RNA Interference

Diane Carrera
MacMillan Group Meeting
January 21, 2009

Lead References:

Nobel Lectures: Fire, A. Z. *Angew. Chem., Int. Ed.* **2007**, *46*, 6966
Mello, C. C. *Angew. Chem., Int. Ed.* **2007**, *46*, 6985
Nature Insight: *Nature* **2004**, *431*, 338

Overview

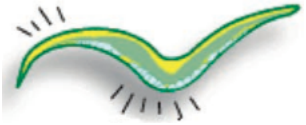
- Introduction to RNAi
  - What is it?
  - Why should we care about RNAi?
  - Biochem Basics
- Discovery of RNAi
- Elucidation of RNAi Mechanism
  - Identifying RISC components
  - Other RNAi triggers
- RNAi and Chromatin Modification
- Possible Therapeutic Applications

What is RNA Interference?

- RNA interference(RNAi) is the knockdown of gene expression by small RNA fragments

c. elegans

RNA trigger



normal *unc-22 expression*

supressed *unc-22 expression*

- 1998 – Mello and Fire publish a seminal *Nature* paper elucidating the trigger for the RNAi process
- 2006 – Mello and Fire awarded Nobel Prize in Physiology and Medicine

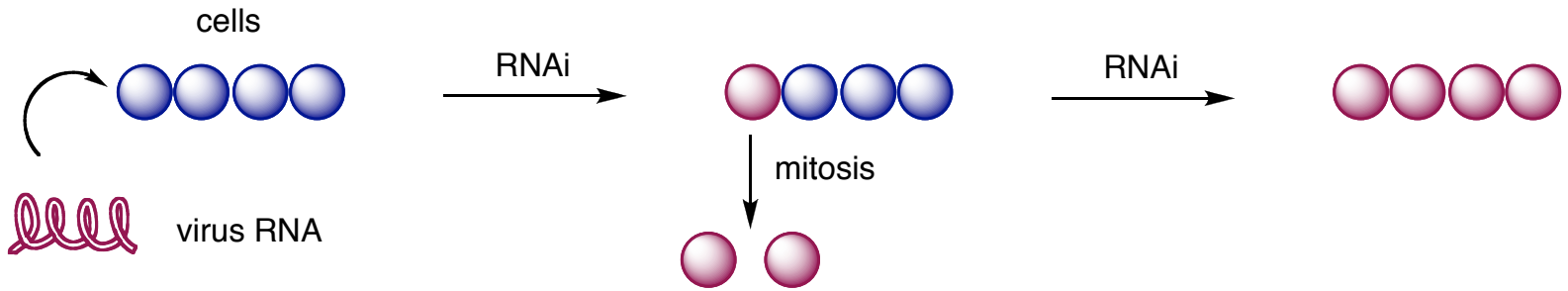
Andrew Fire
Stanford

Craig Mello
UMass

Why is RNAi Important?

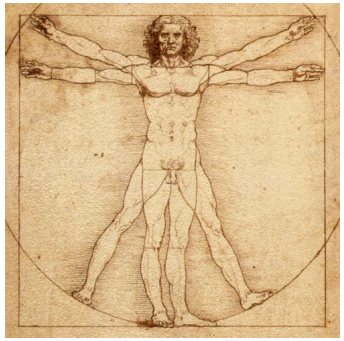
■ Provides valuable insight into evolution

- found in all eukaryotes except some fungi ⇒ ancient biochemical mechanism
- the original form of cellular immunity, signal can also spread horizontally and vertically



- indicates that gene expression is the key to evolving complex organisms

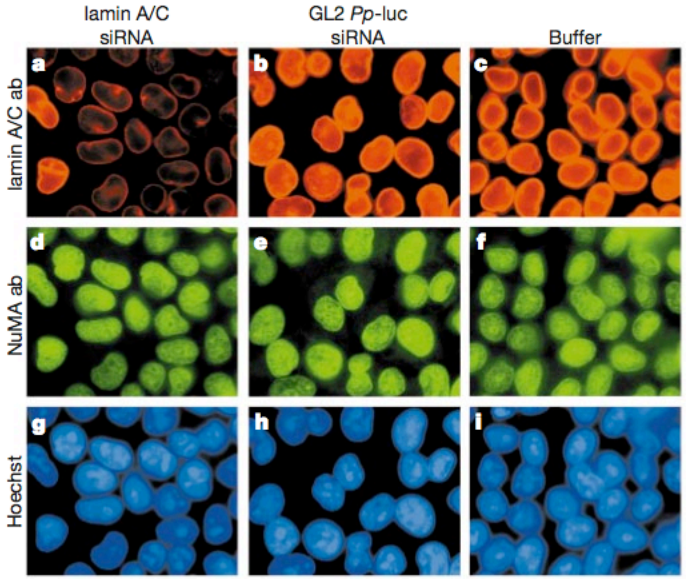
	Human	Nematode
protein encoding genes	21×10^3	19×10^3
miRNAs	677	154



Why is RNAi Important?

■ Invaluable tool for functional genomics

- Knockdowns are easier than knockouts ⇒ Saves time and money
- Allows for study of function with a variety of controls: positive, negative, rescue



- Many public databases (UPenn, Cold Spring Harbor, MIT) now exist for the design of interfering RNAs to target specific genes

■ Medicine and Biotech

Science "Breakthrough of the Year" 2002

Fortune "Billion Dollar Breakthrough"

Alnylam® PHARMACEUTICALS

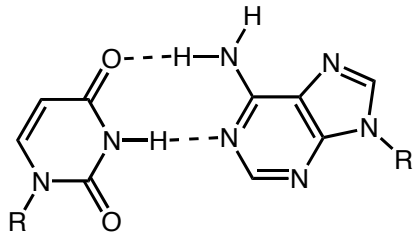
Sirna Therapeutics™

Quark

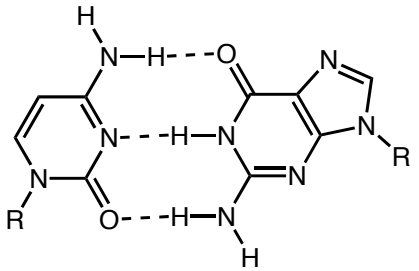
Calando PHARMACEUTICALS

- DNA and RNA are information containing biopolymers

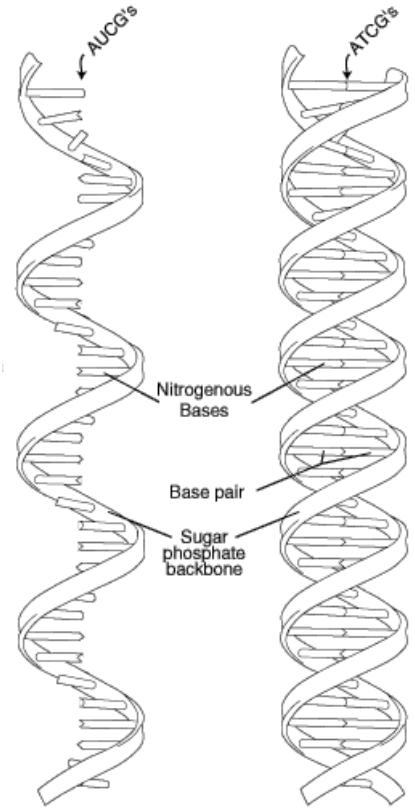
Uracil (U) Adenine(A)



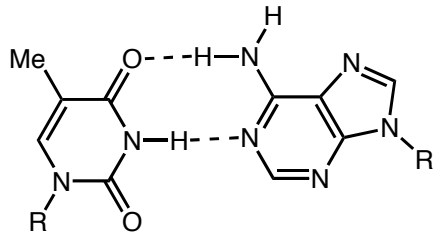
pyrimidines purines



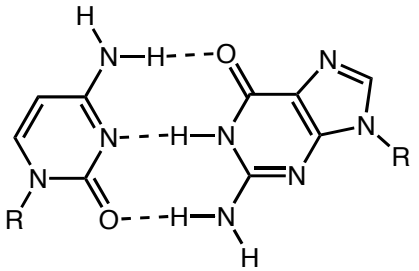
Cytosine (C) Guanine (G)



Thymine (T) Adnenine(A)

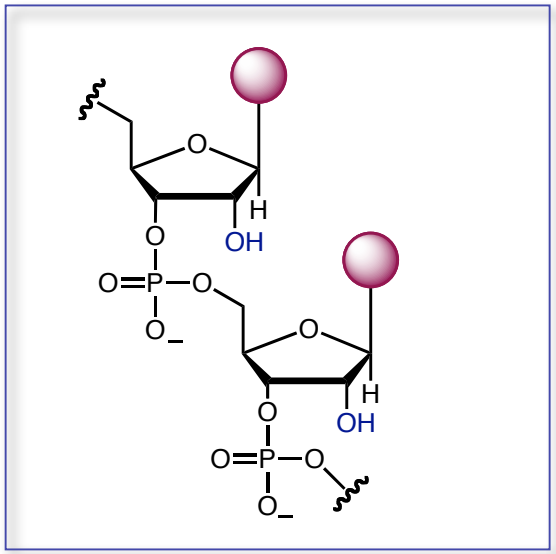


pyrimidines purines



Cytosine (C) Guanine (G)

■ DNA and RNA are information containing biopolymers

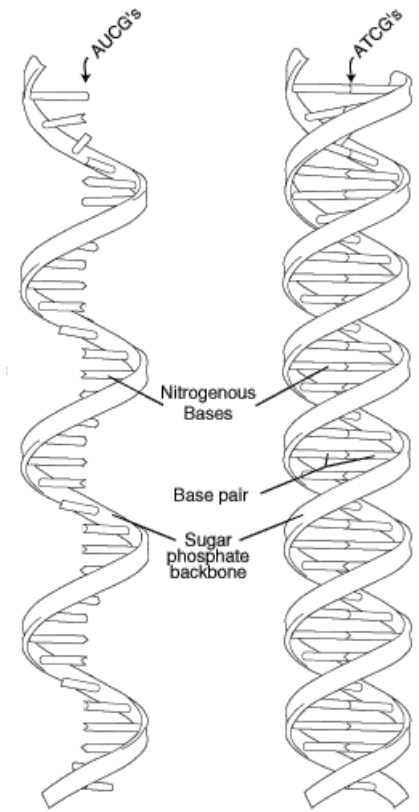


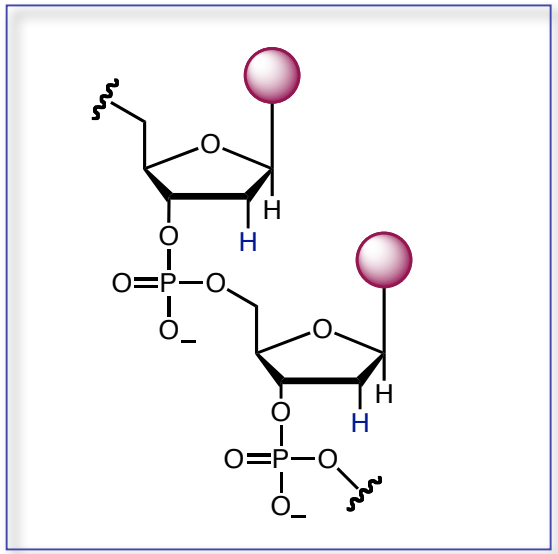
backbone: ribose

prone to hydrolysis

⇩

short term
information storage





backbone: deoxyribose

stable to hydrolysis

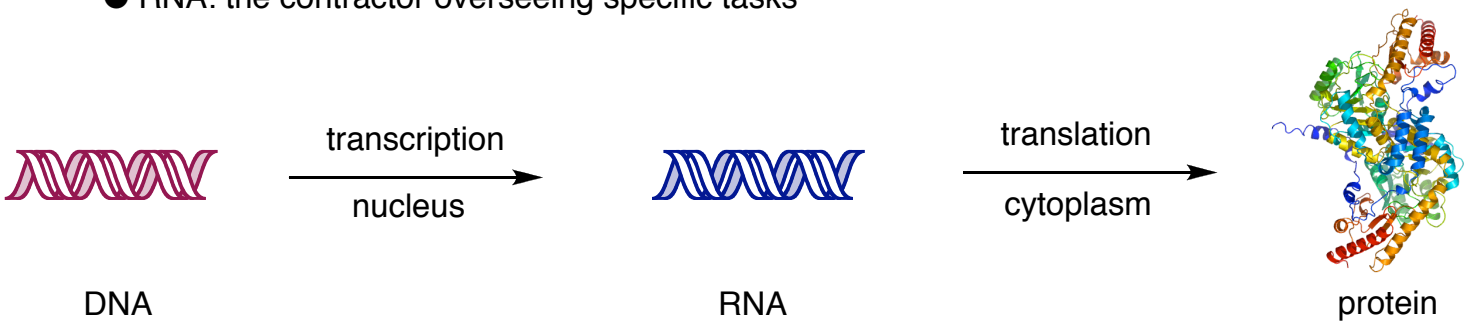
⇩

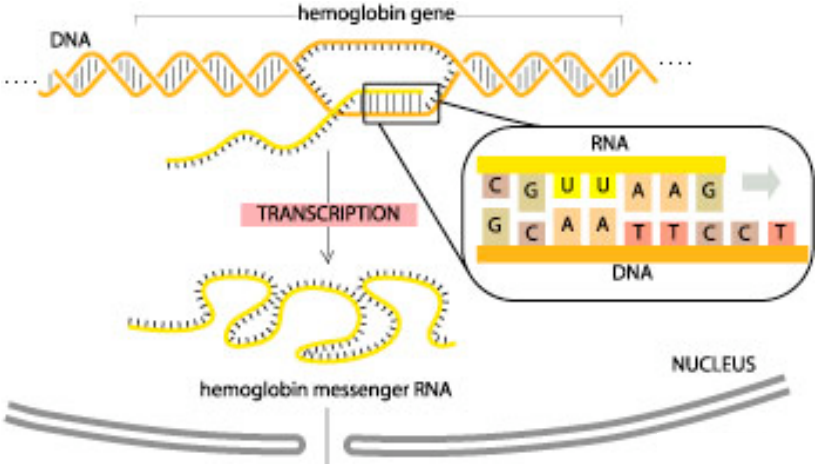
long term
information storage

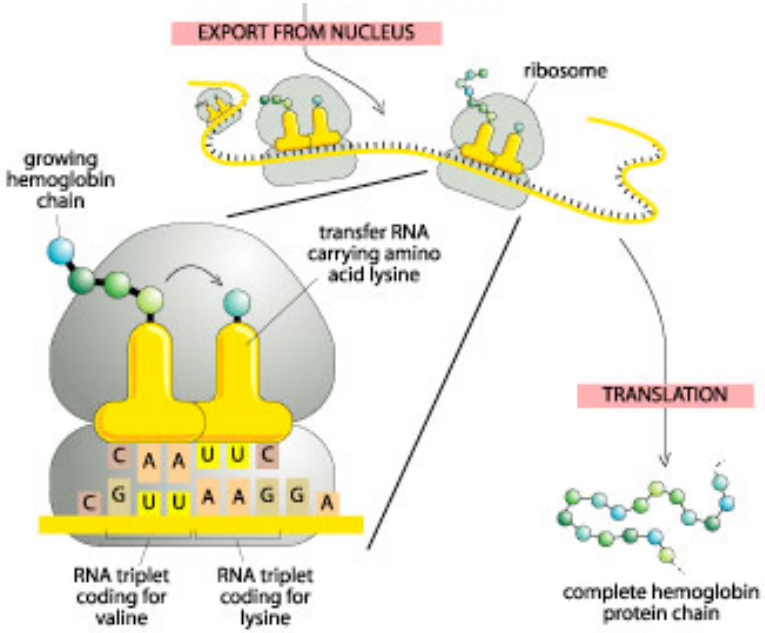
Basics of Gene Expression

■ Gene Expression is comprised of Transcription & Translation with distinct roles for DNA & RNA

- DNA: the architect with the master set of blueprints
- RNA: the contractor overseeing specific tasks



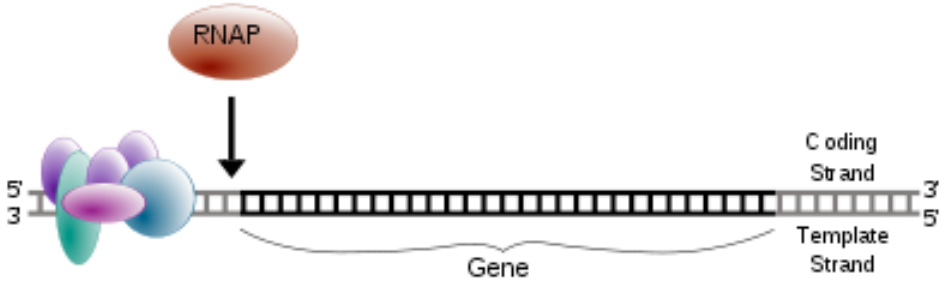




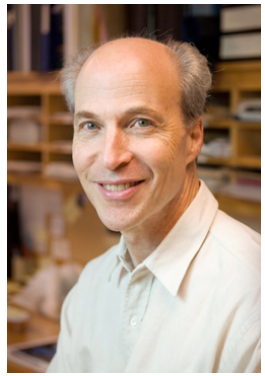
Transcription

■ Transcription is the transfer of information from DNA to messenger RNA (mRNA) and has 3 distinct stages

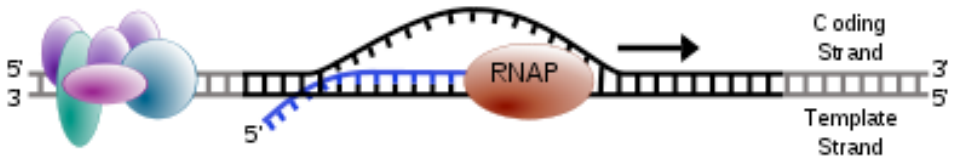
Initiation: RNA polymerase enzyme (RNAp) inserts into DNA double helix



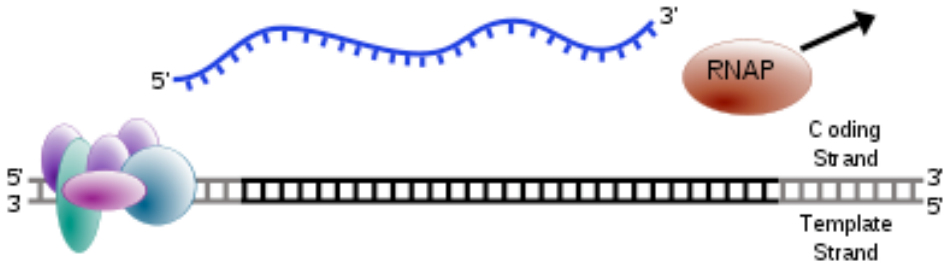
RNAp: 2006 Nobel Prize in Chemistry, Roger Kornberg



Elongation: RNAp builds a strand of mRNA from the template DNA strand



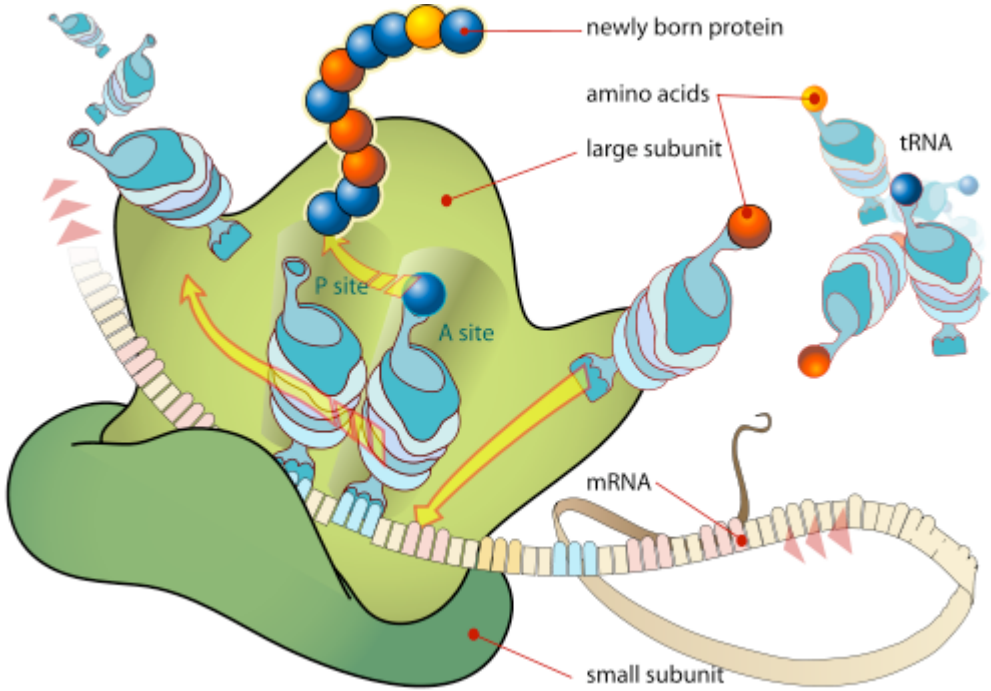
Termination: mRNA synthesis is complete, RNAp and mRNA are released into solution and double helix reforms

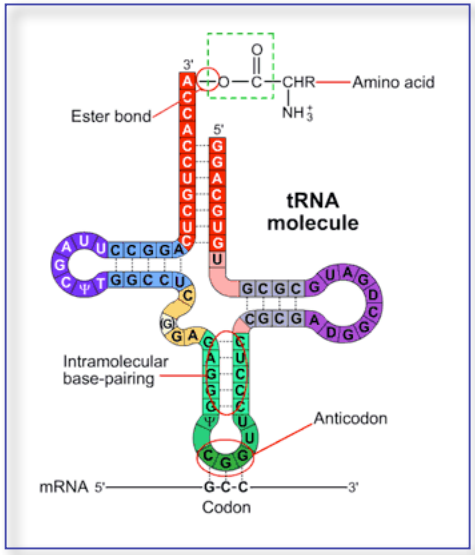


Translation

■ Translation is the synthesis of proteins in 4 distinct stages using the mRNA template

4. Termination: peptide released upon reaching stop codon (UAA, UAG, UGA)





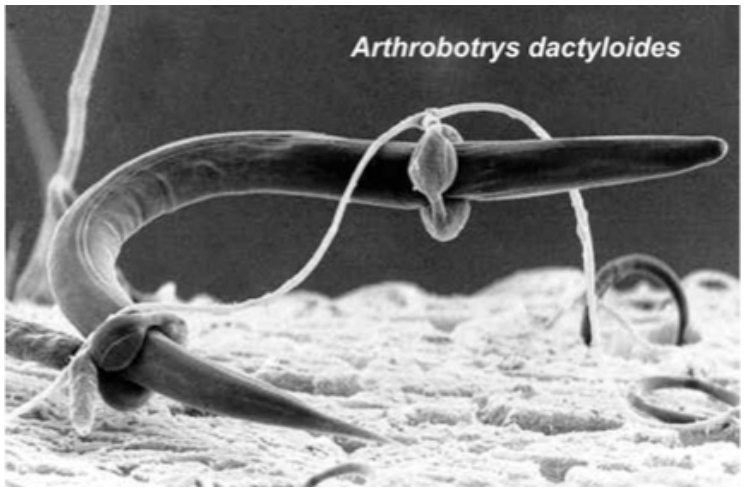
1. Activation: amino acids bind to transfer RNA (tRNA)

3. Elongation: peptide chain grows as amino acids are brought in by tRNAs with anticodons corresponding to mRNA codons

2. Initiation: small subunit of ribosome binds 5' end of mRNA

The Biochemist's Best Friend

■ *Caenorhabditis elegans* (*C. elegans*) is an ideal system for studying gene expression



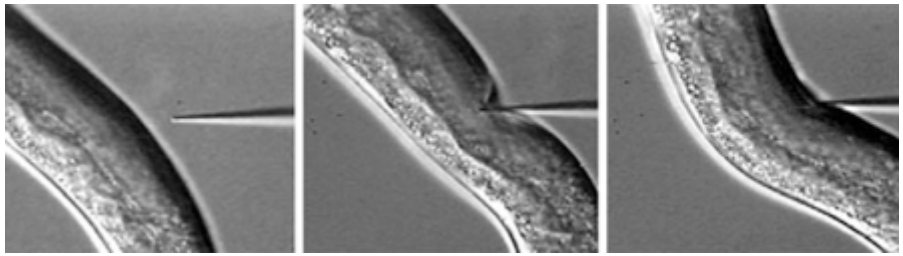
- nematode roundworm
- 1mm in length
- lives in soil

⇨ the developmental timeline of every cell has been mapped

phenotypic response to gene expession is well defined

unc-22 repression	→	twitching
par-1 repression	→	symmetrical cell division

■ Microinjection technique allows for the direct introduction of macromolecules into the animal

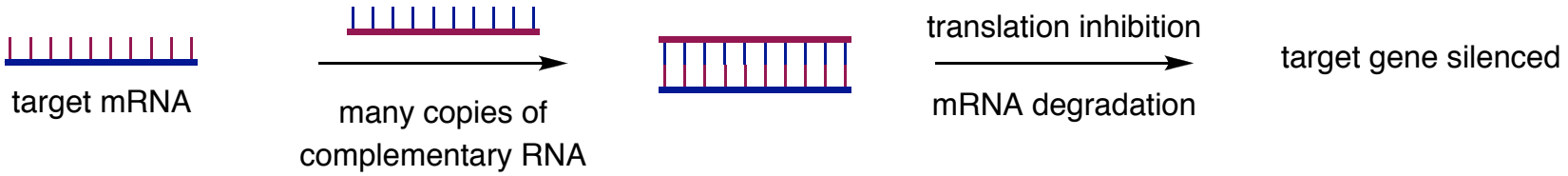


- ability to affect large populations
- RNA, DNA and proteins can all be injected

Unexplained Results with Antisense

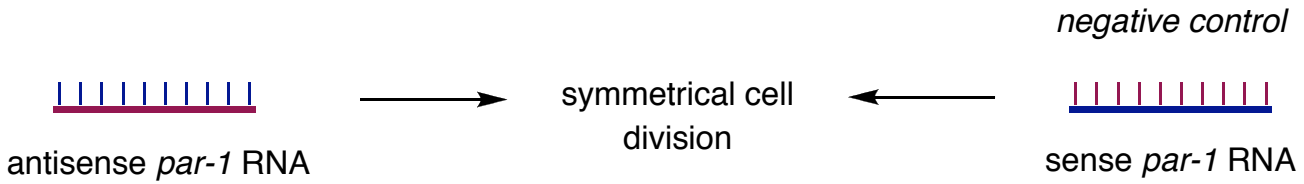
- In the 1990's, antisense looks like the next big thing

antisense mediated silencing



- Some experiments are giving unexpected results

Guo & Kemphues discover that both sense and antisense strands mediate silencing



Cell **1995**, *81*, 611

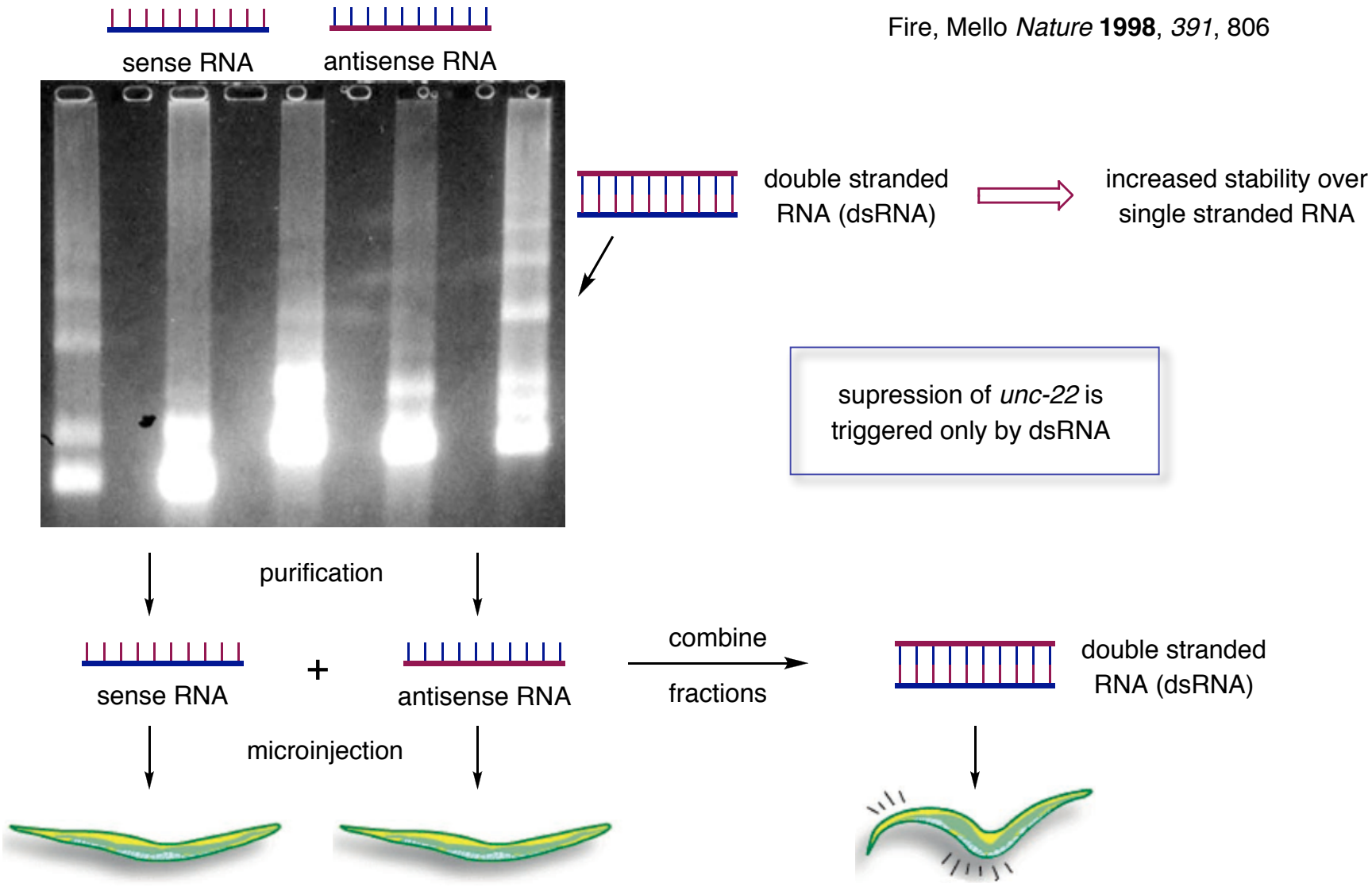
antisense experiments with *unc-22* give same result

supression effects persist and are passed on through germline

Fire *Development* **1991**, *113*, 503
Kuwabara *Genetics* **1996**, *144*, 597

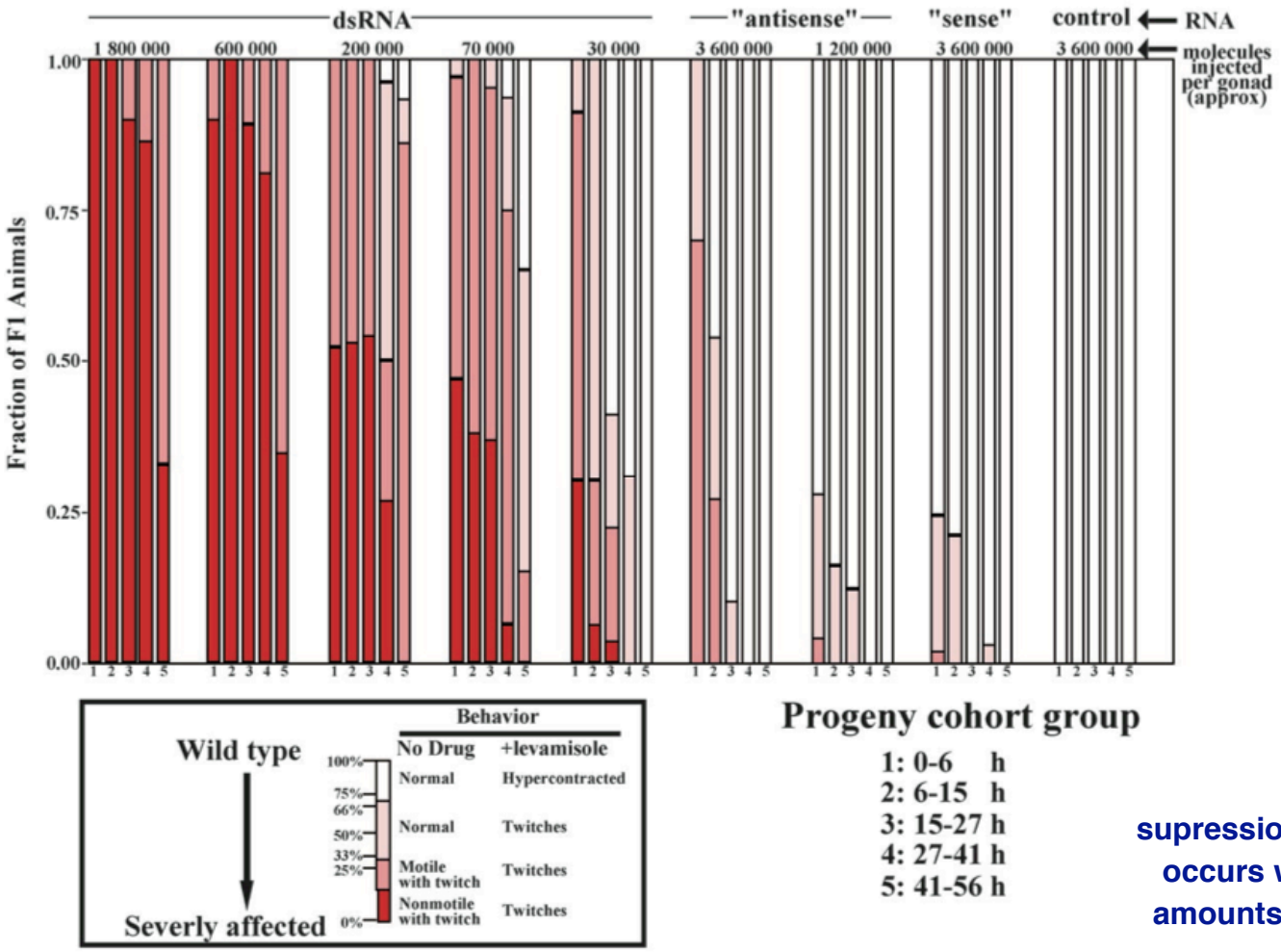
Finding the Trigger

- Electrophoresis shows RNA preparations are contaminated with dsRNA



A Very Potent Trigger

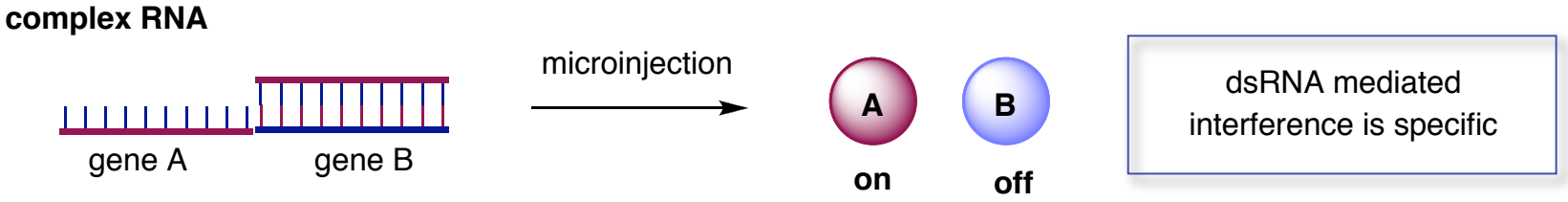
- Dilution studies show supression is obtained with as little as a few molecules per cell



supression of *unc-22* occurs with small amounts of dsRNA

Vital Control Exeriments

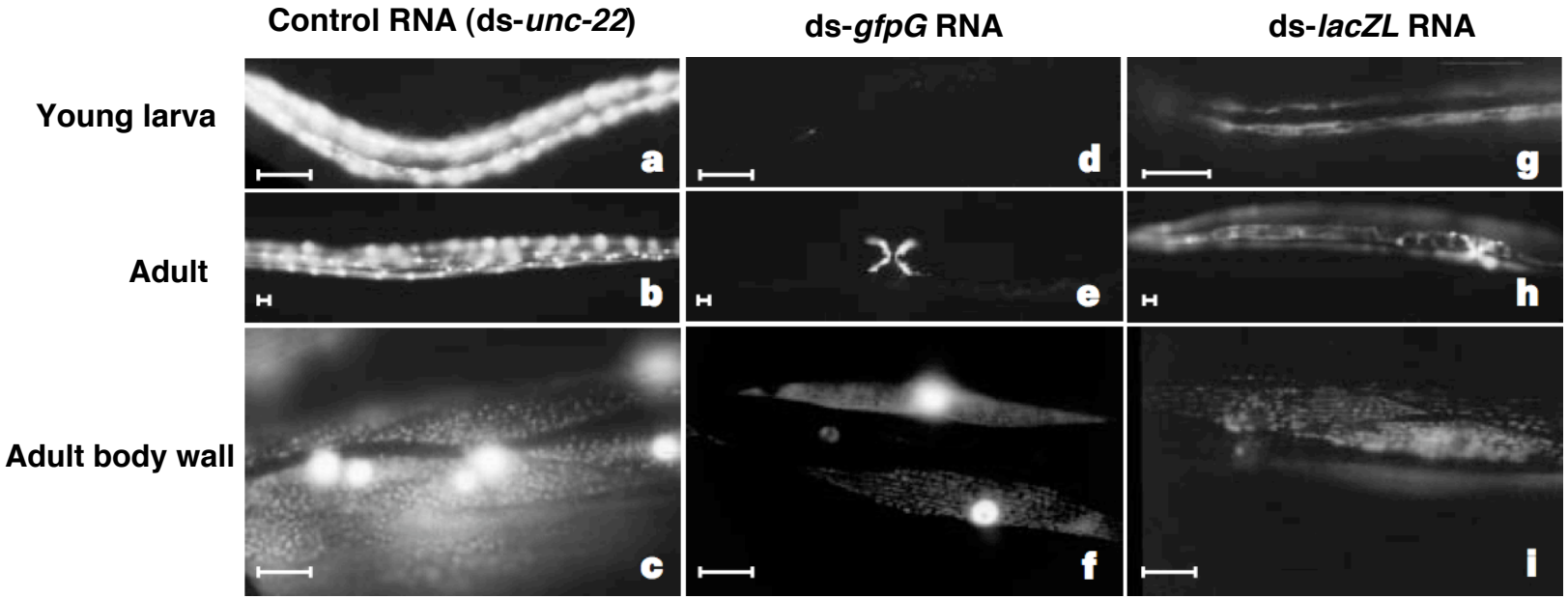
■ Control experiments reveal dsRNA interference is gene specific and spreads throughout the worm cells



GFPG - mitochondrial gfp

LACZ - nuclear gfp

⇒ **non-striated muscles are only unaffected cells**



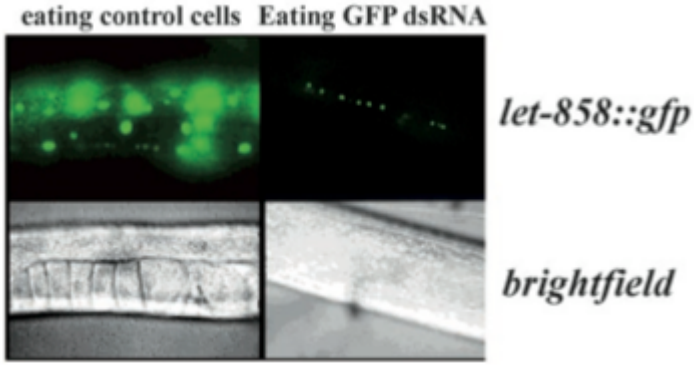
RNAi: So Easy a First Year Can Do It

- Injection into gonads is not required for RNAi to be effective

improperly placed needles still lead to interference

dsRNA	Injection Site	Phenotype	Progeny Phenotype
none	gonad or cavity	no twitching strong GFP	no twitching strong GFP
unc22	gonad body-cavity	weak twitchers weak twitchers	strong twitchers strong twitchers
gfpG	gonad body-caity	weak GFP weak GFP	absent GFP absent GFP
lacZL	gonad body cavity	weak nuclear GFP weak nuclear GFP	absent nuclear GFP absent nuclear GFP

- Ingestion of dsRNA expressing *E. coli* and soaking also work



"Do experiments that your advisor would never condone or suggest" – Fire, Nobel lecture

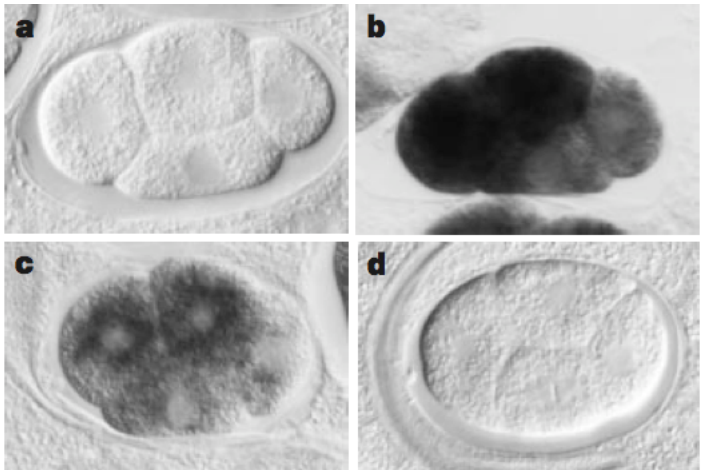
Timmons, Fire *Nature* **1998**, *395*, 854
Mello *Science*, **1998**, *282*, 430

What's in the Black Box?

- Early experiments reveal target mRNA degradation is initiated by dsRNA

mex-3 mRNA can be detected by staining

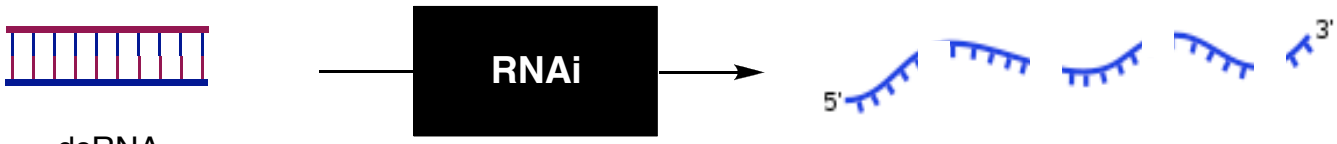
negative control
no staining in wild type
without hybridization



positive control
staining in wild type
with hybridization

***mex-3* antisense RNA**

***mex-3* dsRNA**



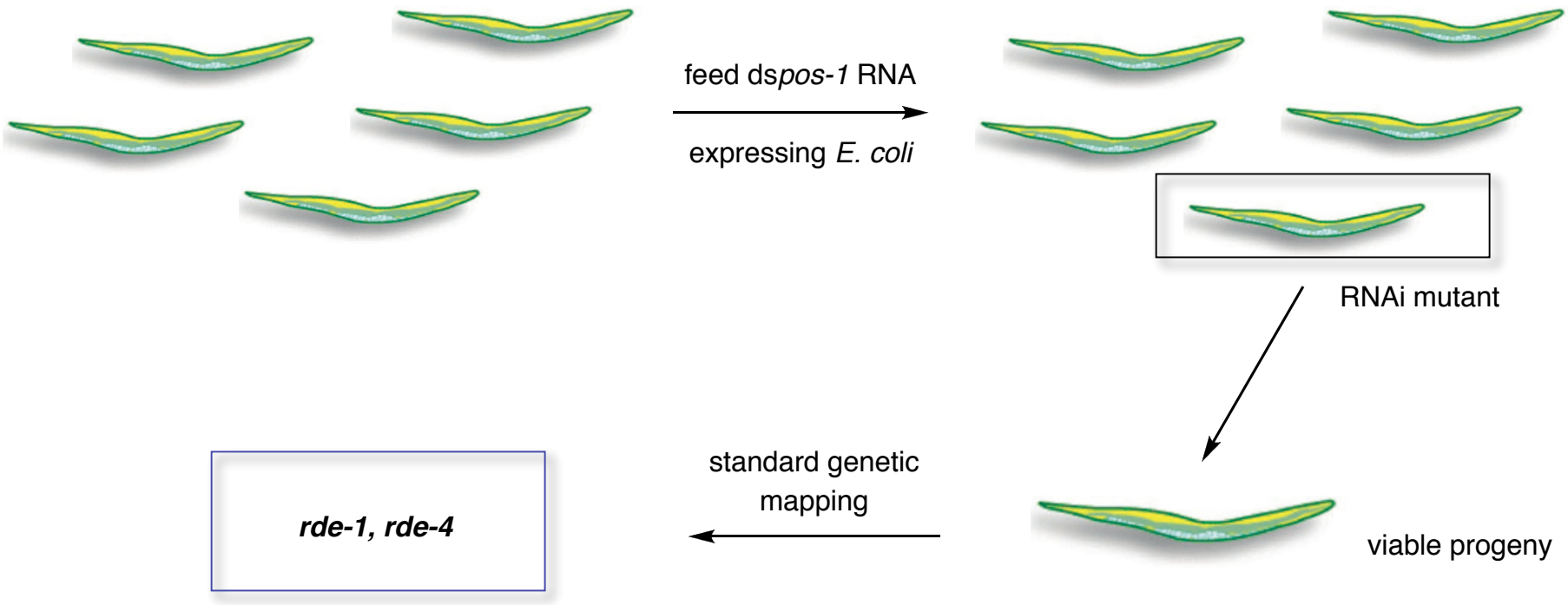
dsRNA

degraded mRNA

What's in the Black Box?

- A clever experiment reveals which genes are involved in RNAi

pos-1 expression is required for C. elegans survival



Fire, Mello *Cell* **1999**, *99*, 123

- *Rde-1* identified as a member of the little known argonaute family of proteins

What's in the Black Box?

- Two groups take the lead in working out the RNAi biochemical pathway

Nature **1998**, *391*, 806

Mello & Fire establish dsRNA as trigger for RNAi

Nature **2000**, *404*, 293

Hannon defines RISC complex as cause of mRNA degradation

Cell **2000**, *101*, 25

Tuschl & Sharp find mRNA is cleaved into 22 nt fragments

Greg Hannon
Cold Spring Harbor

1998

2000

1999

Sharp & Tuschl replicate RNAi in vitro

Genes Dev. **1999**, *13*, 3191

2001

Hannon defines roles of Dicer & Slicer, identifies Argonaute in RISC

Nature **2001**, *409*, 363
Science **2001**, *293*, 1146

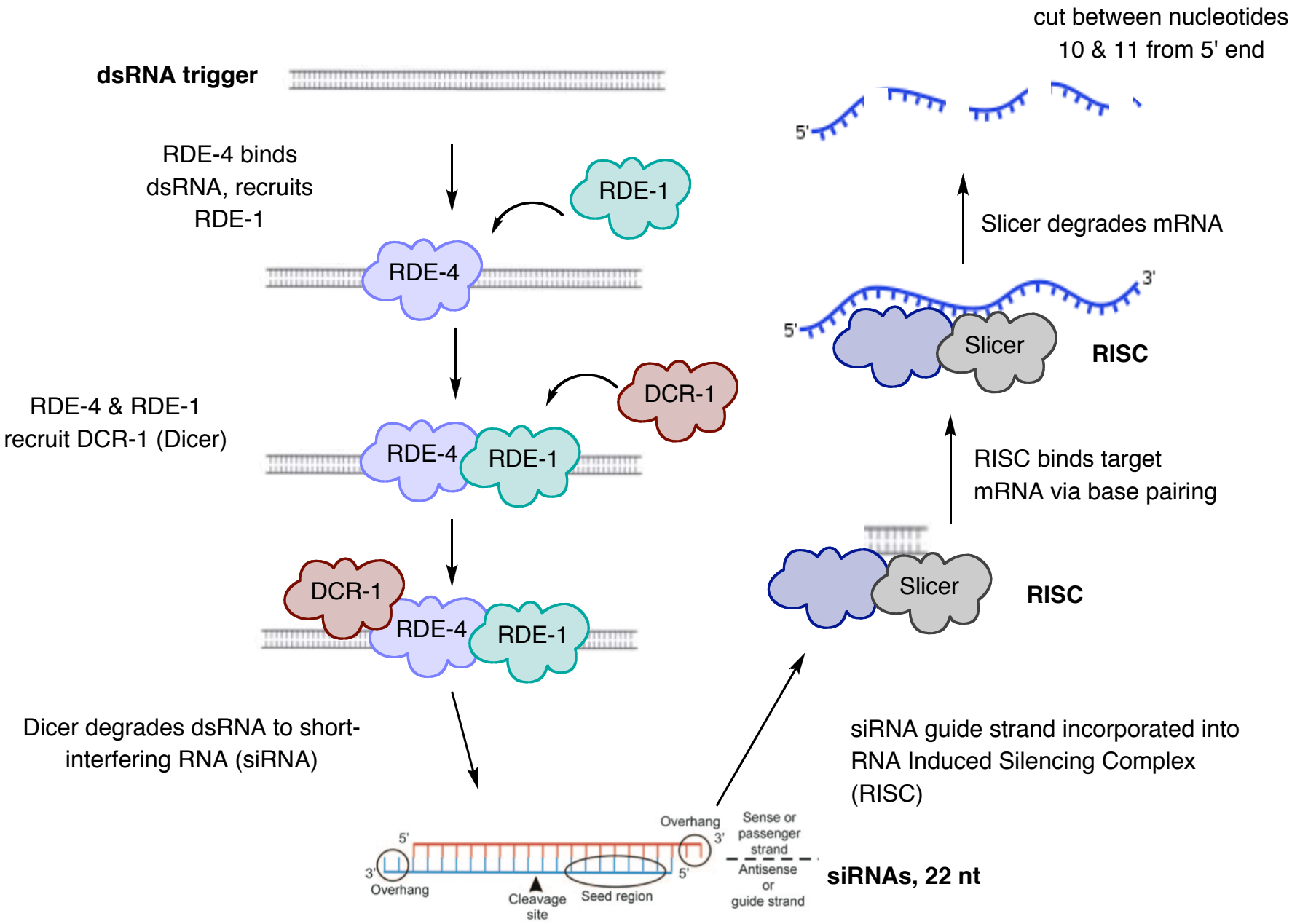
Tuschl determines structures of Dicer dsRNA products, siRNA

Gene Dev. **2001**, *15*, 188
Nature **2001**, *411*, 494

Phil Sharp
MIT

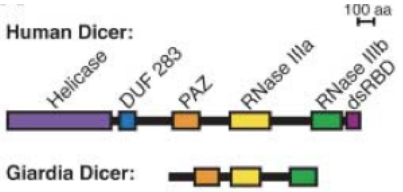
Thomas Tuschl
MIT, Gottingen, Rockefeller

Overall Scheme of RNAi process

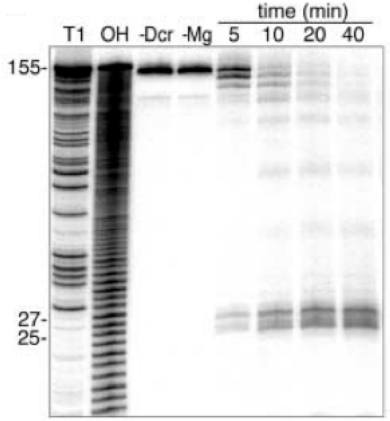


Crystal Structure Reveals Dicer Mechanism

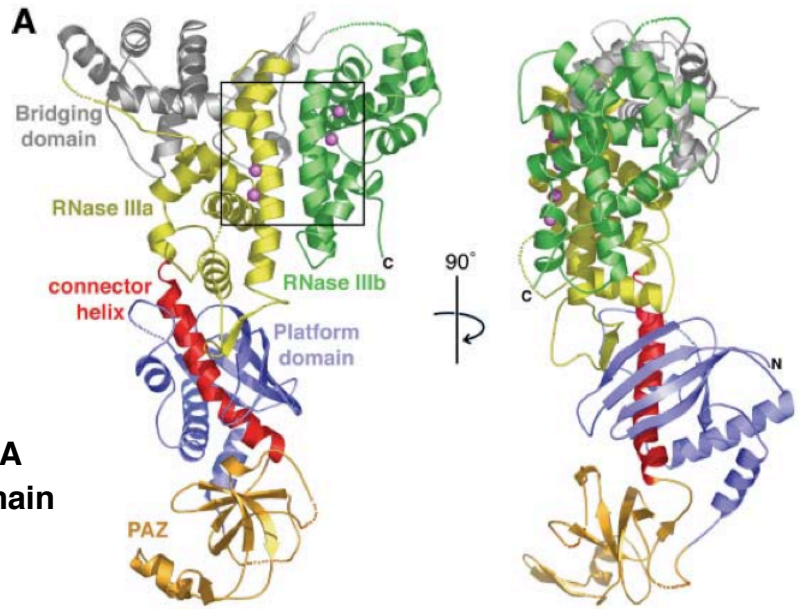
- Doudna *et. al.* is able to obtain a crystal structure of *G. intestinalis* Dicer enzyme



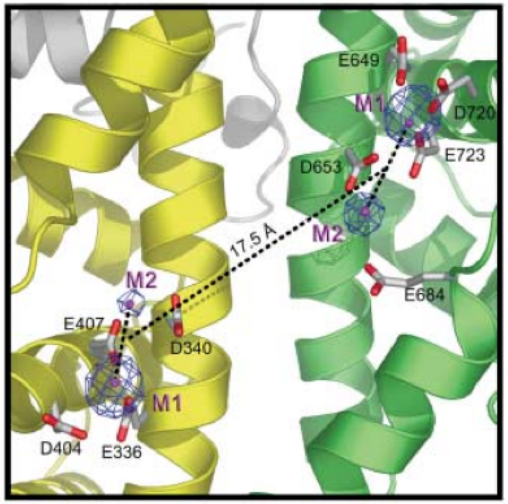
Dicing requires both enzyme and Mg^{2+} to produce siRNAs 25-27 nt in length



two RNase III domains



contains dsRNA binding PAZ domain



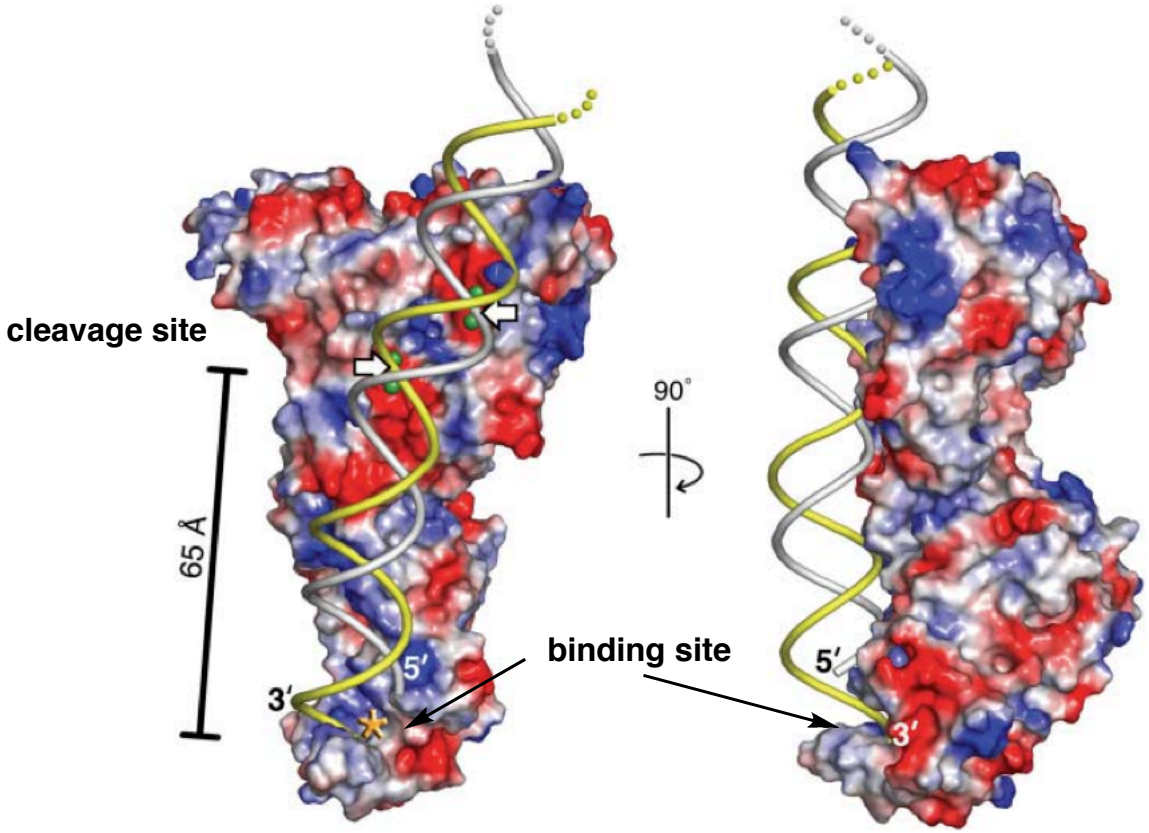
Glu and Asp perform cleavage in active site

A distance of 17.5 Å between the RNase III domains fits the width of the major groove of dsRNA

Science **2006**, *311*, 195

Crystal Structure Reveals Dicer Mechanism

- Analysis reveals Dicer is in effect a molecular ruler



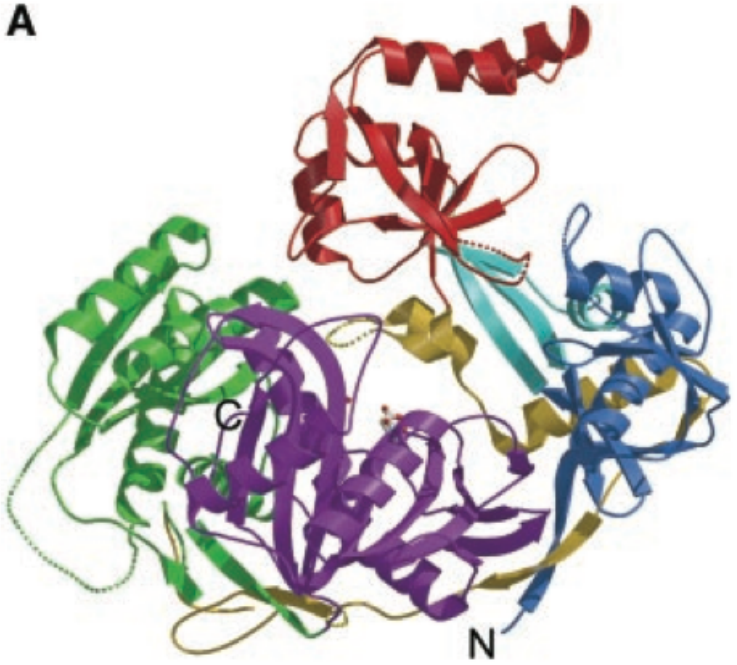
A distance of 65 Å between the PAZ and RNase III domains corresponds to the length of an siRNA fragment containing 25 bp

3' two nucleotide overhang recognition by PAZ OB fold is conserved across many species

Crystal Structure of Argonaute Reveals Slicer Mechanism

- Argonaute has been closely linked to RNAi across all species studied

As seen with Dicer, Slicer also requires Mg^{2+} for activity



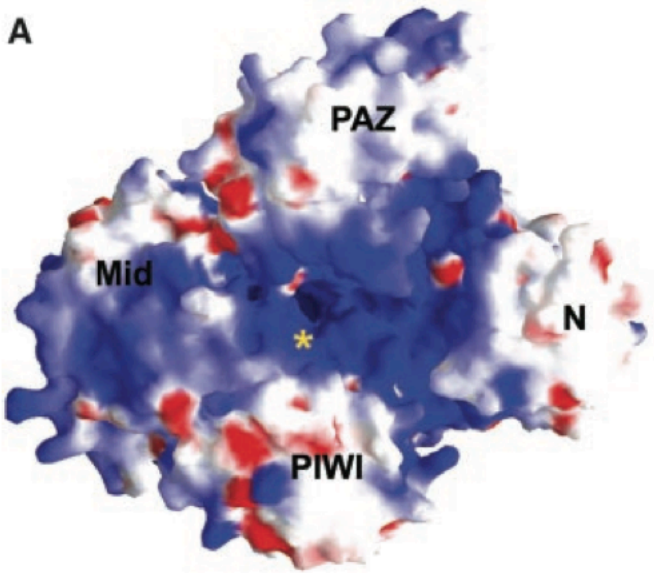
positively charged groove in crescent formed between base (N-terminal, middle and PIWI domains) and overhanging PAZ domain

⇨ **phosphate binding**

homology to sugar binding lac-repressor

RNA binding

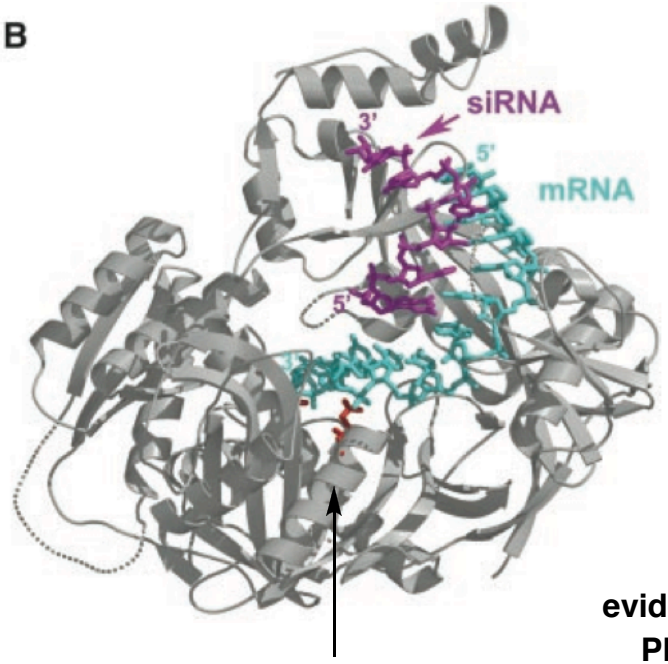
RNase H-like catalytic domain with Asp-Asp-His



Joshua-Tor *Science* **2004**, *305*, 1434

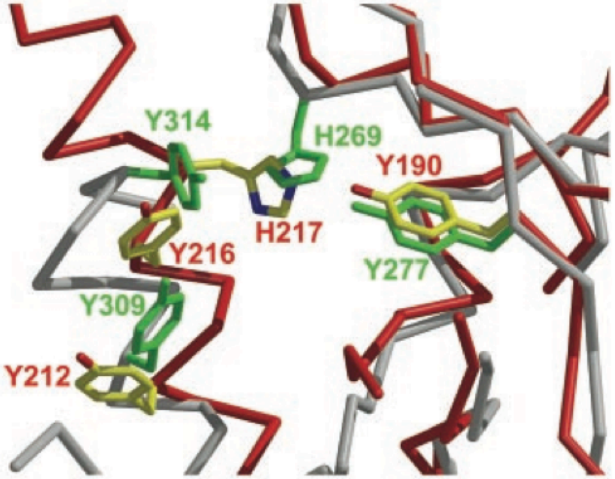
Crystal Structure of Argonaute Reveals Slicer Mechanism

- Modelling of siRNA guide strand and mRNA places scissile bond in active site



active site (PIWI)

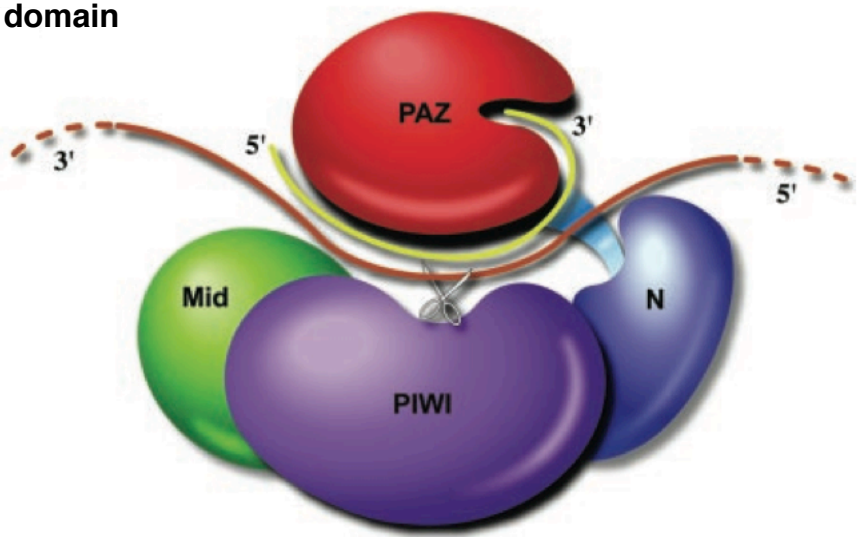
PAZ binds phosphate between 2 overhanging nucleotides of guide strand via H-bonding with His & Tyr residues



human Ago1

Pf Ago

evidence for binding 5' end with PIWI and/or middle domain



base pairing with the guide strand positions mRNA target for cleavage in PIWI domain

Leemor Joshua-Tor
Cold Spring Harbor

RNAi is also Triggered by microRNAs

- Ambros discovers the first endogenous short RNA (microRNA) via forward genetics in 1993

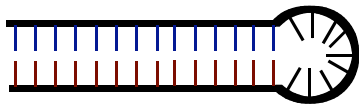
C. elegans mutant with abnormal embryonic development

lin-4, let-7

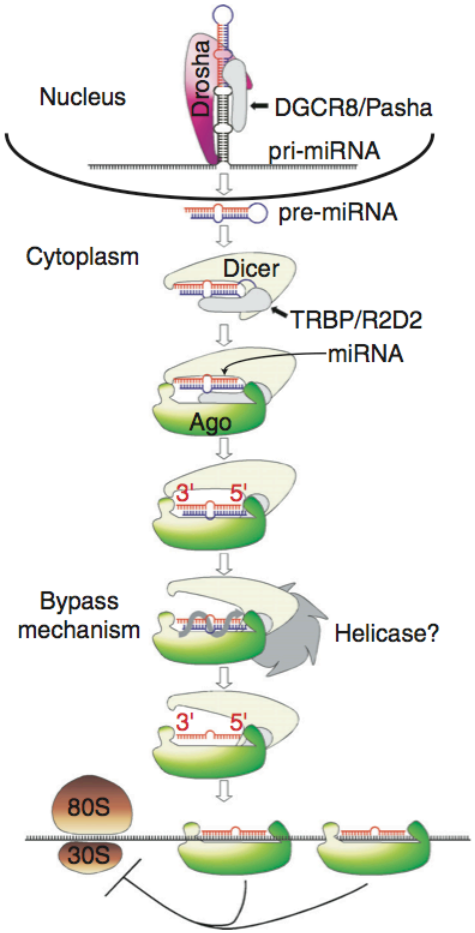
Cell, **1993**, *75*, 843

trancription

translation



short hairpin RNA
70 nt long



miRNAs use the RNAi pathway to regulate gene expression, controlling cell development throughout animal life cycles

c-myc overexpression ⇨ lymphoma

E2F1

miRNA knockout → **cancer**

miRNA → **controlled cell growth**

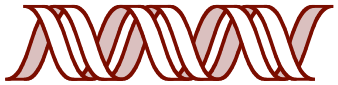
Asuragen

EXIQON

miRagen Therapeutics
Harnessing microRNA biology to treat cardiovascular disease

miRNAs Are Correlated with Complexity

- miRNAs are found in all animals and attempts to find all miRNA encoding genes are ongoing



human genome

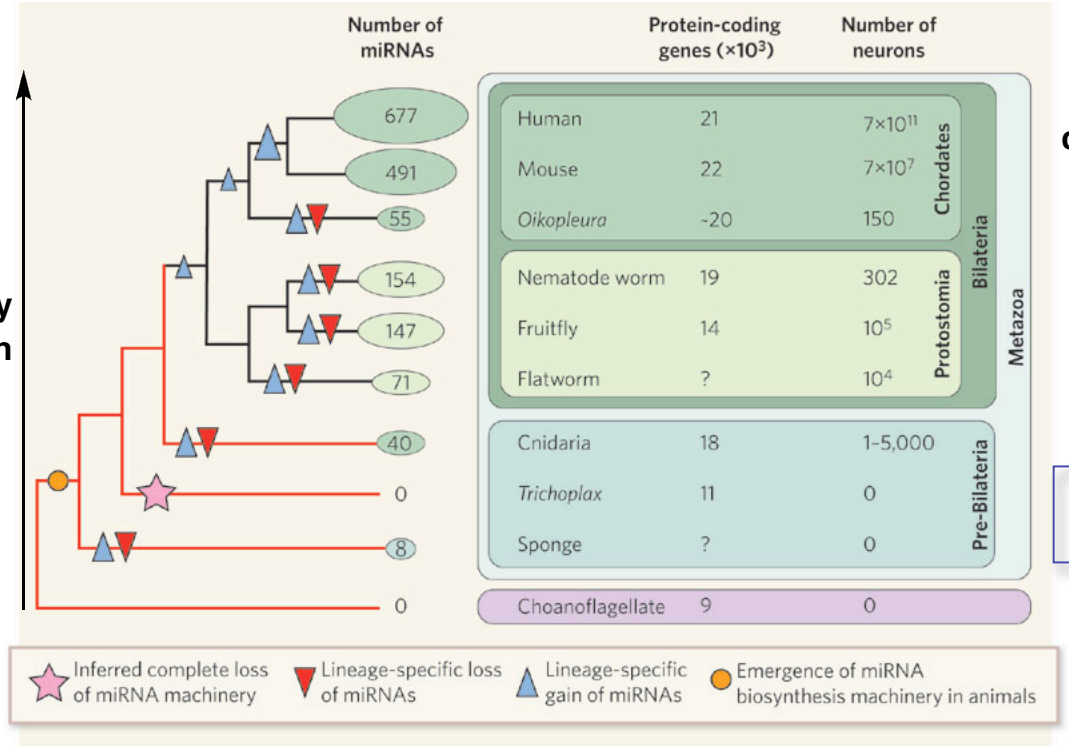
cDNA sequencing

forward genetics

reverse genetics (knockdown)

>500 miRNA encoding genes
(>1000 postulated)

complexity tracks with # miRNAs



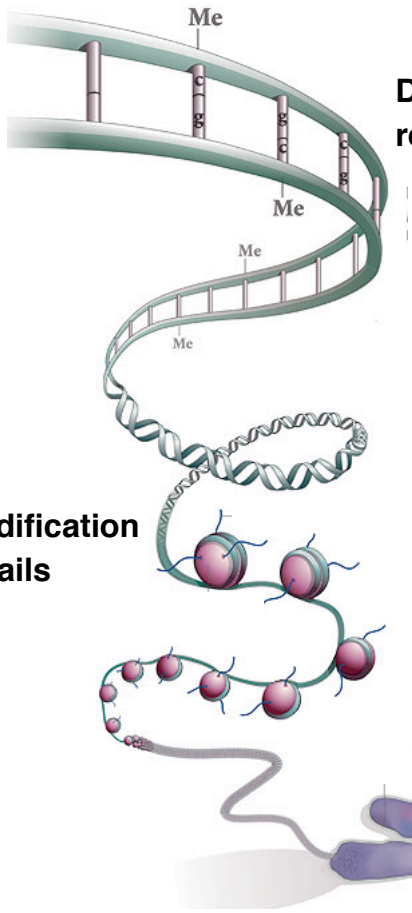
Bartel proposes miRNA regulation could explain why complex organisms have the same number of genes as simple ones

Nature, **2008**, *455*, 1193

gene regulation ⇨ evolution

RNAi Also Directs Heterochromatin Formation

■ Discovered in 1928, heterochromatin is darkly staining, covalently modified chromatin that does not unwind at any time in the cell cycle



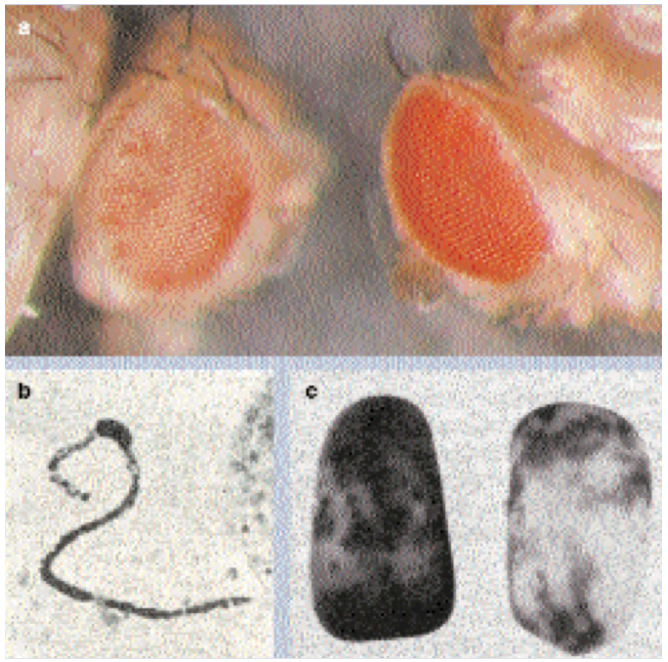
DNA methylation results in silencing

histone modification occurs on tails

methylation
acetylation
phosphorylation
ubiquitinylation

chromosome

has a regulatory effect on nearby genes,
ie fruitfly & maize variegation

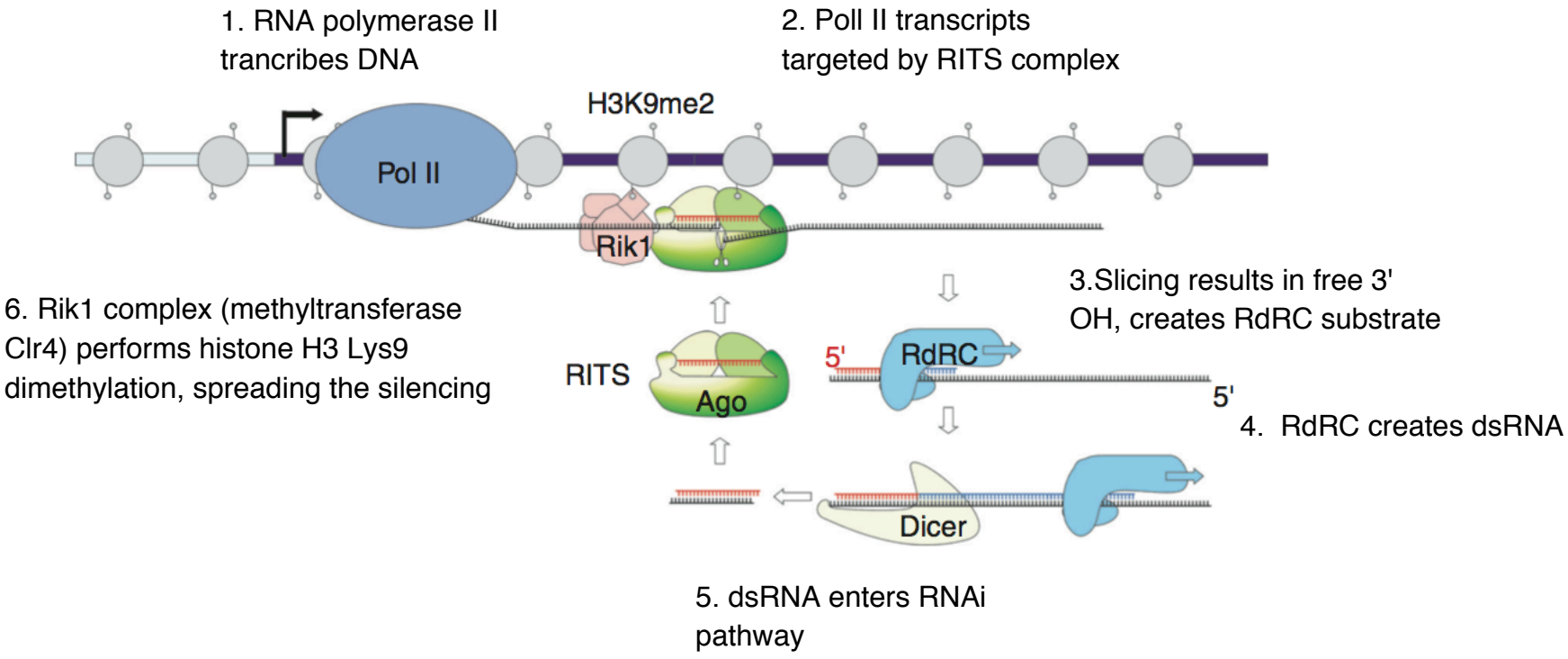


comprised of short repeating sequences, heterochromatin produces repeat associated siRNAs (rasiRNAs) that induce covalent modification of DNA & histone

RNAi Also Directs Heterochromatin Formation

■ Though its exact mechanism is still unclear, the RNA-induced transcriptional silencing (RITS) complex plays the role of RISC and contains an argonaute protein

Joshua-Tor *Nature Chemical Biology* **2006**, *3*, 36



chromatin modification ⇒ **inherited gene regulation** ⇒ **evolution**

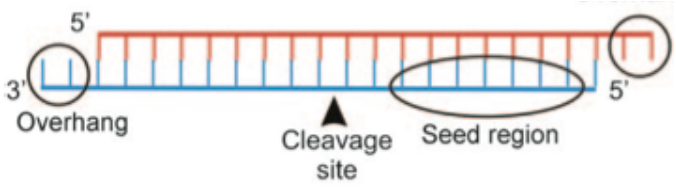
Therapeutic Possibilities for RNAi

■ Using RNAi as an experimental and therapuetic tool requires several considerations

Innate immune response (interferon) provides viral immunity

Design of siRNA

passenger strand (sense)



guide strand (antisense)

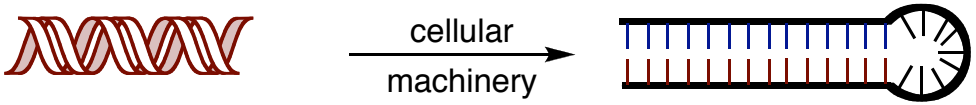
- strand with lower 5' thermodynamic stability (A–T) is incorporated into RISC
- seed region has greatest effect on mRNA recognition
- cleavage site between nt 10 & 11 from 5' end

4 Rules for effective siRNAs

1. Get right strand into RISC (design algorithms)
2. Target several alleles (phenotypic correlation)
3. Use low concentrations (limit off target effects)
4. Use rescue experiments (important control)

Therapeutic Possibilities for RNAi

■ Endogenous miRNAs have inspired the design of short hairpin RNAs (shRNAs)



synthetic DNA

shRNA

advantage: longer lasting supression, easy delivery

disadvantage: need to desgin DNA construct

HIV

- RNAi has been demonstrated to stop spread of HIV in mice
- high mutation rate poses problems, cellular cofactors are other possible targets (NF-κB, CD4, CXCR4, CCR5)

Hepatitis C

- blocking has been demonstrated but it is temporary due to virus mutation

Cancer

- Alnylam compound in clinical trials for treatment of liver cancer
- contains 2 siRNAs in lipid nanoparticle (Tekmira) targeting KSP & VEGF

Genetic Disease

- Amyotrophic lateral sclerosis treatment targeting single nucleotide mutant of *sod1*
- Huntingtons disease

Therapeutic Possibilities for RNAi

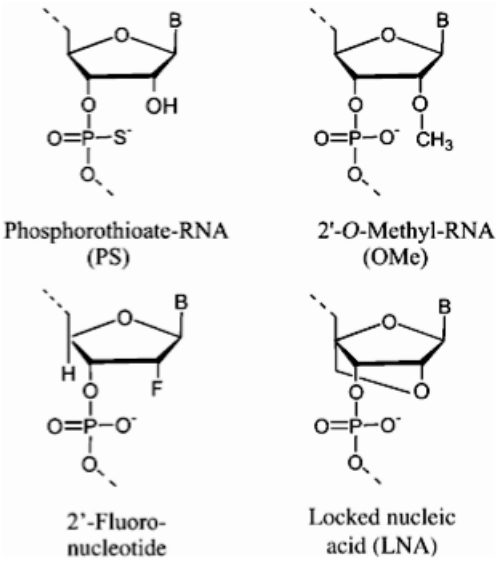
■ The path to commercial therapies that utilize RNAi is not necessarily clear

Can the RNAi pathway be saturated?

- therapies hijack the native RNAi machinery which we know is used in gene expression
- RISC saturation has been shown *in vitro*

How can we delivery these therapies?

- this issue has plagued antisense for over 20 years
- many options exist (backbone modification, virus vectors, transfection) but none work perfectly
- for now targets are set low (liver)



What about off target effects?

- supression of cofactors may result in disruption of normal cellular processes
- chromatin modification has not yet been identified in humans but endogenous RNAs point to the possibility of its existence

Kurreck *Angew. Chem. Int. Ed.* **2009**, *48*, ASAP

Conclusions

- RNAi is an important part of the cellular machinery that provides viral immunity and a mechanism for the control of gene expression

- A variety of RNA triggers function in the RNAi mechansim result in gene suppression that can be both temporary and permanent

  dsRNA, siRNA, miRNA, shRNA

- Recent studies have implied that RNAi could be an important factor in evolution due to its key role in epigenetics

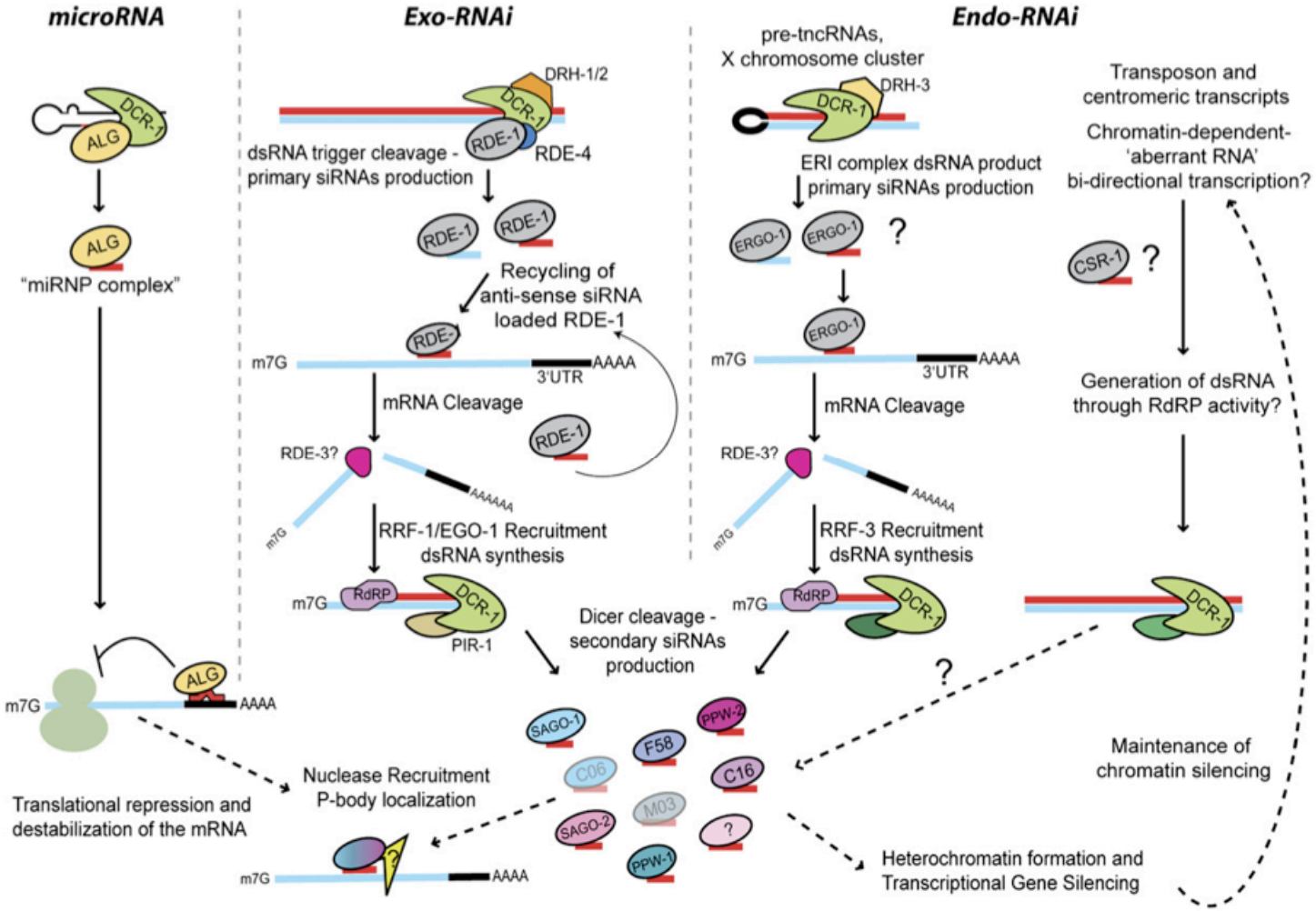
  miRNA, chromatin modification

- Potential therapeutic applications of RNAi include treatments for viruses, cancer and heritable disease

- The field is relatively young and much remains to be discovered

RNAi is Actually Quite Complicated

- RNAi is still an emerging field with many unanswered questions and potential applications



***C. elegans* contains 27 argonaute proteins, all of which are involved in RNAi**

Mello *Cell*, **2006**, *127*, 747