20.109

LABORATORY FUNDAMENTALS IN BIOLOGICAL ENGINEERING

MODULE 2

EXPRESSION ENGINEERING

Lecture # 2

Leona Samson

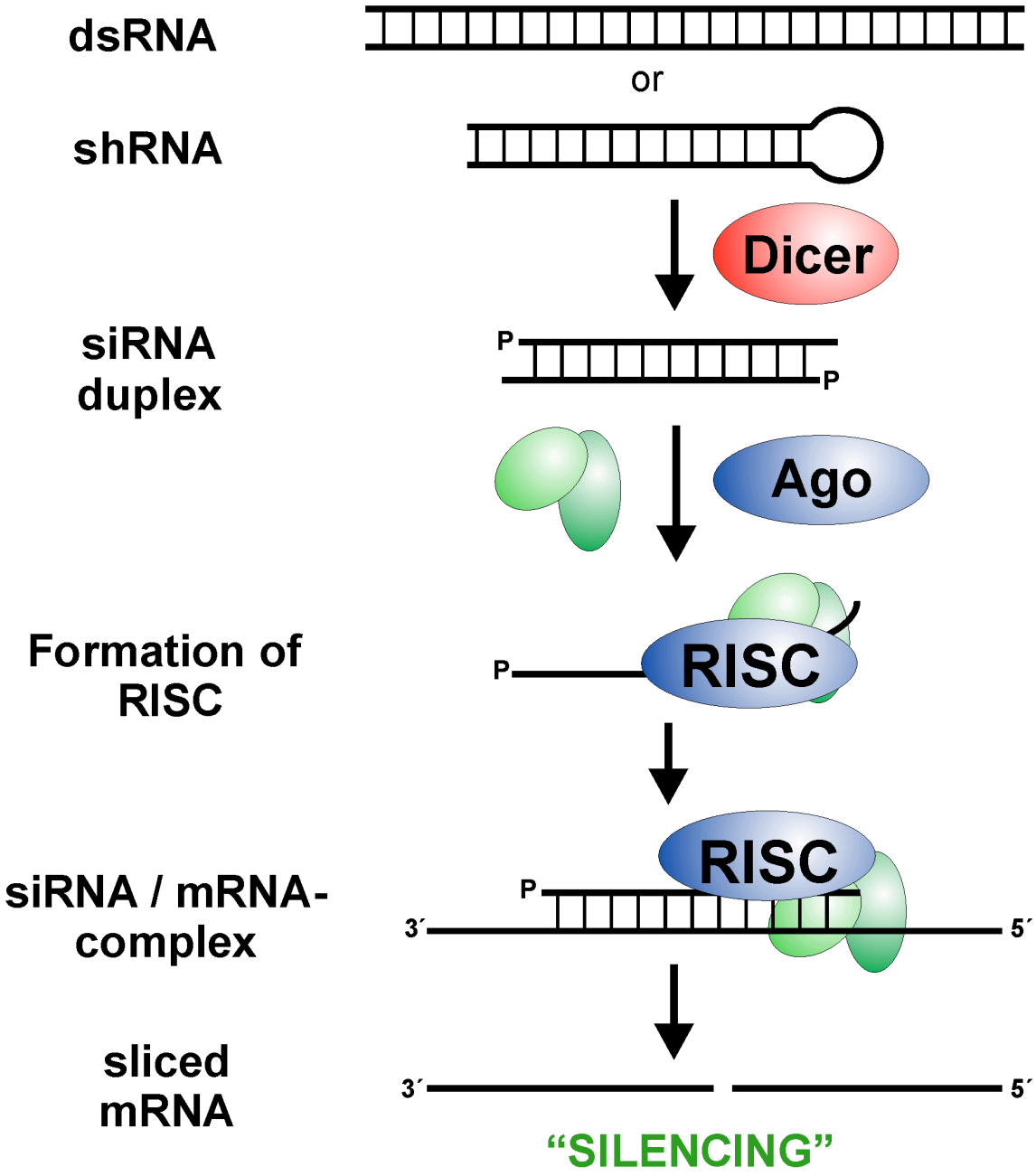
March 12th 2009

Snapshot of the next four weeks

We will eliminate the expression of various genes using

(i) RNA interference technology
(ii) Cultured mouse ES cells
(iii) Chemiluminescent proteins
(iv) DNA microarrays

RNA Interference



RISC = RNA induced sliencing complex

http://www.gene-quantification.de/siRNA-mechanism.png

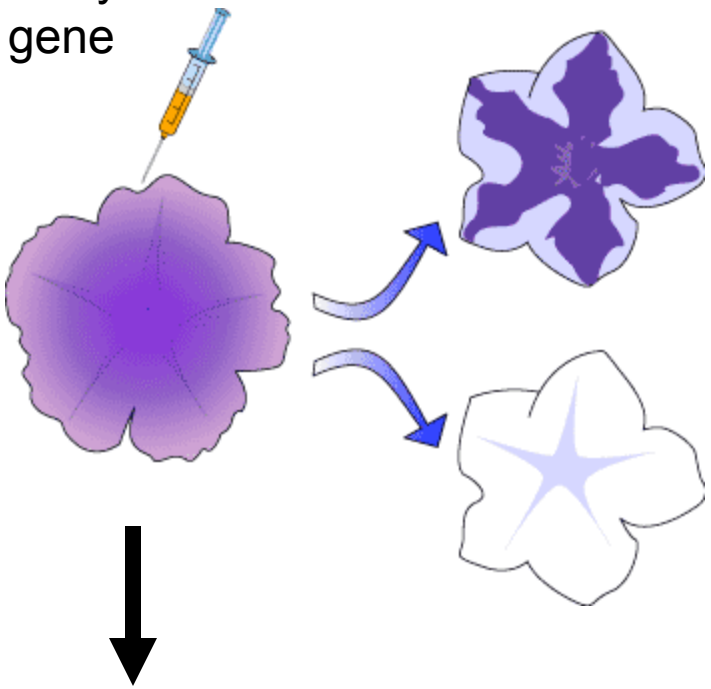
RNA interference first discovered in Petunias!

Called PTGS, for "Post Transcriptional Gene Silencing"

Color changes can be induced by RNAi, or PTGS..

Post transcipt-ional gene silencing

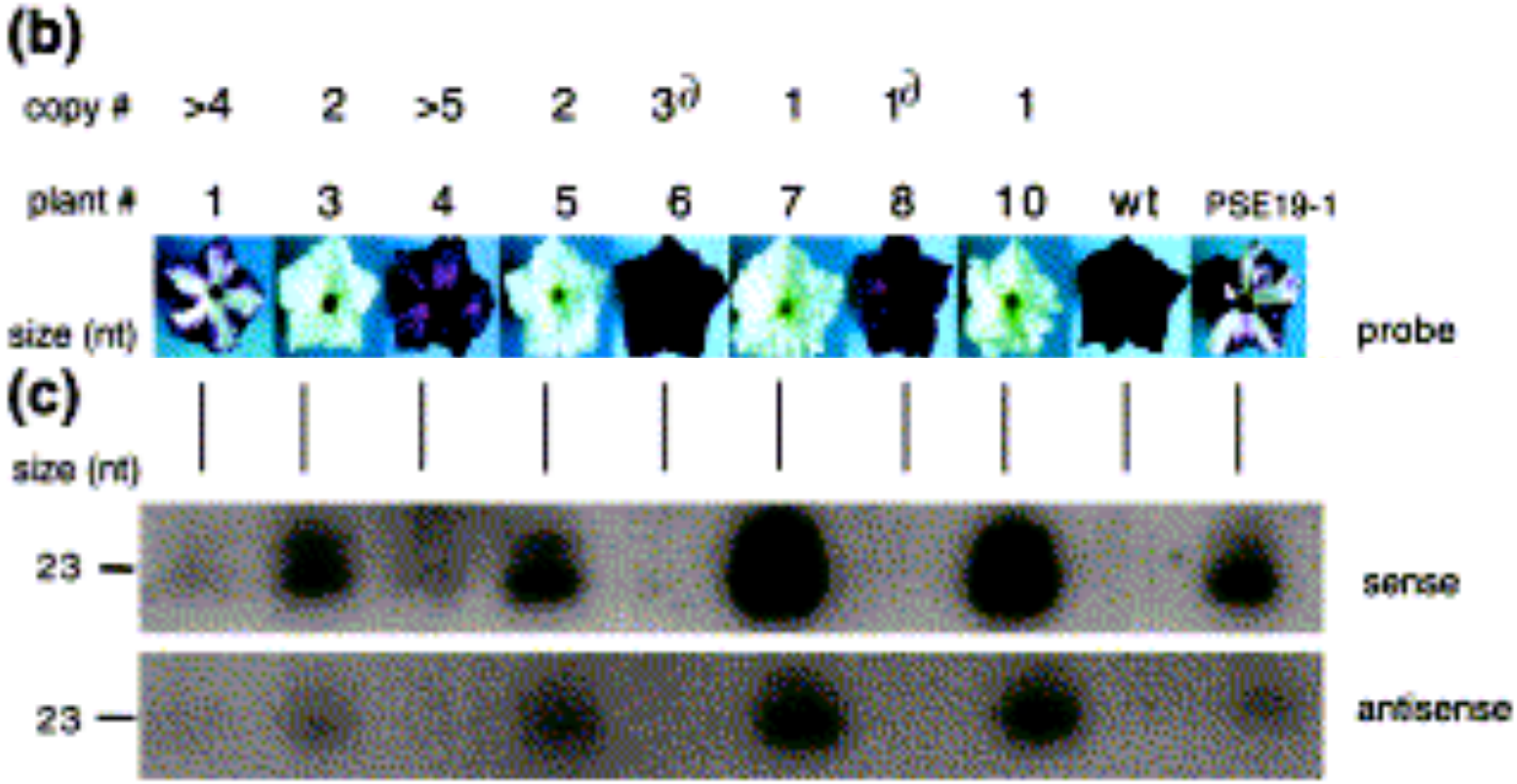
Add in an extra pigment biosynthesis gene



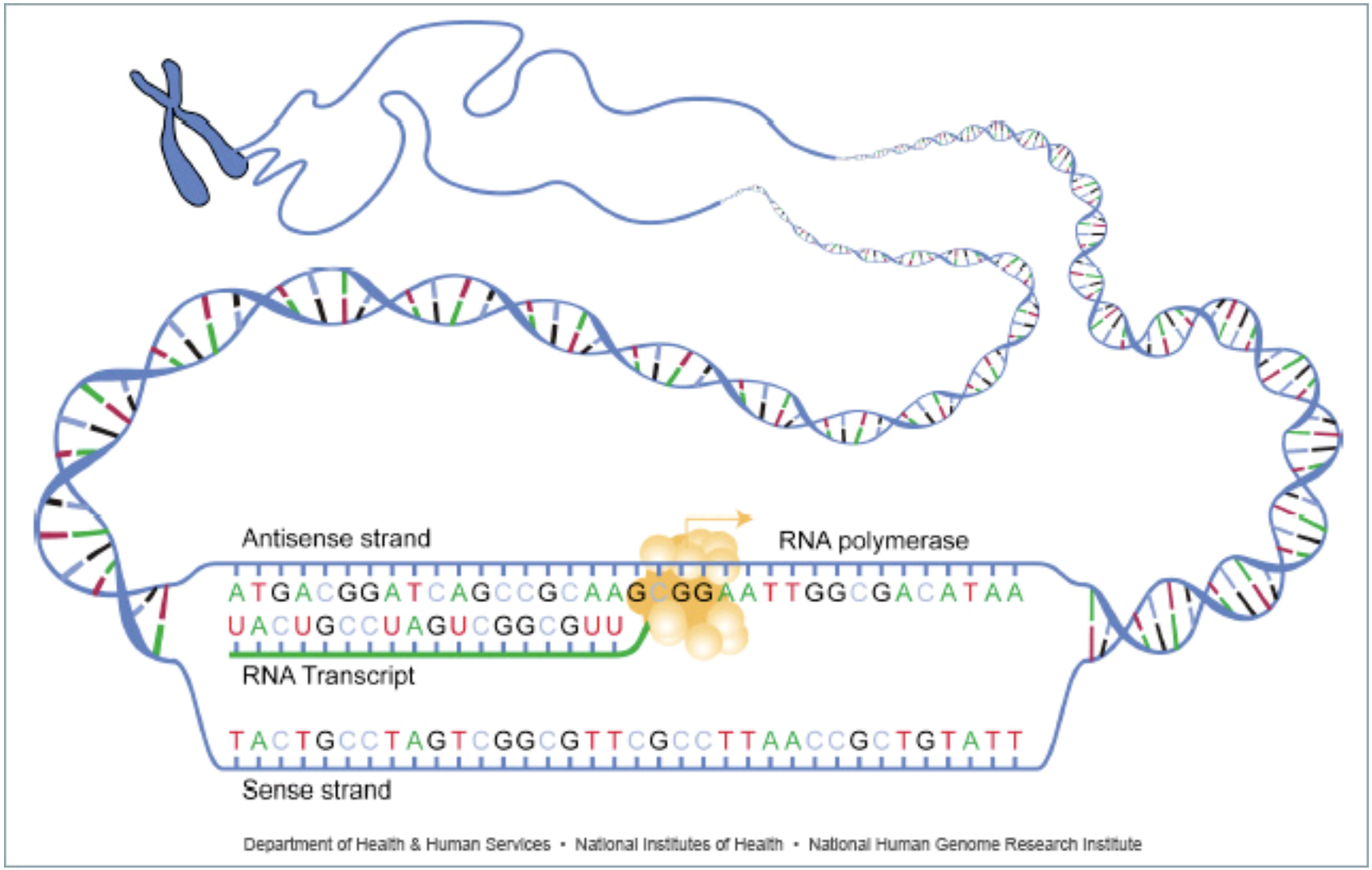
Obtained result – less purple!!

Expected result – deeper purple

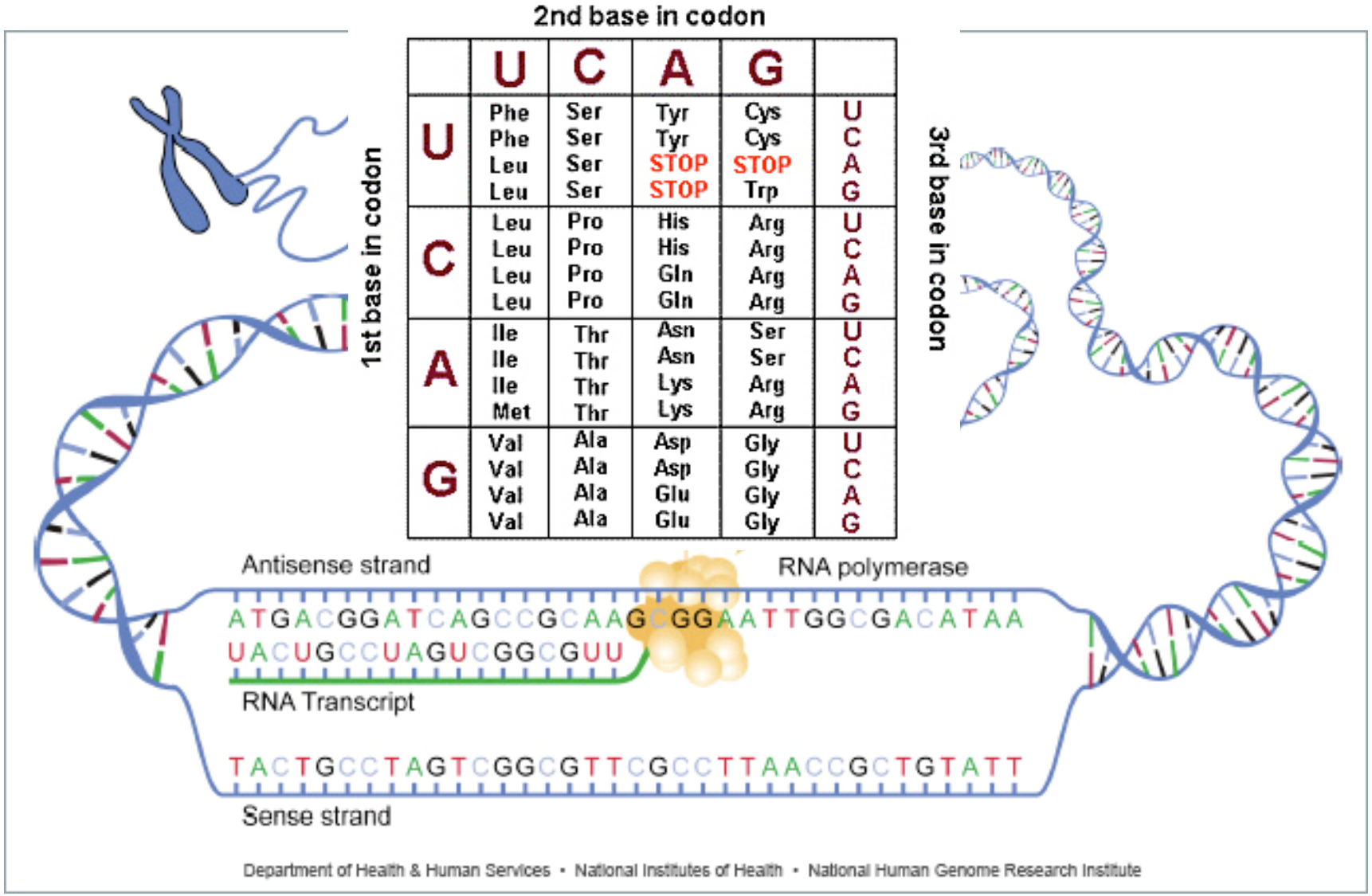
Small (21-23 nts) RNA duplexes, with the same sequence as in the silenced gene, were identified as being responsible for knocking down expression



Sense and Antisense Nucleic Acid Strands



Sense and Antisense Nucleic Acid Strands



	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

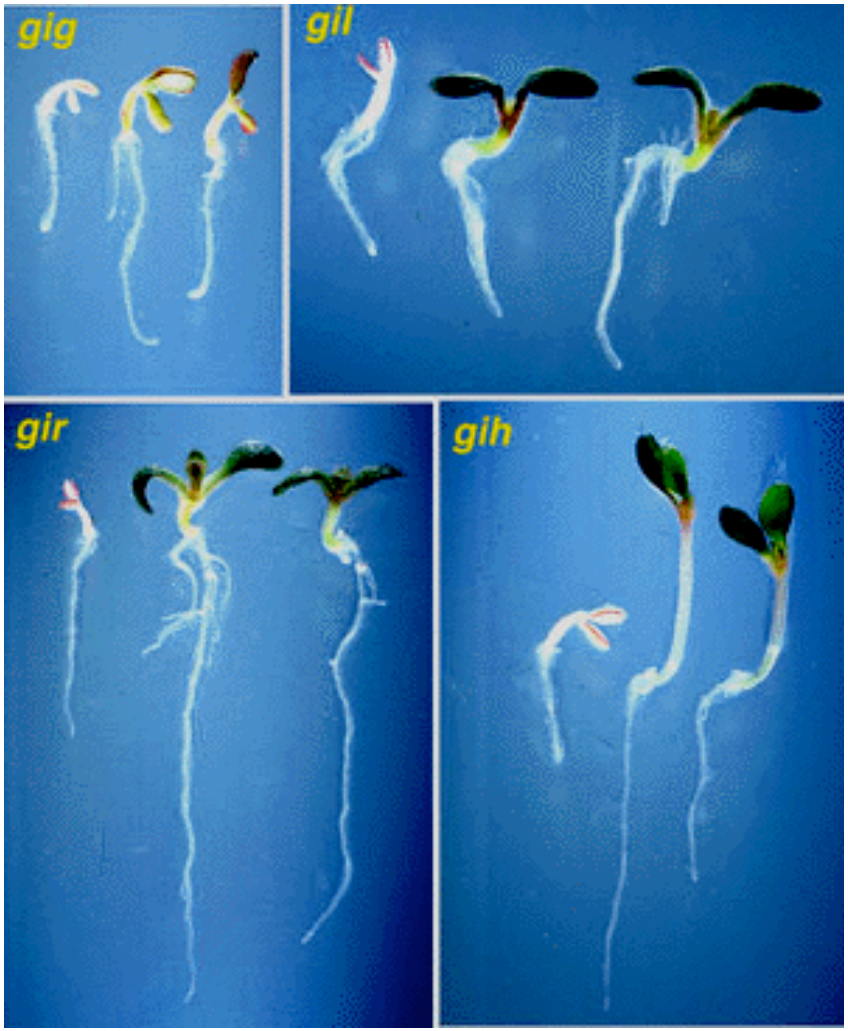
So what other organisms can do this thing called PTGS?

"Post Transcriptional Gene Silencing"

Or

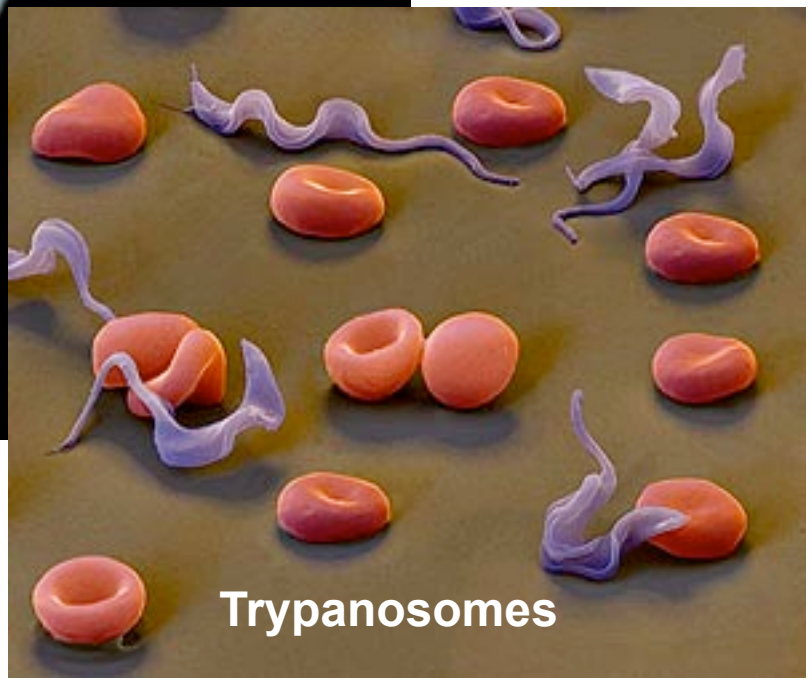
"RNA interference"

Arabidopsis thaliana

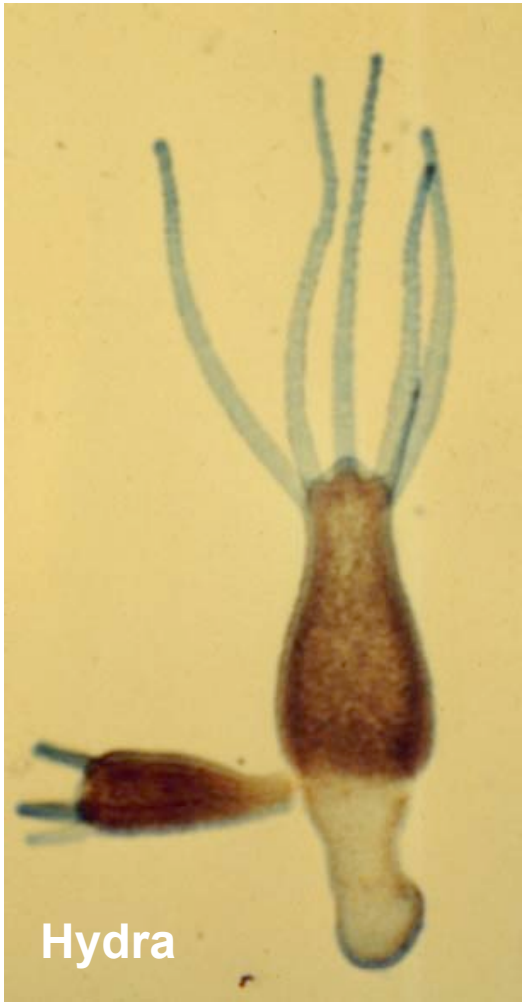


Planaria

Protozoa can use RNAi for gene silencing



Trypanosomes



Hydra

RNAi can operate in insects too!

The nematode worm ***Caenorhabditis elegans***

C. elegans

C. Elegans **grow on agar dishes with** ***E. coli*** **bacteria as a source of food.**

If they eat ***E. coli*** **expressing dsRNA molecules…this creates specific knock-down of gene expression!**

Functional genomics

RNA sets the standard

Thomas Tuschl

One way of finding out what genes do is to inactivate them, and to study the effects, in 'model' organisms. That has now been done for many thousands of worm genes in two large-scale analyses.

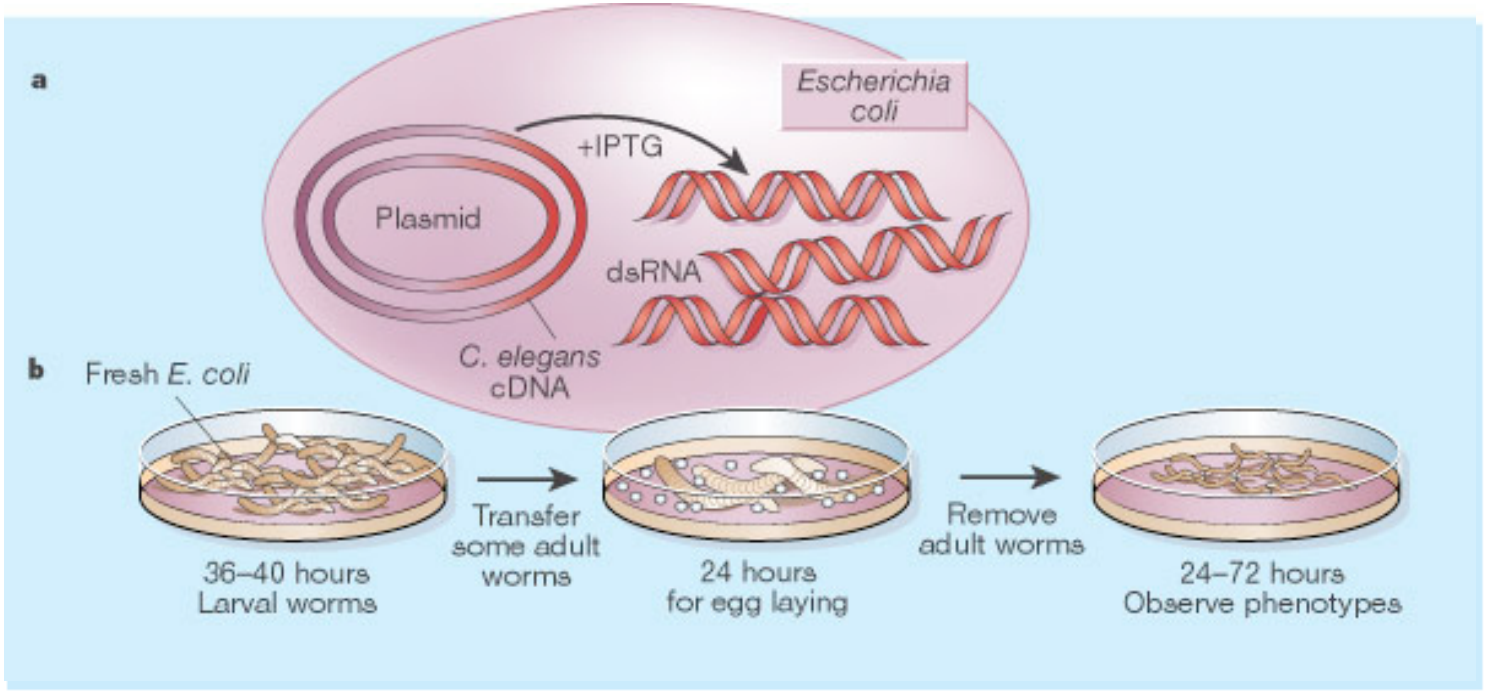


Figure 1 Gene screening by double-stranded-RNA-mediated interference (RNAi). Kamath *et al.*[1] and Ashrafi *et al.*[2] used the following technique to silence the expression of 16,757 genes individually in *Caenorhabditis elegans*. **a,** DNA molecules (plasmids) encoding a double-stranded RNA (dsRNA) of choice are inserted into *Escherichia coli* bacteria. Incubation with isopropylthio-β-galactoside (IPTG) induces production of the dsRNA. **b,** Worms at the latest larval stage are placed on a lawn of *E. coli*, and allowed to feed. Several adult worms are then placed onto new plates seeded with the same bacteria to lay eggs. The offspring are monitored for embryonic death and post-embryonic phenotypes, such as slow larval growth or movement disorders.

Silenced 16,757 genes, individually!!!!

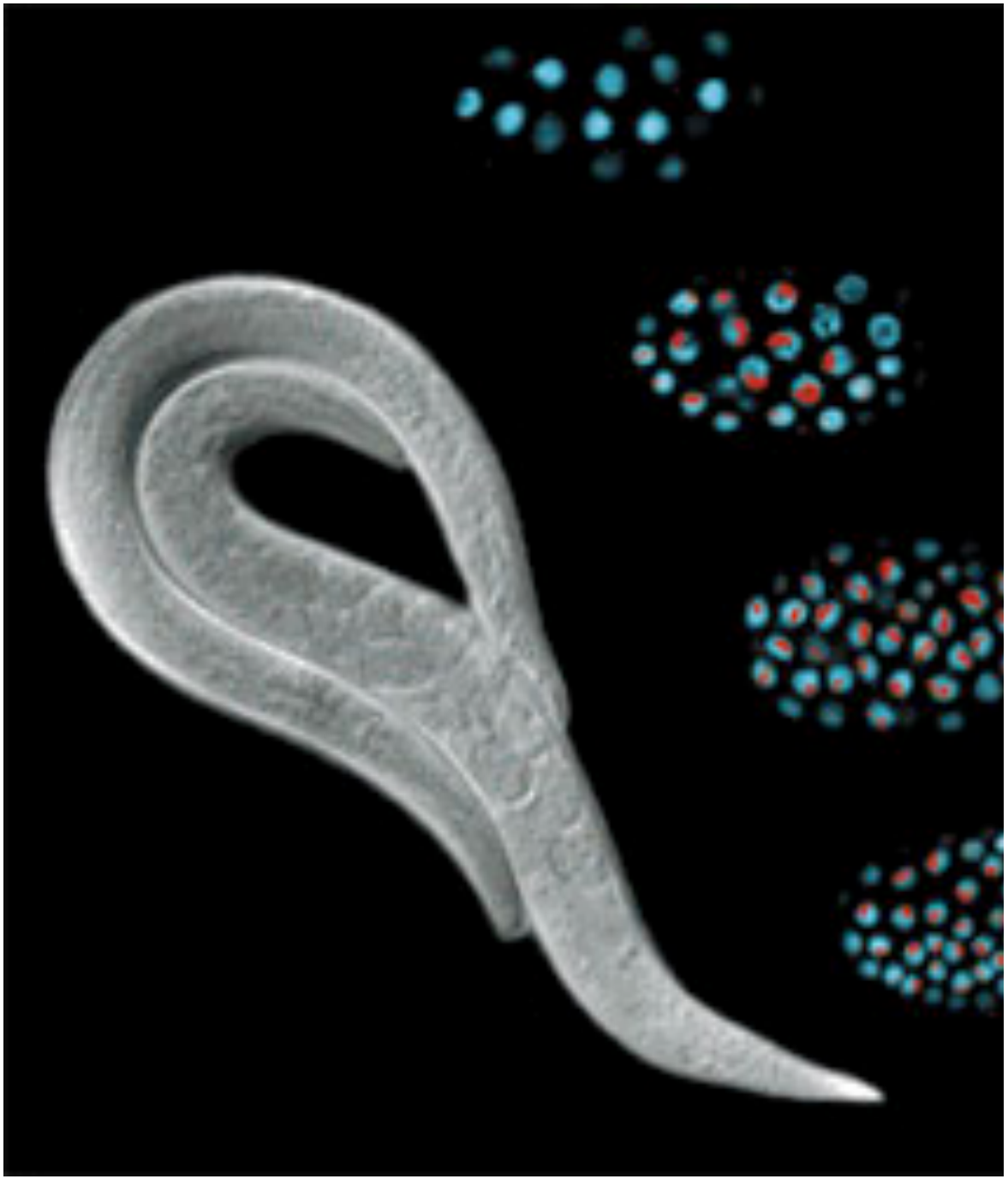
http://www.nature.com/cgi-taf/DynaPage.taf?file=/nature/journal/v421/n6920/full/421220a_fs.html&content_filetype=pdf

Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi

Ravi S. Kamath*†, Andrew G. Fraser*†§, Yan Dong*, Gino Poulin*, Richard Durbin‡, Monica Gotta*§, Alexander Kanapin‖, Nathalie Le Bot*, Sergio Moreno*¶, Marc Sohrmann‡§, David P. Weichman*, Peder Zipperlen* & Julie Ahringer*

Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes

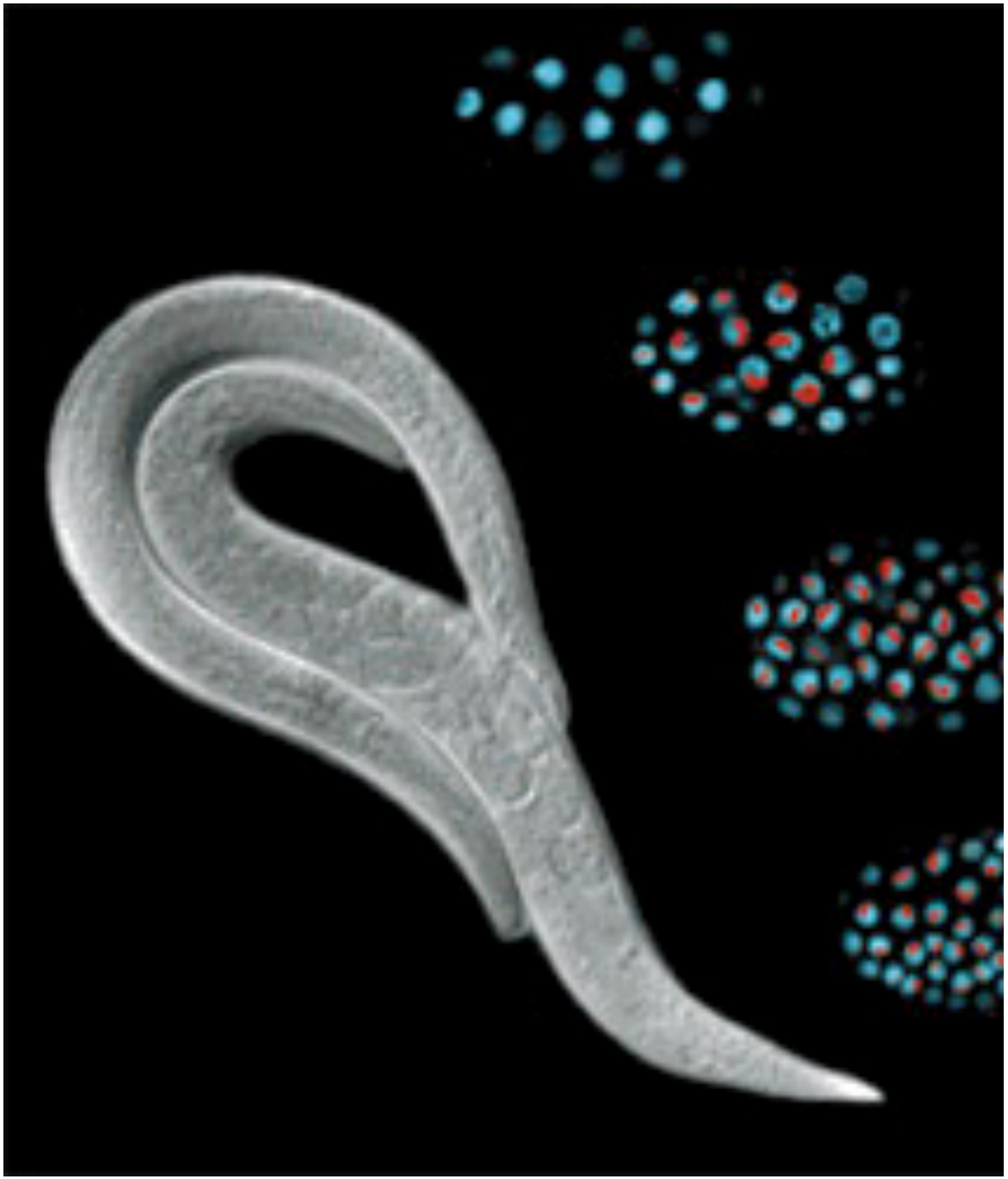
Kaveh Ashrafi*†, Francesca Y. Chang*, Jennifer L. Watts‡, Andrew G. Fraser§, Ravi S. Kamath§, Julie Ahringer§ & Gary Ruvkun*†



19,757 genes

16,757 have been inactivated by RNAi

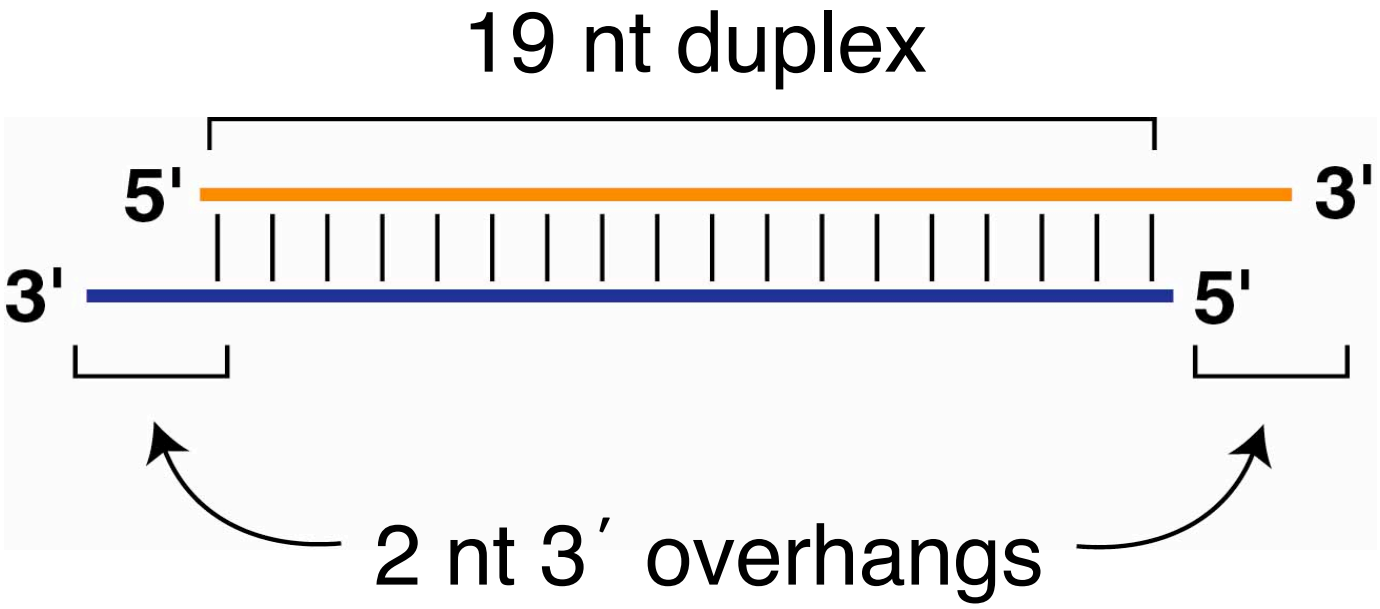
10% display spontaneous phenotype; this 10% is enriched for conserved genes



19,757 genes

16,757 knock down mutants were screened for body fat content

305 knock downs had increased body fat, 112 genes had decreased body fat...new targets for obesity?



This dsRNA species found in plants, *C. elegans* and *Drosophila melanogaster* undergoing gene silencing….but how to prove it is responsible?

Purified them and showed *in vitro* silencing in *Drosophila* extracts; used synthetic dsRNA oligo to achieve same thing!

So…what about RNAi in mammalian cells…

May 2001…the first report…

letters to nature

Nature **411**, 494 - 498 (2001); doi:10.1038/35078107

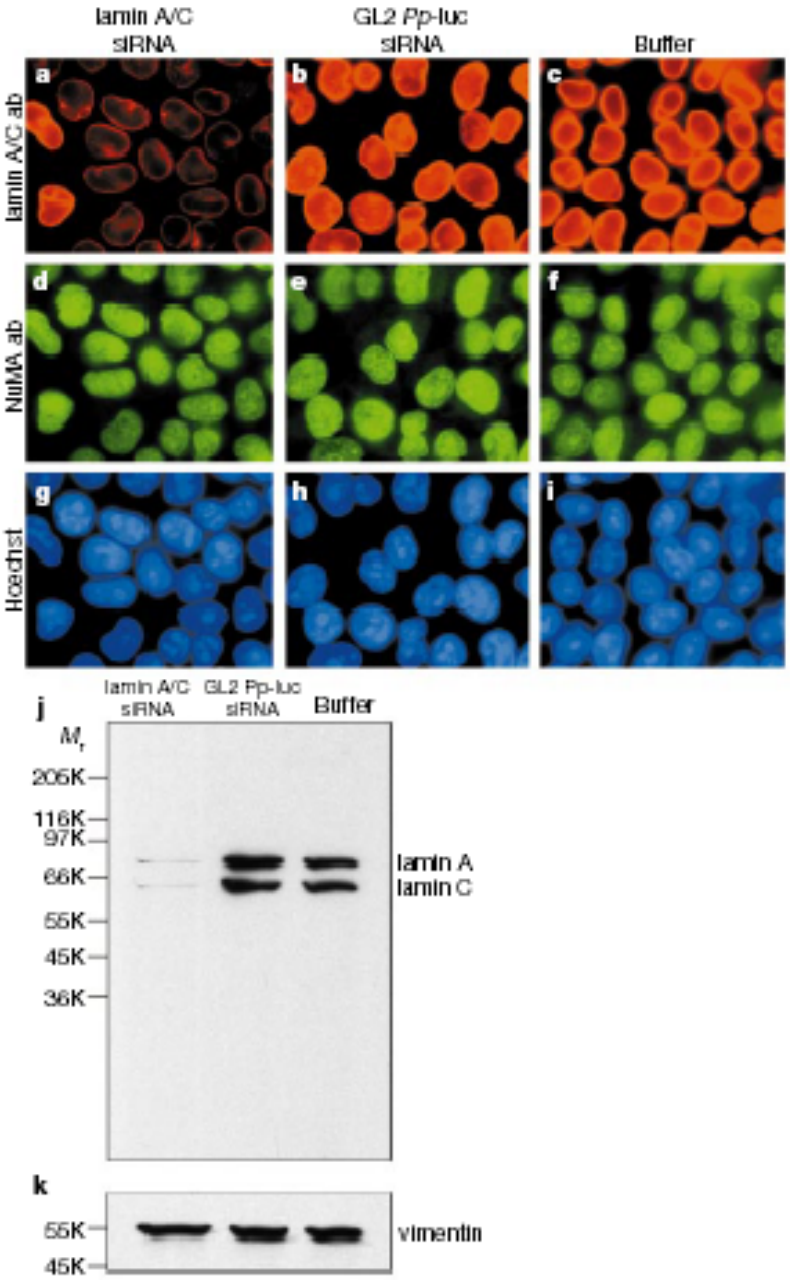
Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells

SAYDA M. ELBASHIR*, JENS HARBORTH†, WINFRIED LENDECKEL*, ABDULLAH YALCIN*, KLAUS WEBER† & THOMAS TUSCHL*

* Department of Cellular Biochemistry; and
† Department of Biochemistry and Cell Biology, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

Correspondence and requests for materials should be addressed to T.T. (e-mail: ttuschl@mpibpc.gwdg.de).

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The mediators of sequence-specific messenger RNA degradation are 21- and 22-nucleotide small interfering RNAs (siRNAs) generated by ribonuclease III cleavage from longer dsRNAs. Here we show that 21-nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney (293) and HeLa cells. Therefore, 21-nucleotide siRNA duplexes provide a new tool for studying gene function in mammalian cells and may eventually be used as gene-specific therapeutics.



Duplexes of 21–nucleotide RNAs mediate RNA interference in cultured mammalian cells

Sayda M. Elbashir*, Jens Harborth†, Winfried Lendeckel*, Abdullah Yalcin*, Klaus Weber† & Thomas Tuschl*

** Department of Cellular Biochemistry; and † Department of Biochemistry and Cell Biology, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany*

NATURE | VOL 411 | 24 MAY 2001 | www.nature.com

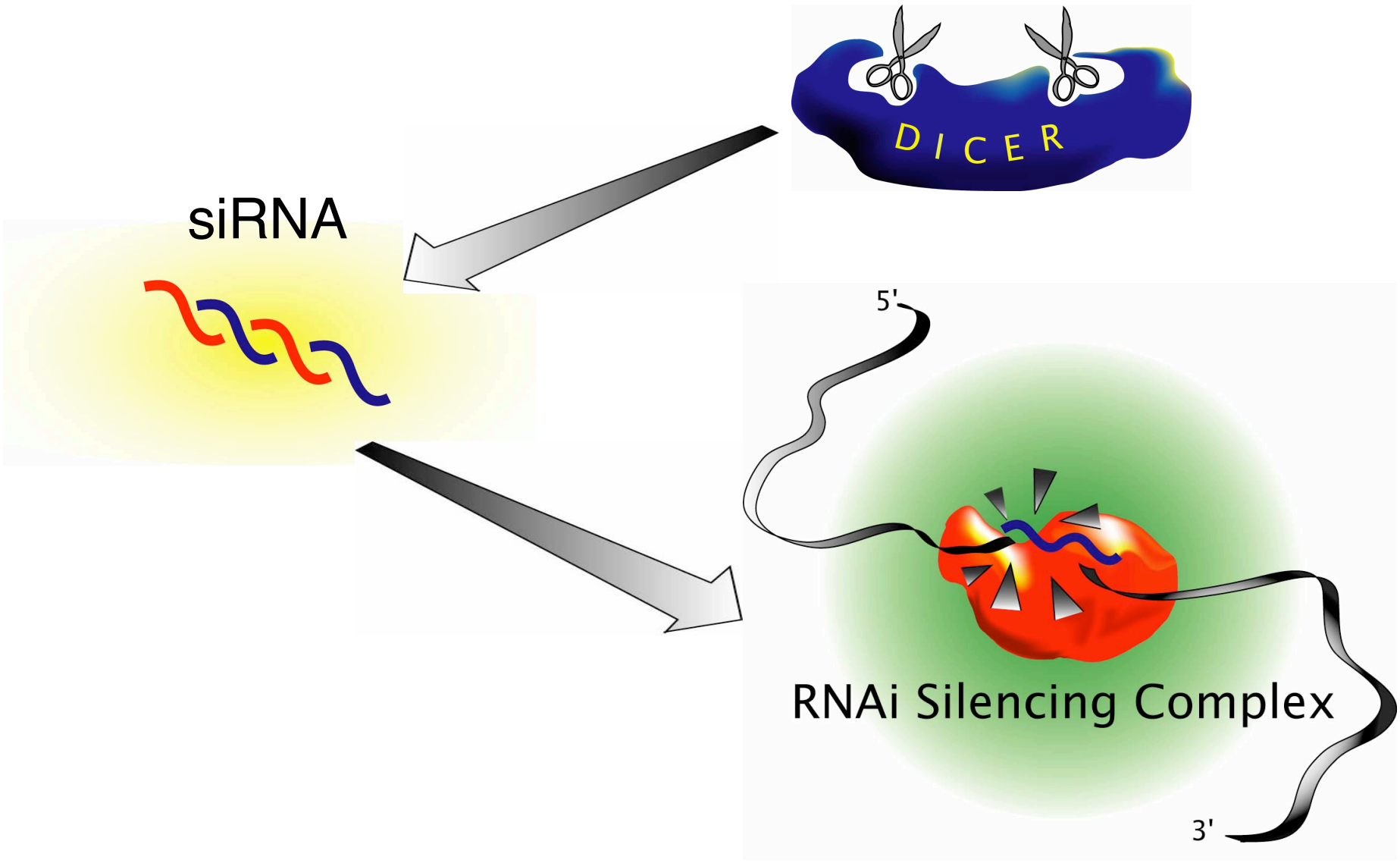
Figure 4 Silencing of nuclear envelope proteins lamin A/C in HeLa cells. Triple fluorescence staining of cells transfected with lamin A/C siRNA duplex (**a**, **d**, **g**), with GL2 luciferase siRNA duplex (nonspecific siRNA control) (**b**, **e**, **h**), and with buffer only (**c**, **f**, **i**). **a**–**c**, Staining with lamin A/C specific antibody; **d**–**f**, staining with NuMA-specific antibody; **g**–**i**, Hoechst staining of nuclear chromatin. Bright fluorescent nuclei in **a** represent untransfected cells. **j**, **k**, Western blots of transfected cells using lamin A/C- (**j**) or vimentin-specific (**k**) antibodies. The Western blot was stripped and re-probed with vimentin antibody to check for equal loading of total protein.

How does RNAi work in mammalian cells?

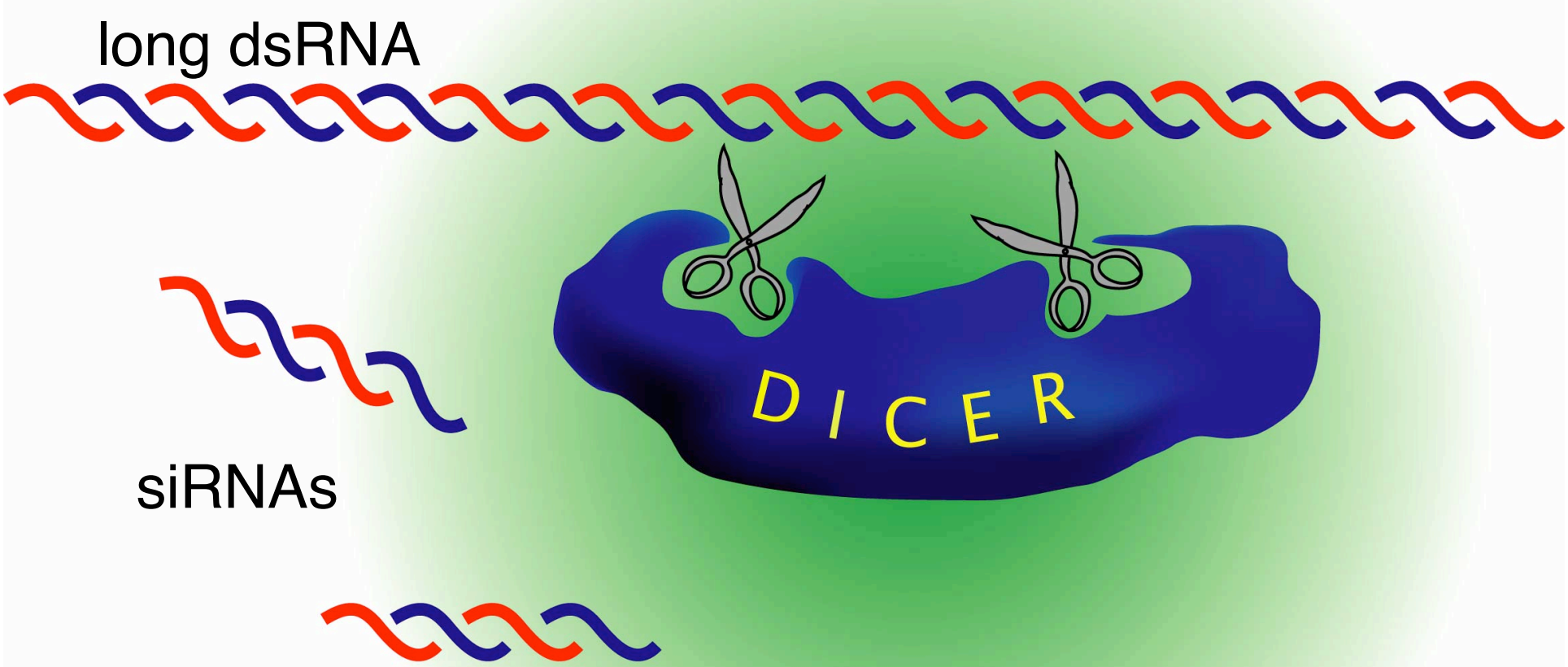
RNAi works postranscriptionally……..

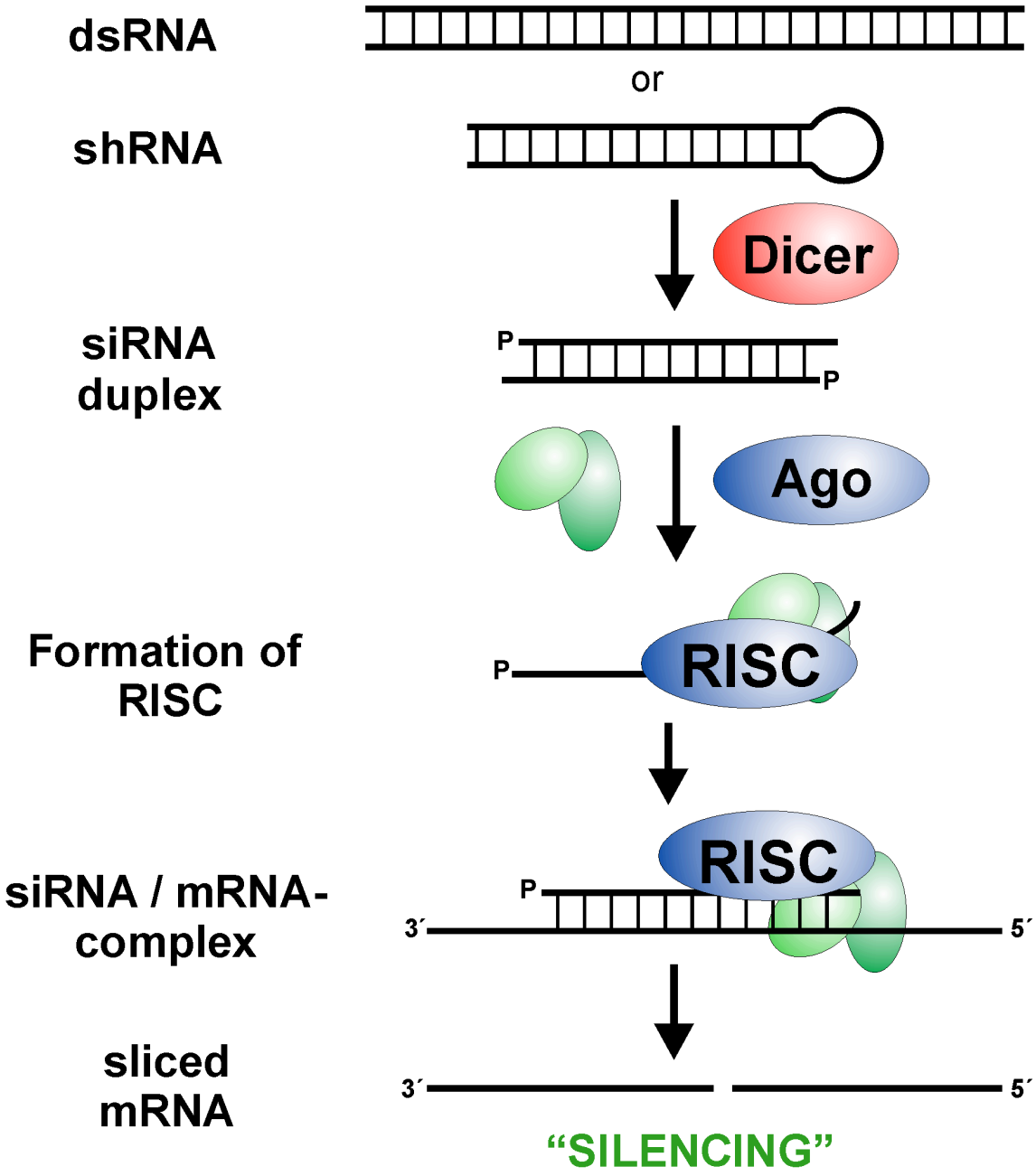
in key two steps!

Model for RNAi



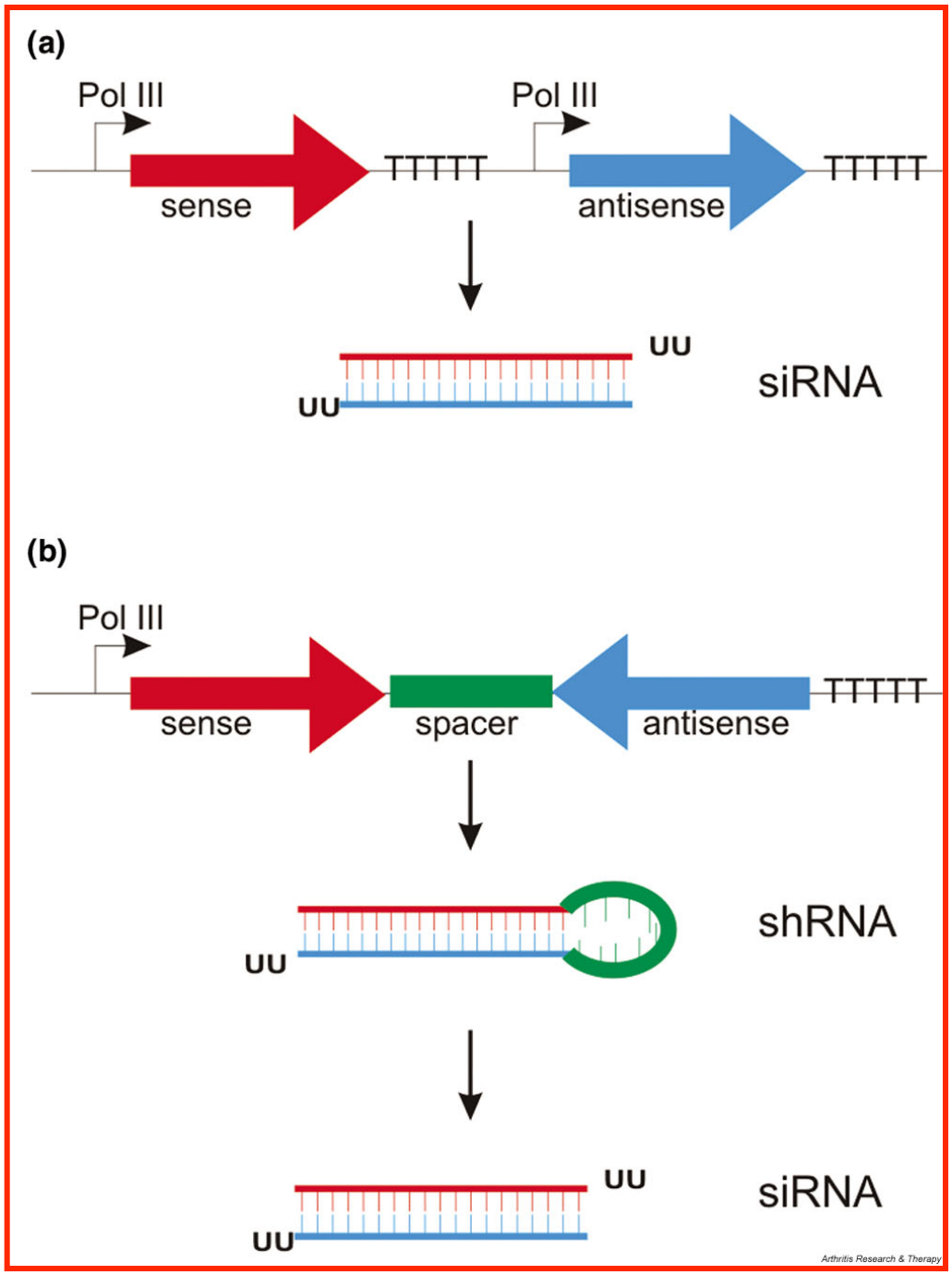
Dicer contains two RNAse III domains





RISC = RNA induced sliencing complex

http://www.gene-quantification.de/siRNA-mechanism.png

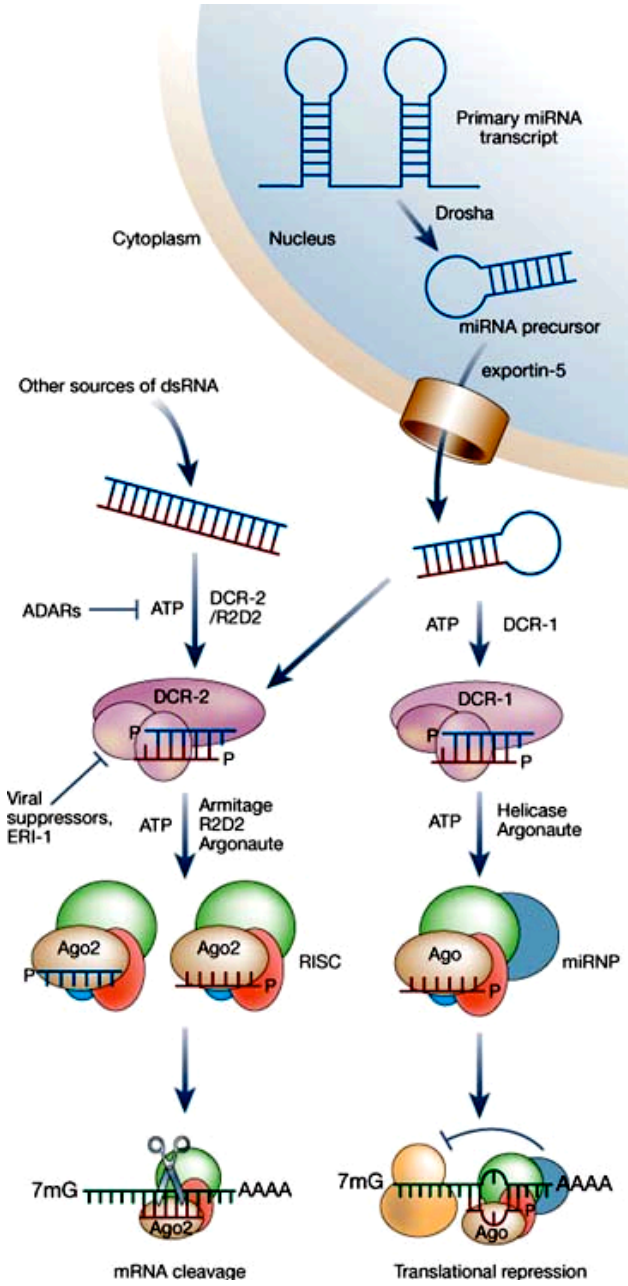


Endogenous expression of siRNAs in mammalian cells.

(a) Sense and antisense strand of the siRNA duplex are expressed from separate promoters.

(b) siRNA duplex is expressed as a stem-loop structure (small hairpin RNA [shRNA]) from a single promotor. Sense and antisense strands are separated by a loop-forming spacer. The construct is further processed by Dicer within the cell to form a functional siRNA. In both cases transcription is terminated by six consecutive thymidine residues.

Rutz and Scheffold *Arthritis Res Ther* 2004 **6**:78 doi:10.1186/ar1168



NATURE | VOL 431 | 16 SEPTEMBER 2004 | www.nature.com/nature

Mechanisms of gene silencing by double-stranded RNA

Gunter Meister & Thomas Tuschl

mRNA cleavage

Translational Repression

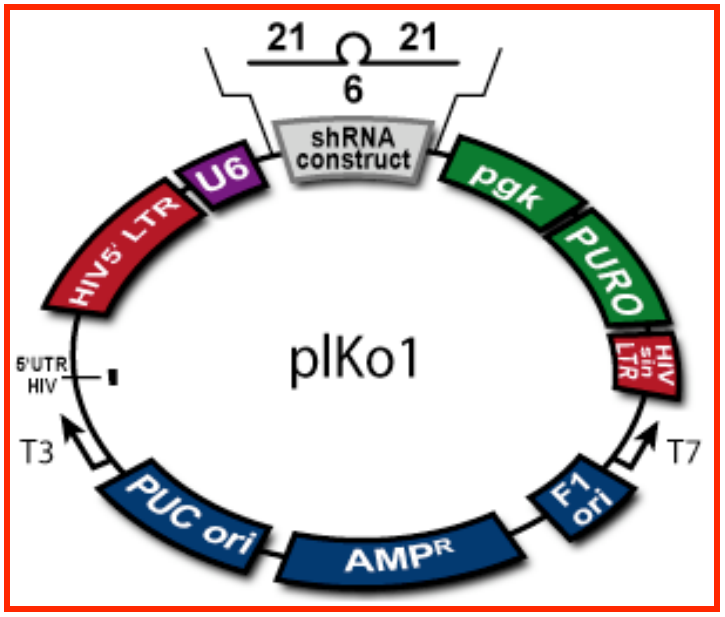
(Chromatin modification)

letters to nature

Nature **428**, 427 - 431 (25 March 2004); doi:10.1038/nature02370

A resource for large-scale RNA-interference-based screens in mammals

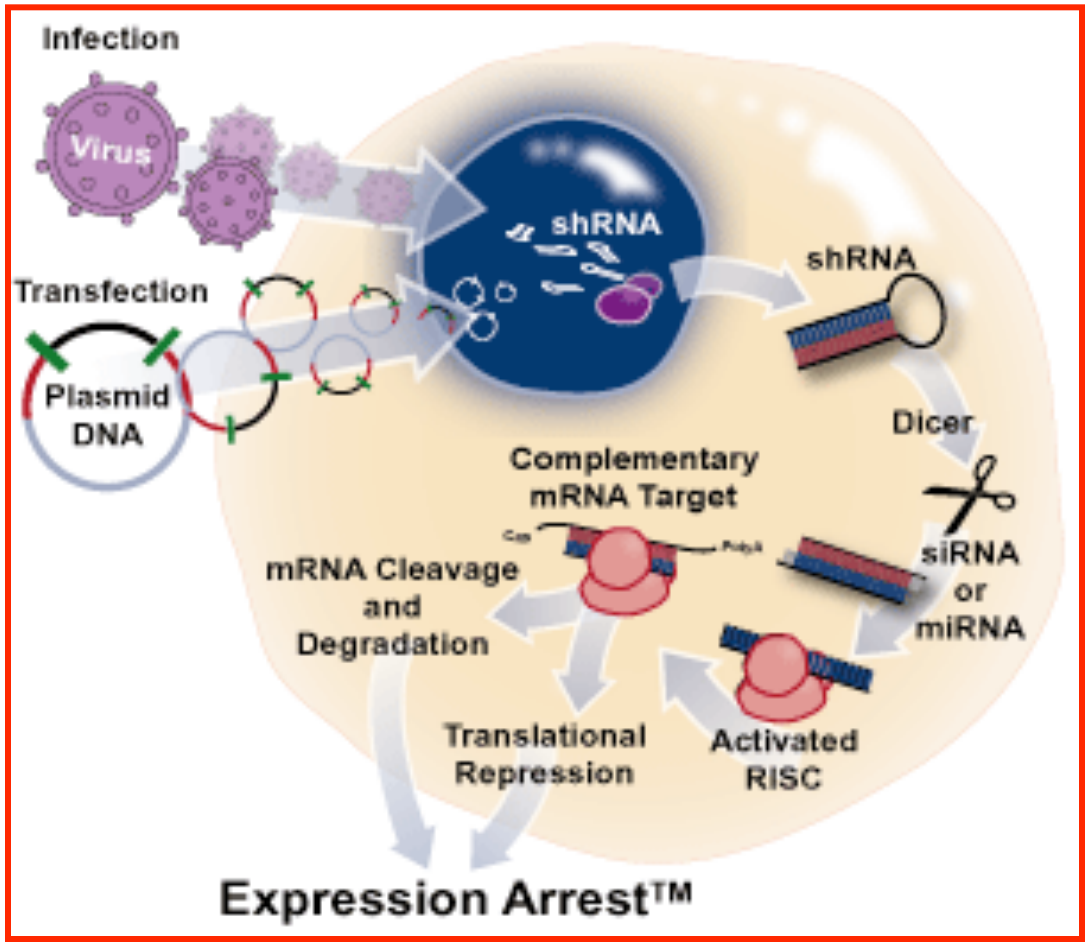
PATRICK J. PADDISON[1,*], JOSE M. SILVA[1,*], DOUGLAS S. CONKLIN[1,*,†], MIKE SCHLABACH[2,†], MAMIE LI[2], SHOLA ARULEBA[1], VIVEKANAND BALIJA[1], ANDY O'SHAUGHNESSY[1], LIDIA GNOJ[1], KIM SCOBIE[1], KENNETH CHANG[1], THOMAS WESTBROOK[2,†], MICHELE CLEARY[3], RAVI SACHIDANANDAM[1], W. RICHARD MCCOMBIE[1], STEPHEN J. ELLEDGE[2,†] & GREGORY J. HANNON[1]



Human shRNA Library (Lentiviral Vector)

The RNAi Consortium (TRC) lentiviral shRNA libraries

Comprehensive RNAi resource enabling transient, stable and *in vivo* RNAi studies.



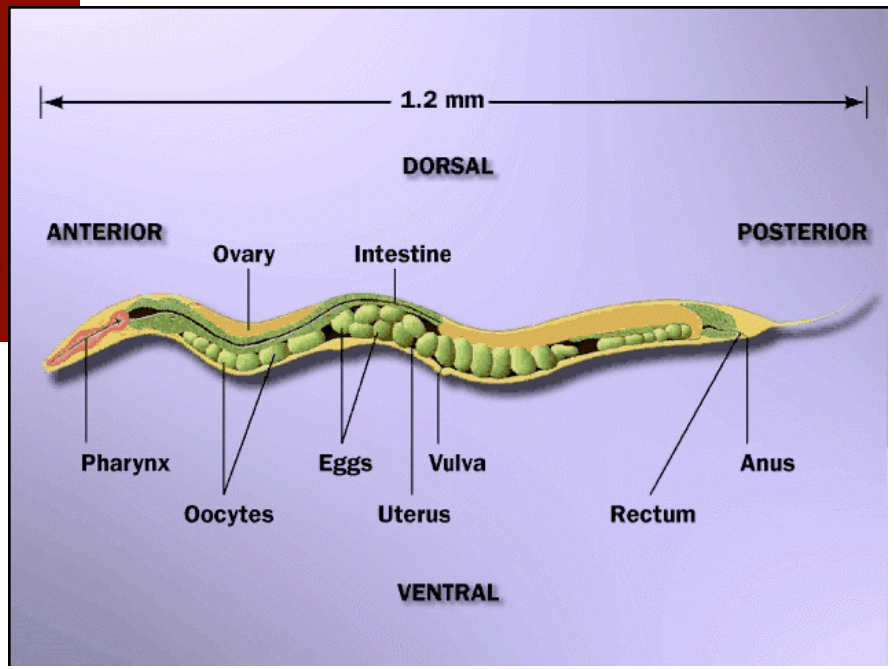
BioCat

Catalyze Your Success.

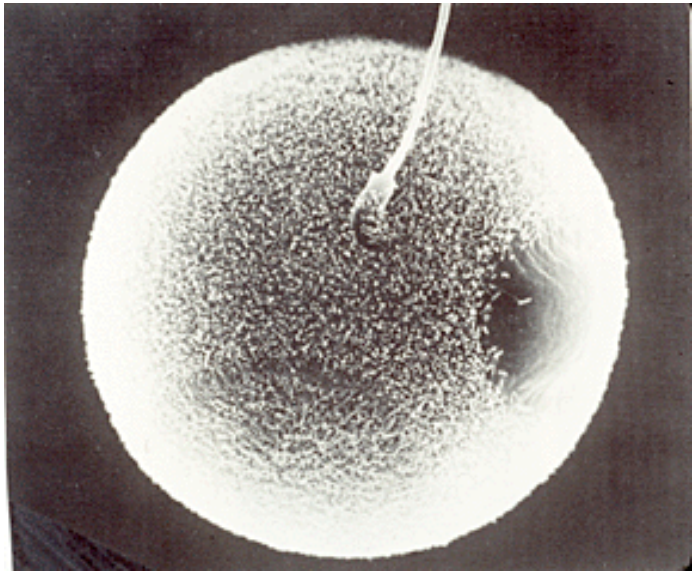
The nematode worm

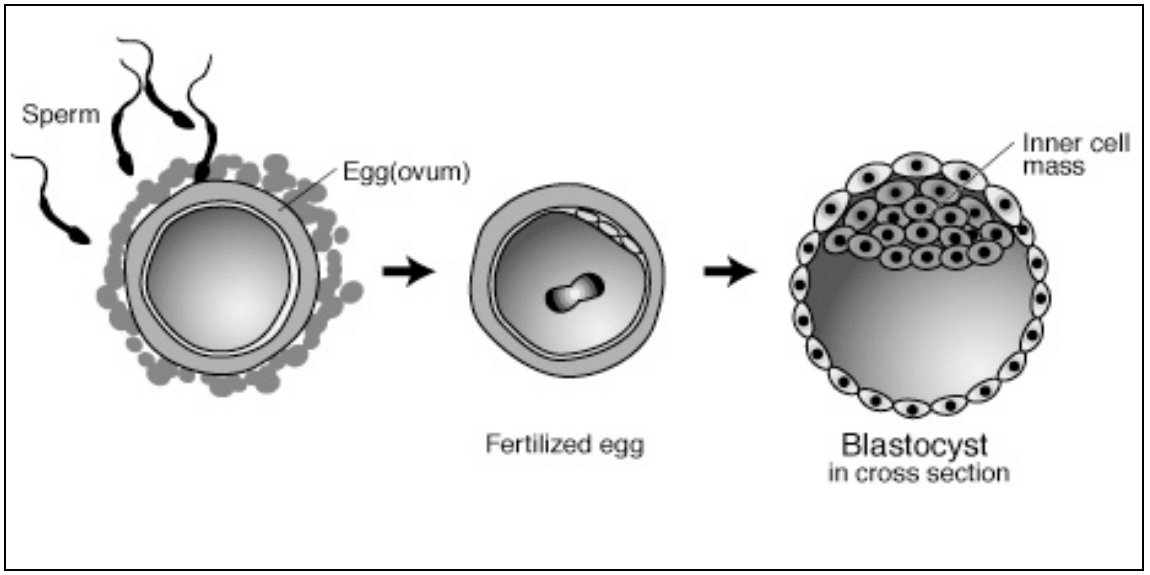
Caenorhabditis elegans

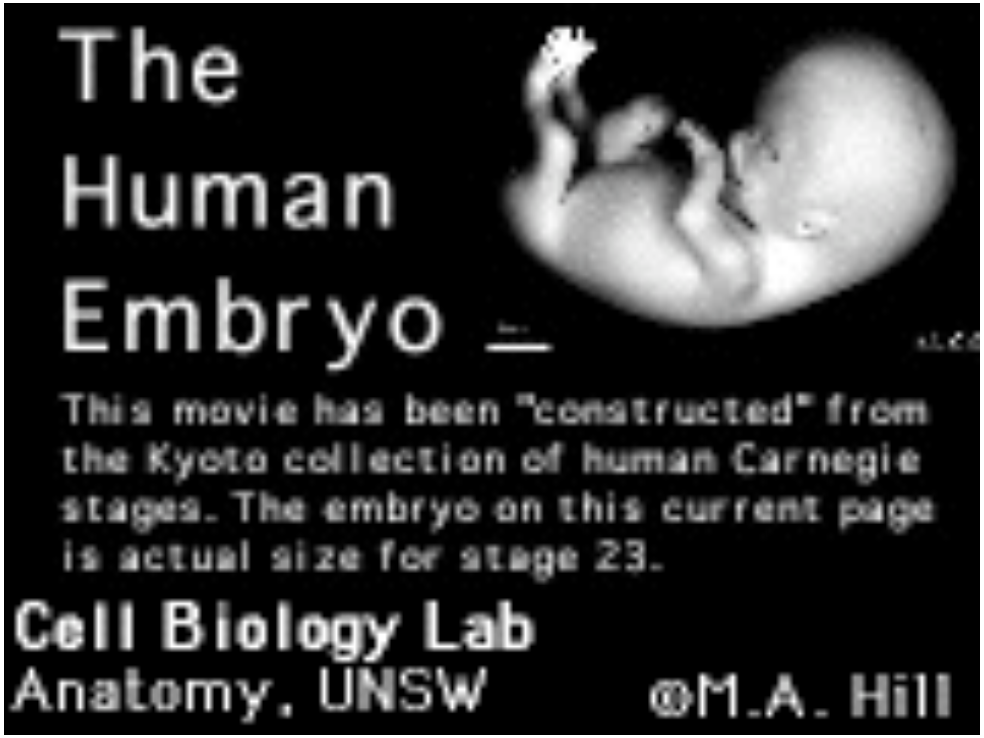
C. elegans



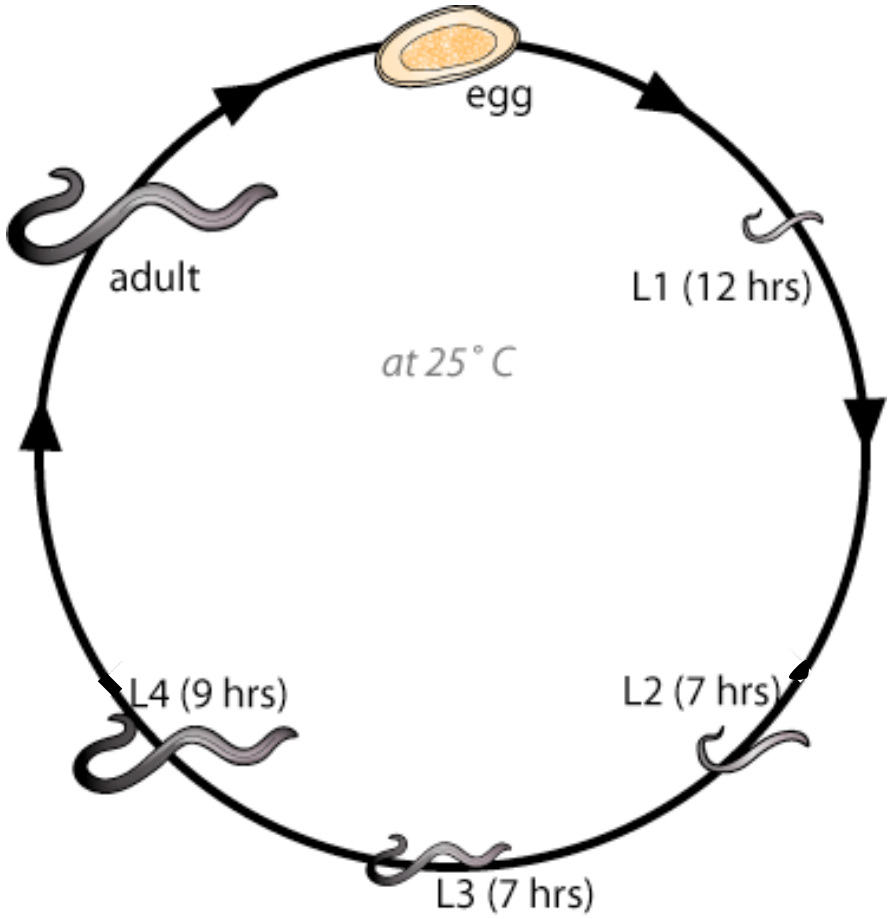
Basically has many of the bits and pieces that we have – a way to eat and excrete, and a way to reproduce.



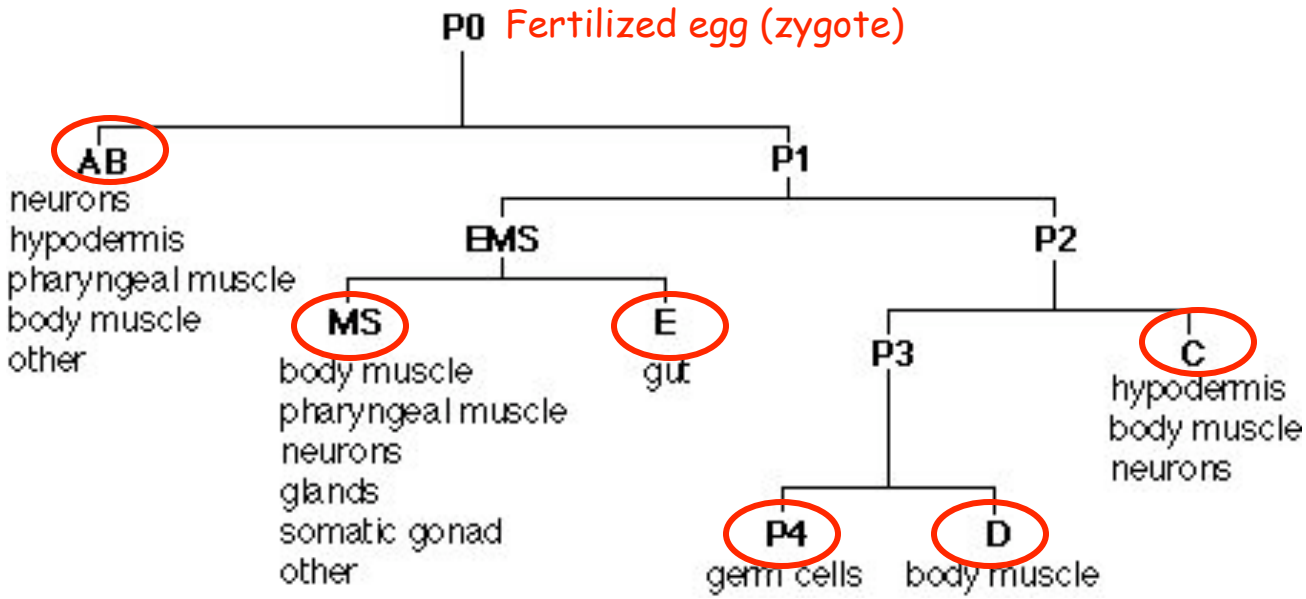

Sperm
Egg(ovum)
Fertilized egg
Inner cell mass
Blastocyst
in cross section

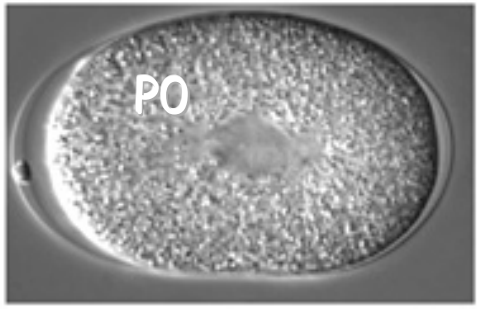

The Human Embryo
This movie has been "constructed" from the Kyoto collection of human Carnegie stages. The embryo on this current page is actual size for stage 23.
Cell Biology Lab
Anatomy, UNSW
©M.A. Hill

The *C. elegans* Life Cycle – only 50 h

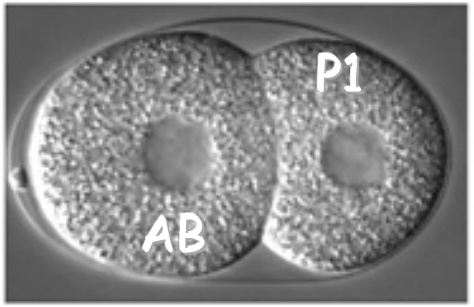


Pattern of early cell divisions from the *C. elegans* zygote to form six Founder Cells

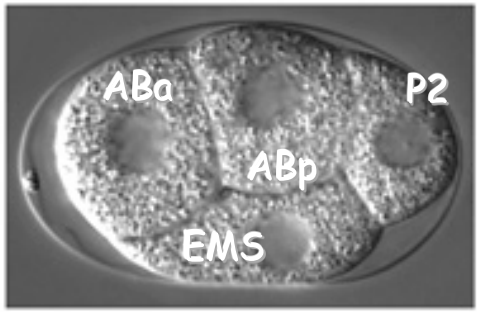




Fertilized egg (zygote)

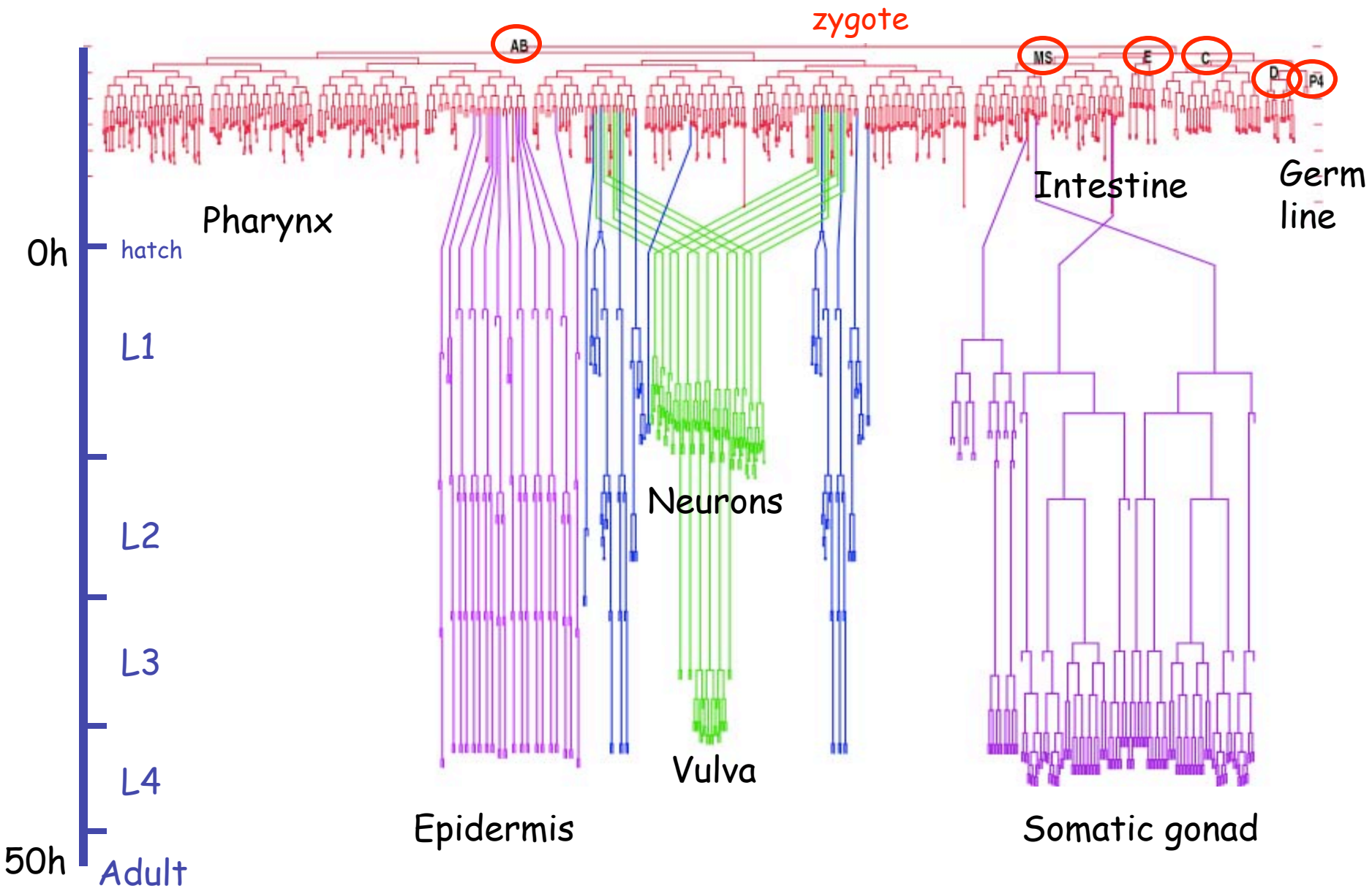


First cell division



Subsequent divisions

Full lineage of the entire *C. elegans* body



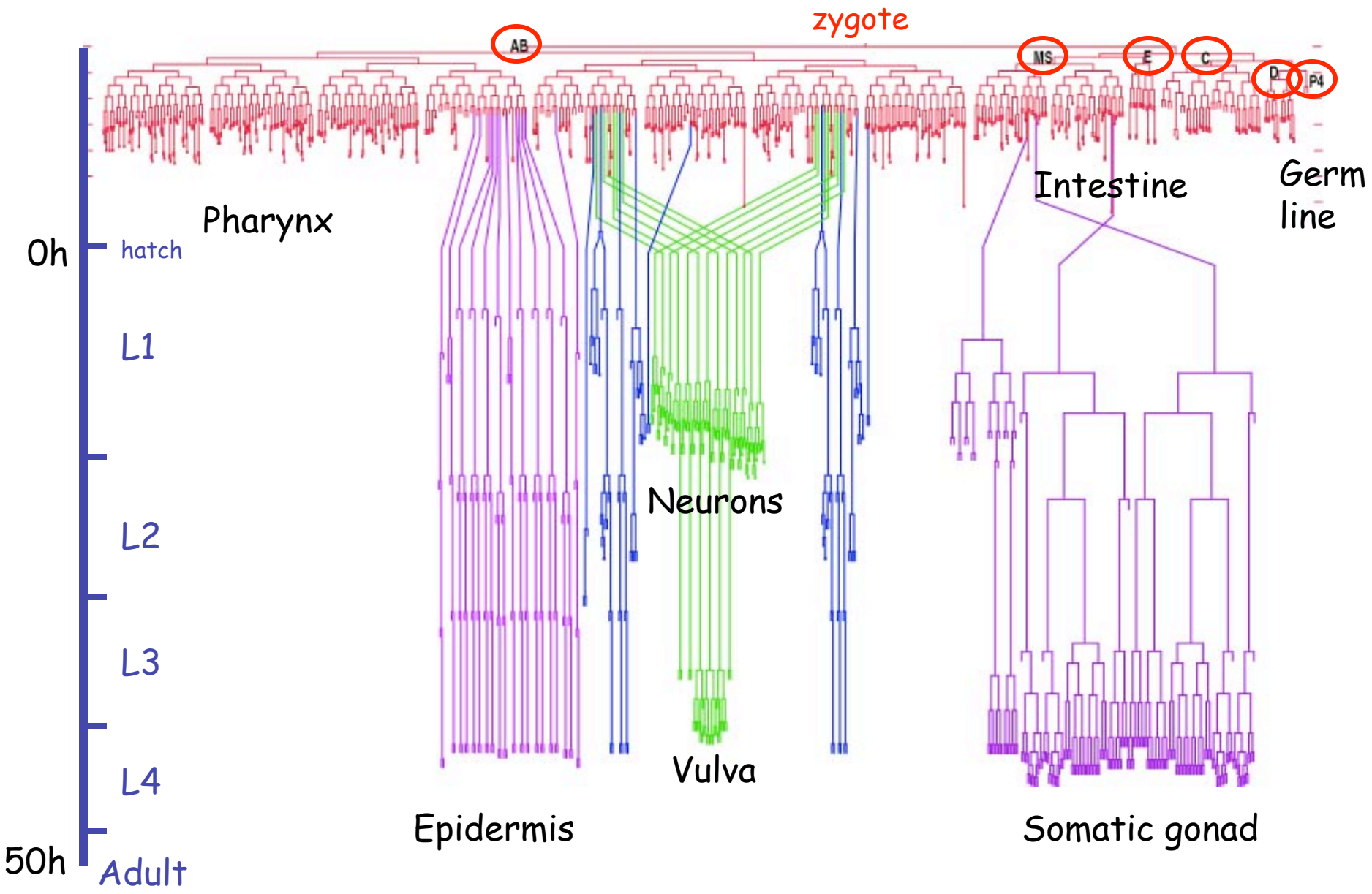
2002 Nobel Prize in Physiology or Medicine

John Sulston

Sydney Brenner

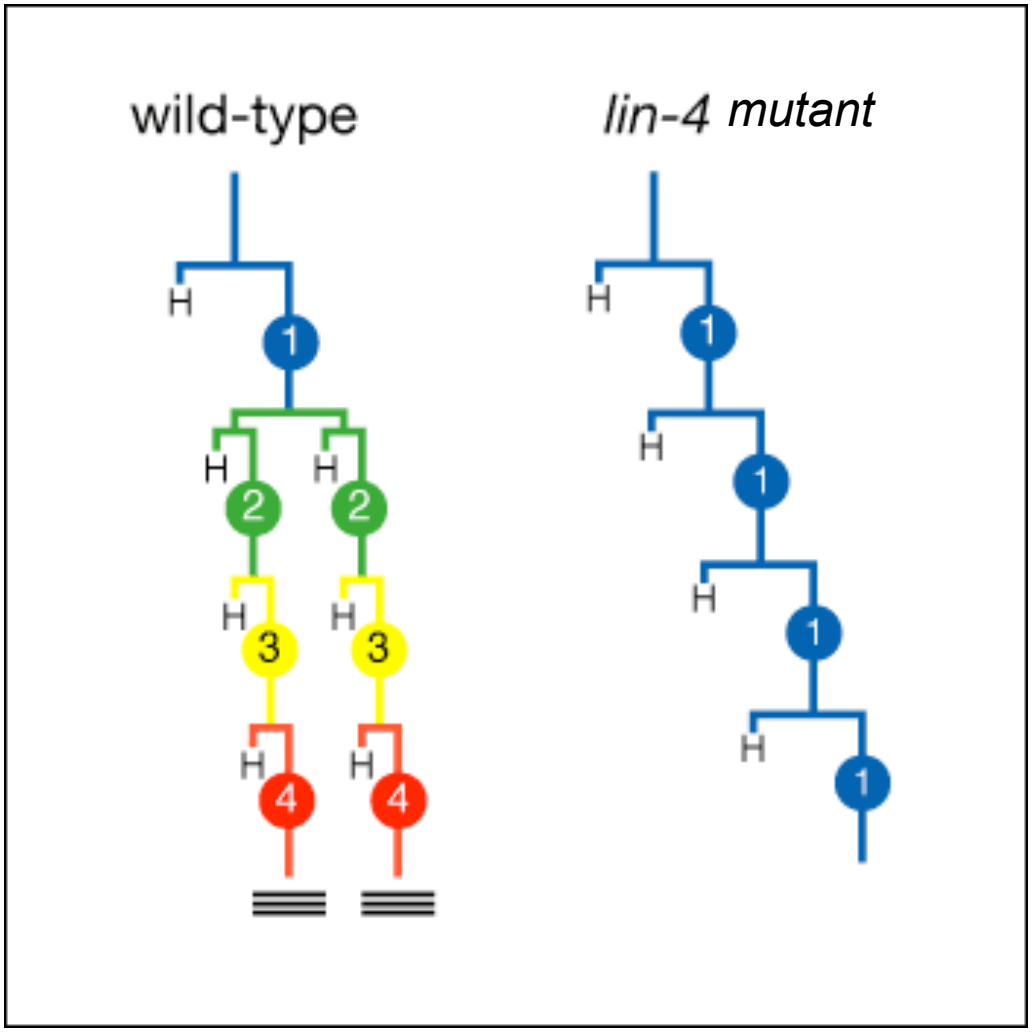
Bob Horvitz (MIT)

Full lineage of the entire *C. elegans* body



Heterochronic Mutants

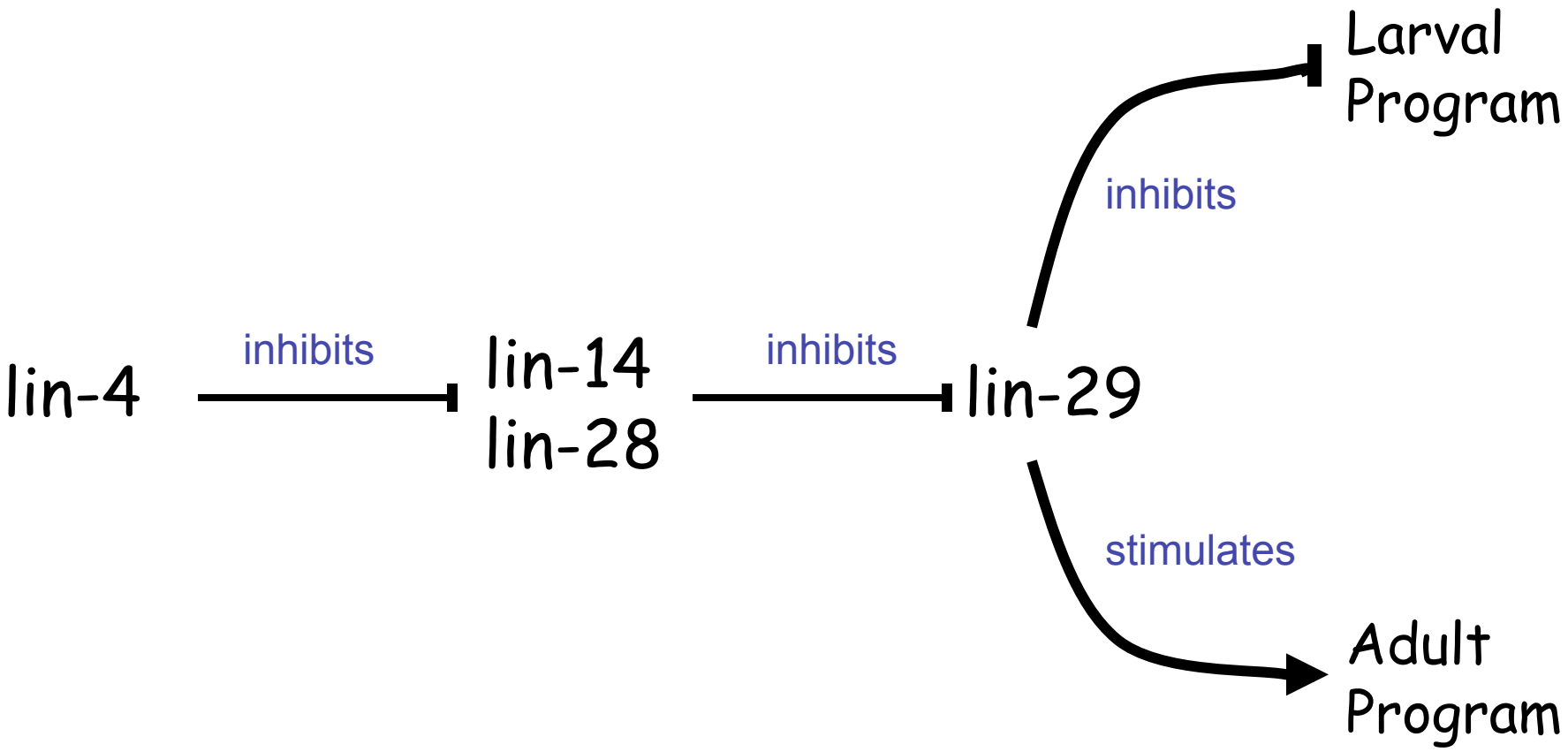
Misregulation of the another lineage

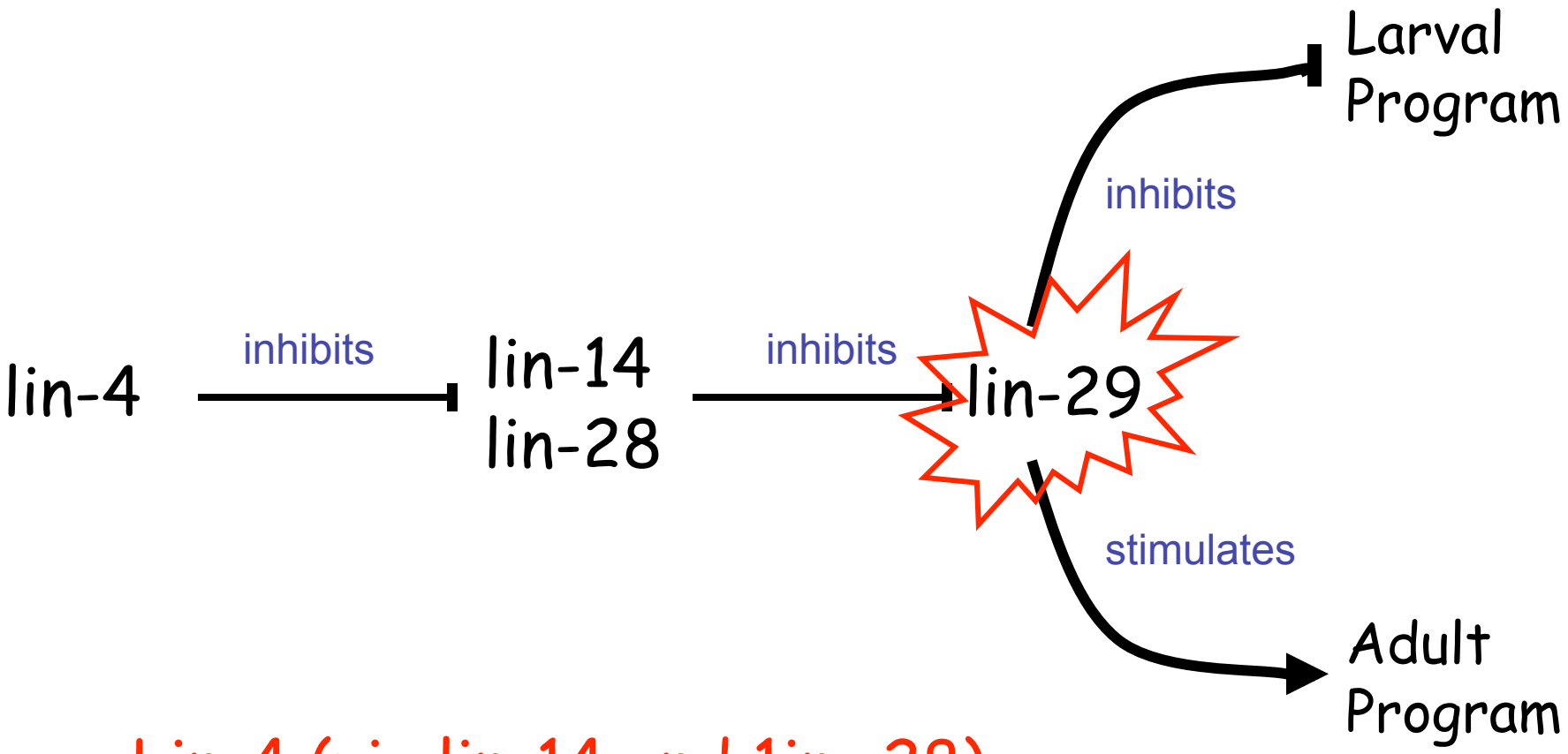


These mutants have been called HETEROCHRONIC mutants – they affect the relative timing of major developmental events

The existence of such mutants indicates that there is coordination of the temporal sequence of many cell fates an animal develops

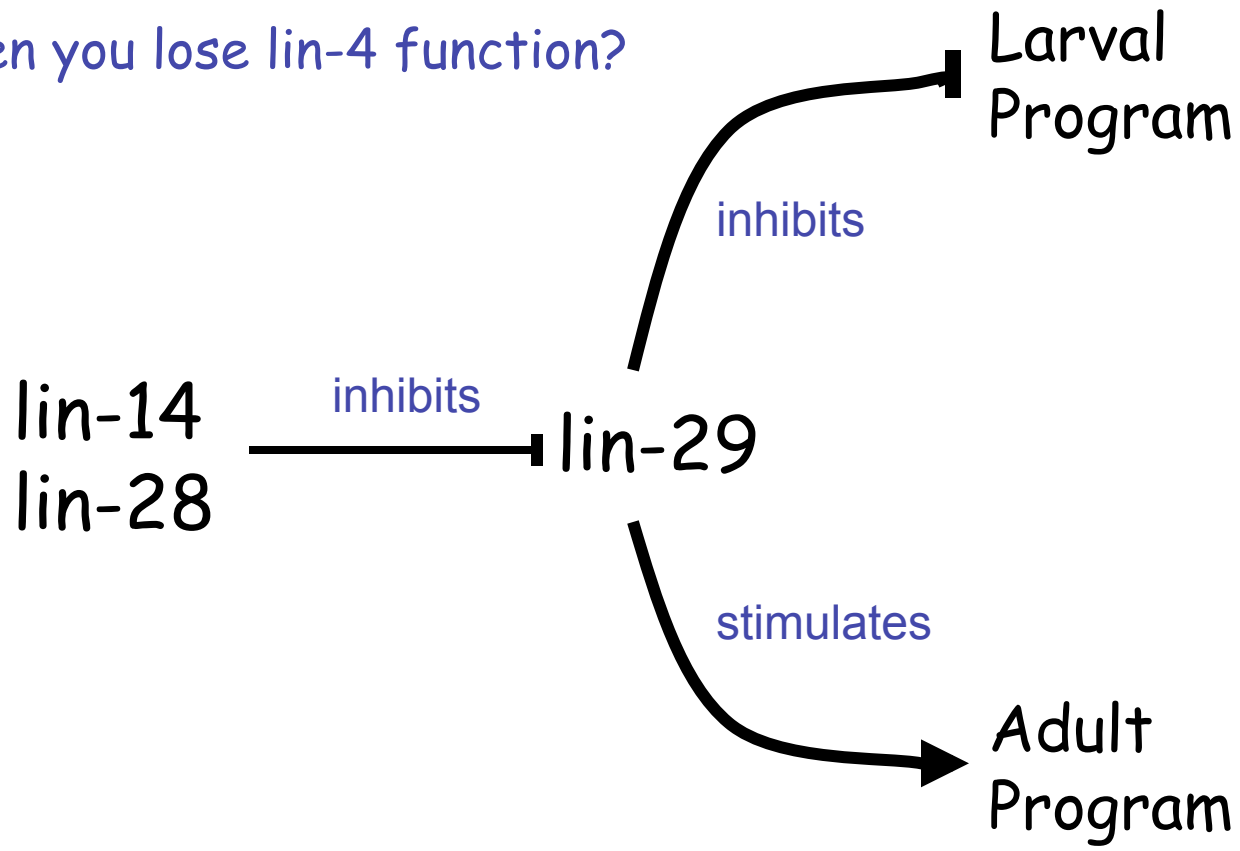
This temporal coordination is genetically regulated


lin-4
inhibits
lin-14
lin-28
inhibits
lin-29
inhibits
Larval
Program
stimulates
Adult
Program



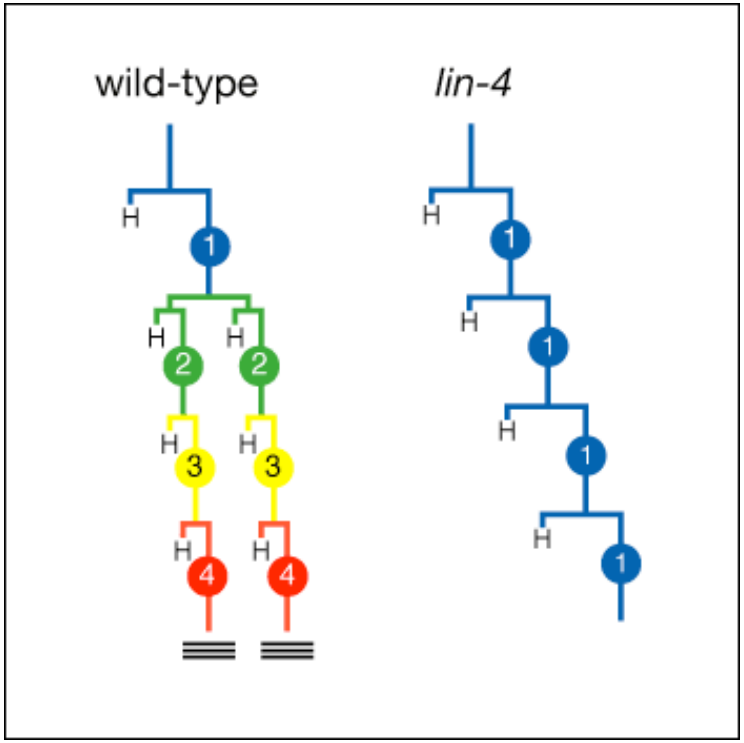
Lin-4 (via lin-14 and 1in- 28) regulates the switch from larval program to adult program

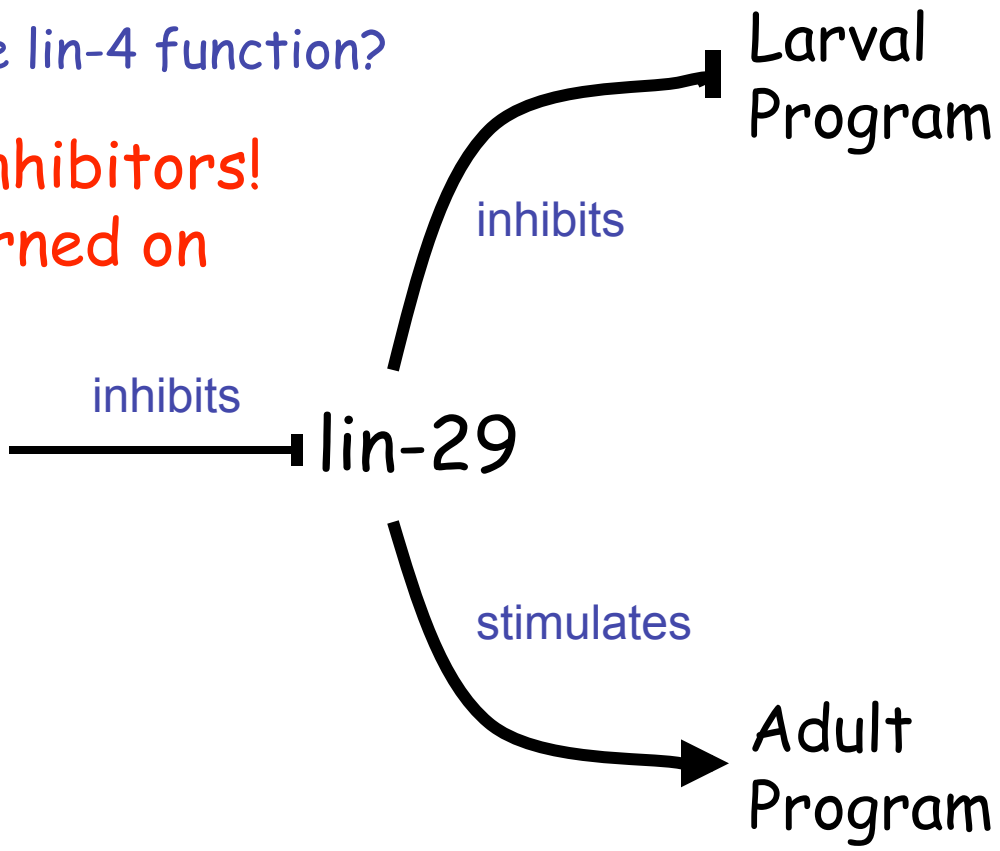
What happens in when you lose lin-4 function?

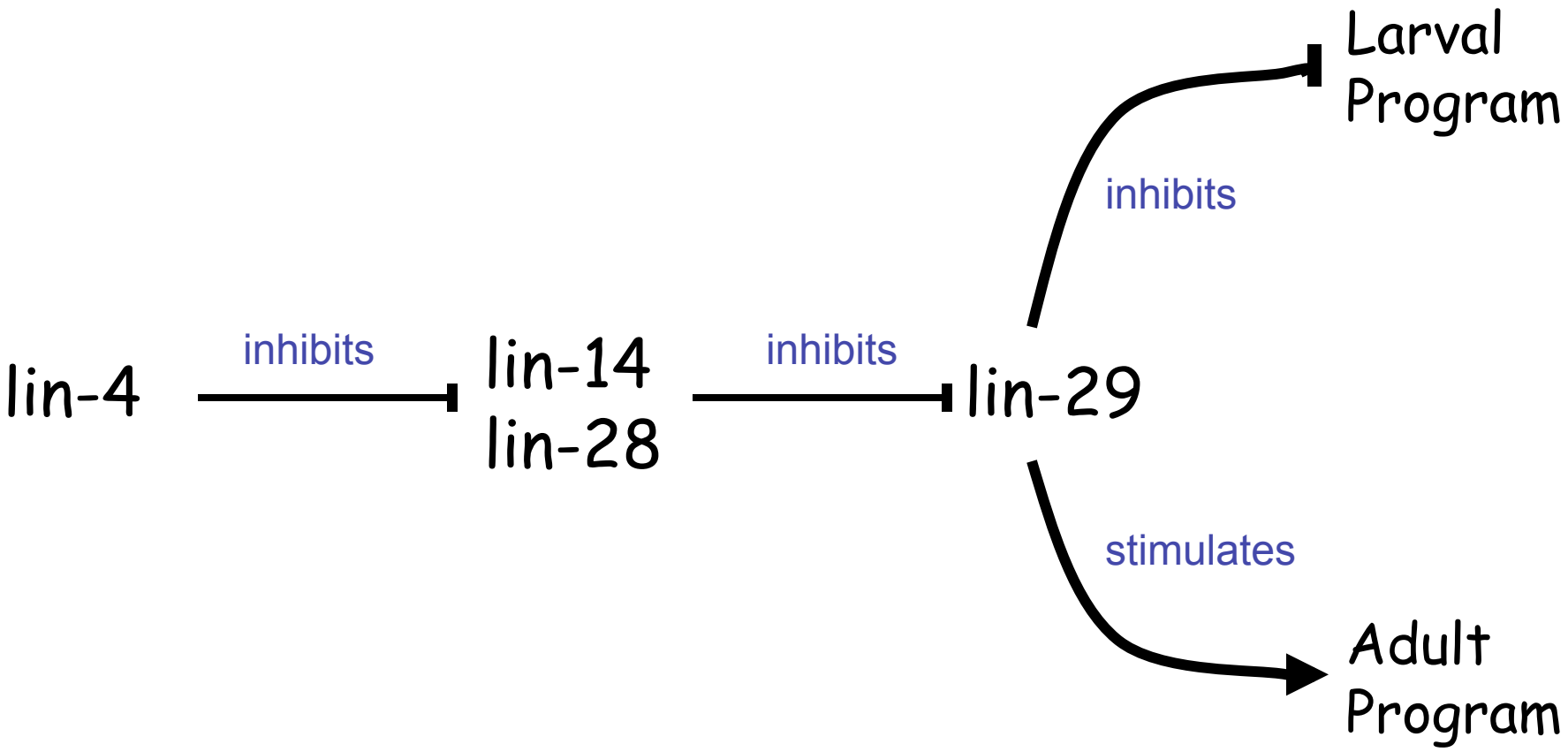


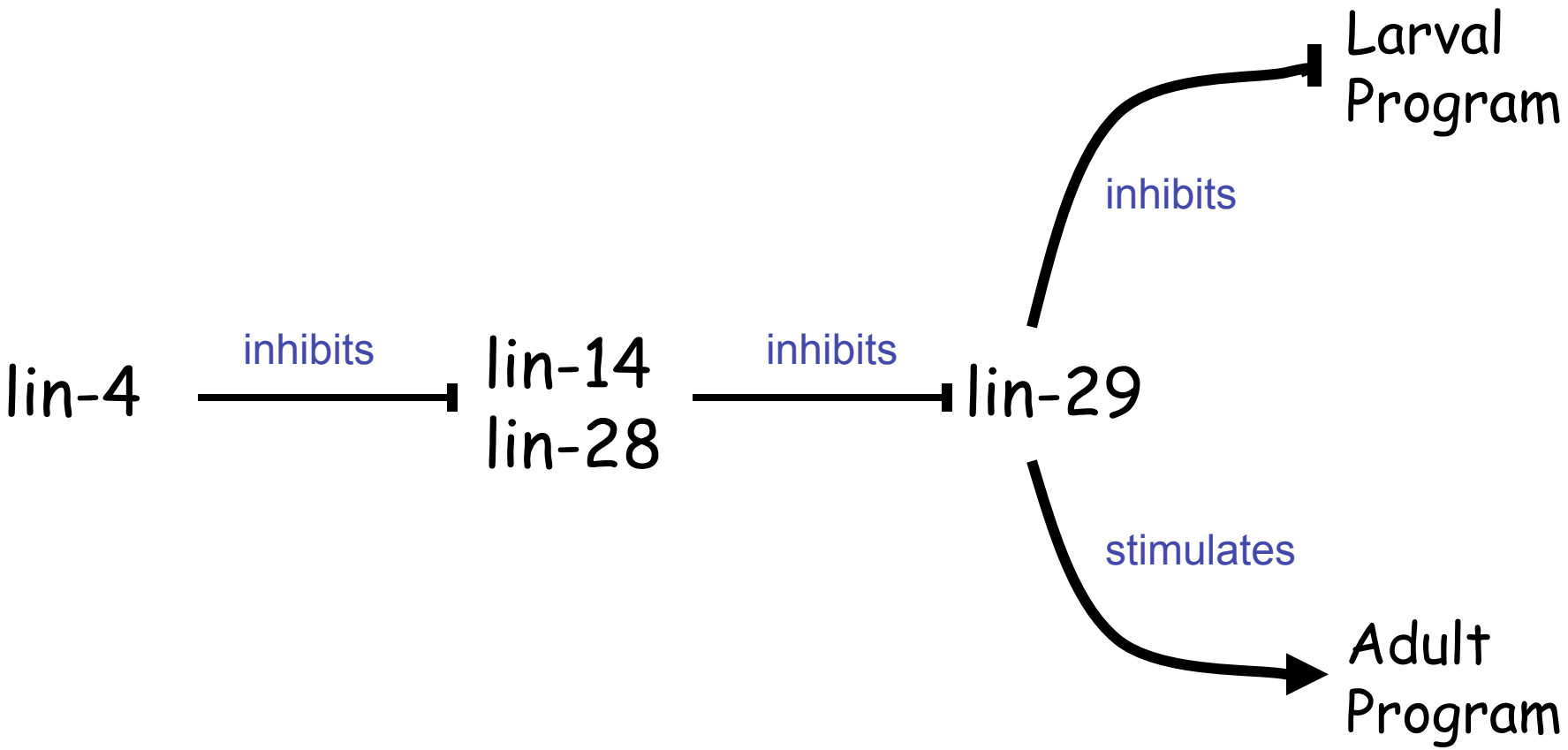
What happens in when you lose lin-4 function?

You can't inhibit the inhibitors!
lin-29 never gets turned on





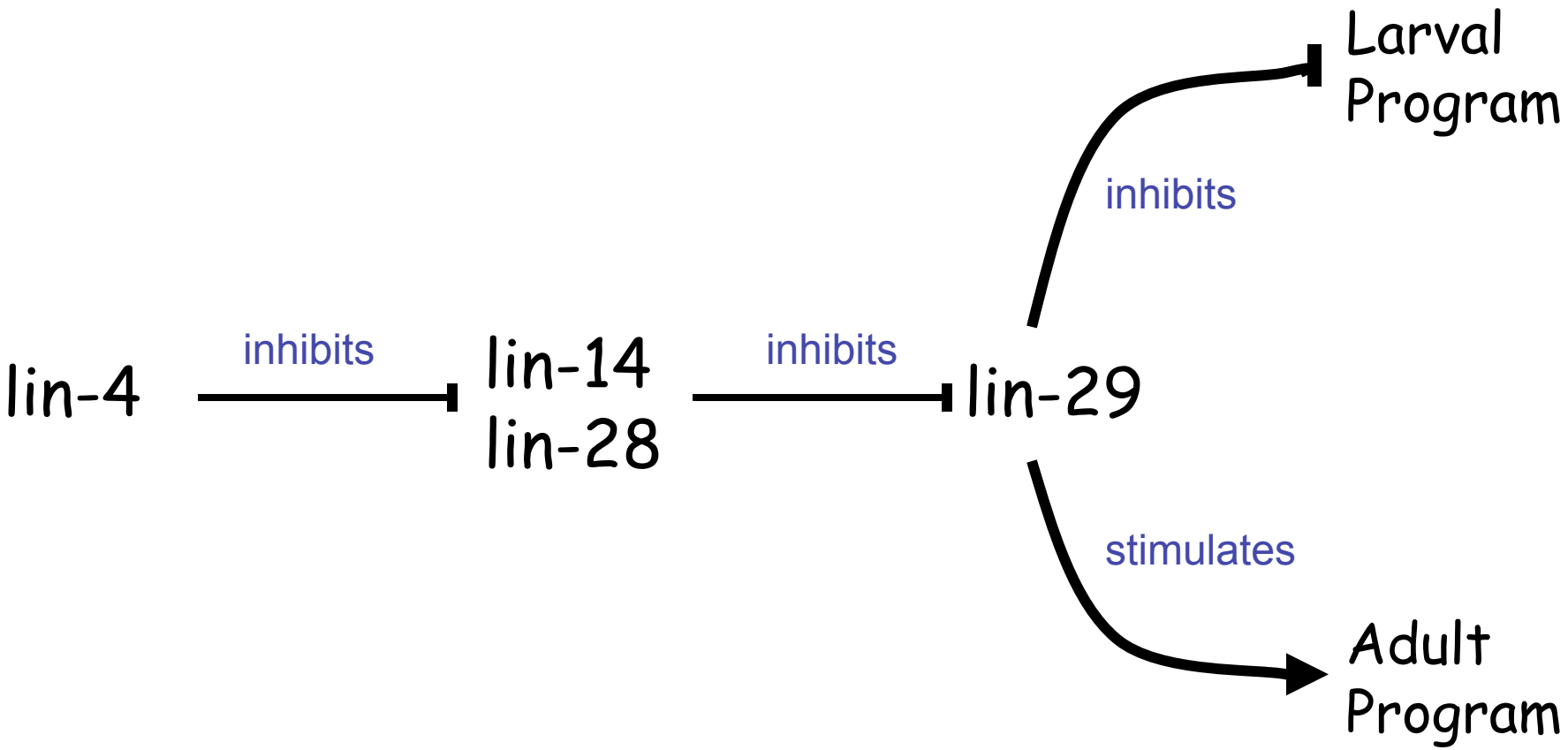

lin-4
inhibits
lin-14
lin-28
inhibits
lin-29
inhibits
Larval
Program
stimulates
Adult
Program



lin-14 encodes a nuclear protein

lin-28 encodes another protein

lin-29 encodes a Transcription factor

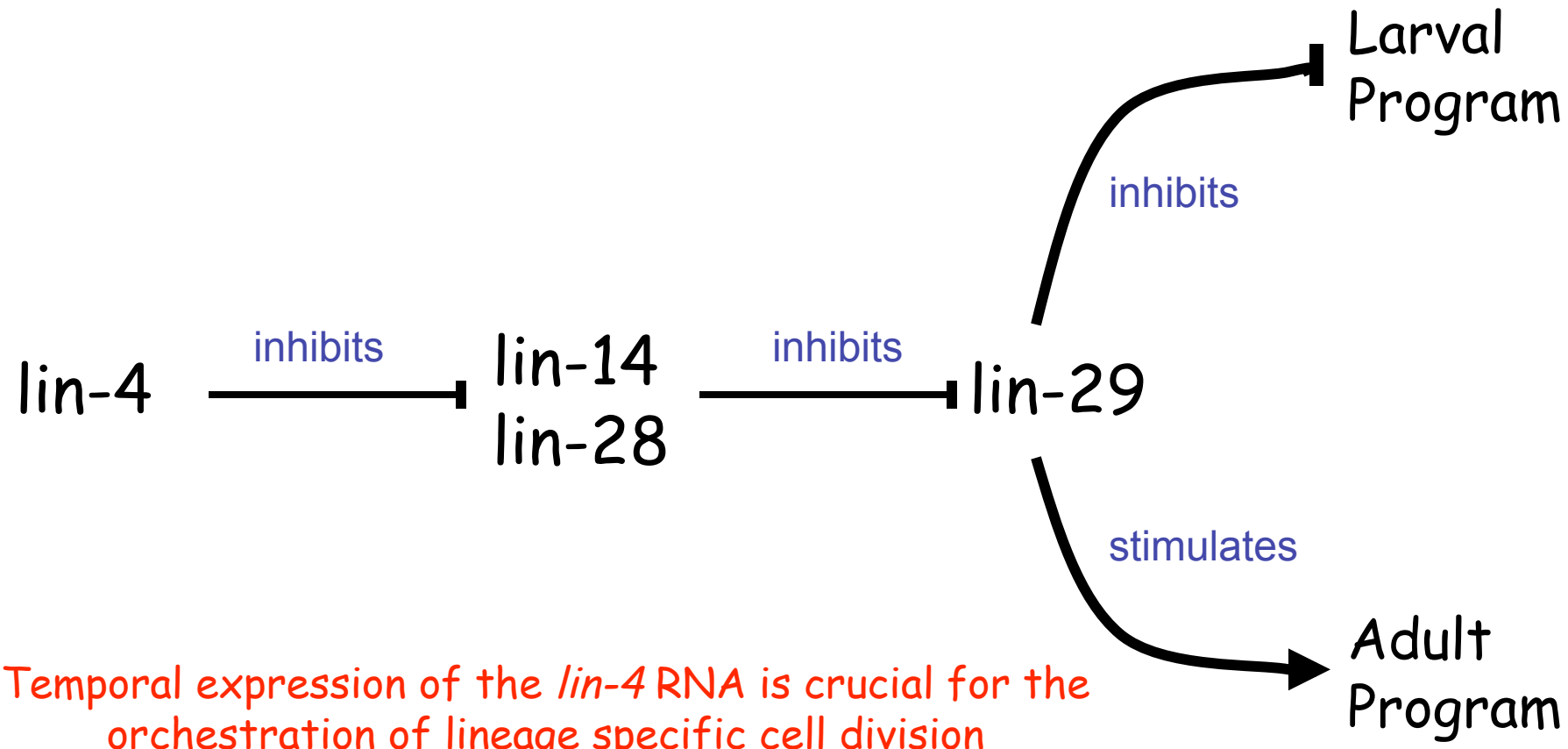


lin-4 encodes small RNAs, 61nt and 21 nt

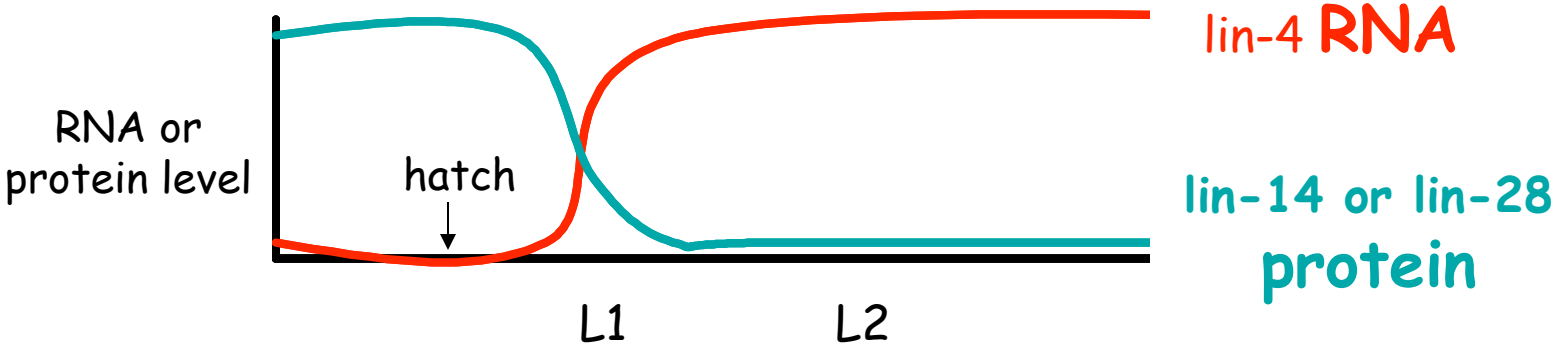
lin-14 encodes a nuclear protein

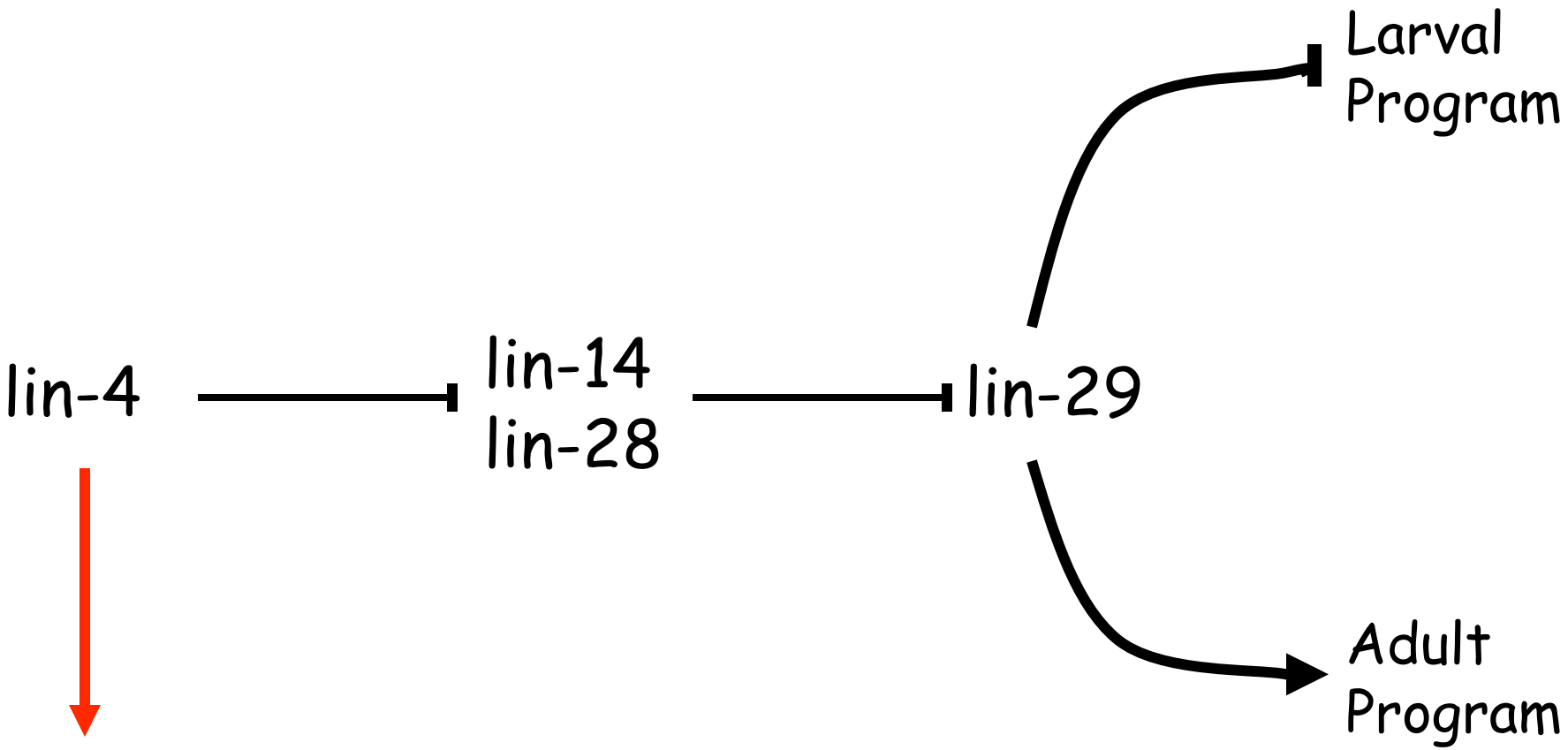
lin-28 encodes another protein

lin-29 encodes a Transcription factor



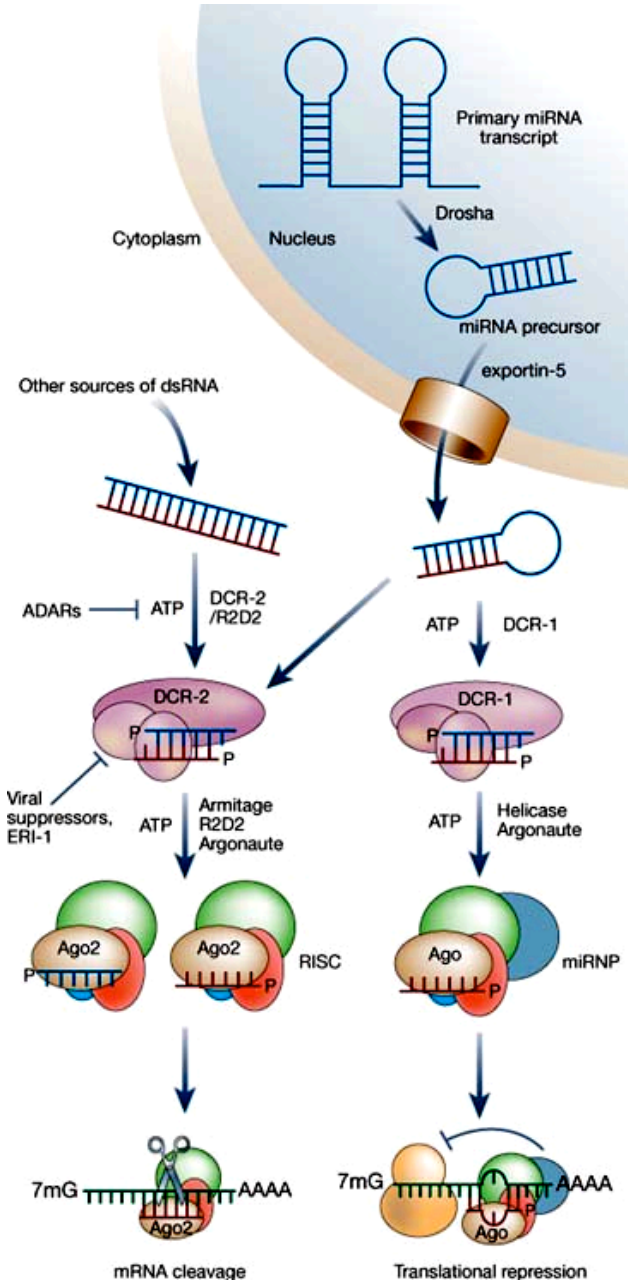
Temporal expression of the *lin-4* RNA is crucial for the orchestration of lineage specific cell division





This was the first microRNA regulatory molecule to be discovered

microRNAs are widespread in nature, including humans, and are very often involved in development and differentiation



NATURE | VOL 431 | 16 SEPTEMBER 2004 | www.nature.com/nature

Mechanisms of gene silencing by double-stranded RNA

Gunter Meister & Thomas Tuschl

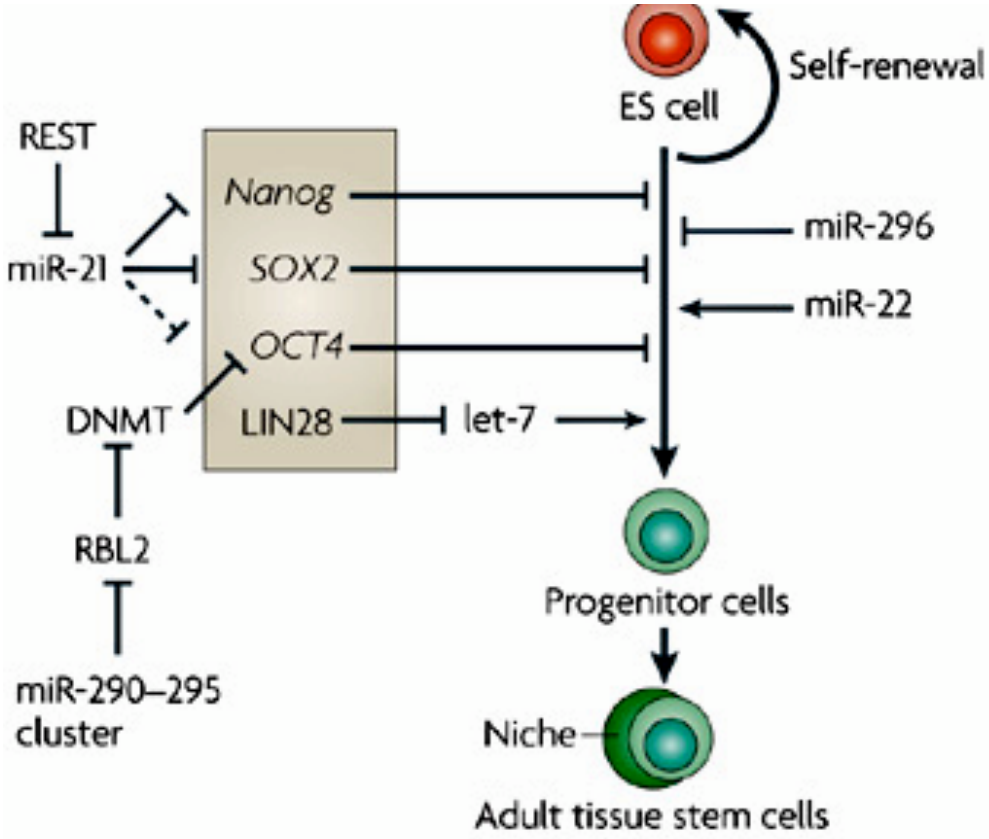
mRNA cleavage

Translational Repression

(Chromatin modification)

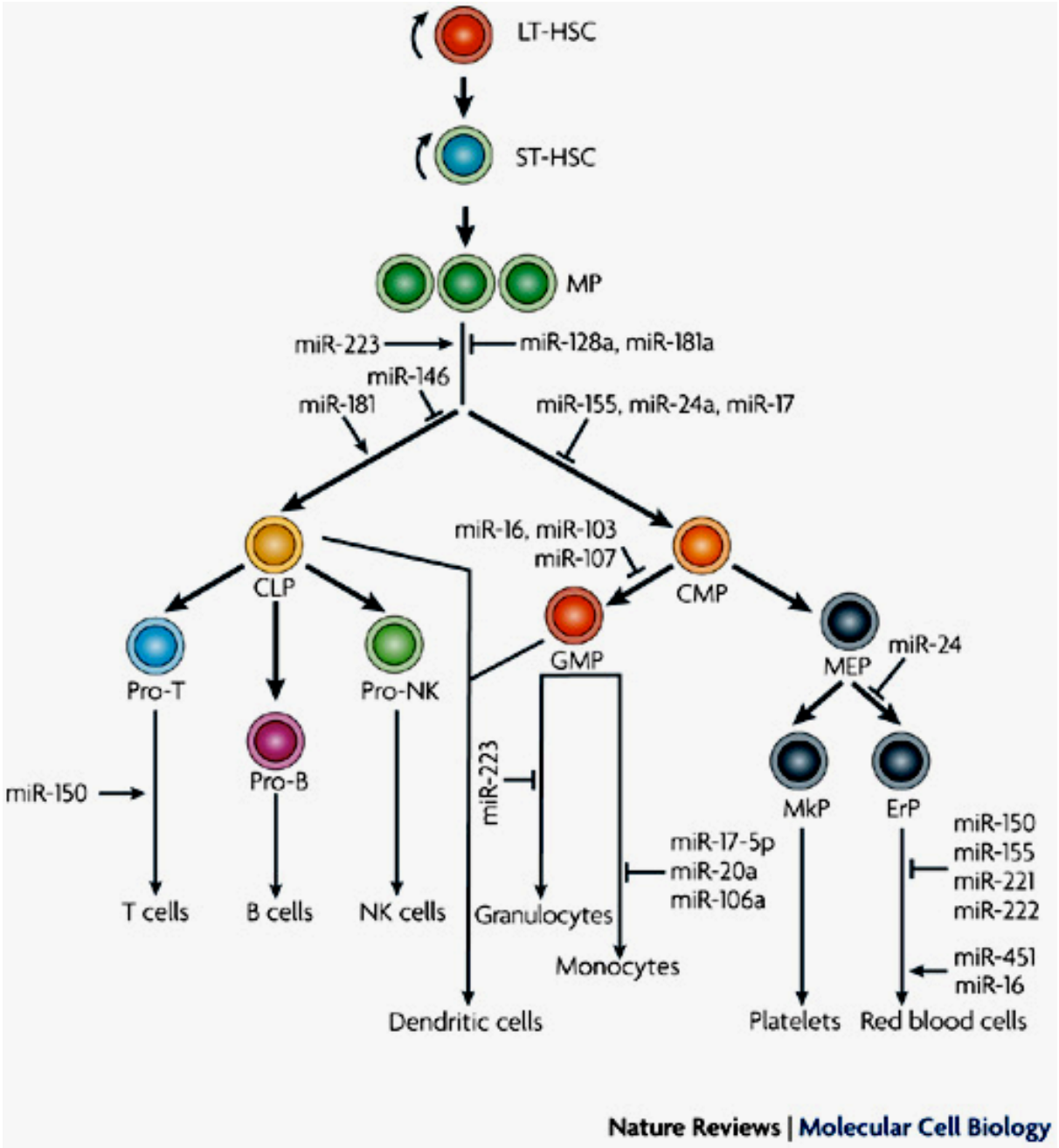
MicroRNAs: key regulators of stem cells

Vamsi K. Gangaraju and Haifan Lin

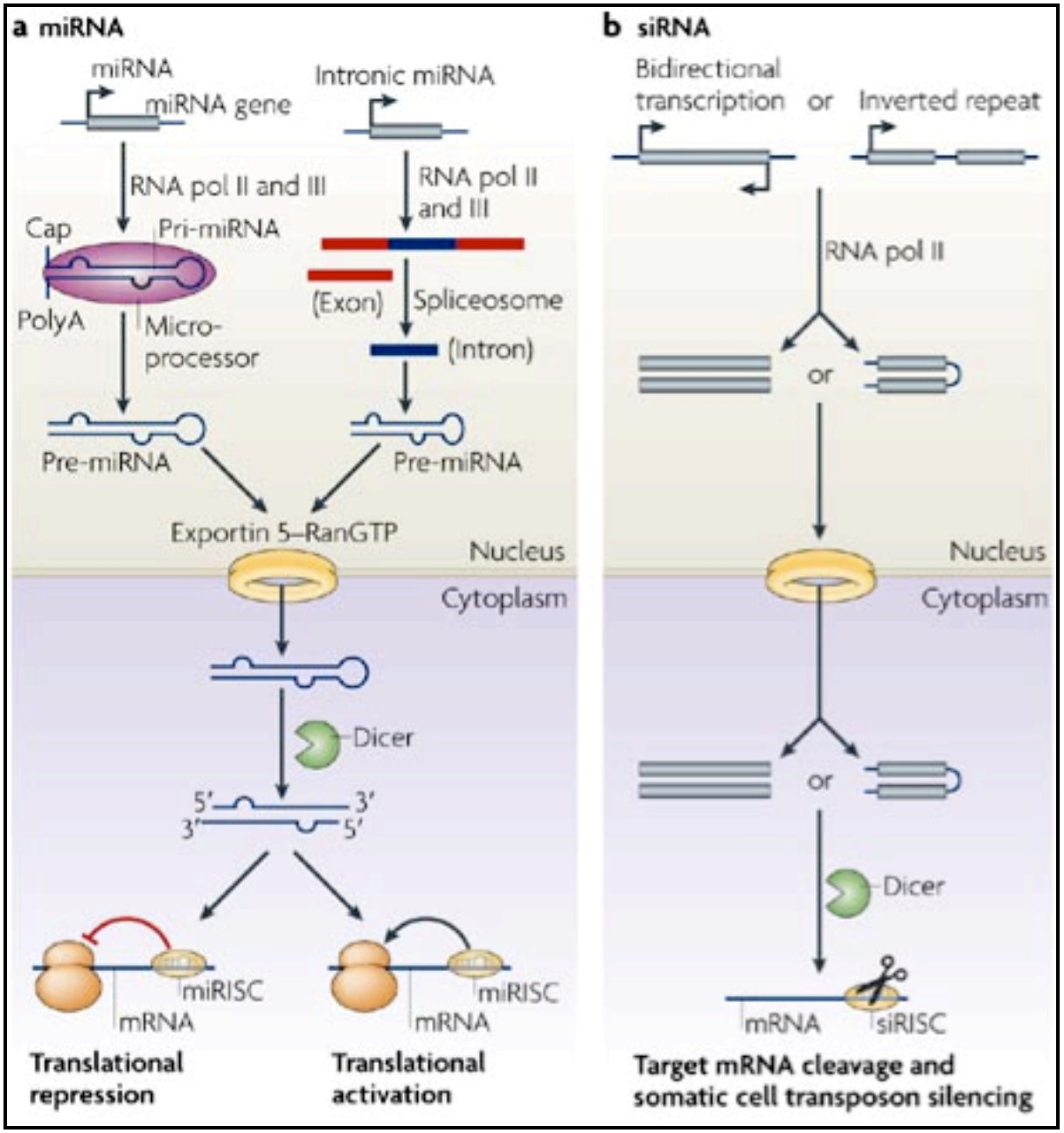


miR = micro RNA

Nature Reviews | Molecular Cell Biology
116 | FEBRUARY 2009 | VOLUME 10

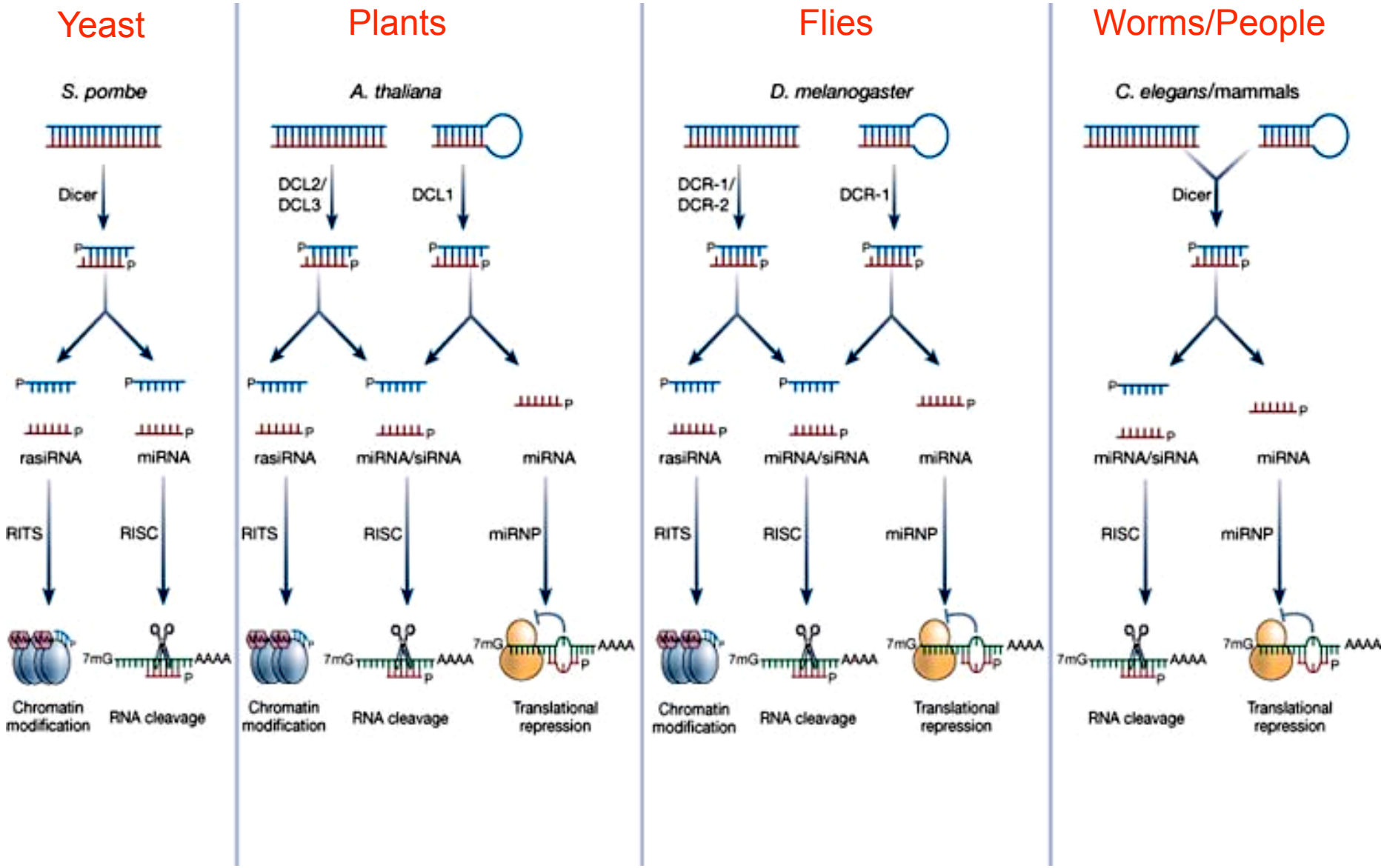


miRNAs and blood cell development



Dicer and RISC playing crucial roles in miRNA function – just as in siRNA silencing

Mechanisms differ slightly between organisms



Practical Aspects of RNAi

- biological research
  - defining gene function (gene knockout)
    - genome-wide RNAi projects
  - defining biochemical pathways
    - microarray screening of RNAi knockouts

- therapeutic treatment
  - cancer
  - viral infection
  - parasitic infection