gene regulators

July 2002
Plant miRNAs are discovered

July 2002
siRNAs are revealed as triggers of RNAi in mice

September 2002
Small RNAs guide the production of heterochromatin at centromeres

November 2002
miRNAs implicated in cancer

September 2003
It is clear that miRNA maturation begins in the nucleus

November 2003
Dicer shown to be required for mouse embryogenesis, and perhaps for stem cell production

March 2004
Human genome-wide RNAi libraries become available

April 2004
Animal viruses found to encode miRNAs

August 2004
First "investigational new drug" application filed for a therapeutic siRNA

September 2004
Argonaute is revealed as the RNAi endonuclease, "Slicer"

June 2005
miRNAs shown to act as oncogenes

July 2005
Primate-specific miRNAs identified

Biological Role of RNA Interference

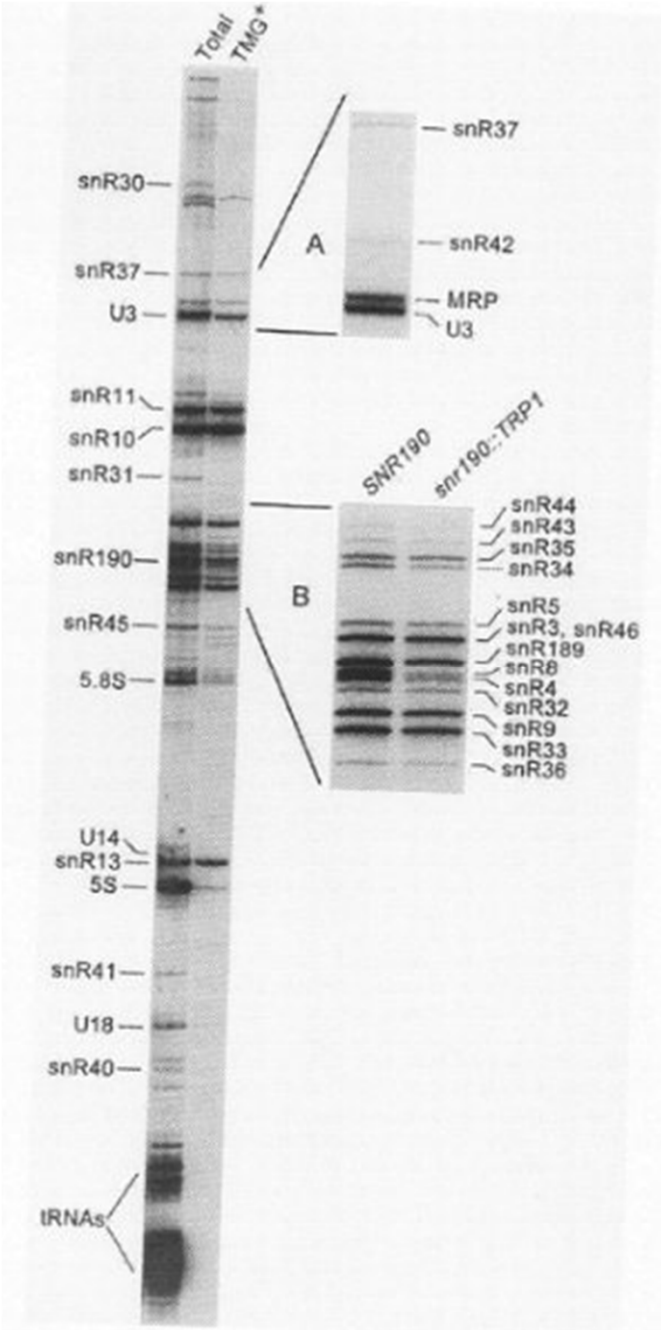
One idea: An immune defense mechanism against nucleic acids.
Evidence: Elimination of RNAi in worms led to increase in transposable elements.

Evolution of RNA Interference

The fact that an ancient eukaryote – *Trypanosoma brucei* – shows RNAi suggests that it is an ancient trait.

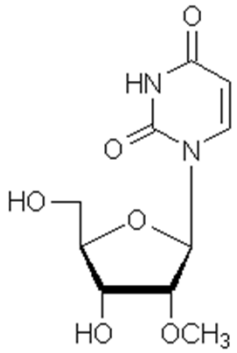
The close relationship of argonaute (slicer) with viral reverse transcriptases, transposases and integrases led to the idea that siRNAs may originally have been primers for the replication of early genomes.

Yeast snoRNAs

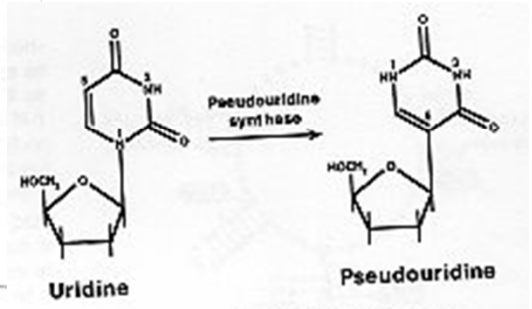


Nucleotide Modifications in rRNAs

2’ O-methyl uridine



Pseudouridine



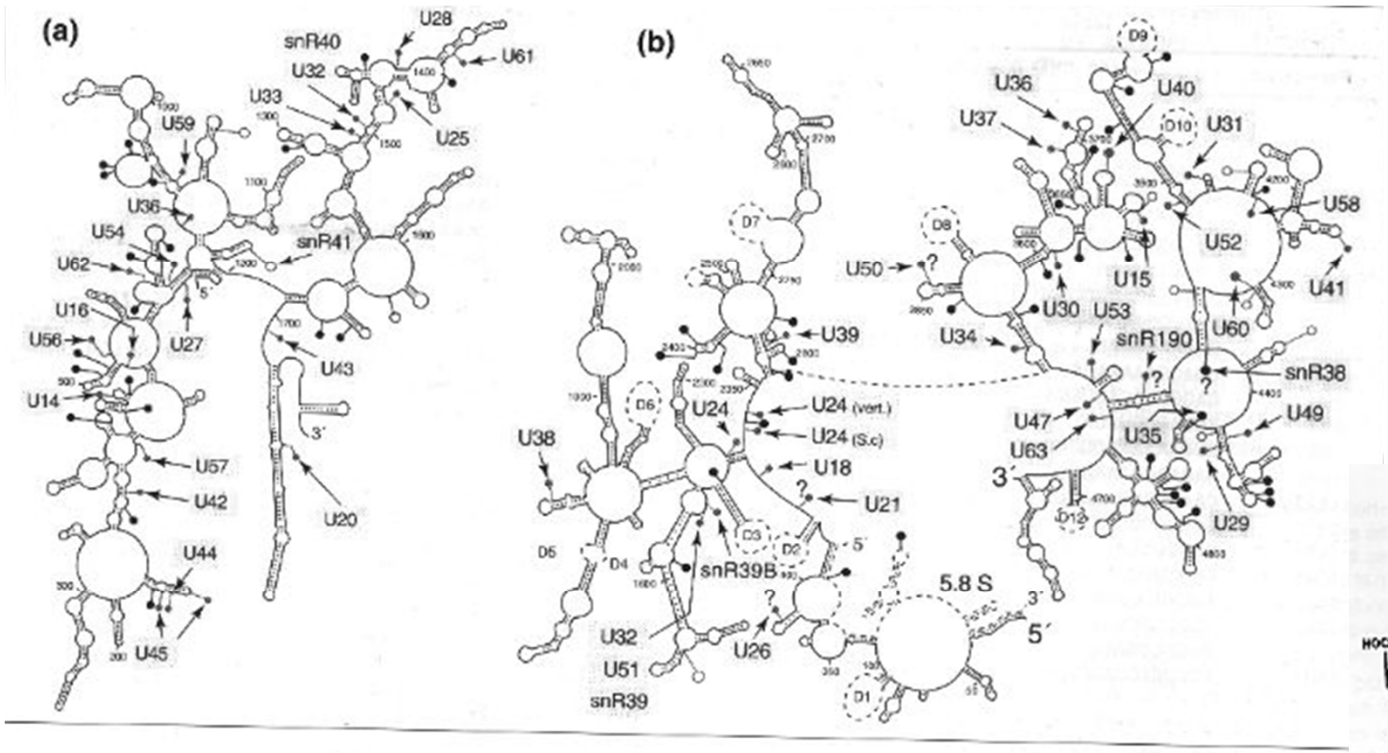


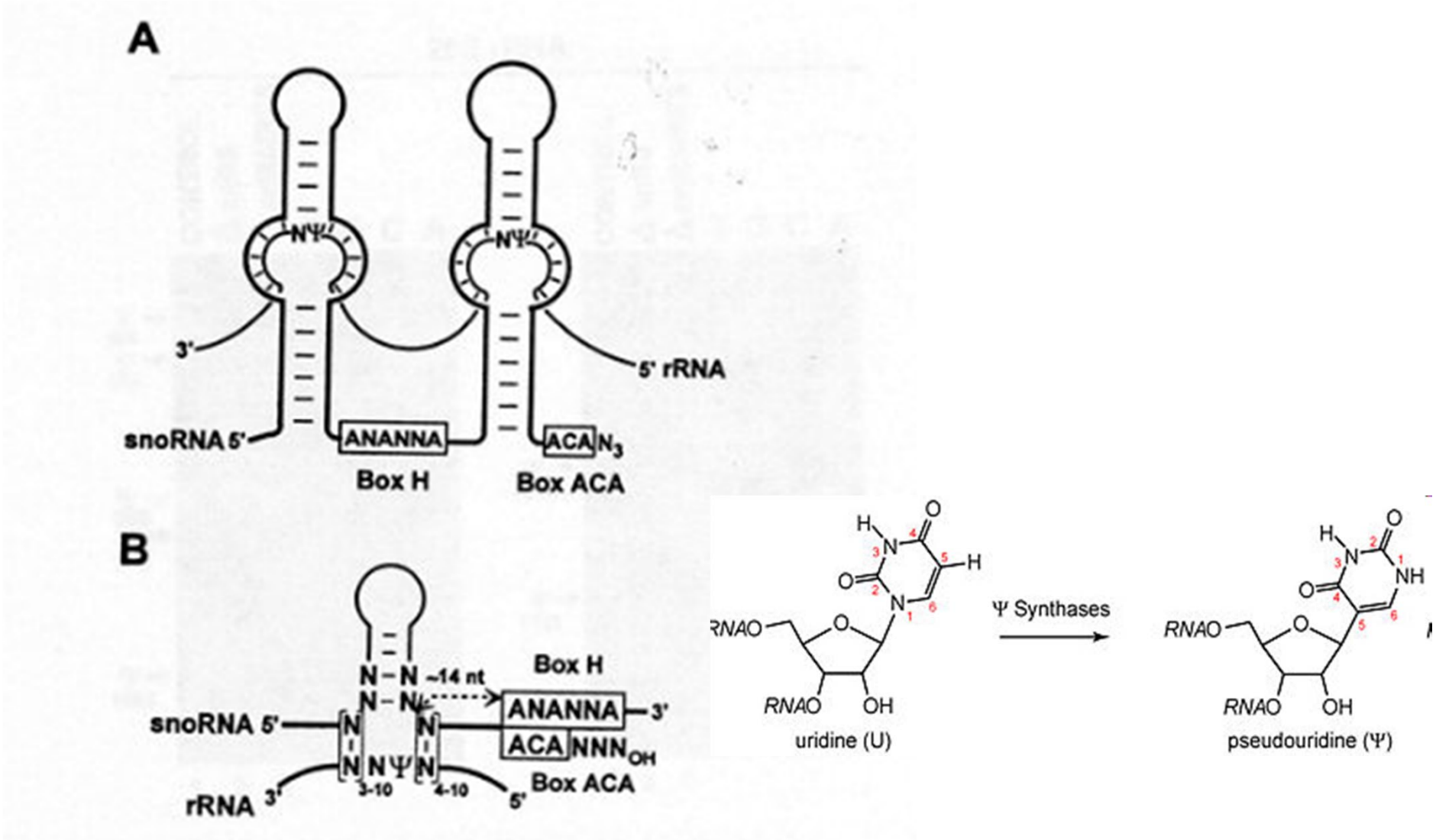
Figure 2

Location of ribose-methylation sites in the secondary structure of mature rRNAs. All ribose-methylation sites characterized in human rRNAs are indicated (filled circles) and presumptive sites are denoted by question marks (see Table I). The few sites of ribose methylation present in yeast, but absent in vertebrates, are also shown (open circles). Only the universally conserved core structure of rRNAs is represented (t… evolutionarily variable D domains are depicted by dots).

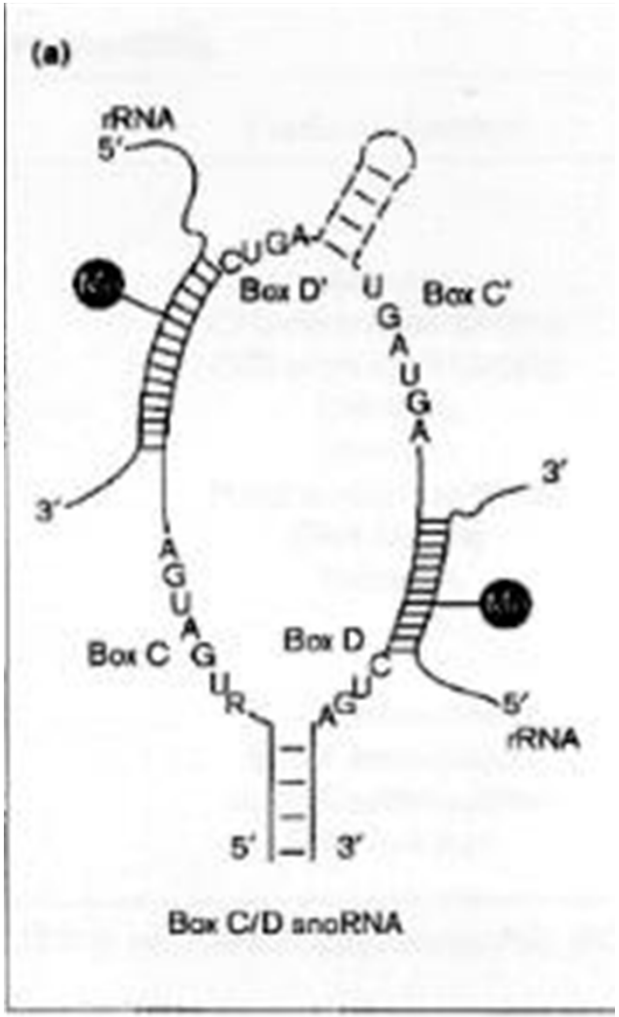
Tamas Kiss
INSERM

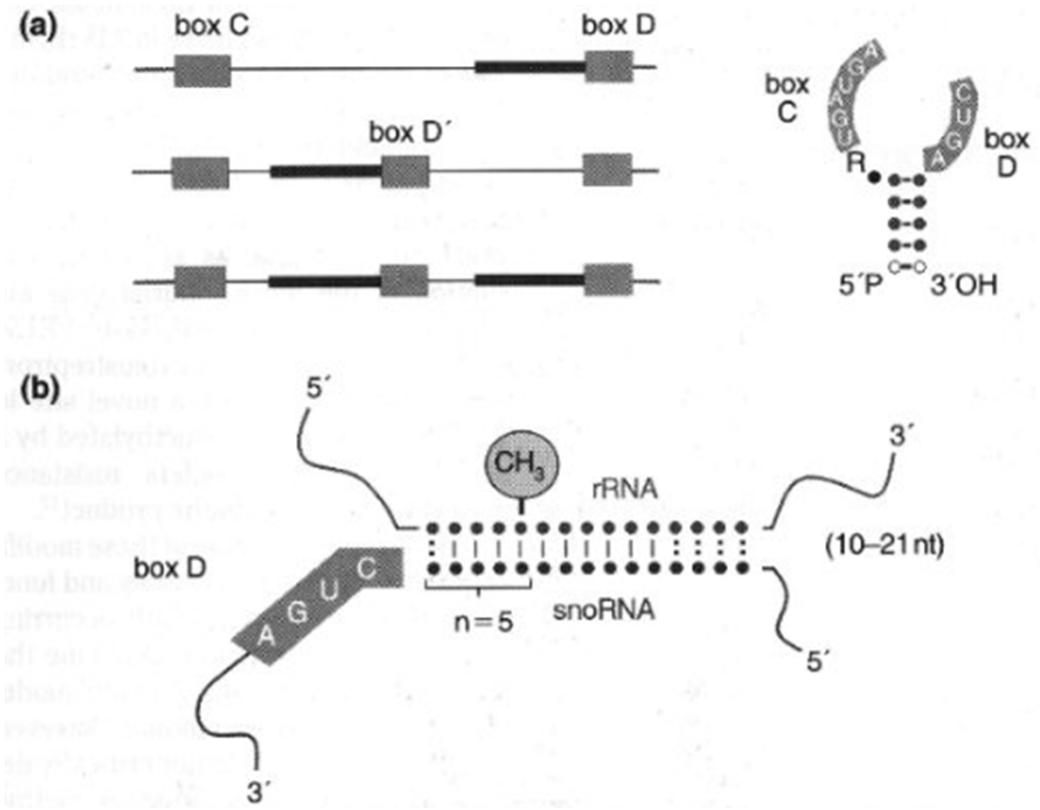
Skip Fournier
U of Mass.

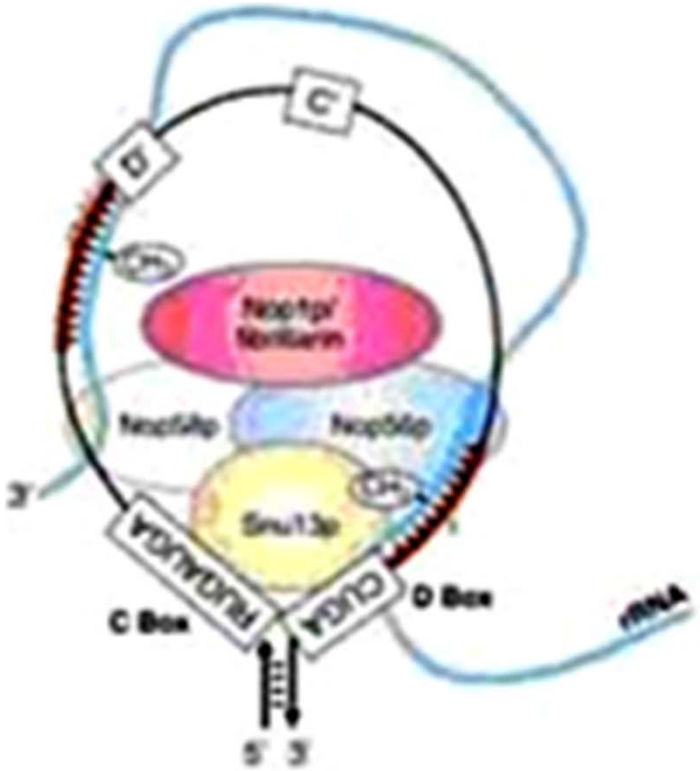
Box H/ACA snoRNAs guide pseudouridylation of specific residues



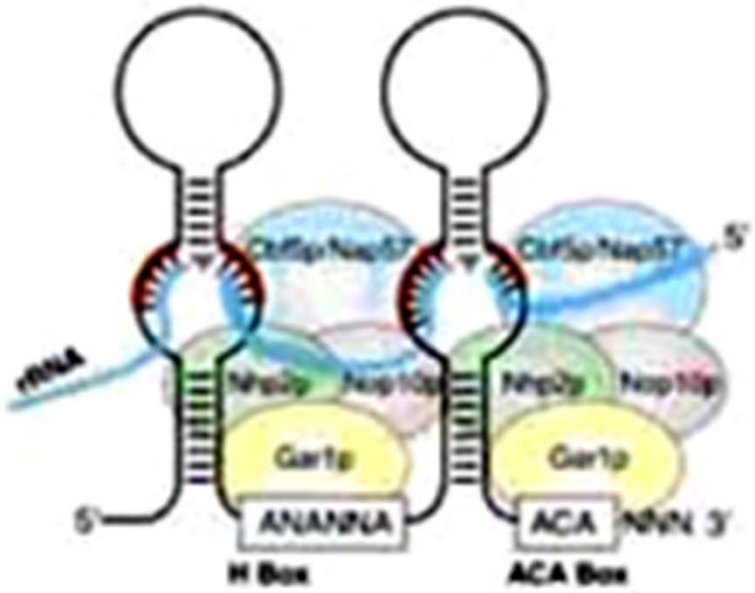
Box C/D snoRNAs guide specific methylations



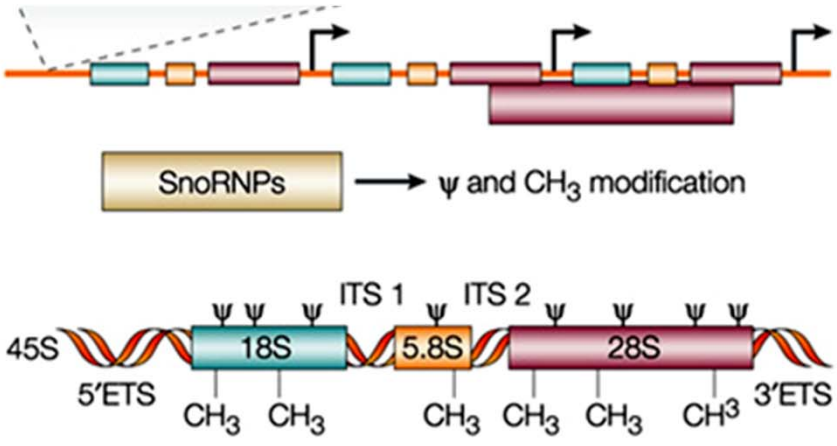




C/D snoRNP



H/ACA snoRNP



A to I (=G) Editing in Mammals

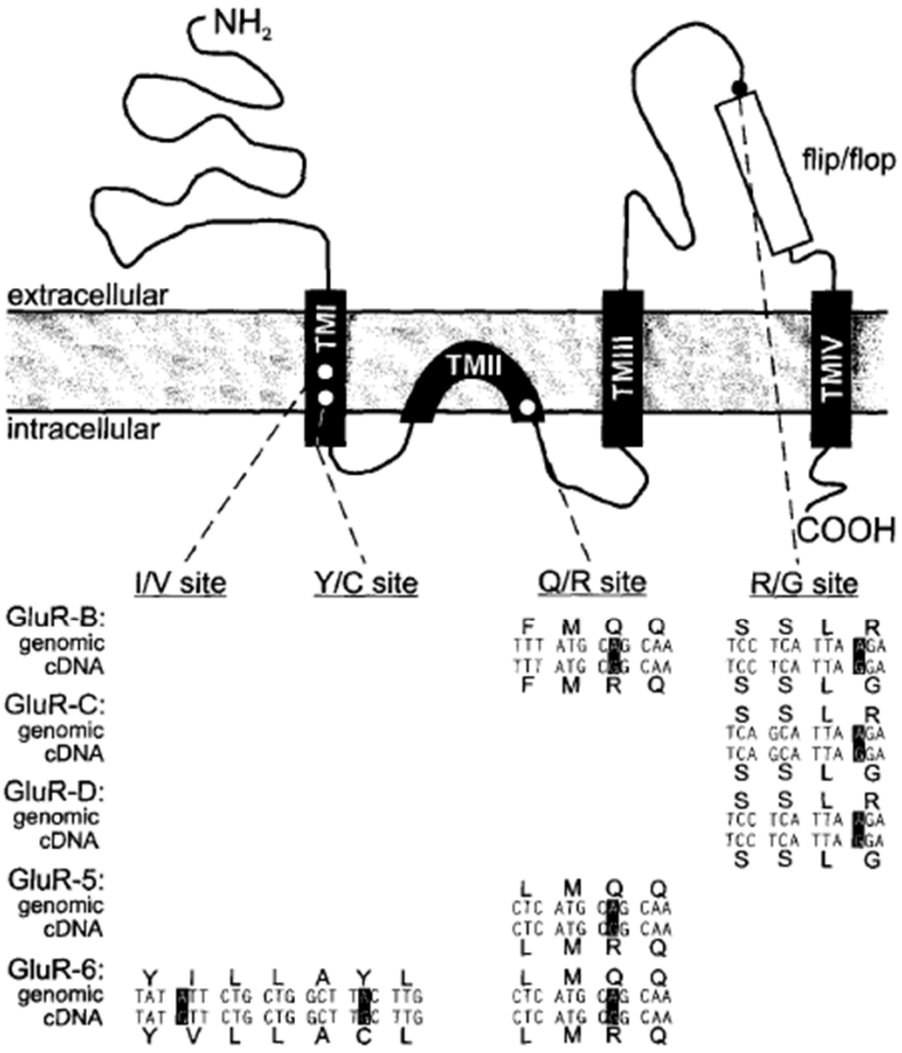


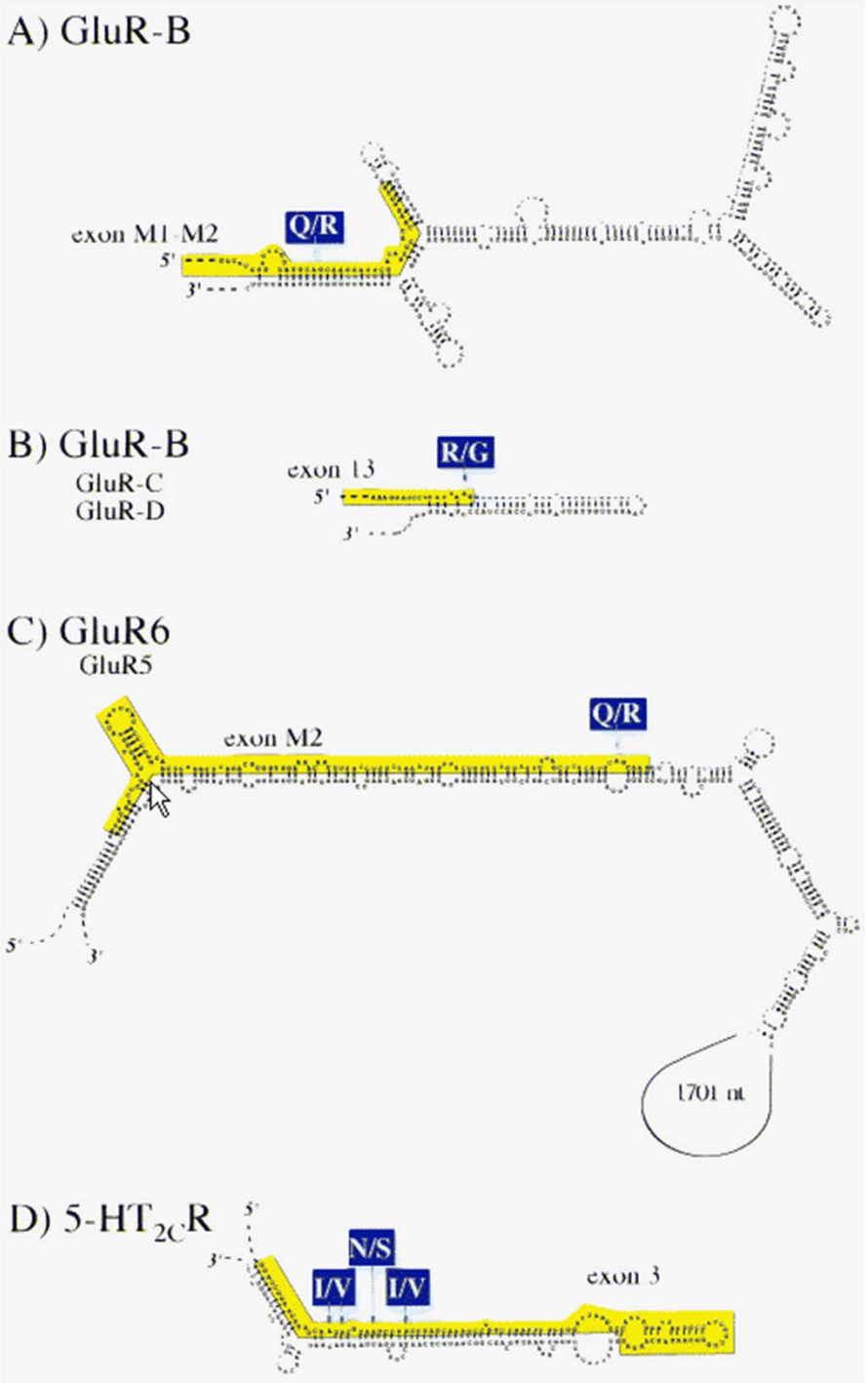
Figure 3 A summary of the editing events for mRNAs encoding non-NMDA glutamate receptor subunits. A schematic representation of the proposed topology for GluR-A is presented indicating the relative positions of editing sites within non-NMDA receptor subunits; the toplogy of subunits other than GluR-A (Hollman et al 1994) have not yet been determined. The genomic, cDNA, and amino acid sequences surrounding the editing sites are presented and modified nucleosides are presented in inverse lettering.

ADAR – Adenosine deaminase acting on RNA

Roles

Regulation of protein function
Alteration of splice sites
Interactions with noncoding RNAs
Effects on RNAi
Effects on miRNA prosessing and target selection

Needs short imperfect complementary sequences (9-15 b) in an adjacent intron



Potential therapeutic uses of RNAi

Major problem is delivery system. Possible solutions involve synthetic siRNAs capable of penetrating cells and using viral vectors to express siRNA precursors.

A problem with mammalian cells is the immune response induced by long double stranded RNAs. This was solved by using siRNAs or hairpin RNAs.

Potential for off-target effects. Usually the in vivo potency of the siRNA "drug" is separated by several log orders from the off-target effects.

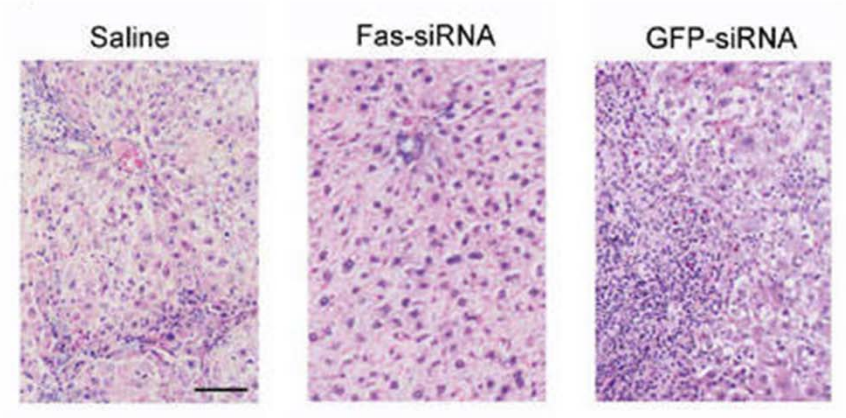
Mouse models have shown good results with several diseases including autoimmune hepatitis, pulmonary infections (influenza and respiratory syncytial virus).

HIV-1 replication was prevented in vitro.

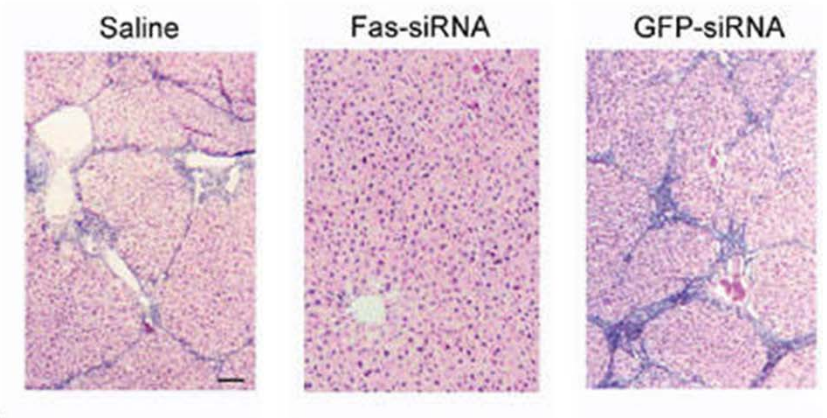
Tumor growth has been suppressed by silencing a kinase.

Prevention of fulminant hepatitis in mouse models

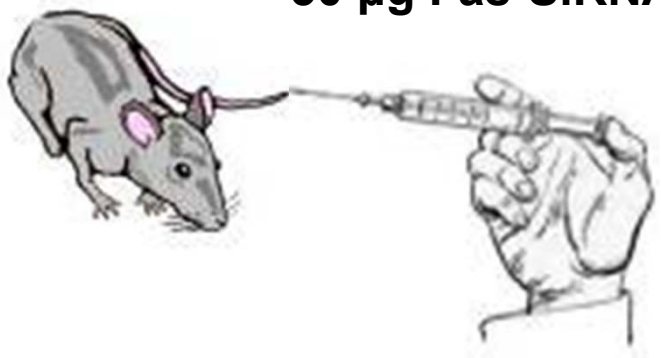
Mice treated with Fas siRNA were challenged after 1 day with ConA.



Mice treated with Fas siRNA 1 day after injection of 6 weekly injections of ConA



50 µg Fas-SiRNA duplex in 1 ml saline



Delivery methods

1. Conjugation of cholesterol to Si RNA

2. Liposomal nanoparticle-mediated delivery (LNP) of siRNA to liver

3. Multicomponent complexes: SiRNA conjugated with PEG plus a liver-targeting ligand (NAG)

Clinical RNAi Pipeline

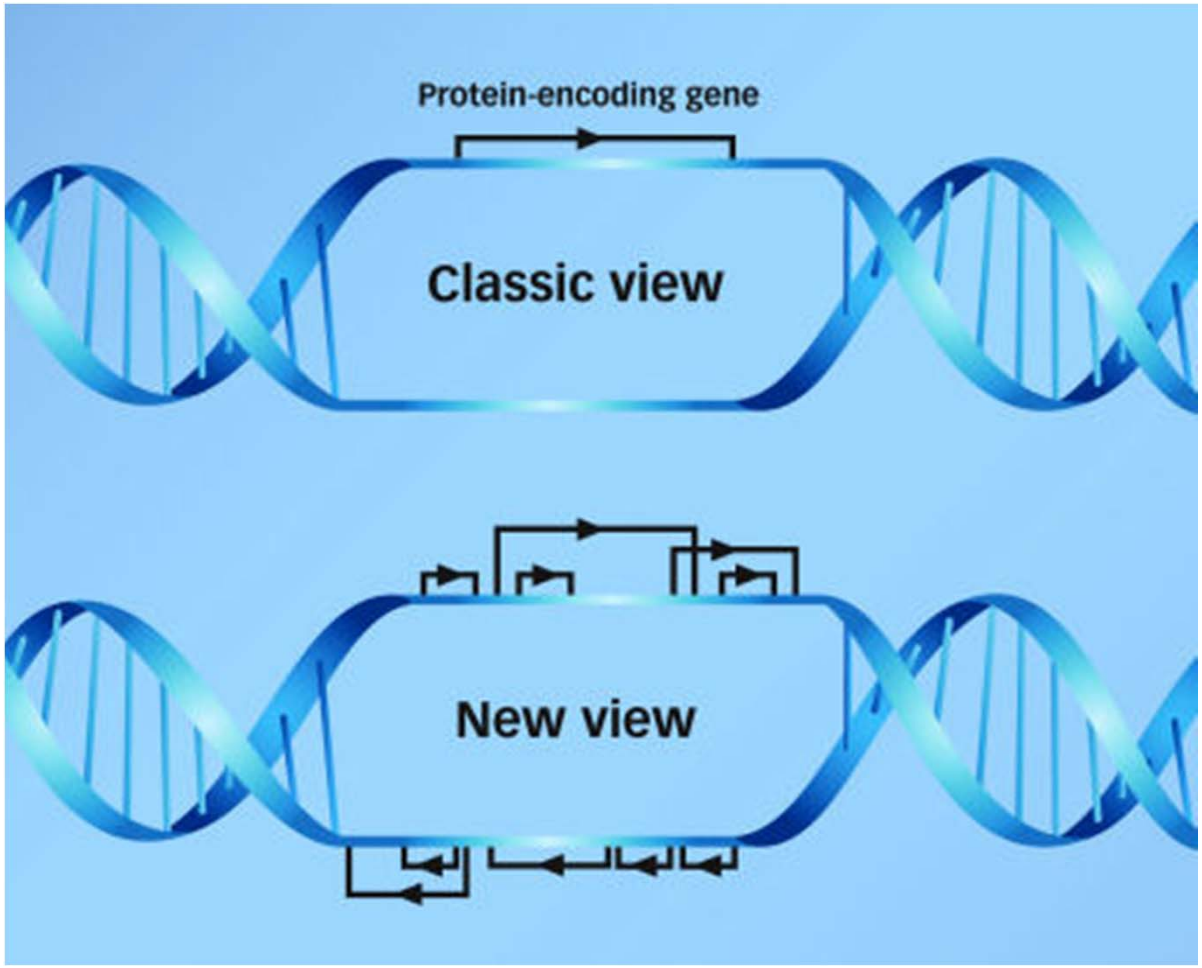
Most advanced program: ALN-SRV capsid.

The global RNA interference pipeline*

Sponsor	Program (clinical stage)	Status	Target	Indication	Number enrolled
Alnylam/Cubist/Kyowa Kirin	ALN-RSV (phase IIb)	Ongoing	RSV nucleocapsid	Adult RSV infection	354
Pfizer/Quark	PF-04523655 (phase II)	Ongoing	RTP801	(1) AMD, diabetic macular edema	244†
Quark	QPI 1002 (phase II)	Ongoing	p53	(1) Acute kidney injury, delayed graft function	56†
Zabecor	Excellair (phase II)	Ongoing	Syk kinase	Asthma	?
Alnylam	ALN-VSP (phase I)	Ongoing	VEGF, KSP	Primary and secondary liver cancer	55†
Calando	CALAA-01 (phase I)	Ongoing	RRM2	Cancer	36†
Silence	Atu-027 (phase I)	Ongoing	PKN3	Cancer (GI, lung other)	33†
Sylentis	SYL040012 (phase I)	Ongoing	β2 adrenergic receptor	Glaucoma	?
Alnylam	ALN-TTR (phase I)	Ongoing	TTR	TTR amyloidosis	Enrollment begins H
Opko	Bevasiranib (phase III)	Terminated	VEGF-A	AMD	522
Allergan/SIRNA	AGN211745 (phase II)	Terminated	VEGFRI	AMD	164
Tekmira	ApoB SNALP (phase I)	Completed	ApoB	Hypercholesterolemia	23
Transderm	TD101 (phase I completed)	Completed	Mutant K6a	Pachyonychia congenita	1
Univ. Duisberg-Essen‡	Bcr-abl (phase I completed)	Unknown	Bcr-abl oncogene	CML	1

AMD = age-related macular degeneration; CML = chronic myeloid leukemia; GI = gastrointestinal; KSP = kinesin spindle protein; PKN = protein kinase N3; RRM2 = ribonucle
VEGF = vascular endothelial growth factor; VEGFRI = vascular endothelial growth factor receptor I.

Discovery of microRNAs has led to a new concept of the genome



WMAP – Microwave Anisotropy Probe