RNAi and miRNAs

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Eric Lai
laie@mskcc.org
1017C RRL

“the central dogma”: DNA-->RNA-->protein

however, many types of functional RNAs are known

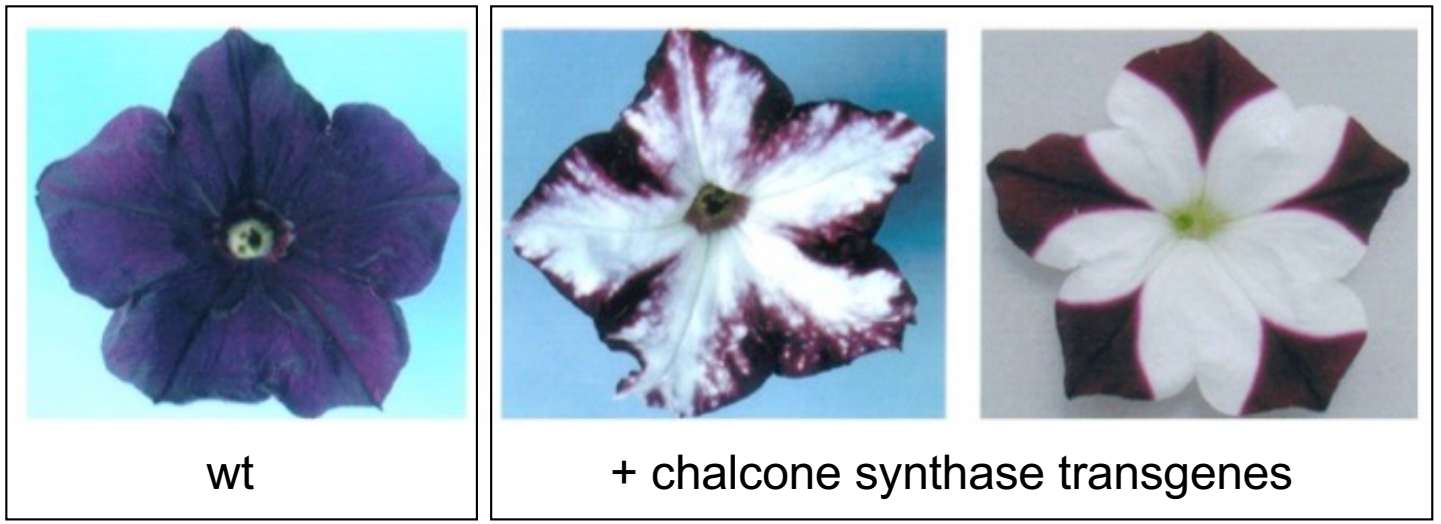
- tRNA (transfer RNA): translation
- snoRNA (small nucleolar RNA): Direct rRNA modification
- rRNA (ribosomal RNA): Ribosomal RNA is responsible for peptide bond formation in the ribosome
- Group I Introns: class of RNA introns that catalyze their own splicing
- Riboswitches: RNA motifs that control transcript activity by allosteric binding of metabolites
- RNaseP RNA: Component of RNaseP, which edits tRNA
- SRP RNA (signal recognition molecule): part of RNP that transports secreted proteins to ER
- Telomerase RNA: Structured RNA that provides sequence template for telomere sequences
- >50 small ncRNAs in prokaryotes: many are regulatory RNAs
- long “mRNA-like ncRNAs”: Xist, H19, bxd, etc; diverse regulatory or scaffolding functions
- miRNA/siRNA/piRNA: <30nt RNAs in Argonaute effectors
- CRISPR/Cas9: phage defense system in bacteria

and more to come: a still ongoing expansion in the types of functional non-coding RNAs known across all life forms

RNAi and miRNAs

- how RNAi and miRNAs were found
- basic mechanisms of RNAi and miRNA activity
- defining biological functions for miRNA genes
- using RNAi as a genetic screening tool

in plants: attempts made to produce petunias with deeper pigment
by overexpressing chalcone synthase (Jorgensen and Stuitje)



Van der Krol et al, Plant Cell 1990; Napoli et al, Plant Cell 1990

- instead, transgenic plants show random or sectored pigment loss!
- effect termed “co-suppression” or “post-transcriptional gene silencing (PTGS)”
- process related to control of invasive nucleic acids (e.g. TEs, viruses)
- later associated with accumulation of small RNAs (Hamilton, Science 1999)

US 2017/0196177 A1

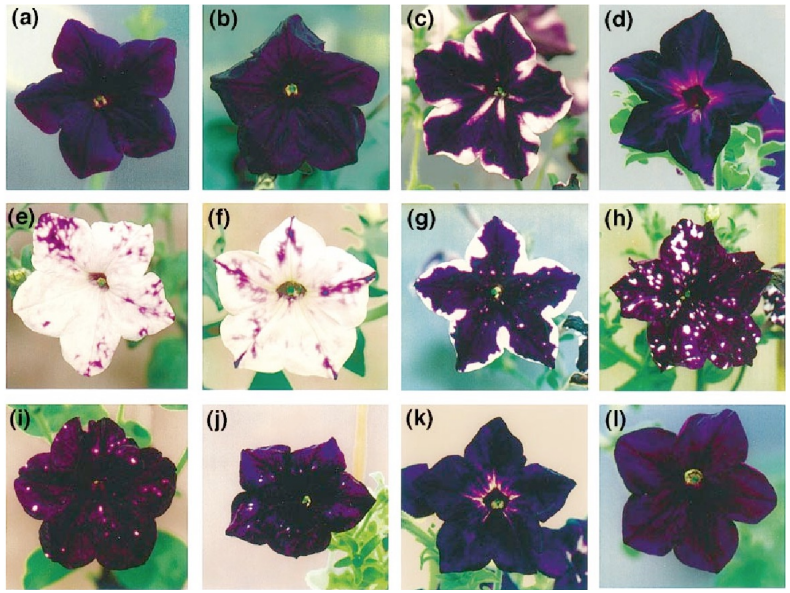
PETUNIA PLANTS HAVING A UNIQUE FLOWER COLOR PATTERN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a continuation-in-part application which claims priority to U.S. Provisional Application No. 62/316,271 filed on Mar. 31, 2016, the contents of which are hereby incorporated in their entirety.

10. Molecular Background of the Spotted Flower Color Pattern in *Petunia*

[0070] The *Petunia* variety 'Can Can' as mentioned above exhibits a bicolor color pattern similar to other star-type Petunias. In this group (of star-type *Petunia*), it was shown that the pattern is induced by sequence-specific RNA degradation of Chalcone Synthase, the first specific enzyme in anthocyanin pigment biosynthesis. Please see Morita, Y., et al. "Tandemly arranged chalone synthase A genes contribute to the spatially regulated expression in siRNA and the natural bicolor floral phenotype in *Petunia hybrida*" *The Plant Journal*. 70:739-749 (2012). Sequence-specific RNA degradation occurs during a process called post-transcriptional gene silencing (PTGS) which protects the cell from viral infection. PTGS is triggered by over threshold-levels of viral gene mRNA. Also, over threshold-amounts of plant gene mRNA can trigger PTGS. Please see Baulcombe, D. "RNA silencing in plants" *Nature*. 431:356-363 (2004).



Meanwhile, in animals...

many attempts made to produce gene knockdowns
with antisense RNA

block translation?

Thymidine Kinase Gene Expression:

Izant, J.G. & Weintraub, H. (1984) Inhibition of Thymidine Kinase Gene Expression by Anti-Sense RNA: A Molecular Approach to Genetic Analysis. Cell 36: 1007-1015.

Izant, J.G. & Weintraub, H. (1984) Constitutive and Conditional Suppression of Exogenous and Endogenous Genes by Anti-Sense RNA. Science 229: 345-352.

Kim, S.K. & Wold, B.J. (1985) Stable Reduction of Thymidine Kinase Activity in Cells Expressing High Levels of Anti-Sense RNA. Cell 42: 129-138.

occasio

...so ene activity

B-globin Gene Expression:

Melton, D.A. (1985) Injected Anti-sense RNAs Specifically Block Messenger RNA Translation in vivo. Proc. Natl. Acad. Scie. USA 82: 144-148.

Kruppel Gene Expression:

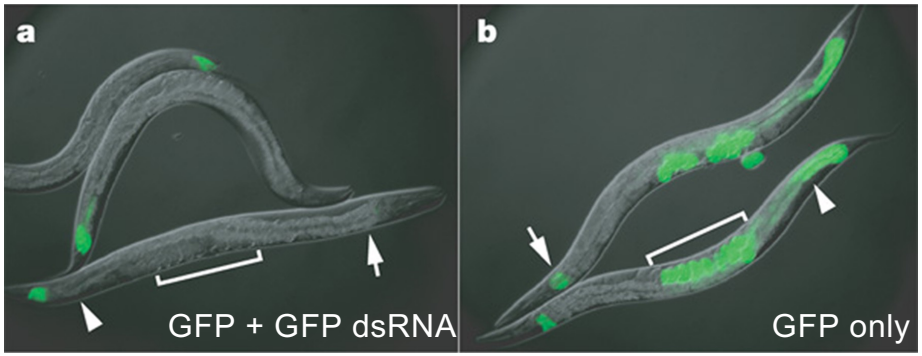
Rosenberg, U.B., Preiss, A., Seifert, E., Jackle, H. & Knipple, D.C. (1985) Production of Phenocopies by Kruppel Antisense RNA Injection Into Drosophila Embryos. Nature 313: 703-706.

Effects of antisense RNA were studied carefully by Fire and Mello

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%)

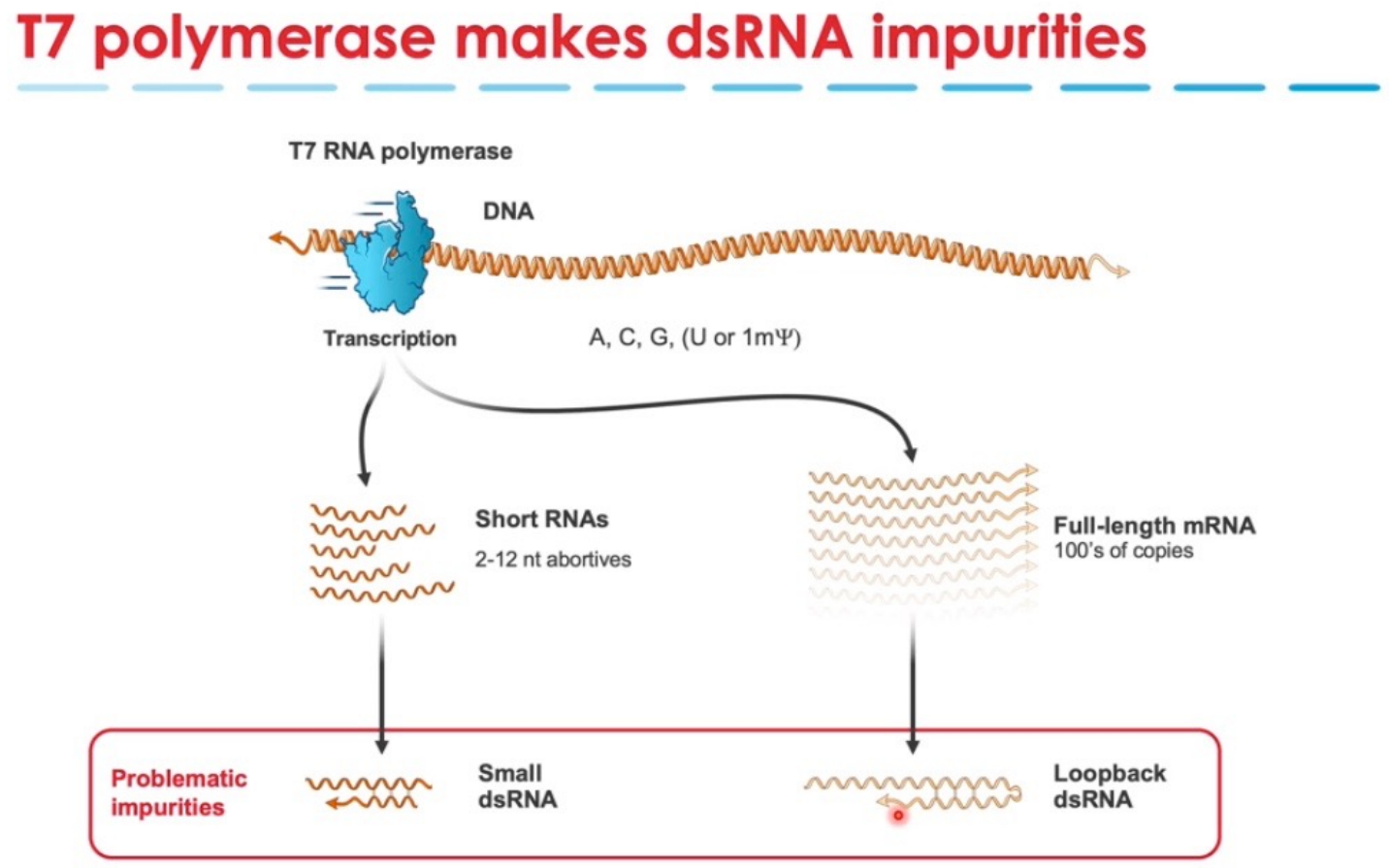
Fire et al, Nature 1998



- critical component for gene knockdown is dsRNA (the “trigger”)
- occasional inhibitory effects of sense or antisense likely due to contaminating dsRNA

Minimization of dsRNA side products is critical for therapeutics

(eg mRNA vaccines!)



Dousis Nat Biotech 2023

moderna

- potent negative regulatory activity of dsRNA
soon extended to diverse experimental systems

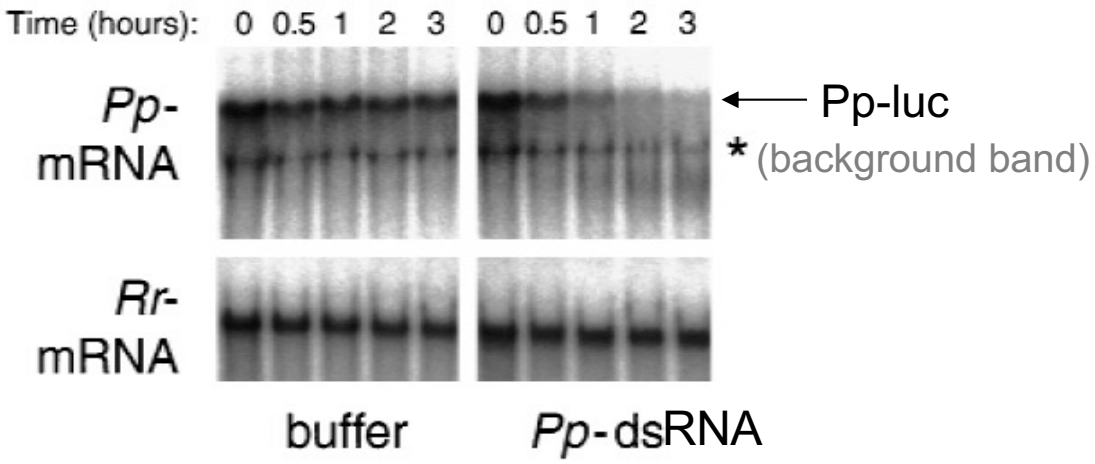
- reflects a fundamental cellular response to dsRNA

- phenomenon termed RNA interference (RNAi)

RNAi can be recapitulated in vitro using cell extracts

- ^{32}P-target and control mRNA
- targeting dsRNA
- cell lysate

incubate and run on gel



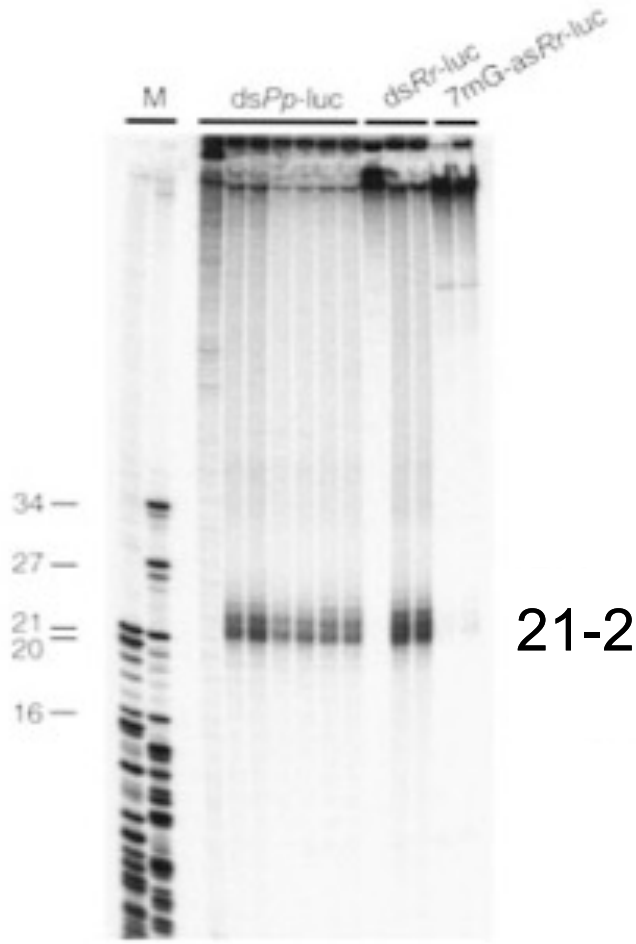
(Tuschl G&D 1999; Zamore Cell 2000)

paves the way for biochemical dissection of RNAi mechanism

dsRNA triggers are processed into 21-22 nt segments (“dicing”)

- (+/- target)
- ^{32}P-dsRNA
- cell lysate

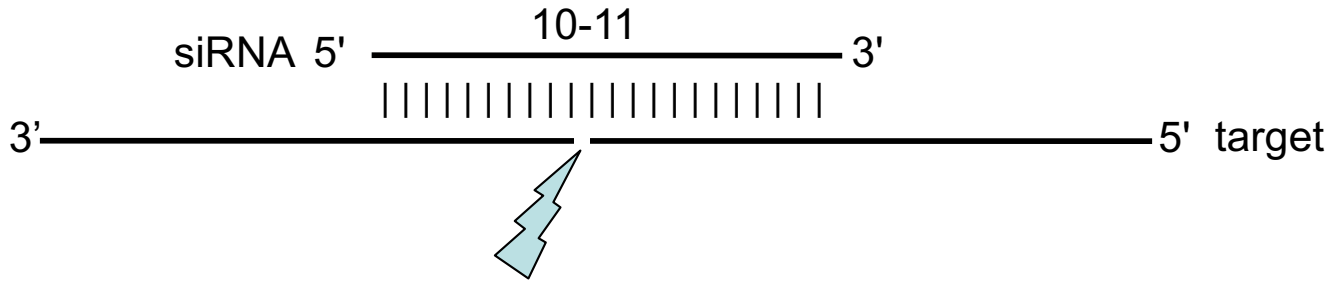
incubate and run on gel



21-22nt siRNAs

Zamore Cell 2000

siRNAs determine cleavage sites on target mRNAs (“slicing”)



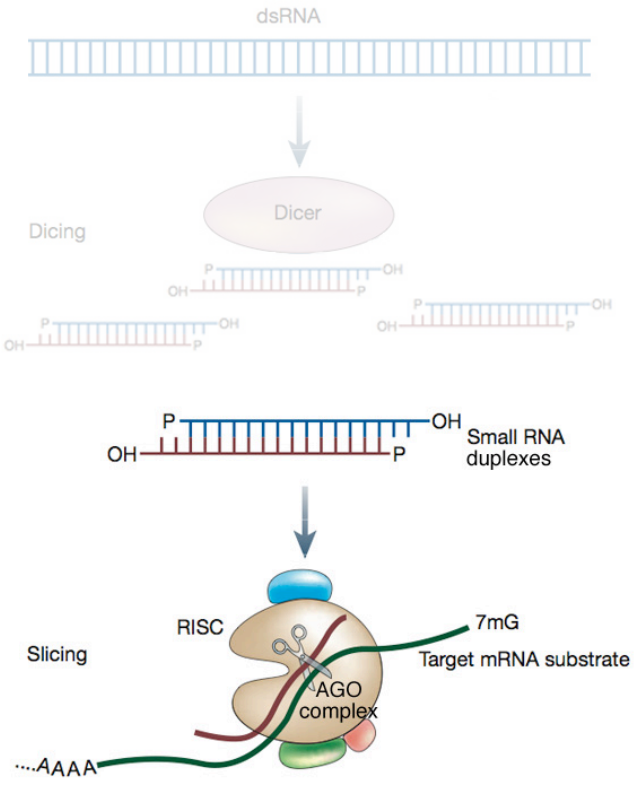
siRNAs assemble into RNA-induced silencing complex (RISC)

target sequences are cleaved between
positions 10 and 11 from the 5’ end of the guide siRNA

Elbashir EMBO 2001

RNAi can be divided into “initiation” and “effector” phases

(dicing) (slicing)



- process dsRNA into siRNA duplexes
- mediated by “Dicer” (RNase III)

- assemble ss-siRNA into RISC (RNA induced silencing complex)
- RISC finds and cleaves targets
- “Slicer”=Argonaute/AGO (RNase H-like)

it is biochemically/genetically possible to bypass the dicing/RNaseIII phase

cloning and sequencing of siRNAs from exogenous dsRNA triggers
also identified endogenously-encoded small RNAs

some derived from transposons,
others shared characteristics with some unusual worm genes...

Cell, Vol. 75, 855–862, December 3, 1993, Copyright © 1993 by Cell Press

Posttranscriptional Regulation of the Heterochronic Gene *lin-14* by *lin-4* Mediates Temporal Pattern Formation in C. elegans

Bruce Wightman,*† Ilho Ha,* and Gary Ruvkun
Department of Molecular Biology
Massachusetts General Hospital
Boston, Massachusetts 02114

Cell, Vol. 75, 843–854, December 3, 1993, Copyright © 1993 by Cell Press

The C. elegans Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*

Rosalind C. Lee,*† Rhonda L. Feinbaum,*‡ and Victor Ambros†
Harvard University
Department of Cellular and Developmental Biology
Cambridge, Massachusetts 02138

lin-4 and let-7 precursor RNAs

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

Brenda J. Reinhart*†, Frank J. Slack*†‡, Michael Basson‡§, Amy E. Pasquinelli*, Jill C. Bettinger‡#, Ann E. Rougvie#, H. Robert Horvitz§ & Gary Ruvkun*

NATURE | VOL 403 | 24 FEBRUARY 2000 | www.nature.com

lin-4 and let-7 mediate negative regulation by forming RNA duplexes with target 3' UTRs

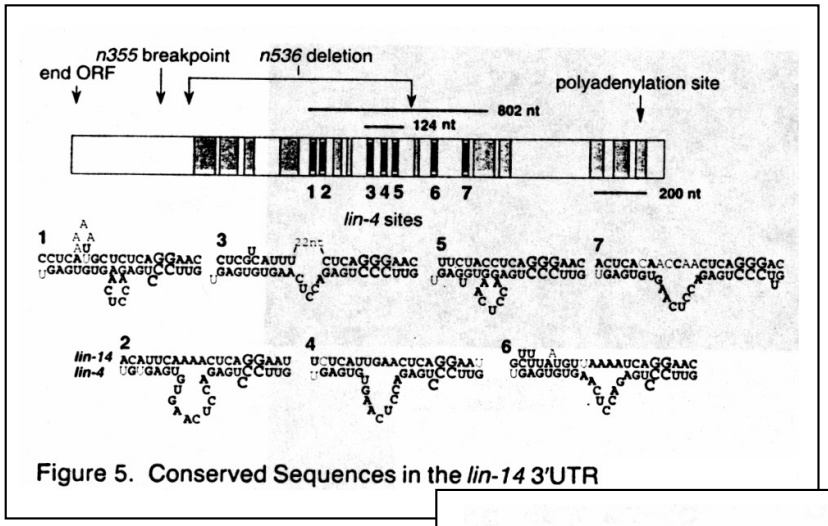
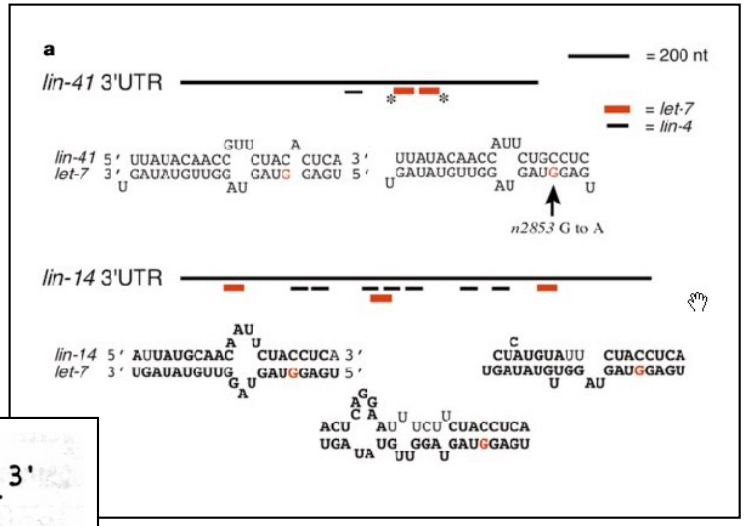
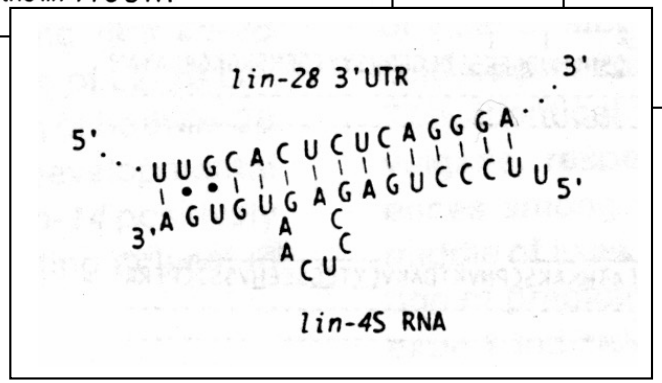


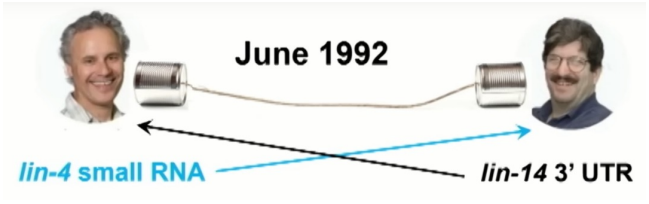
Figure 5. Conserved Sequences in the *lin-14* 3'UTR

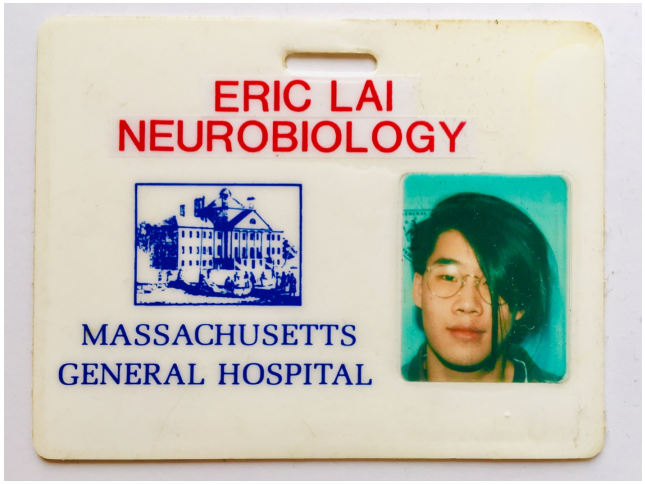




Ambros and Ruvkun labs

- unlike siRNAs, these worm small RNAs have "imperfect" targets



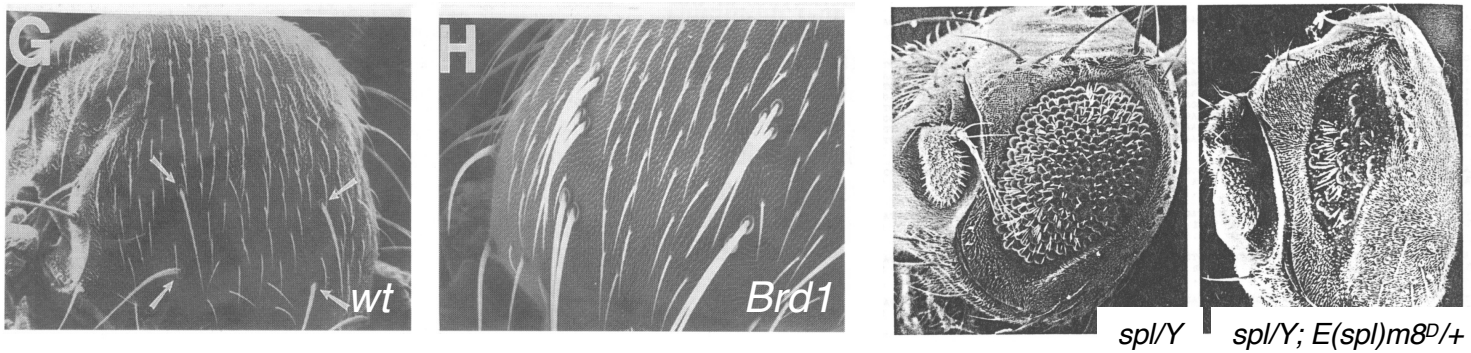


1992-1993

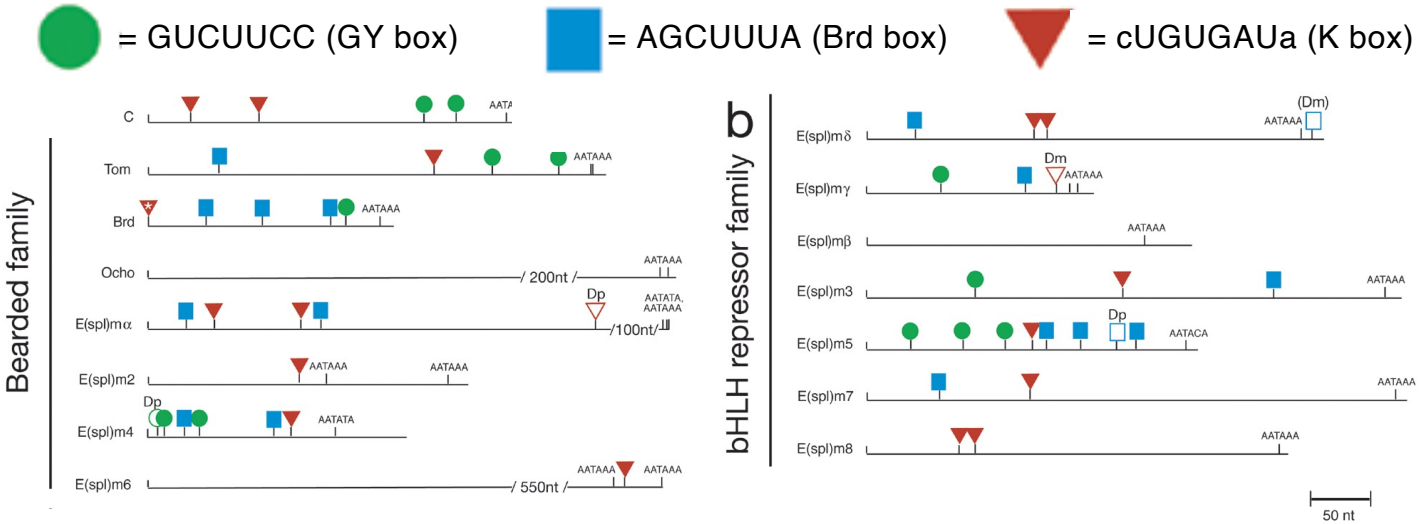
Gary Nob73 Apr 2025

Gain-of-function alleles of Notch target genes

reveal networks of post-transcriptional regulation by ~7nt motifs

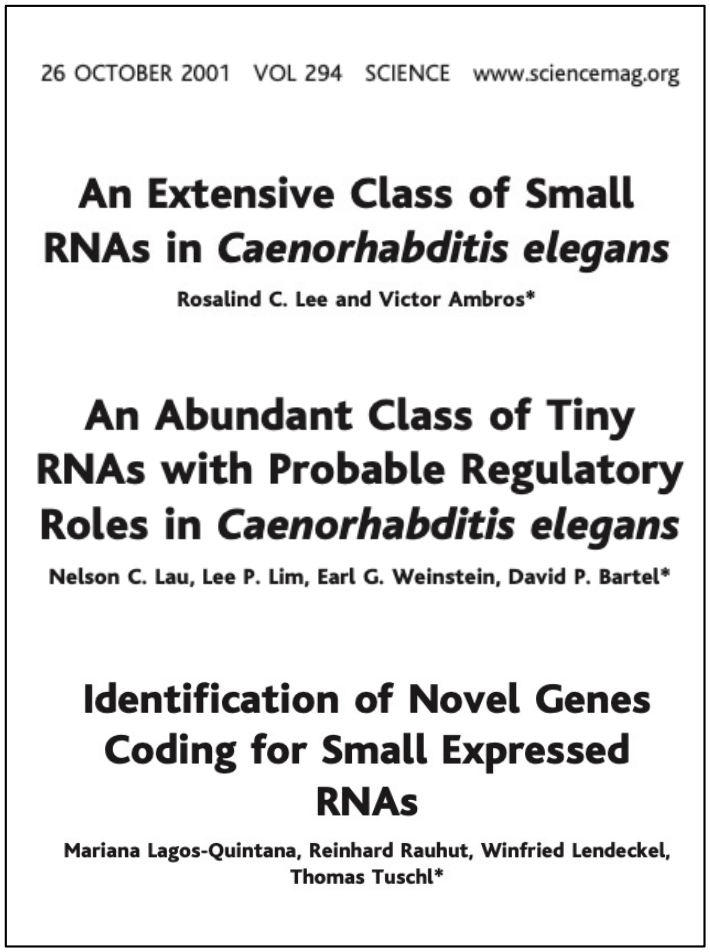


Leviten 1997; Knust 1987



Lai et al, Development 1997, Dev 1998, Cell 1998

“boxes” reveal the regulatory logic of microRNA target sites



26 OCTOBER 2001 VOL 294 SCIENCE www.sciencemag.org

An Extensive Class of Small RNAs in *Caenorhabditis elegans*

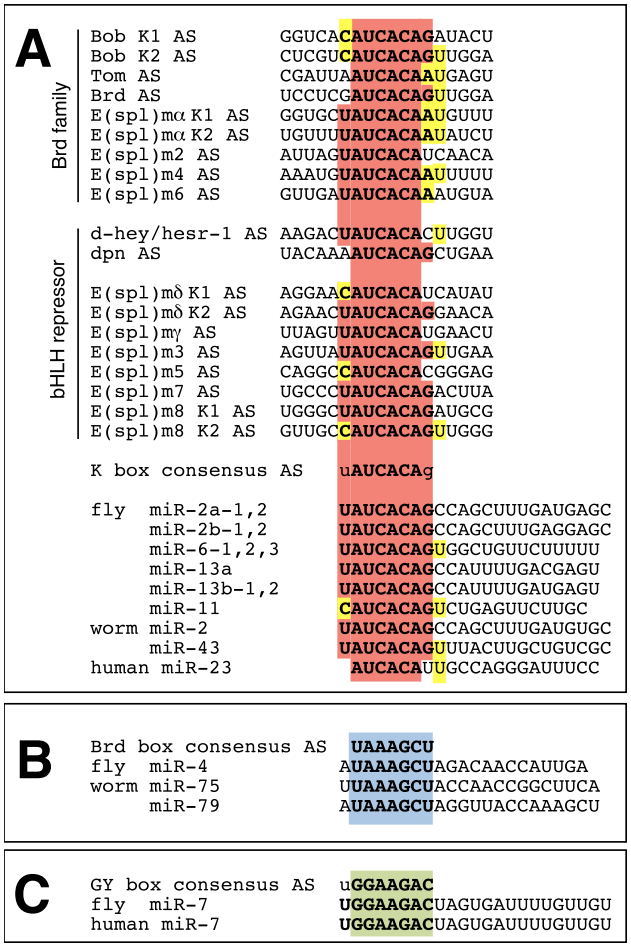
Rosalind C. Lee and Victor Ambros*

An Abundant Class of Tiny RNAs with Probable Regulatory Roles in *Caenorhabditis elegans*

Nelson C. Lau, Lee P. Lim, Earl G. Weinstein, David P. Bartel*

Identification of Novel Genes Coding for Small Expressed RNAs

Mariana Lagos-Quintana, Reinhard Rauhut, Winfried Lendeckel, Thomas Tuschl*

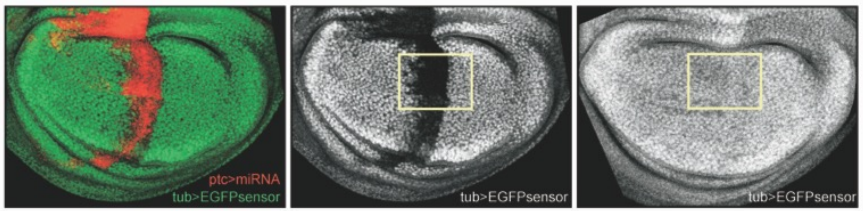


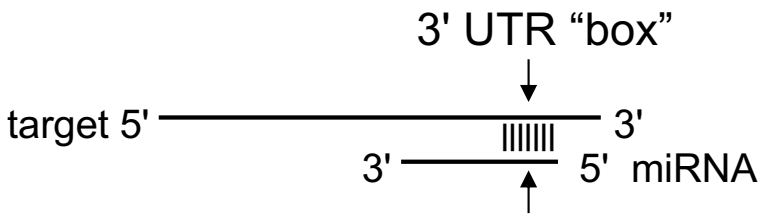
Lai, Nature Genetics 2002, Genes Dev 2005

Animal miRNAs can have limited complementarity with targets

- as little as 7 bp of seed-pairing can suffice for regulation in vivo
- more pairing can be better, but is not necessarily better

“sensor” assay





Brennecke et al, PLoS 2005

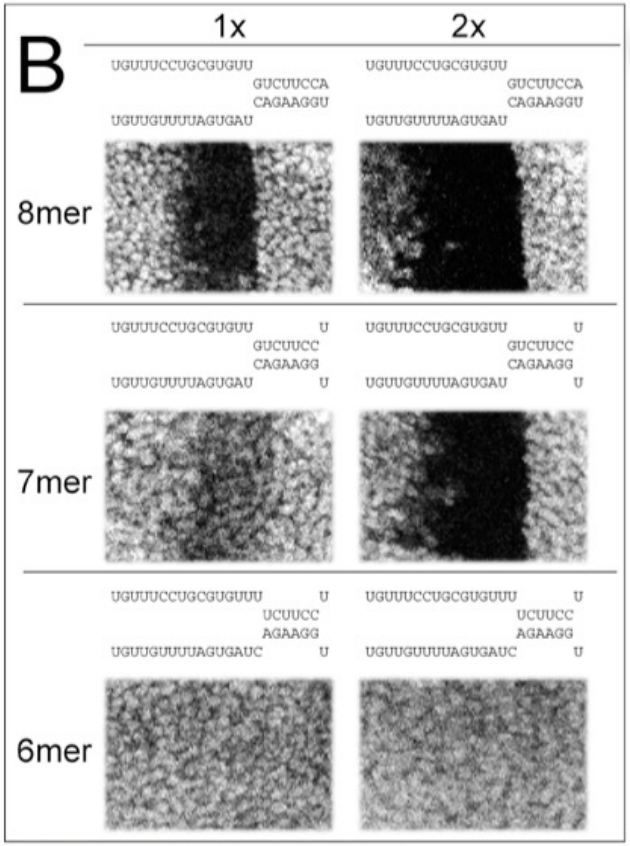
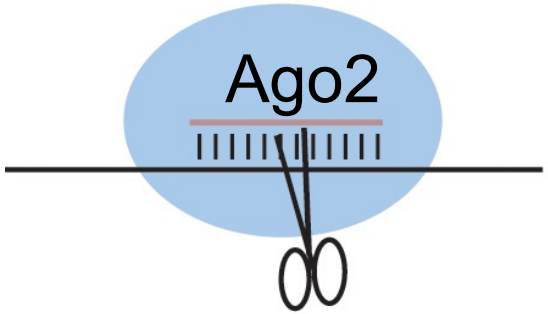
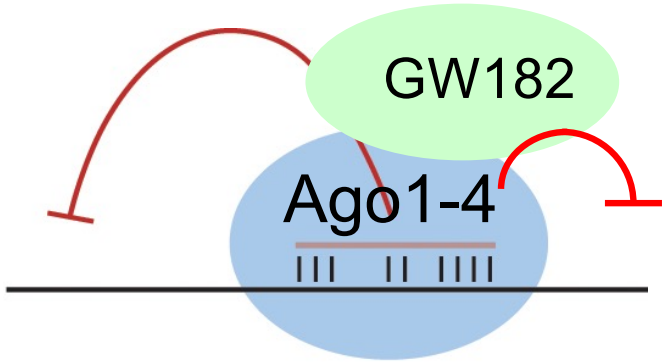


Figure 2. The Minimal miRNA Target Site

Different modes of repression by AGO/small RNA complexes



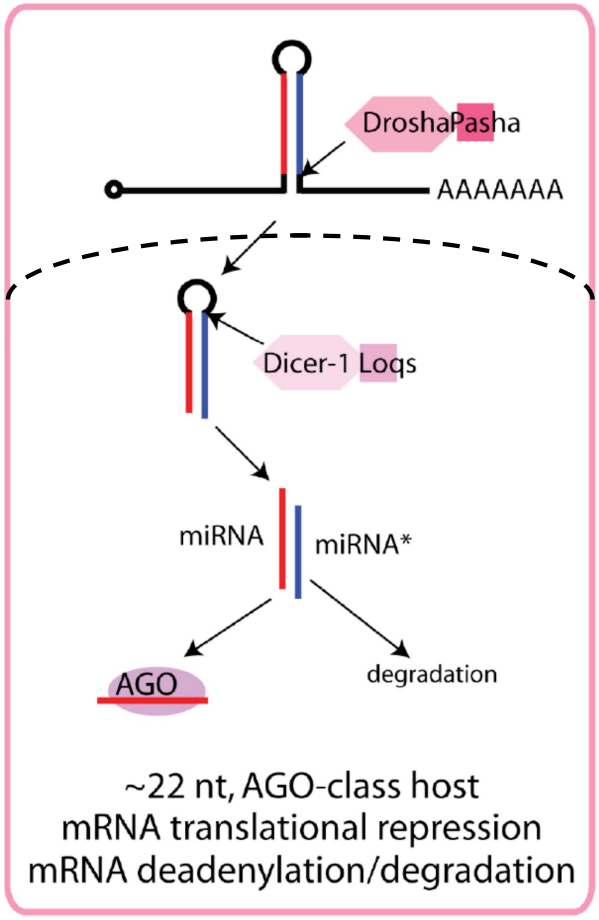
"siRNA"/Slicer complex:
target cleavage



"miRNA"/non-Slicer complex:
GW182 (TNRC6) is an essential cofactor
target mRNA deadenylation/degradation
translational inhibition
relocalization/storage?
etc.

- type of AGO loaded / extent of target complementarity determines regulation
  (4 AGOs in mammals, only Ago2 is a Slicer)

Canonical miRNA biogenesis pathway

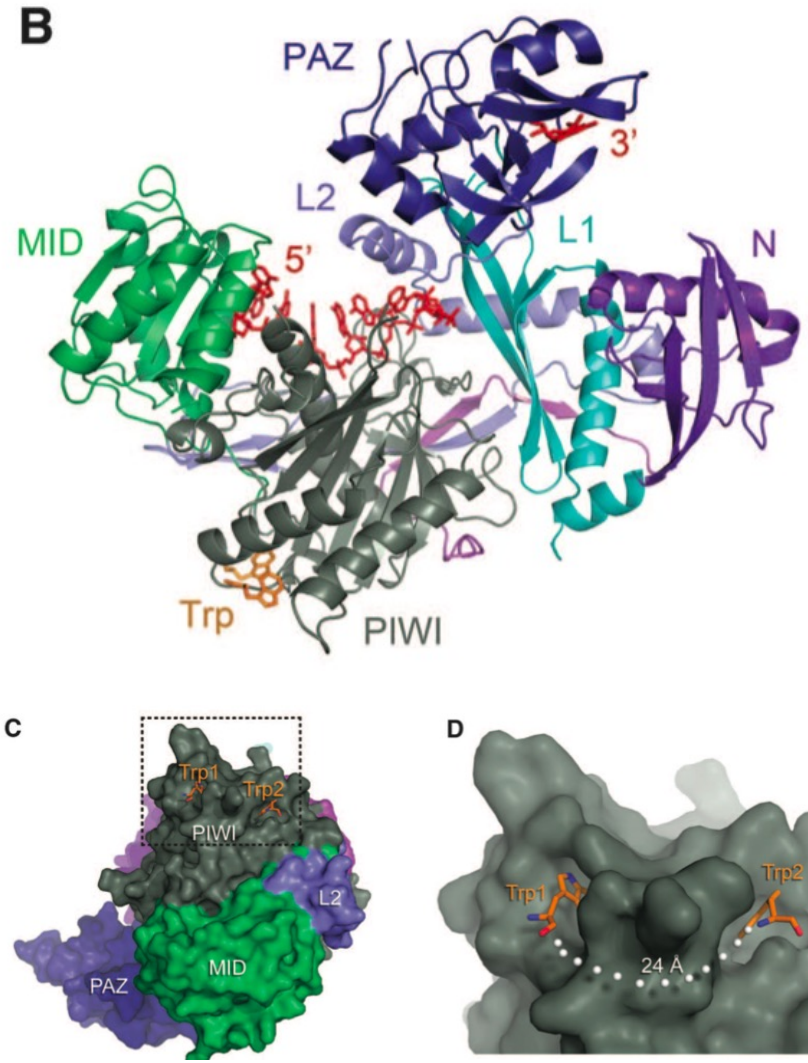


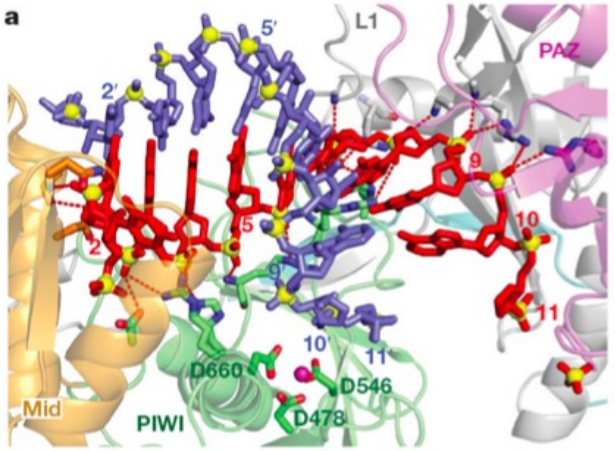
stepwise,
compartmentalized,
cleavage by
two RNase III
enzymes

open questions:

- there are many alternative ways to make miRNAs...
- there are many regulatory steps for miRNA biogenesis and function...

Structural biology of silencing





archeal and mammalian Ago:

- MID binds 5' end sRNA
- PAZ binds 3' end sRNA
- PIWI is RNase H-like domain

- seed (~nt 2-8) is splayed out and adopts a-helix with target

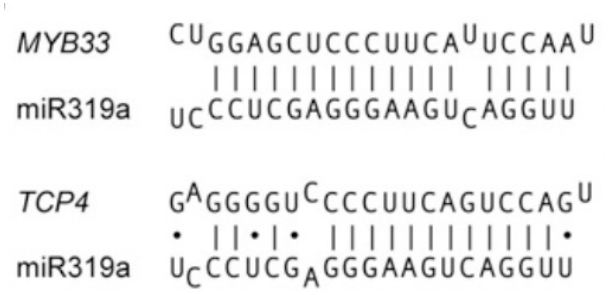
- human Ago2 has tandem hydrophobic W-binding pockets to dock GW182

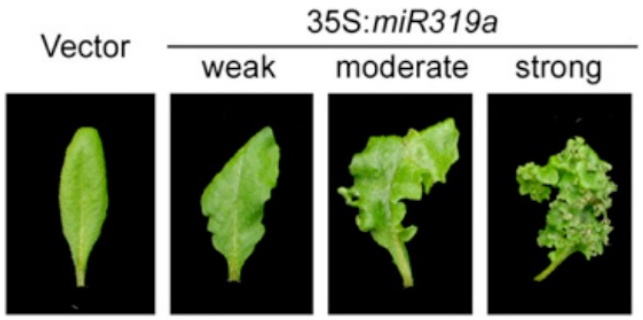
- Microprocessor docks to pri-miRNAs
- Dynamic Dicer structures

Patel, Joshua-Tor, Barford, Macrae, Kim, etc

miRNA biology: >1000 miRNAs known, but what do they do?

- plant miRNAs “easier” to study than animal miRNAs
  mostly highly complementary targets: mostly developmental TFs





- animal miRNAs “hard” to study since target sites as little as 7 nt

- best understood animal miRNAs came from forward genetics

- clear mutant phenotypes placed them into biological contexts
  (independent of their identity as miRNAs)

An example: a fly miRNA+target that controls female behavior

virgin

mated

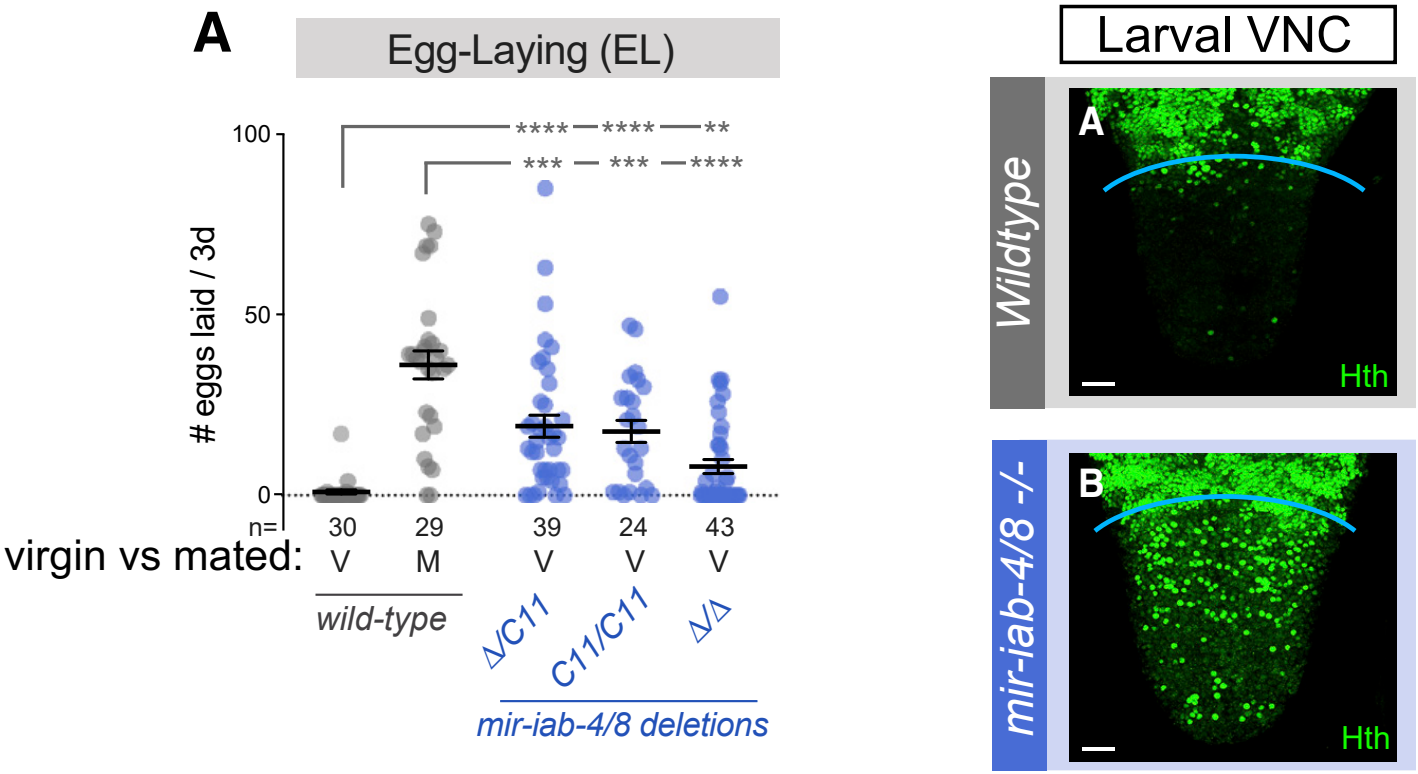
- receptive to males
- suppress egg-laying

- lay fertilized eggs
- change metabolism and behavior

Linking behavior with internal state

Garaulet Dev Cell 2020

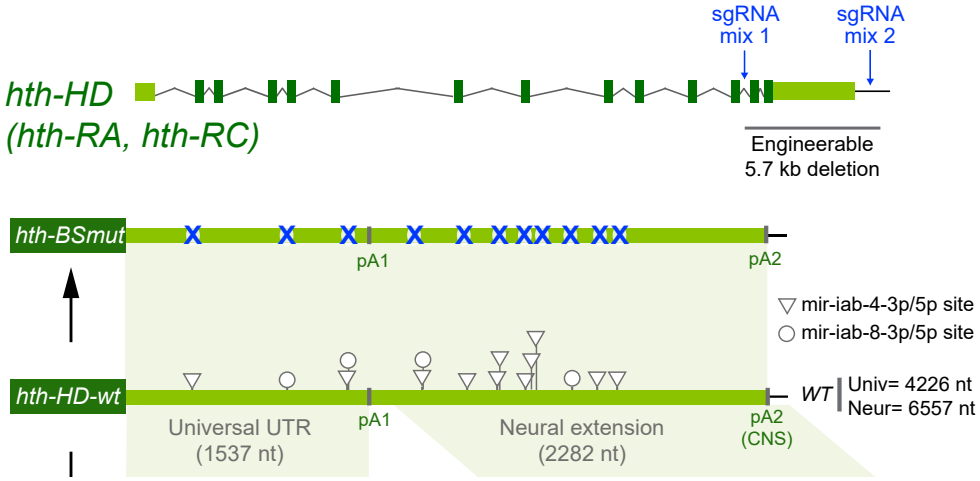
An example: a fly miRNA+target that controls female behavior

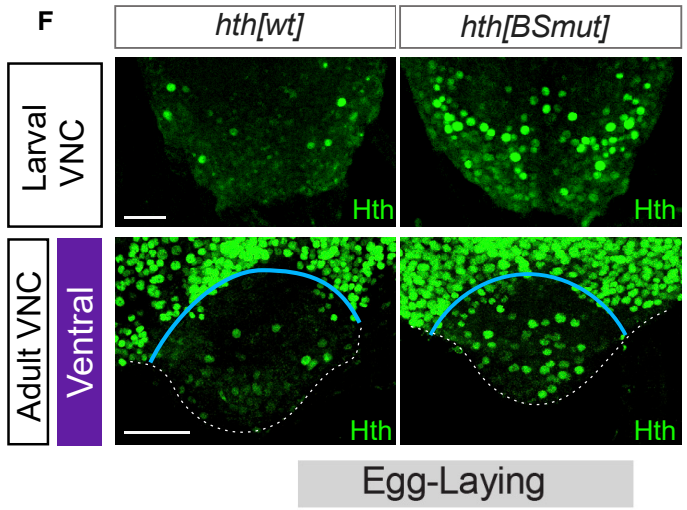


- knockout of *mir-iab-4/8* induces mated behaviors in virgin females
- knockout of *mir-iab-4/8* induces misexpression of Hth in the VNC

Garaulet Dev Cell 2020

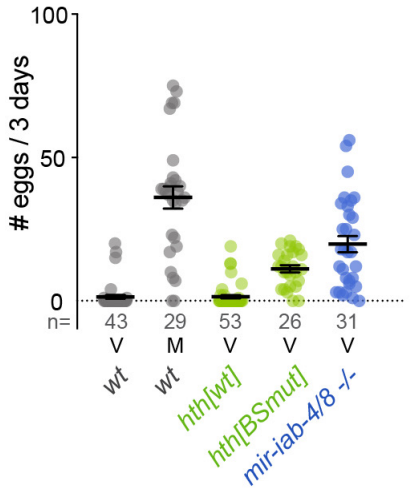
An example: a fly miRNA+target that controls female behavior





- engineerable platform for 3' UTR alleles
- point mutations in *hth* miRNA binding sites
  induce ectopic Hth in VNC
  and induce mated behaviors in virgin females,
  like Δ*mir-iab-4/8*

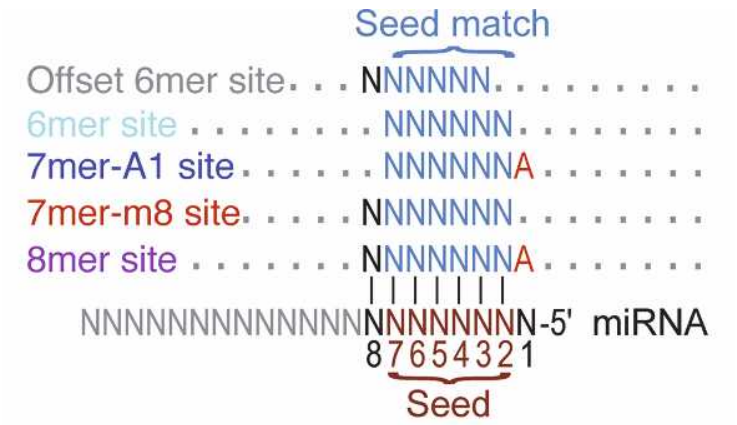
- *hth* is a demonstrable key target of miR-iab-4/8
  is it the only such target??

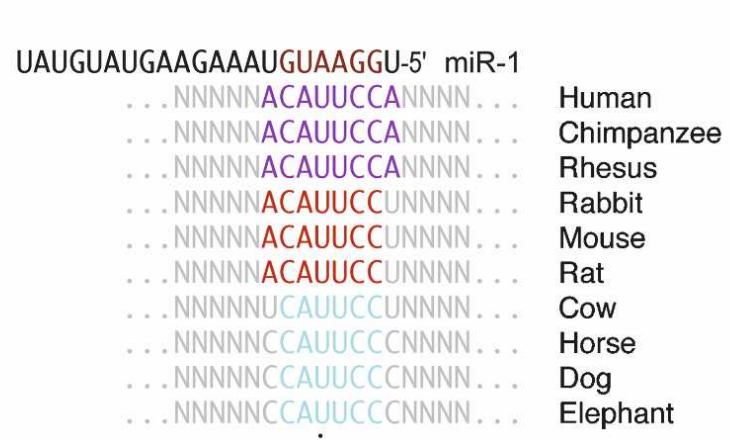


Garaulet Dev Cell 2020

Another viewpoint of animal miRNA function

- conserved seed-pairing provides evidence for biologically constrained miRNA:target interactions





- computational evidence for many (100s) targets per miRNA

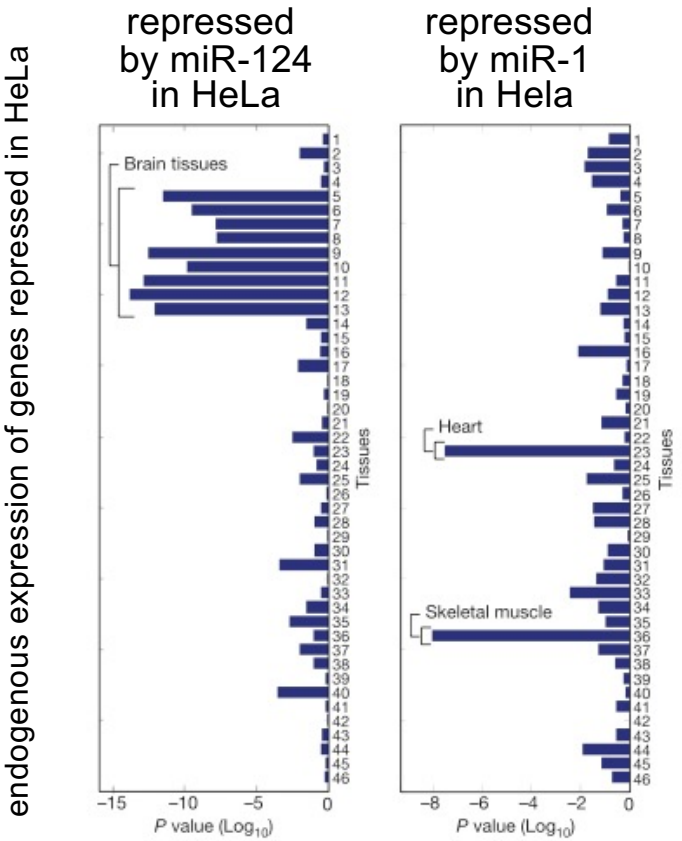
- 10,000s of conserved target sites in most human genes, overall covering most biological processes

Friedman Genome Res 2009

Why so many targets for miRNAs?

experiment: transfect a muscle specific and a brain specific miRNA in HeLa cells, monitor cellular response by microarrays

100-200 genes went down--most had seed matches in their 3' UTRs



• genes targeted by a brain (miR-124) miRNA and a muscle (miR-1) miRNA, are normally expressed at lowest levels in brain and muscle, respectively

Lim Nature 2005

genetically, best understood miRNAs have a “few” important targets

computationally, most animal miRNAs have “lots” of targets

remains to be seen whether target lists represent
100s of equally (but perhaps mildly) important targets,
or a few very important targets + many subtle targets

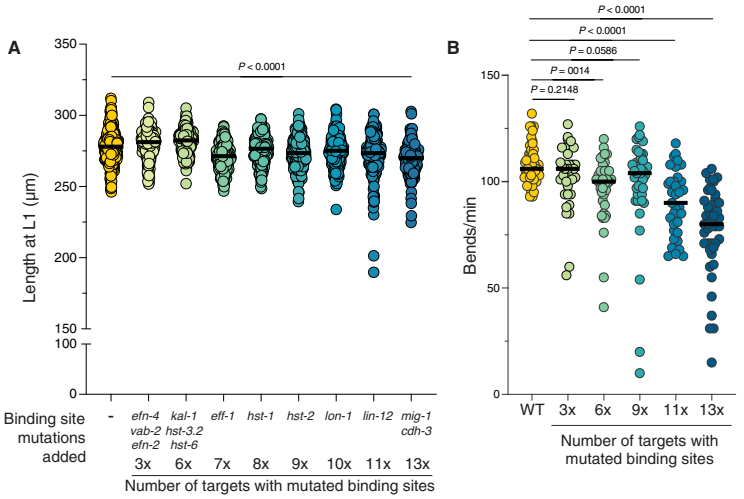
• specific mutation of endogenous miRNA sites is needed
to demonstrate causality of miRNA targeting to phenotype

In vivo CRISPR screening for phenotypic targets of the *mir-35-42* family in *C. elegans*

Bing Yang,[1] Matthew Schwartz,[2] and Katherine McJunkin[1]

An ancient and essential miRNA family controls cellular interaction pathways in *C. elegans*

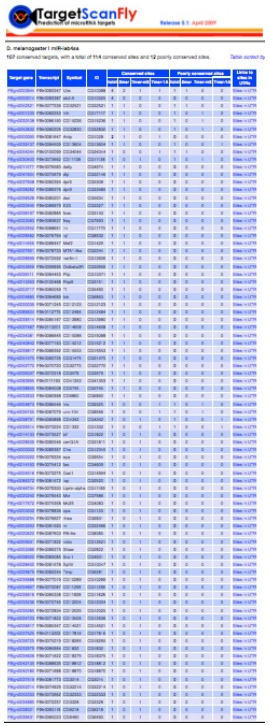
Emilio M. Santillan[1], Eric D. Cormack[1], Jingkui Wang[2], Micaela Rodriguez-Steube[1], Luisa Cochella[1]*



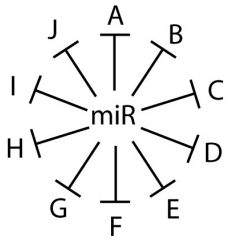
13-way CRISPR mutant animal!

miRNAs mediate their function via target gene regulation

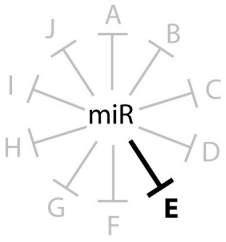
- there are “lots” of conserved targets (100s/miRNA)
- but do they all make equal contribution?
- are some more "important" for phenotype than others?
- how substantial is miRNA regulation for organismal phenotype?



A. Many targets regulated

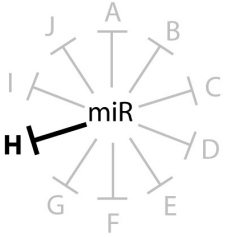


B. A major phenotypic target

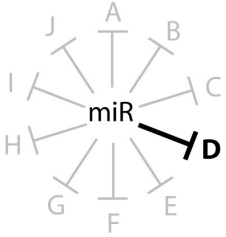


C. Different critical targets in different contexts

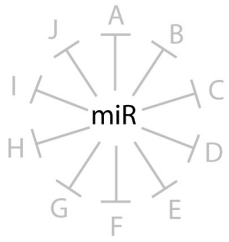
Tissue 1



Tissue 2



D. miRNA is not phenotypically required (in laboratory?)



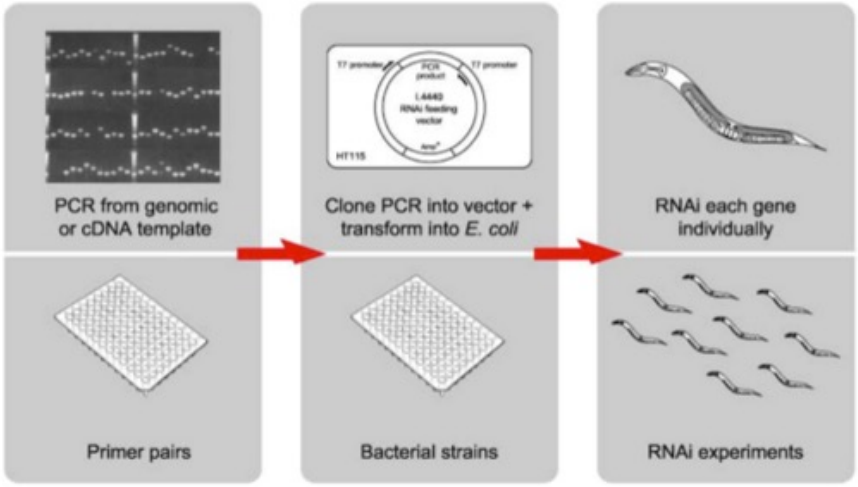
Applications of RNAi:

- on-demand loss-of-function in diverse species
- genomewide reverse/forward genetics
- custom-designed organisms, human therapies

High-throughput, systematic RNAi screening

- in invertebrates, long dsRNA can be effectively used to initiate RNAi
  each dsRNA is converted into many kinds of siRNAs
  -important to make dsRNA to nonconserved domains of genes
  (untranslated regions, gene-specific regions)

- libraries of dsRNA/inverted repeat constructs screenable in worms and flies



genomewide feeding RNAi screens in worms

- multiple genomewide transgenic RNAi collections in flies

RNAi screening in vertebrate/mammalian systems?

long dsRNA in mammals activates the interferon pathway in vivo

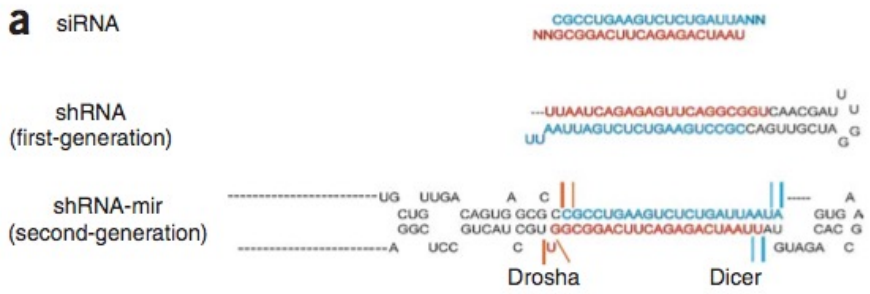
sequence independent RNA destruction/global reduction of translation

can be circumvented by directly using siRNAs

- full siRNA libraries now available for screening mouse and human using transient transfection

Second generation shRNA-mir vectors

endogenous processing using pre-mir backbone, new energy rules



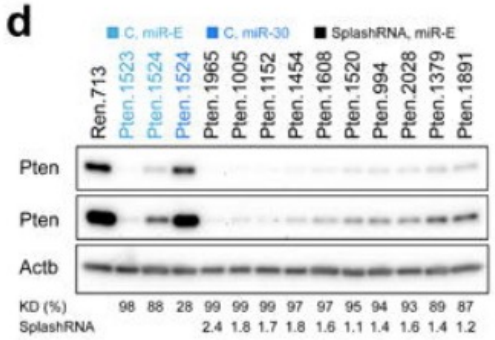
Silva Nature Genetics 2005

- genomewide shRNA-mir plasmids for human and mouse

Third generation shRNA-mir vector

optimized miRNA backbone/prediction classifers for effective single-copy RNAi

- need to validate multiple shRNAs give same phenotype (rule out “off-target”)

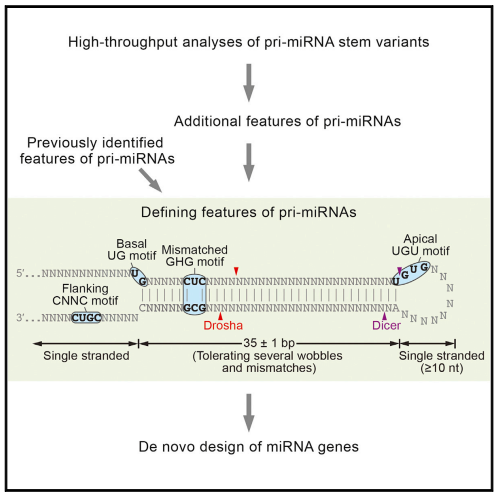


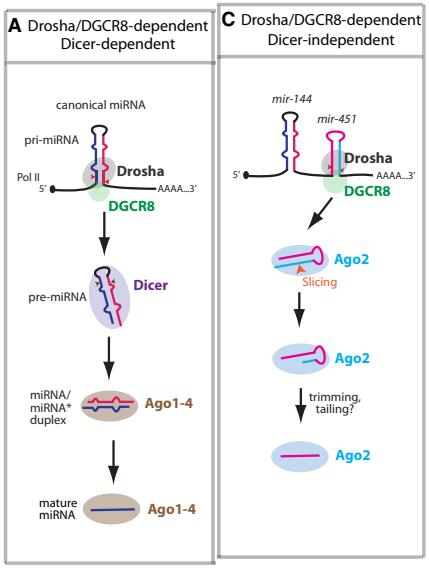
Fellman Cell Reports 2013
Pelossof Nature Biotech 2017
http://splashrna.mskcc.org

Do we know everything about miRNA biogenesis, or still need more mechanistic studies?

The Menu of Features that Define Primary MicroRNAs and Enable De Novo Design of MicroRNA Genes

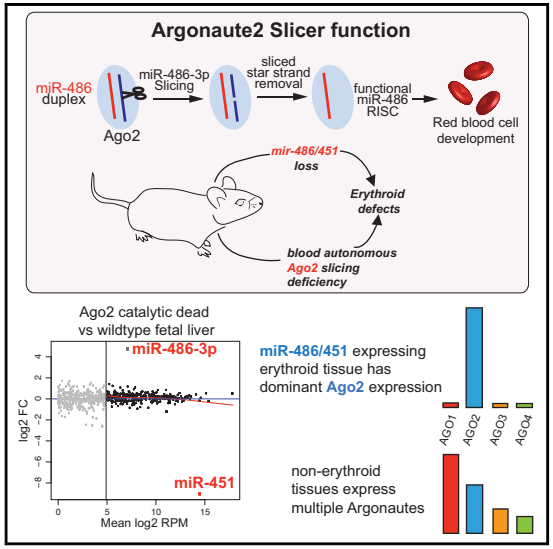
Fang and Bartel 2015





Dual Strategies for Argonaute2-Mediated Biogenesis of Erythroid miRNAs Underlie Conserved Requirements for Slicing in Mammals

Jee and Lai, 2018



Functional parameters of Dicer-independent microRNA biogenesis

Yang and Lai 2012

• improved miRNA biogenesis • requirement for Slicer dependency
• reduce off-target silencing • elimination of miRNA* activity

RNAi genetics: some advantages and disadvantages

(+) systematic reverse genetics
directed screening, know identity of each screened target
(+) synthetic screening, pooled screening possible for complex interactions
(+) powerful for both cell lines and animal settings
(+) (can be) fast, (can be) automated
(+) conditional/reversible (animal models)

(-) partial knockdown
(-) simple knockdowns can fail to reveal specific functions
of broadly used genes (temp sens, gof, domain specific alleles)
(-) poor targeting of proteins with long half-lives
(-) specificity issues, off-target effects
(-) can have limited possibilities for phenotypic assays
(-) still often need to make a mutant (CRISPR/Cas9)

Recently, the first 7 FDA approved RNAi drugs

Aug 2018: patisiran, transthyretin-mediated amyloidosis
Nov 2019: givosiran, for acute hepatic porphyria
Nov 2020: lumasiran, for primary hyperoxaluria type 1
Dec 2021: inclisiran, lowers "bad" cholesterol
July 2022: vutrisiran, 2nd generation hATTR drug
Sept 2023: nedosiran, also for primary hyperoxaluria type 1
Mar 2025: fitusiran, for hemophilia

GIVLAARI®
(givosiran) injection for subcutaneous use
189 mg/mL

LEQVIO®
(inclisiran) injection
284 mg/1.5 mL

these siRNAs target liver or kidney-expressed genes
ongoing challenges: delivery, potency, on-target activity

others submitted for FDA approval or in Phase 3 clinical trials

- improved siRNA function (Lee, *Science* 2024)

Seungjae Lee

Summary

- serendipitous discovery of negative regulatory activity of dsRNA
- RNAi is an ancient mechanism degrade transcripts homologous to dsRNA "triggers", likely as a genome defense
- miRNAs are an adaption of the RNAi pathway to control endogenous genes, may have emerged several times in evolution
- RNAi as a powerful reverse genetic tool and a means to control gene expression, even for human benefit/convenience
- full exploitation of RNAi and miRNAs requires attention to basic mechanisms, endogenous biology, and creativity for new uses

interested in small RNA genomics, regulation, biology?
surprising molecular mechanisms, RNAi therapeutics, impacts on disease/cancer
happy to discuss...
laie@mskcc.org