Small RNAs in Regulation of Eukaryotic Gene Expression

1. Silencing by RNA interference (RNAi)
    - Historical context
    - Molecular pathway (common elements w/ miRNA)
    - Outcomes and functions

2. Regulation by micro RNA (miRNA)
    - Historical context
    - Molecular pathway for miRNA production
    - Outcomes and functions

Discovery of miRNAs in *C. elegans*

- Ambros and colleagues (1993)
- *lin-4* shown to repress *lin-14*, and dependent on sequence in 3'-UTR of *lin-14*
- Subsequent analysis showed that *lin-4* produced a non-coding RNA with complementarity to the critical region in *lin-14*
- Repression in L2 developmental stage was shown to be independent of degradation of the mRNA or even changes in polysome profile
- Now known to be at least 400 miRNAs in humans

Two Key Papers

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*

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Ambros and Horvitz, 1987). Animals carrying a *lin-4* loss-of-function (*lf*) mutation, *lin-4(e912),* display reiterations of early fates at inappropriately late developmental stages; cell lineage patterns normally specific for the L1 are reiterated at later stages, and the animals execute extra larval

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

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Massachusetts General Hospital
Boston, Massachusetts 02114

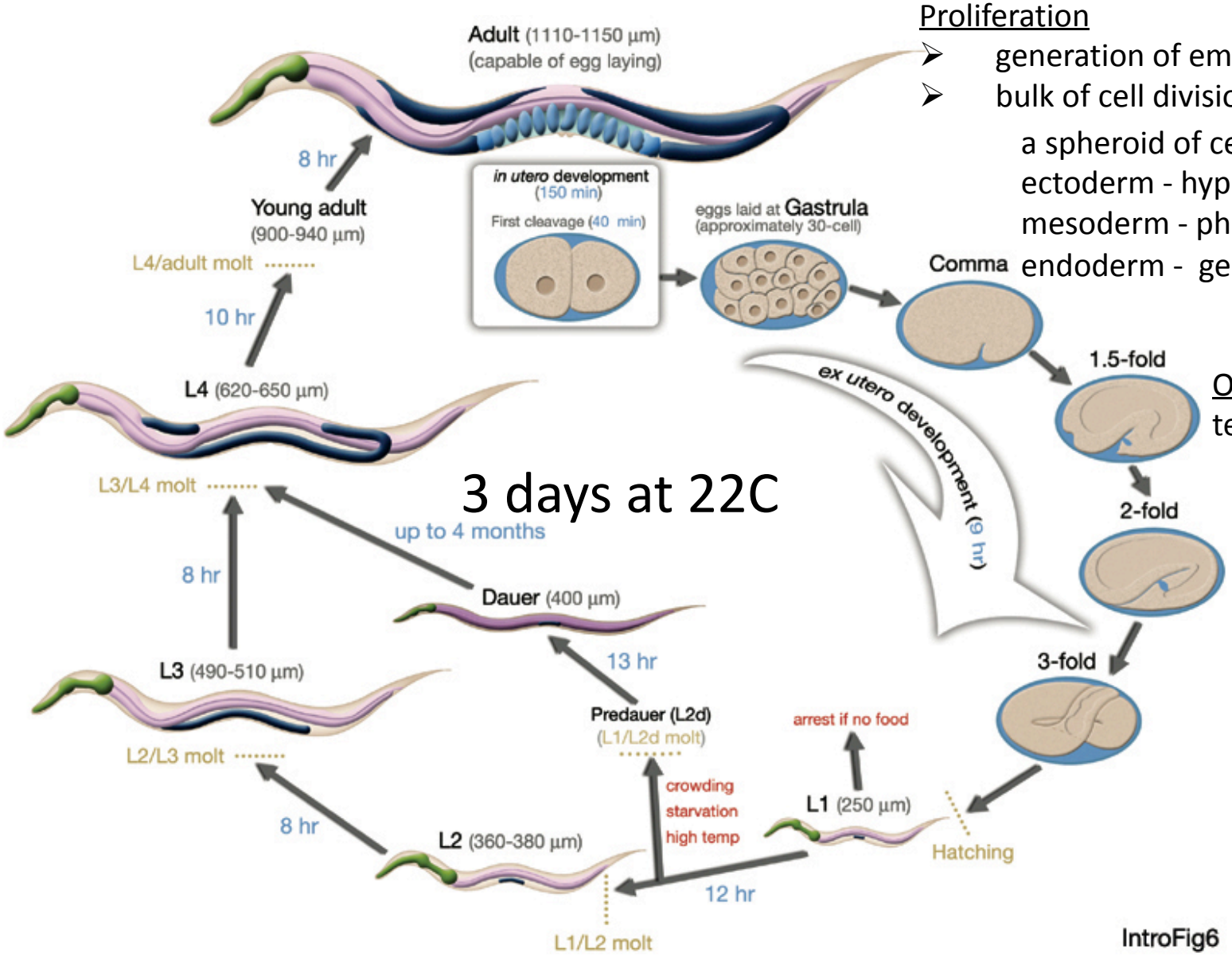
site phenotypes (Ambros and Horvitz, 1987). *lin-14(lf)* alleles cause larvae stage 2 (L2) patterns of cell lineage in a variety of tissues to be executed precociously during the L1 stage (Ambros and Horvitz, 1987). Two *lin-14(gf)*

C. (Caenorhabditus) elegans

1st introduced and used by Sydney Brenner (1963)
to study development and neurology.

- Adults are ~1mm long (small).
- They can be grown on agar plates with lawn of bacteria.
- They have a short generation time- 3 days from egg-laying to adulthood, brood size > 300.
- They are transparent, so internal anatomy can be easily observed.

Life cycle of *Caenorhabditis elegans*



Proliferation

- generation of embryonic founder cells
- bulk of cell divisions and gastrulation.

a spheroid of cells:
ectoderm - hypodermis and neurons,
mesoderm - pharynx and muscle,
endoderm - germline and intestine.

Organogenesis
terminal differentiation

http://www.wormatlas.org

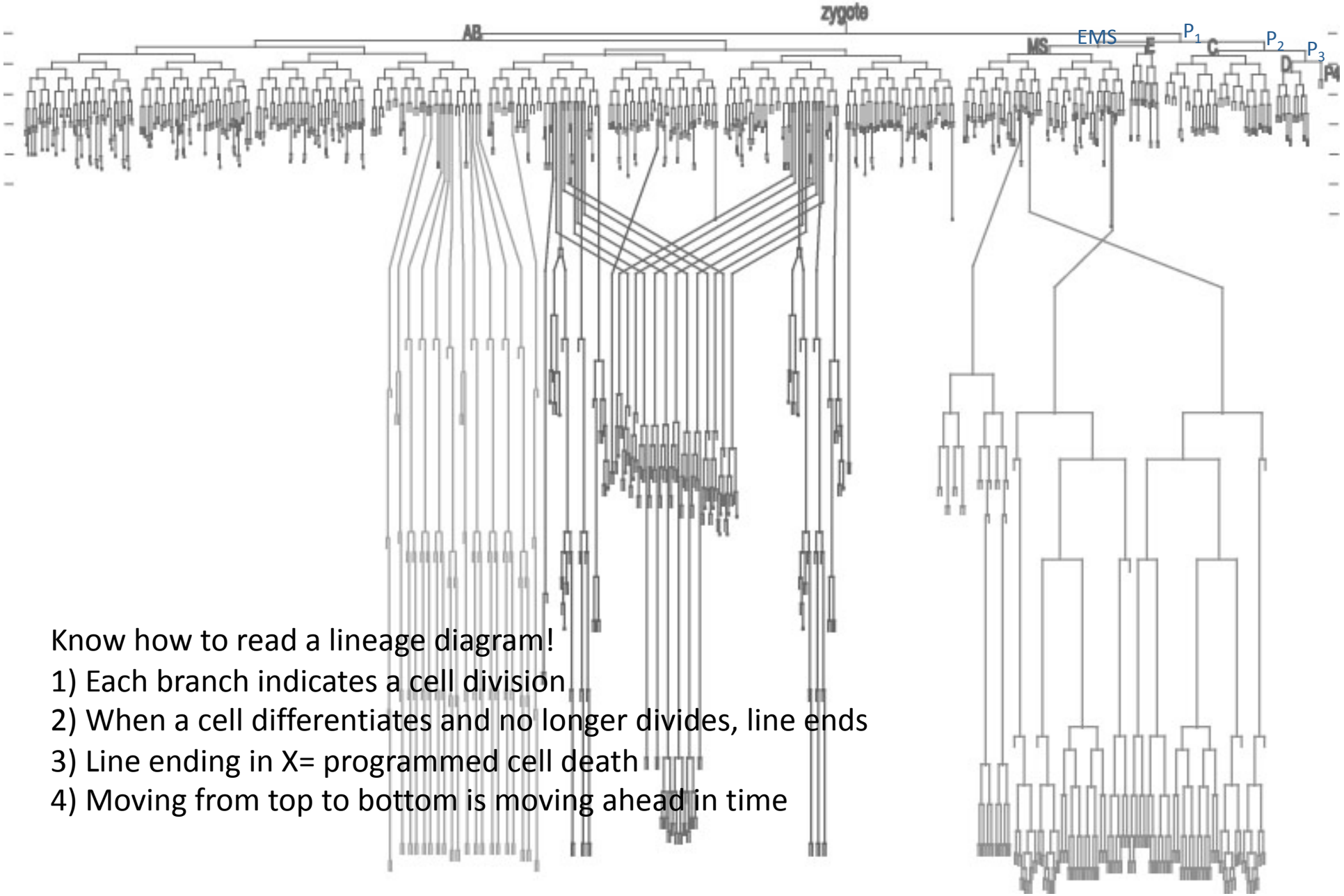
C. elegans cell lineage

- The most important and incredible advantage of worms is that every worm has the exact same number of somatic cells!

- Hatching larva=558 cells
- Males (XO)=1031 somatic cells+ ~1000 sperm
- Hermaphrodite (XX)= 959 somatic cells+ ~2000 eggs and sperm.

- Somatic cells arise by an INVARIANT cell lineage. The divisions are invariant in pattern, timing, and orientation of each division.

Here it is: *C. elegans* cell lineage



Know how to read a lineage diagram!

1) Each branch indicates a cell division
2) When a cell differentiates and no longer divides, line ends
3) Line ending in X= programmed cell death
4) Moving from top to bottom is moving ahead in time

Genes that affect timing and Discovery of microRNAs (miRNAs).

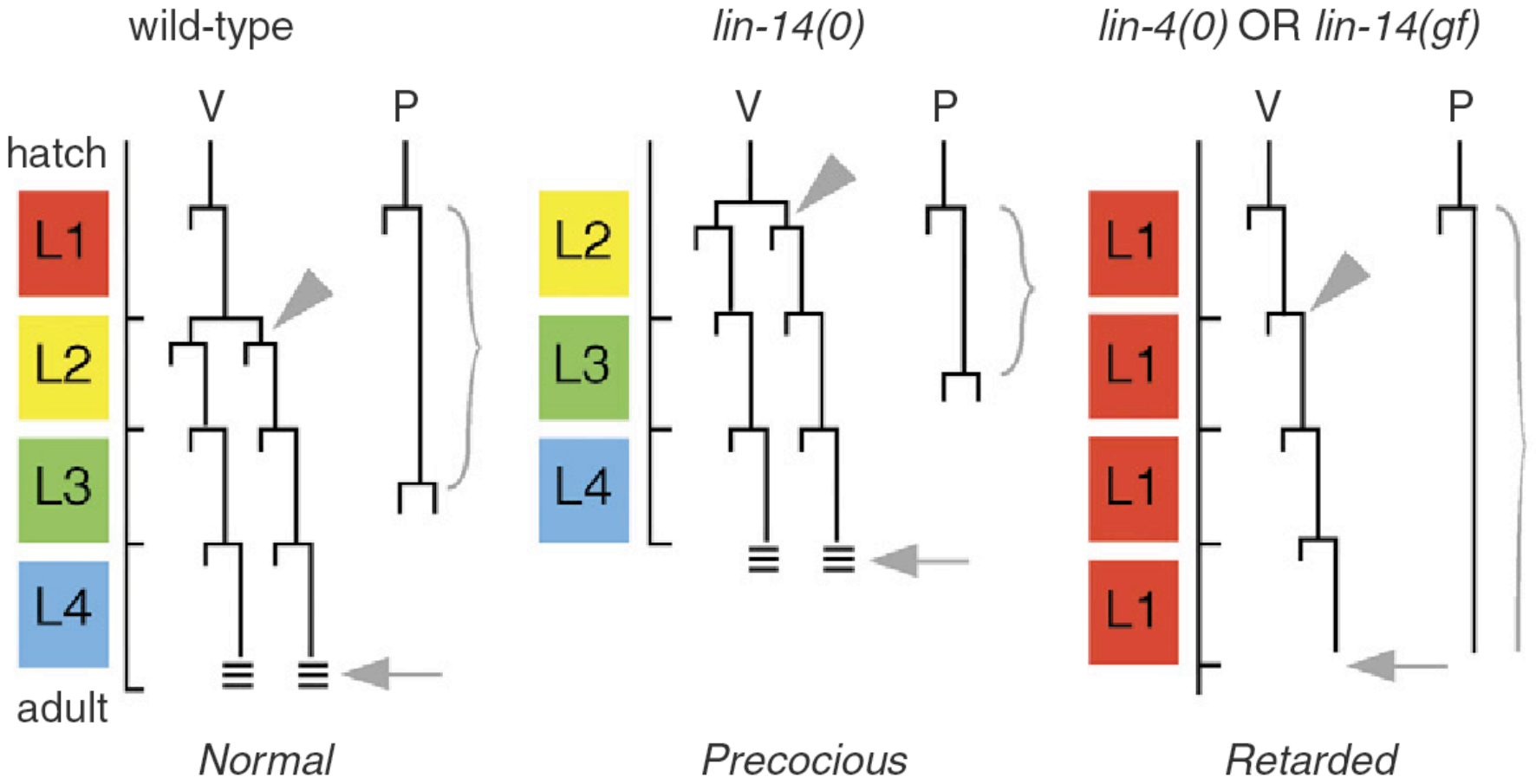
- The definition of **heterochrony** is a change in the relative timing of developmental events. Each scale of development, the cycle of cell divisions, the growth of tissues, the formation of organs, requires proper timing.

- Two general phenotypes are seen in heterochronic mutants —
  - 'precocious,' in which developmental events are skipped,
  - 'retarded,' in which they are repeated.

- A heterochronic mutation may affect different tissues: intestine, epidermis, muscle, and neurons, and
- different kinds of developmental events: a pattern of cell division, a cell cycle lengths, and differentiation.

An example: lin-4 and lin-14 mutants

Hypodermal cell lineage



Moss E. 2007. Current Biology, R425.

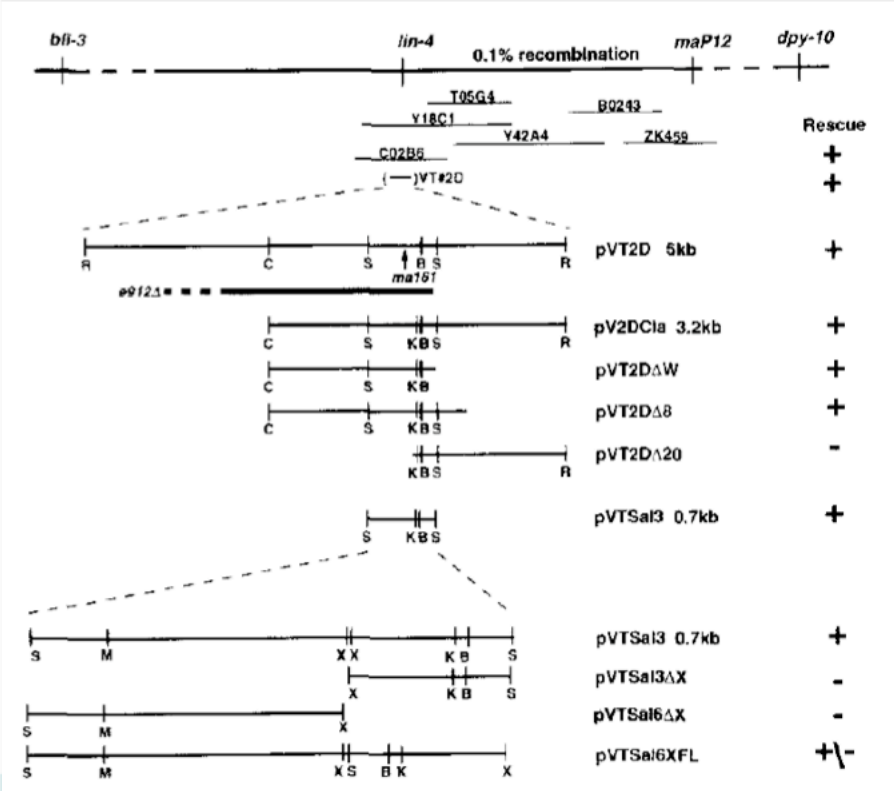
Lin-4 regulates transition from L1 to L2 stage.
Lin-14 gene is a regulator of transcription.

Lin 4 and Lin 14 alleles

Lin 4: Deletion in region LG2
Identity unknown

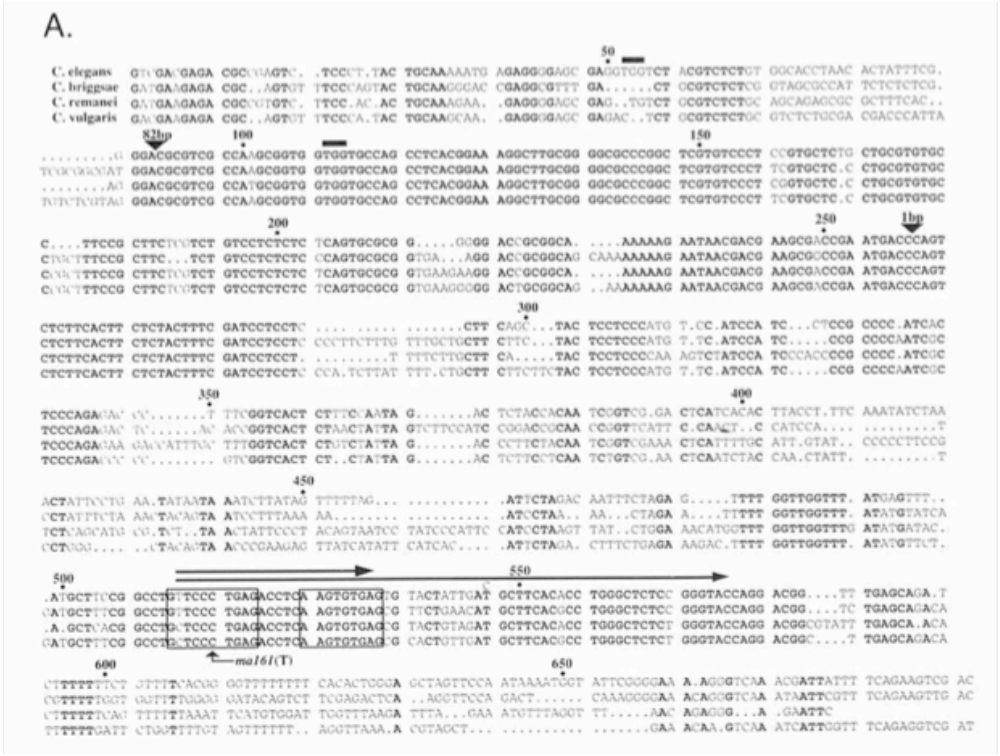
Lin 14: Abnormally high level of lin 14
Mutations in the 3'UTR of lin 14

Positional cloning of lin 4



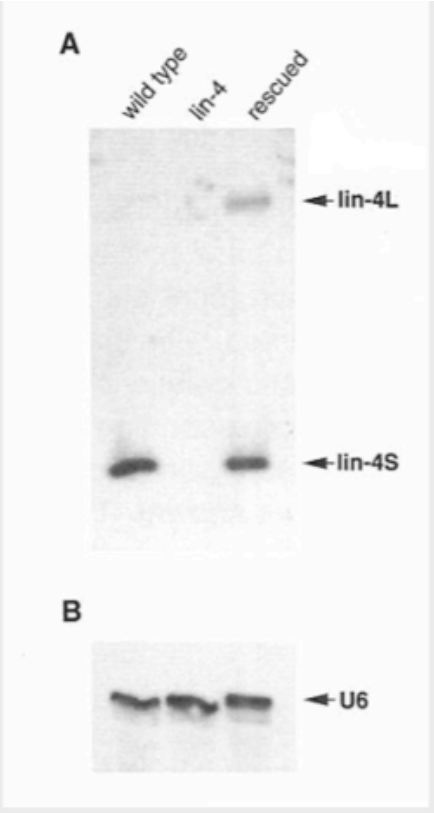
(Lee et al 1993)

Lin 4 is a non coding locus

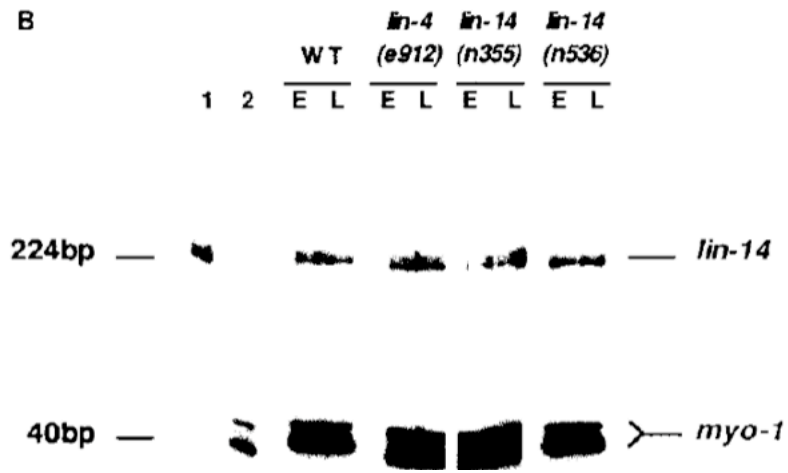


(Lee et al 1993)

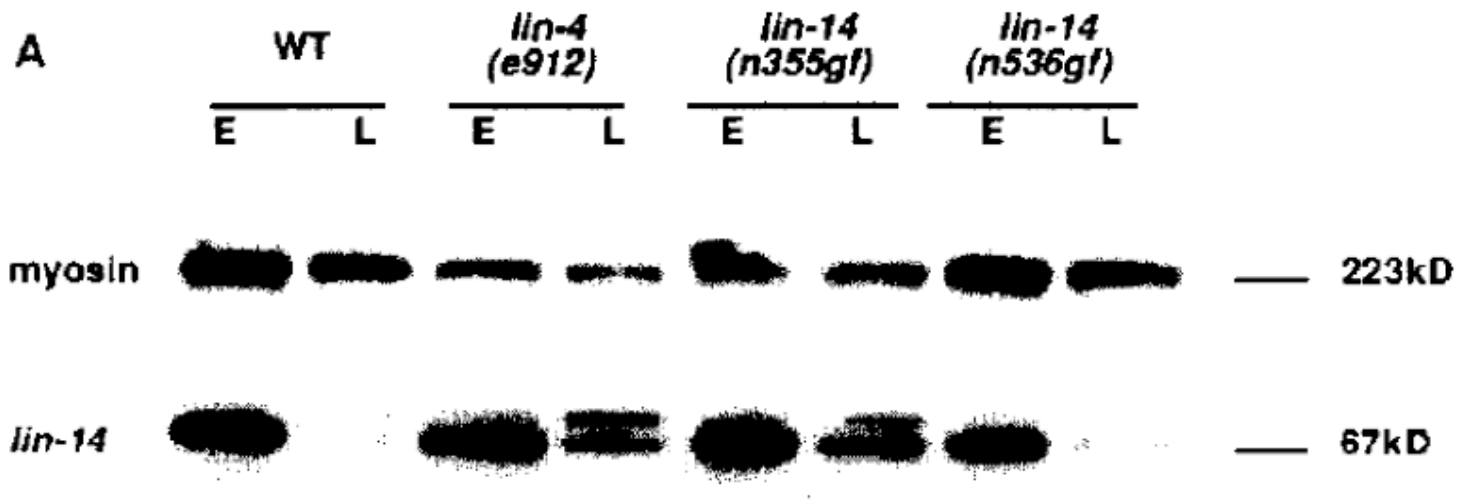
Lin 4 encodes small RNAs



lin-4 does not alter lin-14 mRNA levels

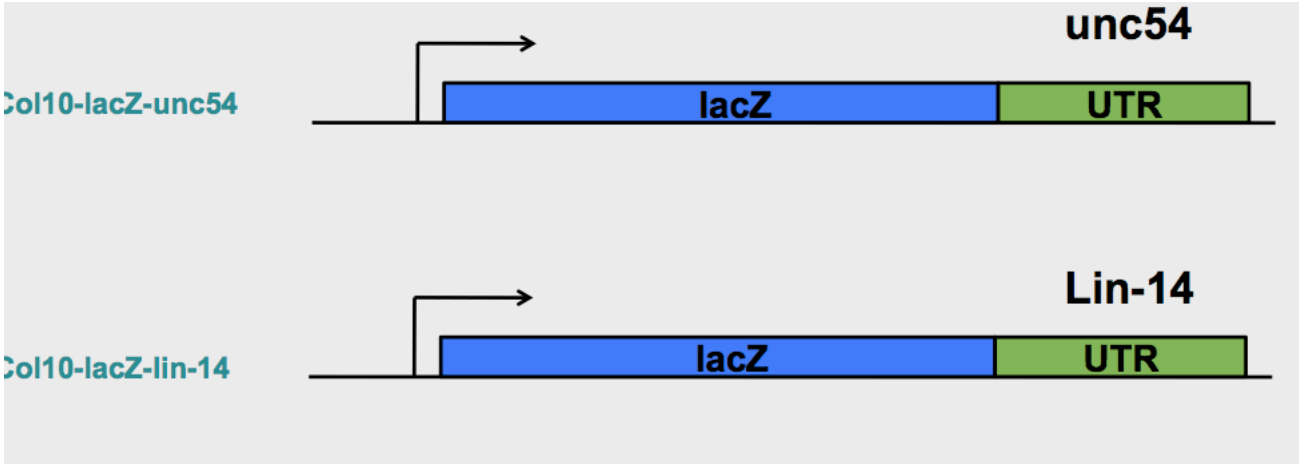


lin-4 alters lin-14 protein levels

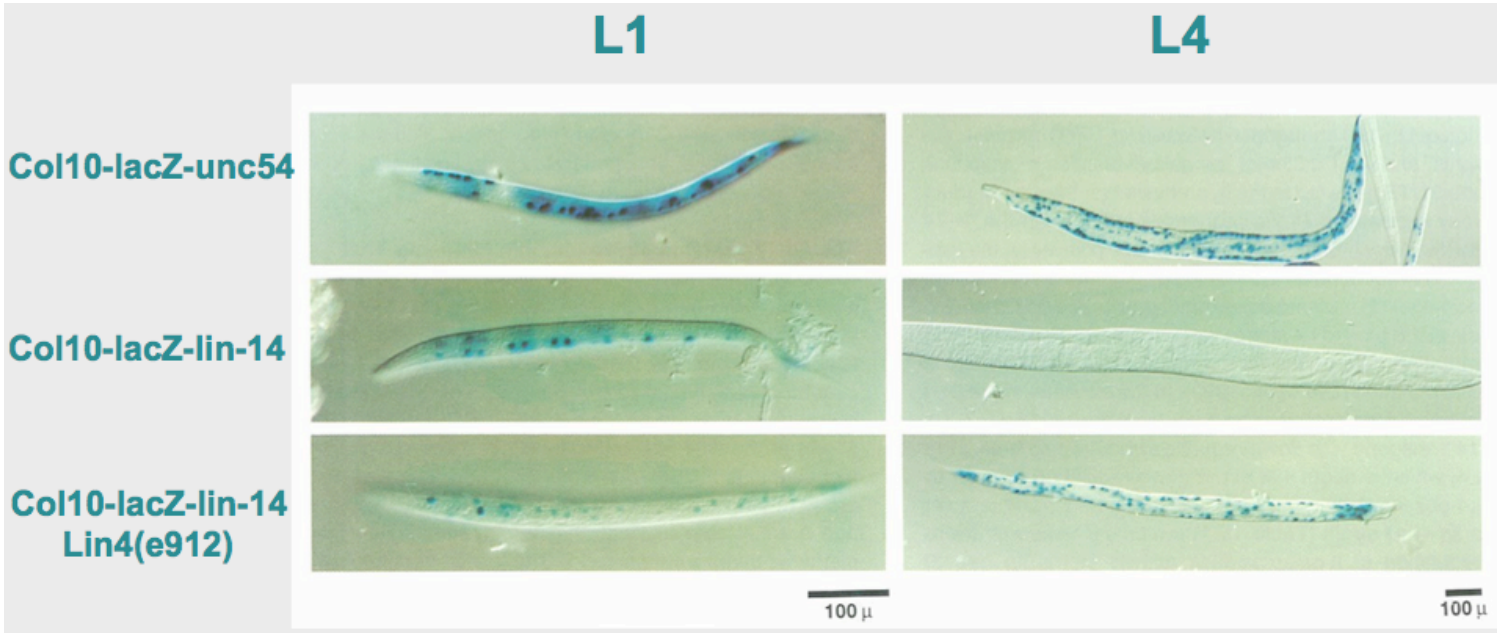


lin-14, encodes a novel nuclear protein and is a putative transcription factor.

Reporter constructs

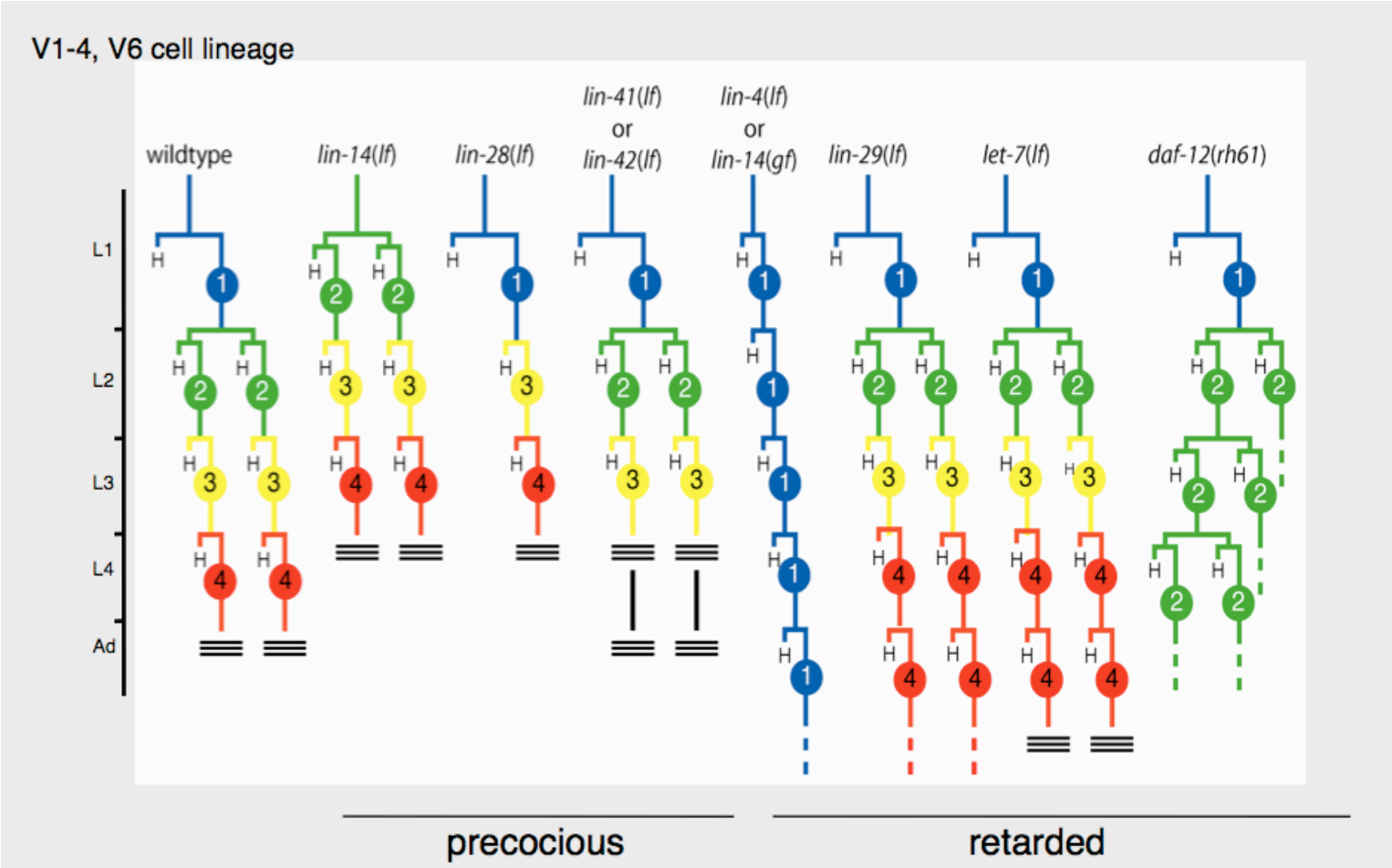


The lin-14 UTR confers temporal regulation to a reporter gene in a lin-4 dependent manner

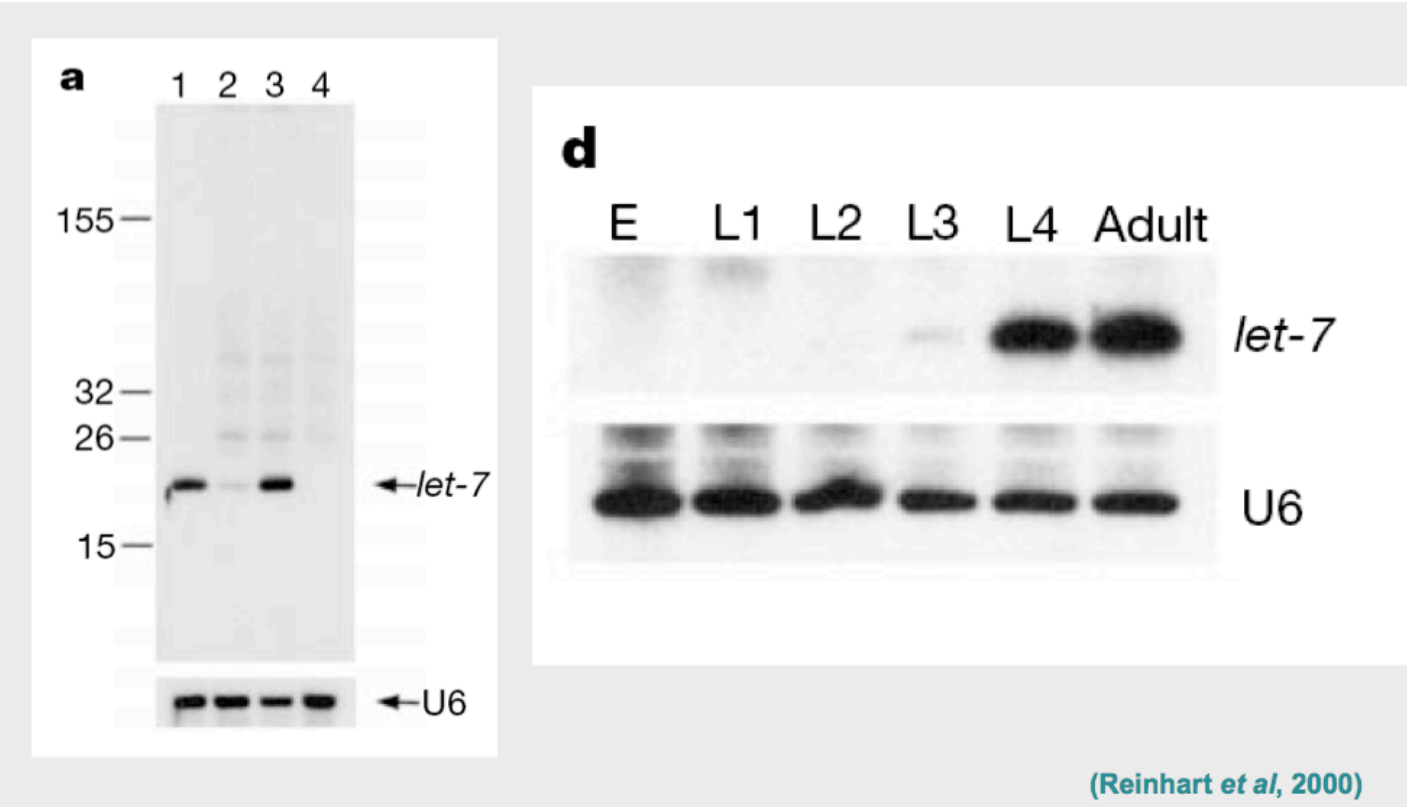


Silencing was conserved
Lin4 was termed short-temporal RNA (stRNA)

let-7 a second stRNA in C. elegans

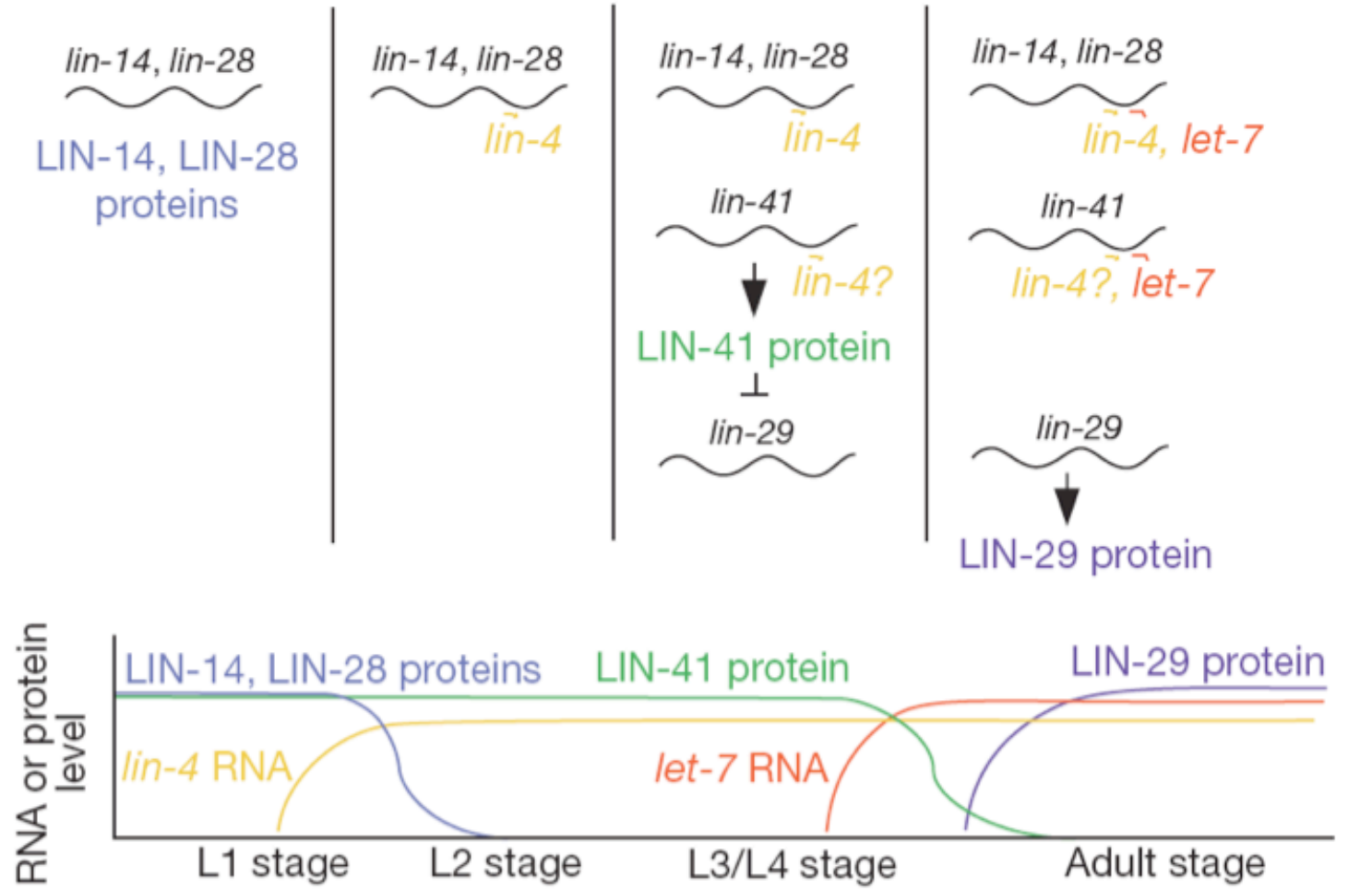


let -7 is an stRNA

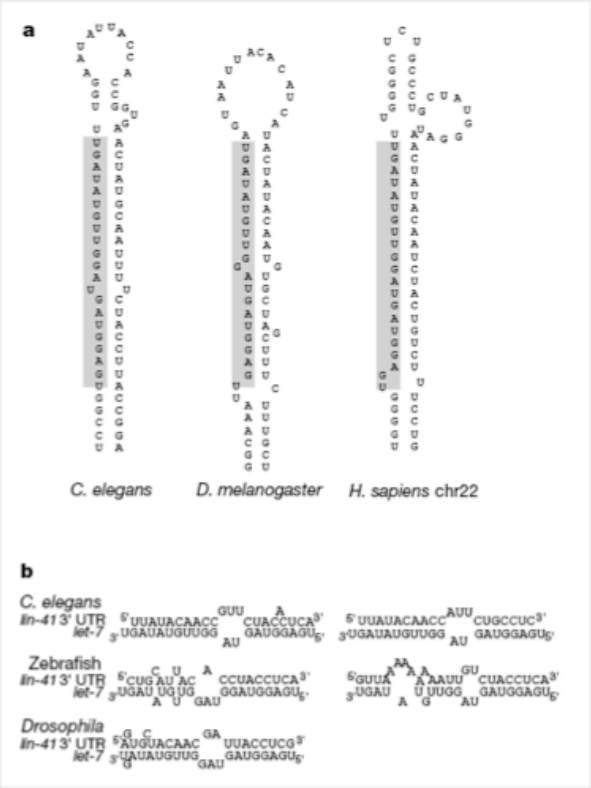


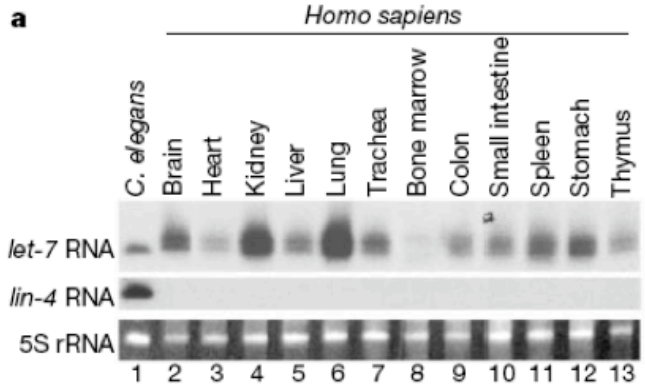
(Reinhart *et al*, 2000)

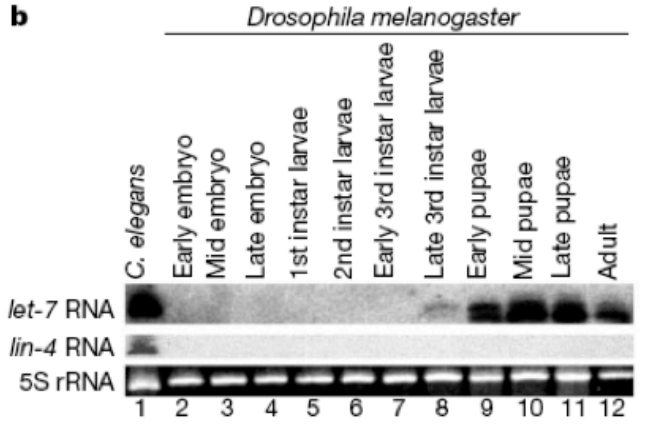
st-RNA and timing of C elegans development



Let7 is conserved

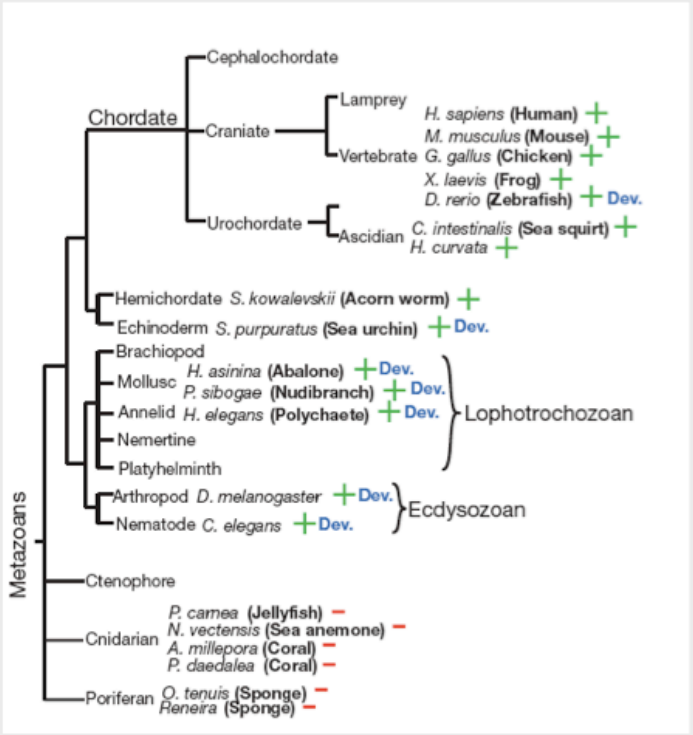






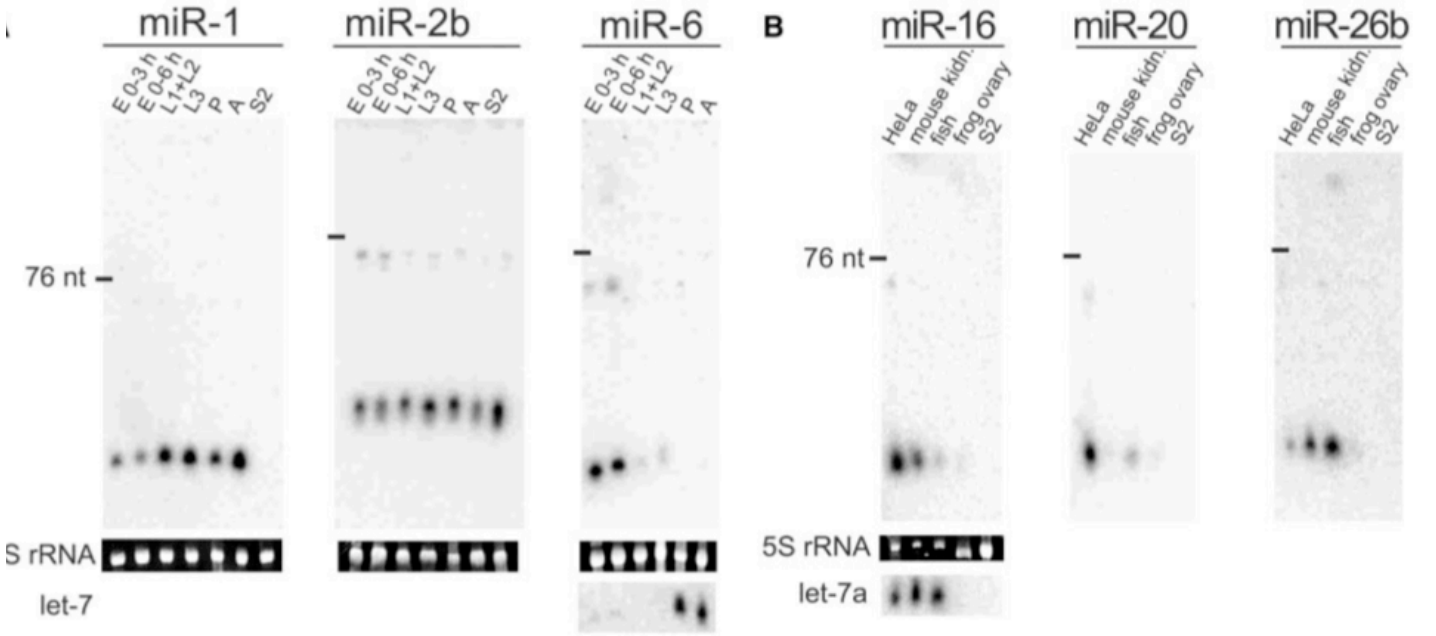
(Pasquinelli *et al*, 2000)

Let7 is highly conserved



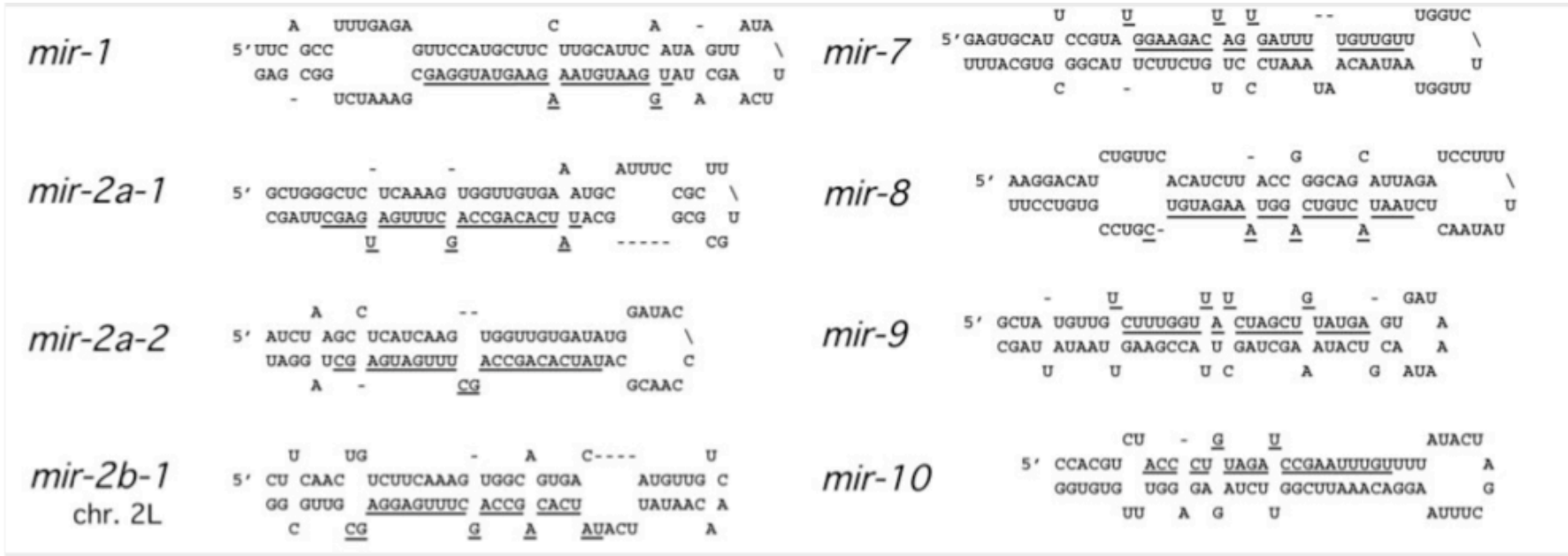
(Pasquinelli *et al*, 2000)

Many genes encode small 21 nt RNAs



microRNAs (mirRNAs) = st-RNAs (Lagos-Quintana *et al*, 2000)

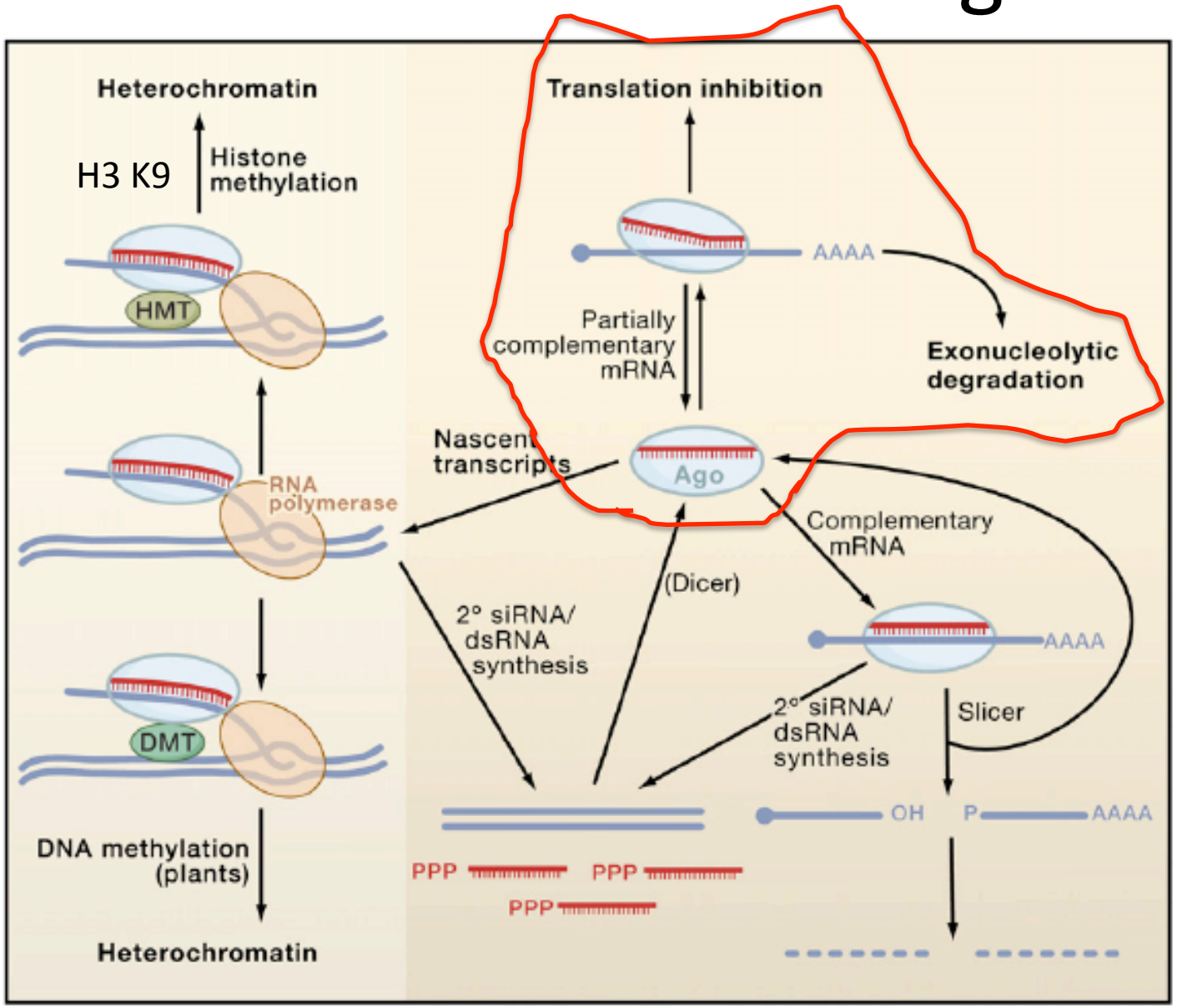
Predicted miRNA precursors



Mechanisms of siRNA Silencing

- Imperfect base-pairing between guide strand and target can give non-degradative silencing (miRNA pathway) with translation inhibition and/or exonucleolytic degration

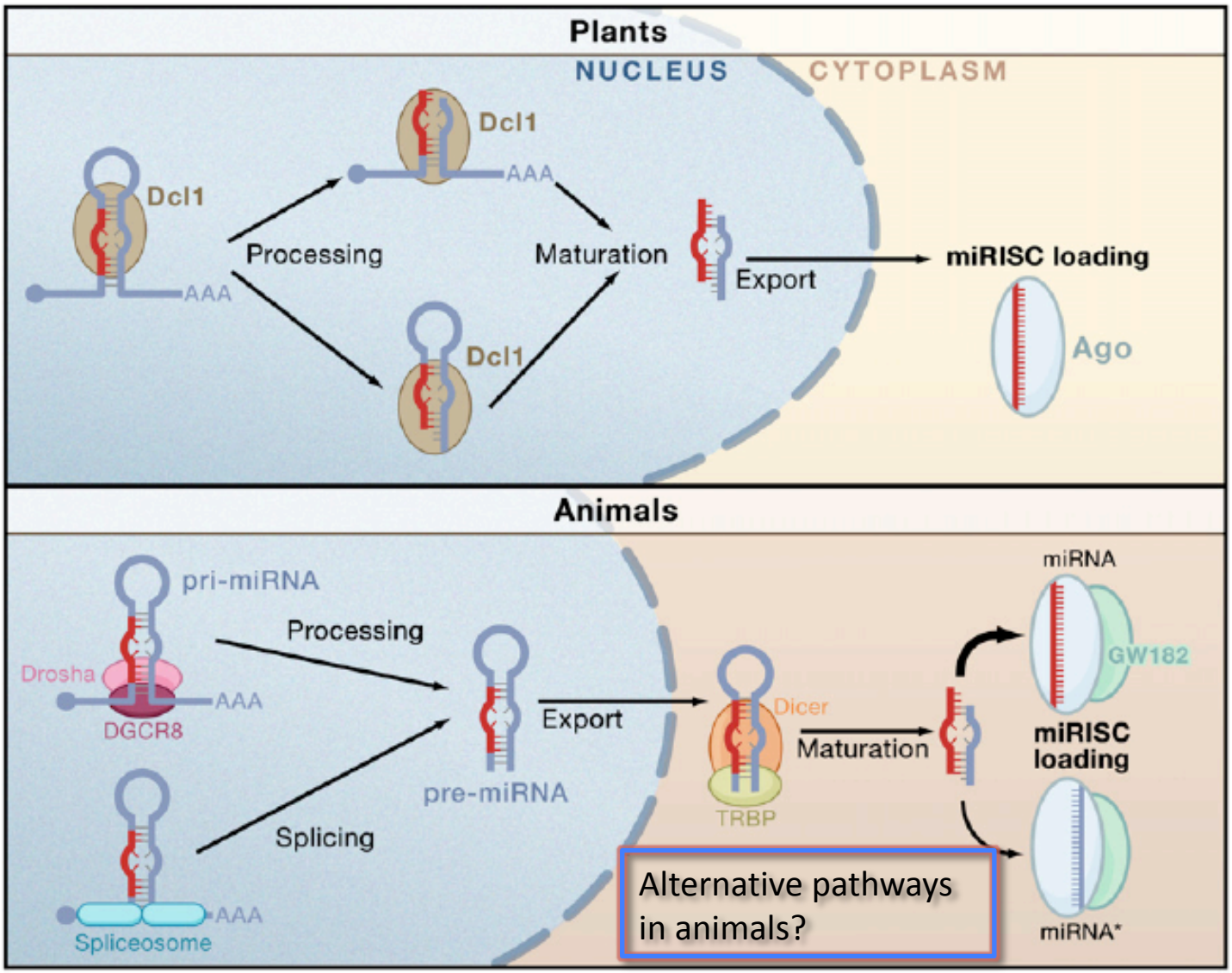
(next lesson)



Carthew and Sontheimer, Cell (2009) 136, 642-655.

miRNA Biogenesis and RISC Assembly

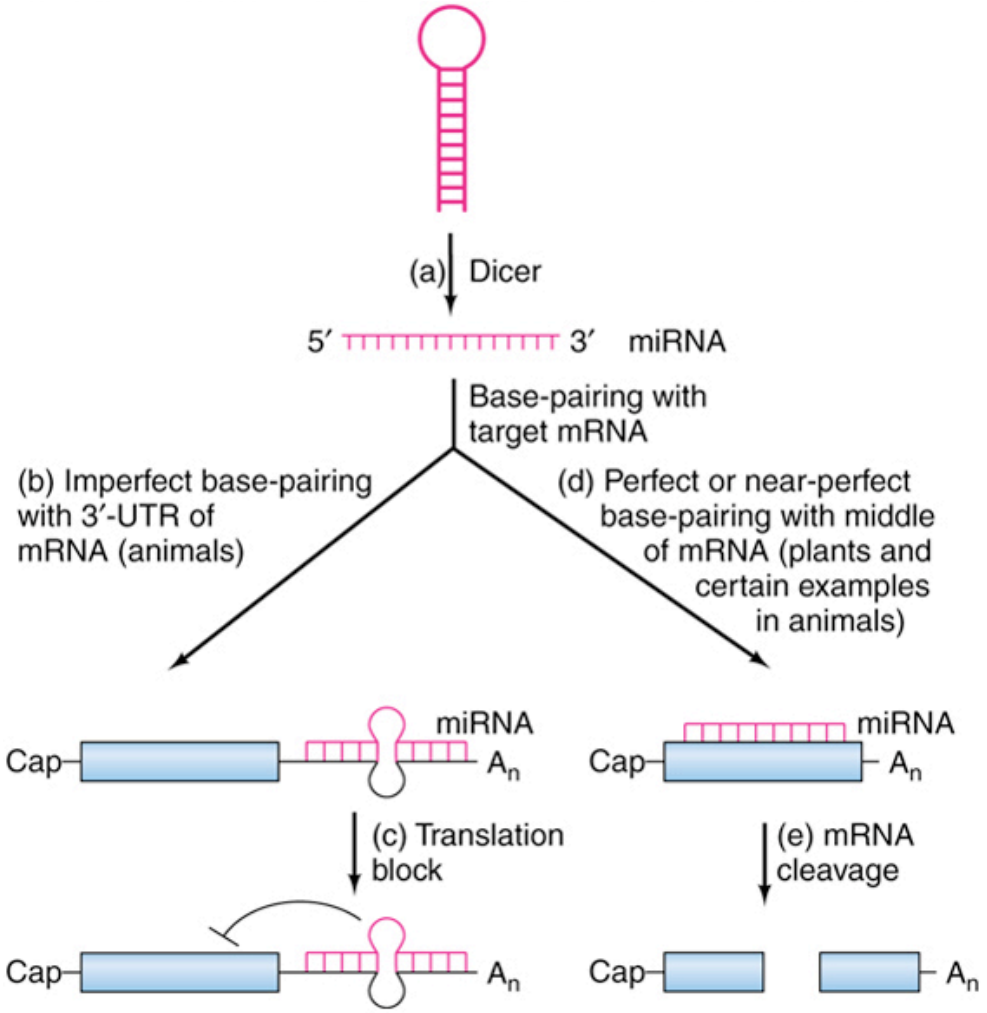
- Transcription is by RNA pol II, and transcripts are capped and polyadenylated
- Processed from an imperfect hairpin within transcript, called pri-miRNA
- In plants, both processing steps to produce short dsRNA are by Dcl1 in nucleus
- In animals, hairpin is excised by Drosha or by splicing in nucleus to produce pre-miRNA
- Then Dicer performs final processing step in the cytoplasm



Carthew and Sontheimer, Cell (2009) 136, 642-655.

Extent of Complementarity Determines Whether miRNA Gives Cleavage or Repression of Target mRNA

- With perfect or near-perfect complementarity, miRNA can act like siRNA, giving cleavage of target
- This pathway operates extensively in plants, less so in animals



Examples of miRNA target sites

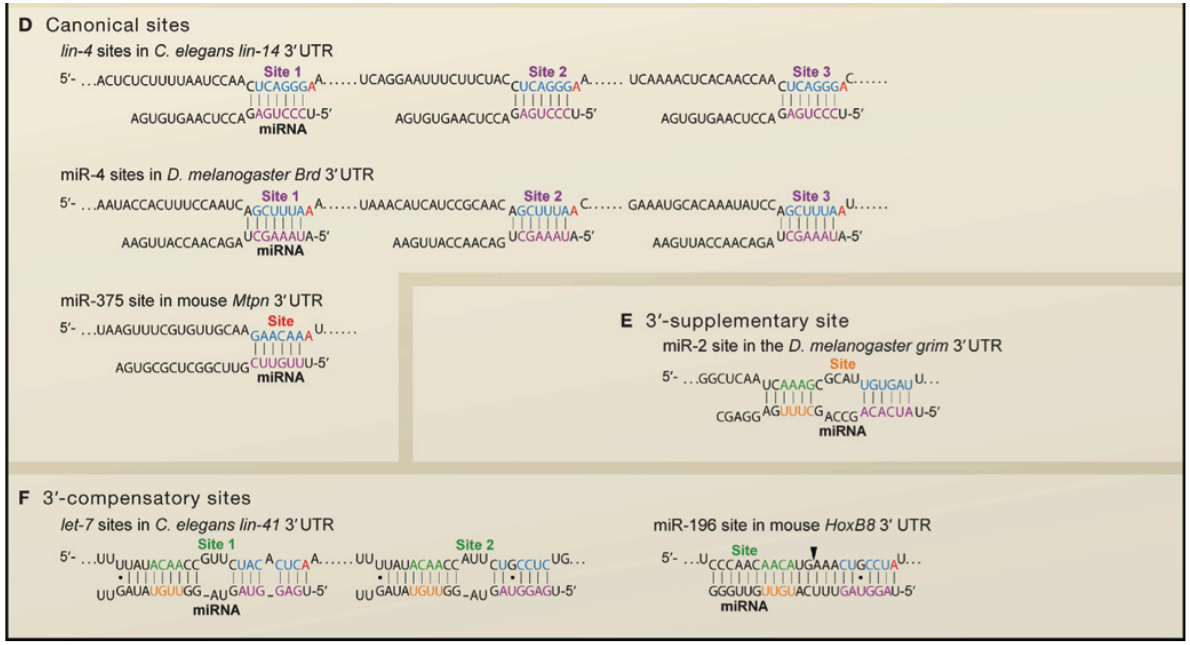


Figure 2. Examples of Conserved miRNA Targets and Sites

Coloring of site types and residues within the miRNAs and targets are as in Figure 1.

(A) Correspondence between the top predicted targets and genetically implicated interactions (Wightman et al., 1991; Abrahante et al., 2003; Johnston and Hobert, 2003; Lai et al., 2005; Mayr et al., 2007; Xiao et al., 2007). The nematode and mammalian predictions are the top TargetScan (release 4.2) predictions for the respective miRNAs, whereas the fly predictions are among several top but essentially equivalent predictions. Many are also among the top three predictions for the respective miRNAs at the PicTar (*lin-14*, *hbl-1*, *Brd*, *Hmga2*), EMBL [*E(Spl)*, *Brd*], and EIMMo (*hbl-1*, *Brd*, *Hmga2*) websites. Sites within cooperative distance of each other are indicated (square brackets).

(B) Additional examples of nematode targets originally identified with assistance from genetics (Moss et al., 1997; Reinhart et al., 2000).

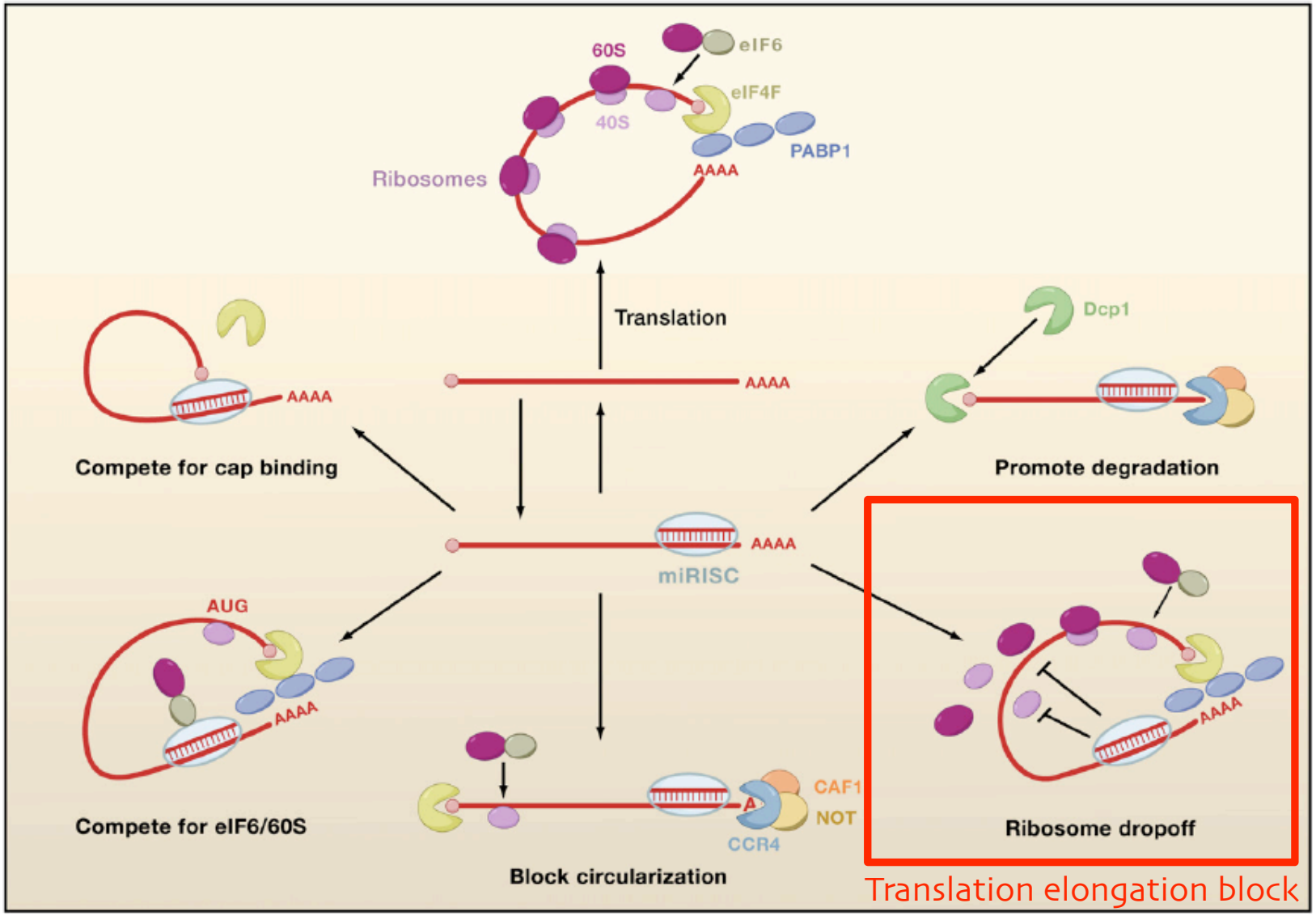
(C) Top ORF target candidate of mammalian *let-7* (Lewis et al., 2005).

(D) Examples of canonical miRNA target sites (Lee et al., 1993; Wightman et al., 1993; Poy et al., 2004; Lai et al., 2005), with sites and pairing reflecting current understanding of target recognition.

(E) A 3′-supplementary site with experimental support (Brennecke et al., 2005).

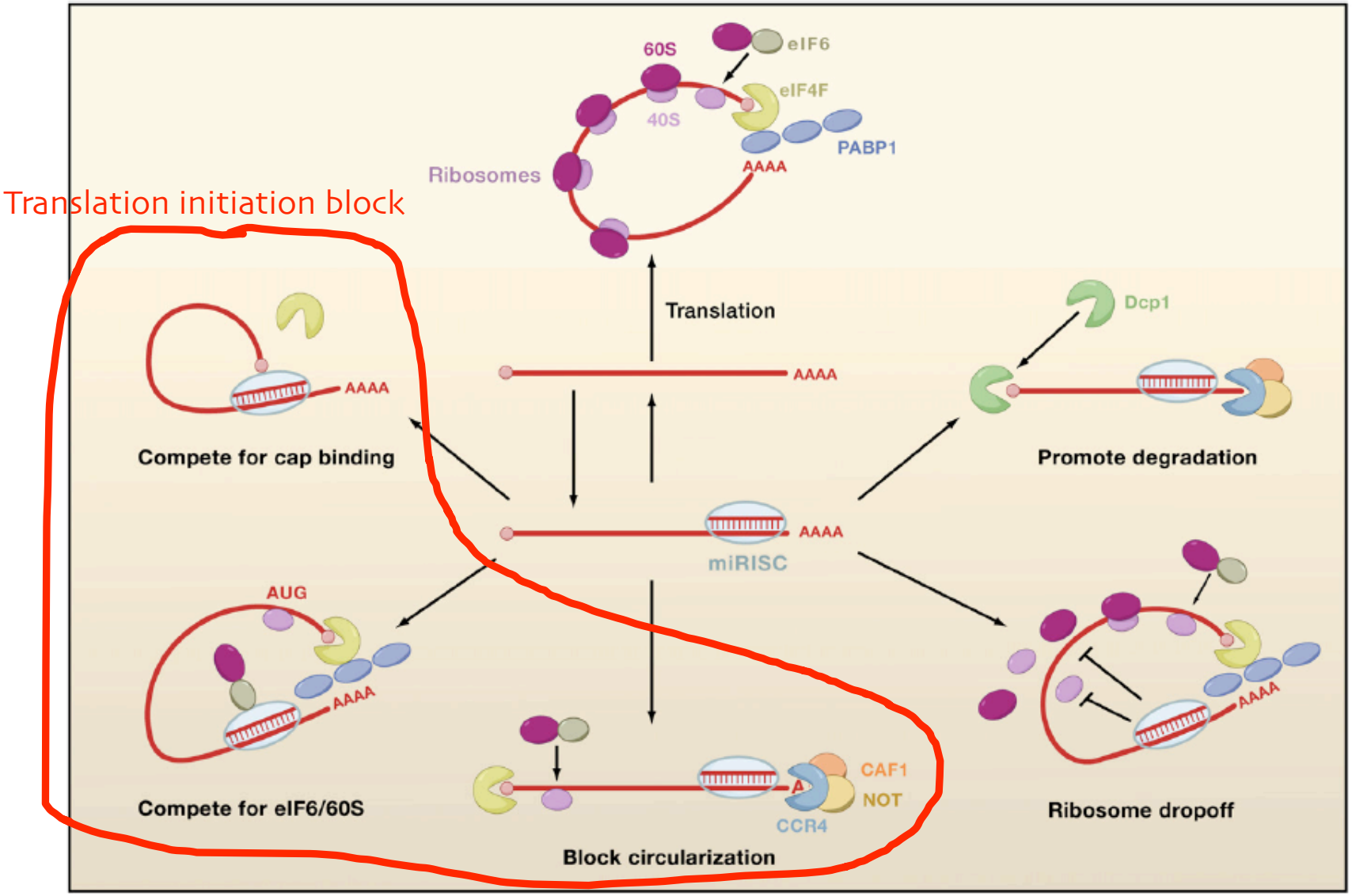
(F) 3′-compensatory sites with experimental support (Vella et al., 2004; Yekta et al., 2004). The position of miRNA-directed cleavage within the *HoxB8* 3′ UTR is indicated (arrowhead).

Possible Mechanisms of Inhibition by miRNA (a controversial issue)



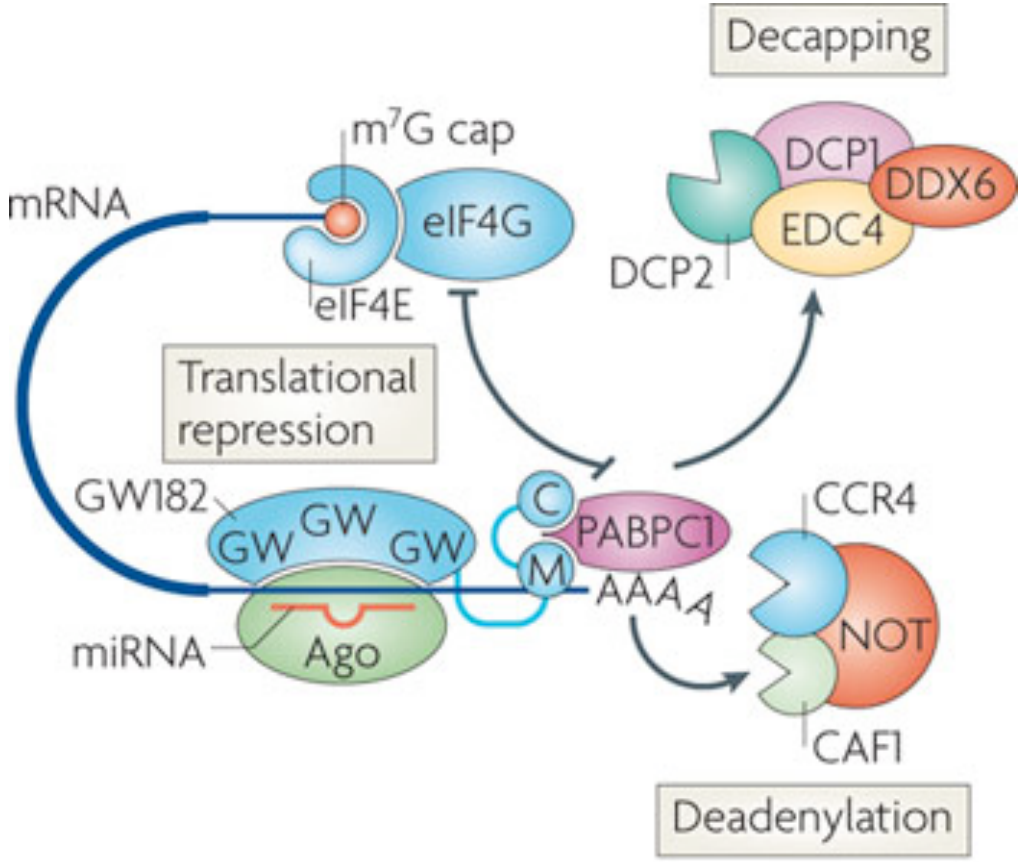
Carthew and Sontheimer, Cell (2009) 136, 642-655.

Possible Mechanisms of Translation Inhibition by miRNA



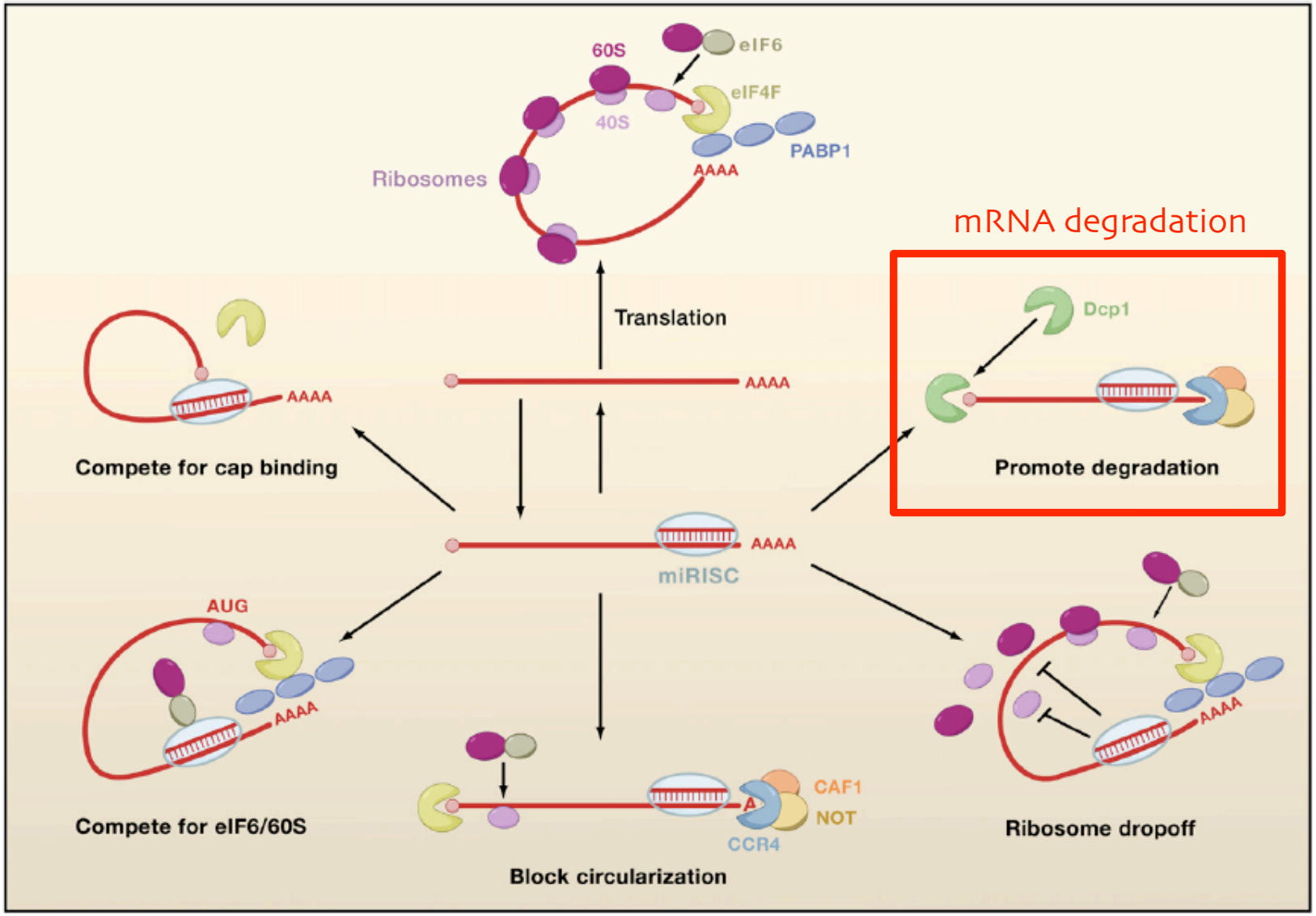
mi-RNA Effects Are Mediated Through GW182 Protein

- GW182 shown to be required for miRNA effects
- Also sufficient: tethering GW182 without Ago gave silencing
- GW182 shown to bind to polyA-binding protein (PABP or PABPC1 here)



Tritschler et al, *Nat. Rev. Cell Mol. Biol.* (2010) 11, 379-384

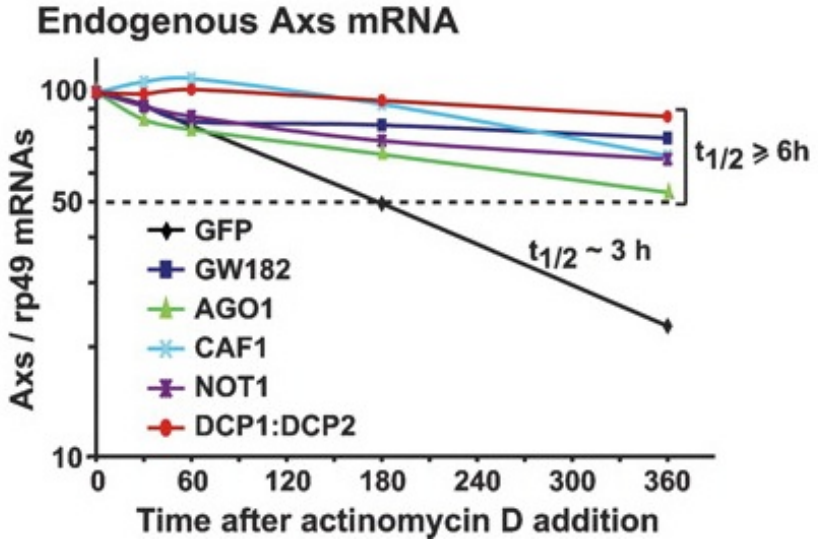
Possible Mechanisms of Translation Inhibition by miRNA



Carthew and Sontheimer, Cell (2009) 136, 642-655.

miRNA-dependent Degradation of mRNA Through Conventional Pathway

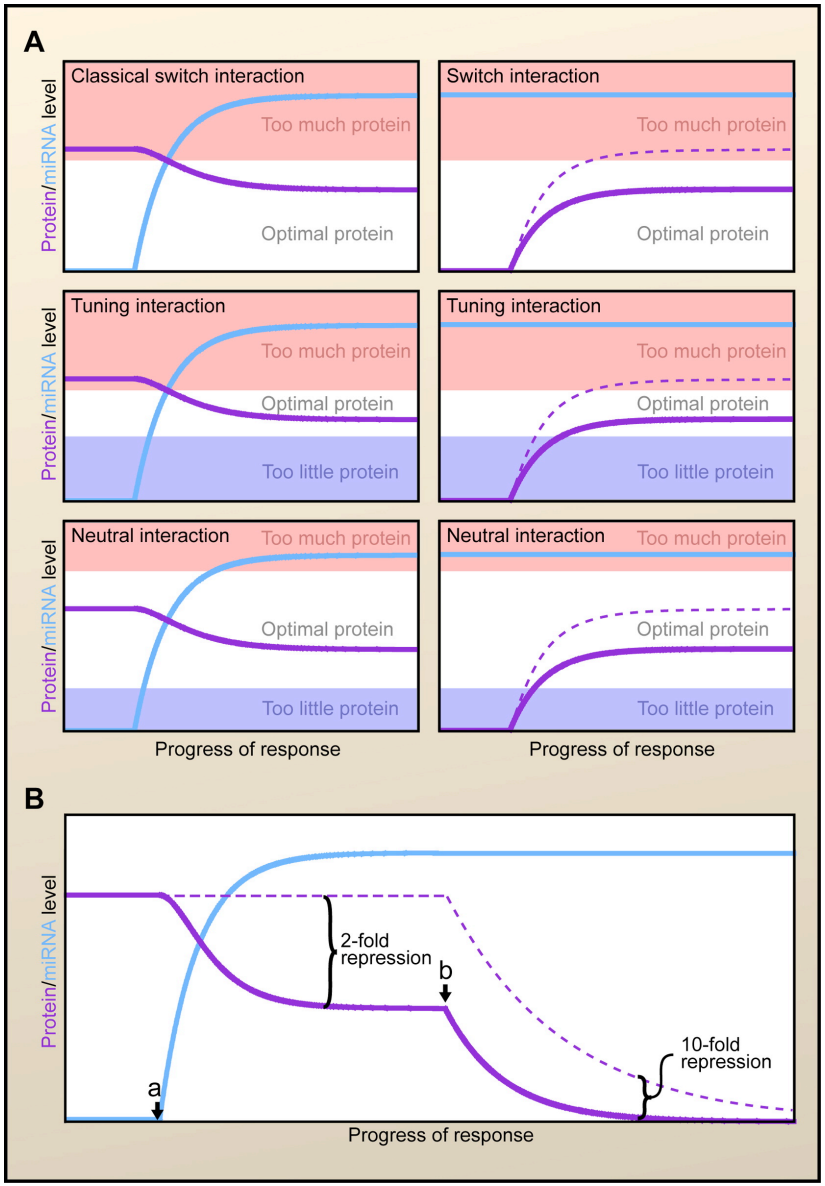
- Izaurralde and co-workers (2006)
- mRNA decay followed in *Drosophila* cells
- Actinomycin D used to block transcription, reporter mRNA level then followed by Northern blot
- Decay of mRNA dependent on GW182, Ago, and deadenylation (NOT1/CAF1) and decapping (DCP) machineries



Behm-Ansmant *et al, Genes Dev.* (2006) 20, 1885-1898

Biological Roles of miRNA-induced Repression of Gene Expression

- Many miRNAs interact with multiple mRNA targets
- mRNA untranslated sequences may evolve in context of milieu of miRNAs that allow fine-tuning of expression levels
- Tuning effect is the same whether miRNA is constitutively expressed or switched on (left vs right panels)
- Small effect can become large if transcription is shut off



Bartel, *Cell* (2009) 136, 215-233

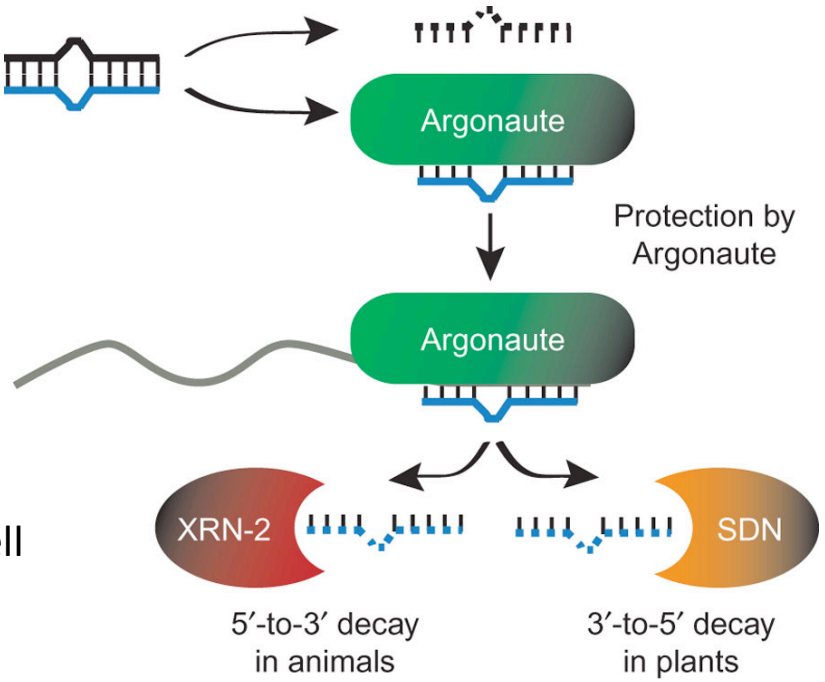
What Governs Turnover of miRNAs?

- Mature and functional miRNAs are protected from degradation by association with Argonaute protein

- Some miRNAs are also modified to give further protection
  - 2'OMe modifications on 3'-most nucleotides
  - adenylation at 3' end
  - uridylation at 3' end

- SDN (small RNA degrading nuclease) first discovered in *Arabidopsis*

- XRN-2 is related to XRN-1 but functions in nucleus
  - seems to facilitate unloading from Argonaute as well as degradation
  - presence in the nucleus suggests that miRNA localization could regulate decay



Kai and Pasquinelli, *Nat. Struct. Mol. Biol*. (2010) 17, 5-10

Key Points

1. Small RNAs, in the forms of siRNAs and miRNAs, play large roles in the regulation of gene expression in eukaryotes. They are important in normal cell metabolism, development, and defense against invaders.

2. siRNAs are produced from longer segments of dsRNA by Dicer, assembed into RISC, and targeted to mRNAs with perfect complementarity, giving silencing by cleavage and degradation of the RNA or by formation of heterochromatin.

3. The pathway for miRNA has many steps in common with that for siRNA. However, miRNAs are processed from hairpin structures by Drosha and then by Dicer, and they most often have imperfect complementarity with their targets, giving effects on translation rather than Ago-mediated cleavage of the mRNA.

Table 1. Tools for Predicting Metazoan miRNA Targets

Tool[a]	Clades[b]	Criteria for Prediction and Ranking	Website URL	Recent Reference
Site Conservation Considered				
TargetScan	m	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility); option of ranking by likelihood of preferential conservation rather than site context	http://targetscan.org	Friedman et al., 2008
TargetScan	f,w	Stringent seed pairing, site number, site type	http://targetscan.org	Ruby et al., 2007; Ruby et al., 2006
EMBL	f	Stringent seed pairing, site number, overall predicted pairing stability	http://russell.embl-heidelberg.de	Stark et al., 2005
PicTar	m,f,w	Stringent seed pairing for at least one of the sites for the miRNA, site number, overall predicted pairing stability	http://pictar.mdc-berlin.de	Lall et al., 2006
EIMMo	m,f,w	Stringent seed pairing, site number, likelihood of preferential conservation	http://www.mirz.unibas.ch/EIMMo2	Gaidatzis et al., 2007
Miranda	m,f,w,+	Moderately stringent seed pairing, site number, pairing to most of the miRNA	http://www.microrna.org	Betel et al., 2008
miRBase Targets	m,f,w,+	Moderately stringent seed pairing, site number, overall pairing	http://microrna.sanger.ac.uk	Griffiths-Jones et al., 2008
PITA Top	m,f,w	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	Kertesz et al., 2007
mirWIP	w	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	http://146.189.76.171/query	Hammell et al., 2008
Site Conservation Not Considered				
TargetScan	m	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility)	http://targetscan.org	Grimson et al., 2007
PITA All	m,f,w	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	Kertesz et al., 2007
RNA22	m,f,w	Moderately stringent seed pairing, matches to sequence patterns generated from miRNA set, overall predicted pairing and predicted pairing stability	http://cbcsrv.watson.ibm.com/rna22.html	Miranda et al., 2006

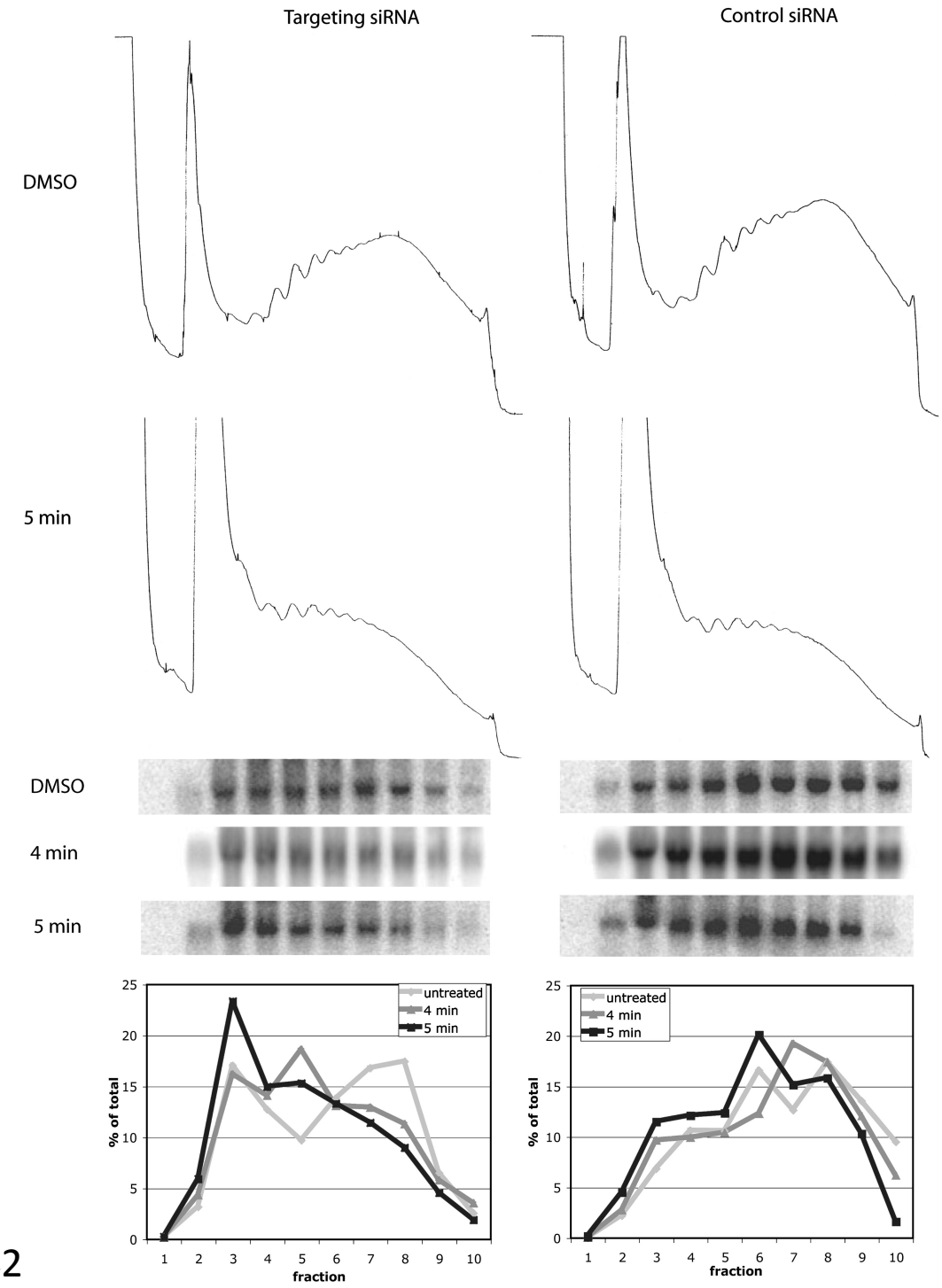
[a]Tools are listed according to criteria for prediction and ranking, which for those tools assessed with recent proteomics results generally correspond to their overall performance (Baek et al., 2008).
[b]Letters indicate predictions provided for the mammalian/vertebrate (m), fly (f), worm (w), or additional (+) clades.

miRNA readings

- Carthew and Sontheimer, Origin and mechanism of miRNAs and siRNAs. Cell (2009) 136, 642-655.
- Jacek Krol, Inga Loedige and Witold Filipowicz The widespread regulation of microRNA biogenesis, function and decay. Nature Reviews Genetics 11 2010, 597
- V. Narry Kim, Jinju Han and Mikiko C. Siomi. Biogenesis of small RNAs in animals. Nature Reviews Mol Cell Biol 10 2009, 126

Evidence For Ribosome Drop-off

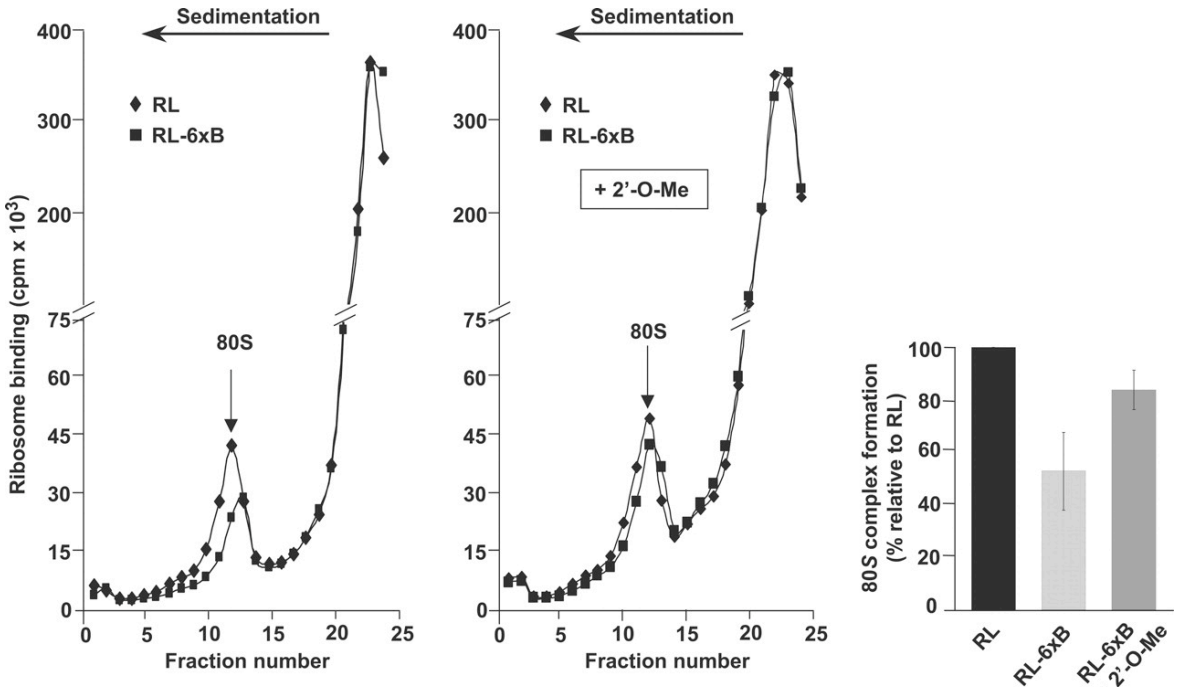
- Sharp and co-workers (2006)
- Human cells transfected with reporter and miRNA mimic (imperfect siRNA) targeted to transfected luciferase gene
- Translation initiation then inhibited with hippuristanol (inhibits eIF4A)
- With miRNA (left), mRNA is removed from polysomes faster than without
- Also found that IRES-containing RNA is inhibited

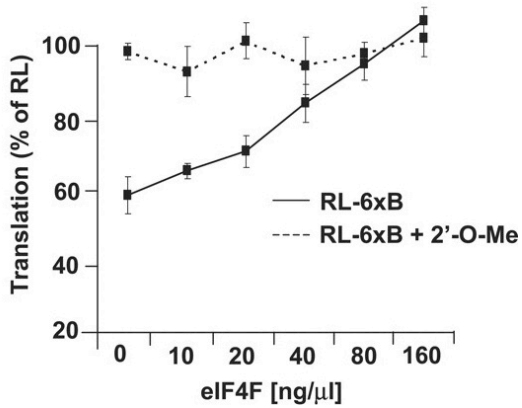


Peterson *et al, Mol. Cell* (2006) 21, 533-542

Does Translational Repression Involve Competition For Cap Binding?

- Sonenberg and co-workers (2007)
- Mouse cell-free extract
- Luciferase mRNA with miRNA binding sites for endogenous let-7 miRNA (RL-6xB)
- Decreased 80S peak monitored by glycerol gradient centrifugation, rescued by comp. oligo to let-7
- Decrease inferred to reflect decreased translation initiation
- Translation also inhibited, and inhibition eliminated with inclusion of IRES in mRNA
- Inhibitory effect on translation reversed by increased eIF4F concentration (at right)





Mathonnet *et al*, *Science* (2007) 317, 1764-1767