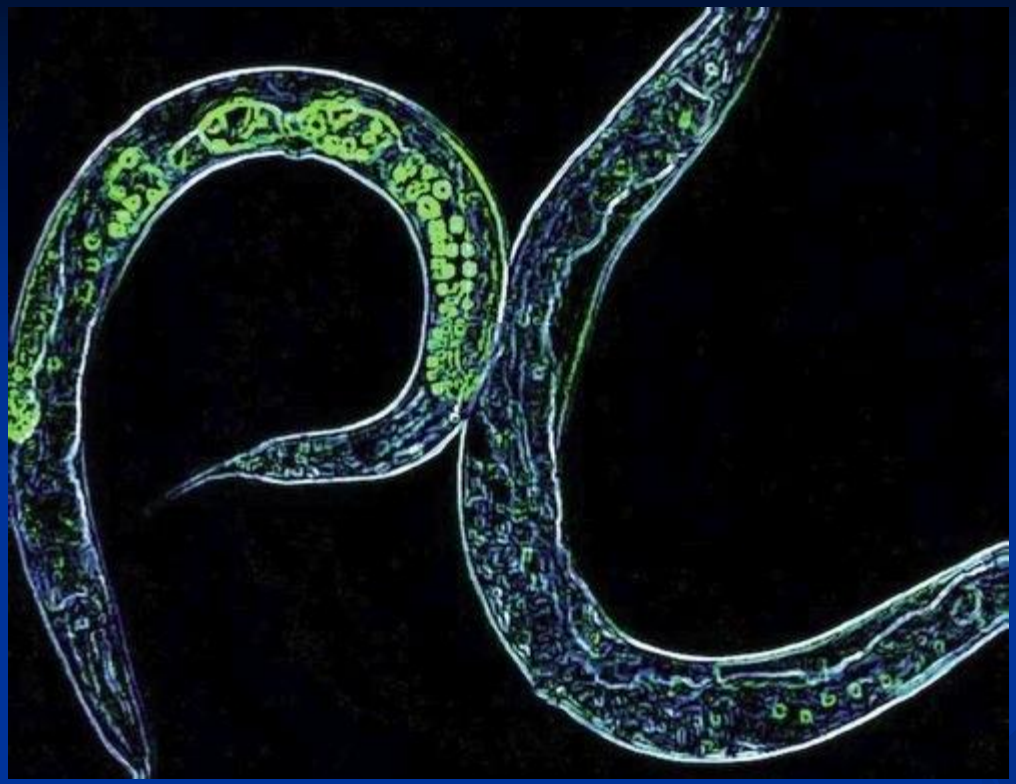


RNA interference

Hans Bluyssen

29-11-2017

1928 report of development of viral (tobacco ringspot virus) resistance in tobacco.

JOURNAL OF AGRICULTURAL RESEARCH

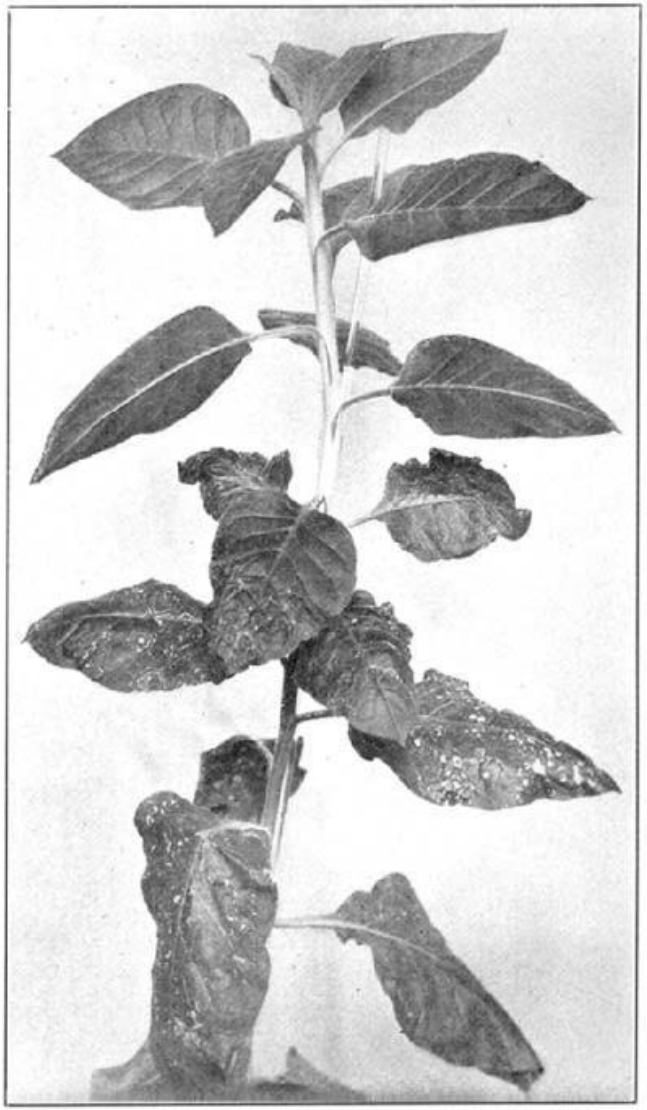
VOL. 37 WASHINGTON, D. C., AUGUST 1, 1928 No. 3

HOSTS AND SYMPTOMS OF RING SPOT, A VIRUS DISEASE OF PLANTS[1]

By S. A. WINGARD[2]

Associate Plant Pathologist, Virginia Agricultural Experiment Station

INTRODUCTION



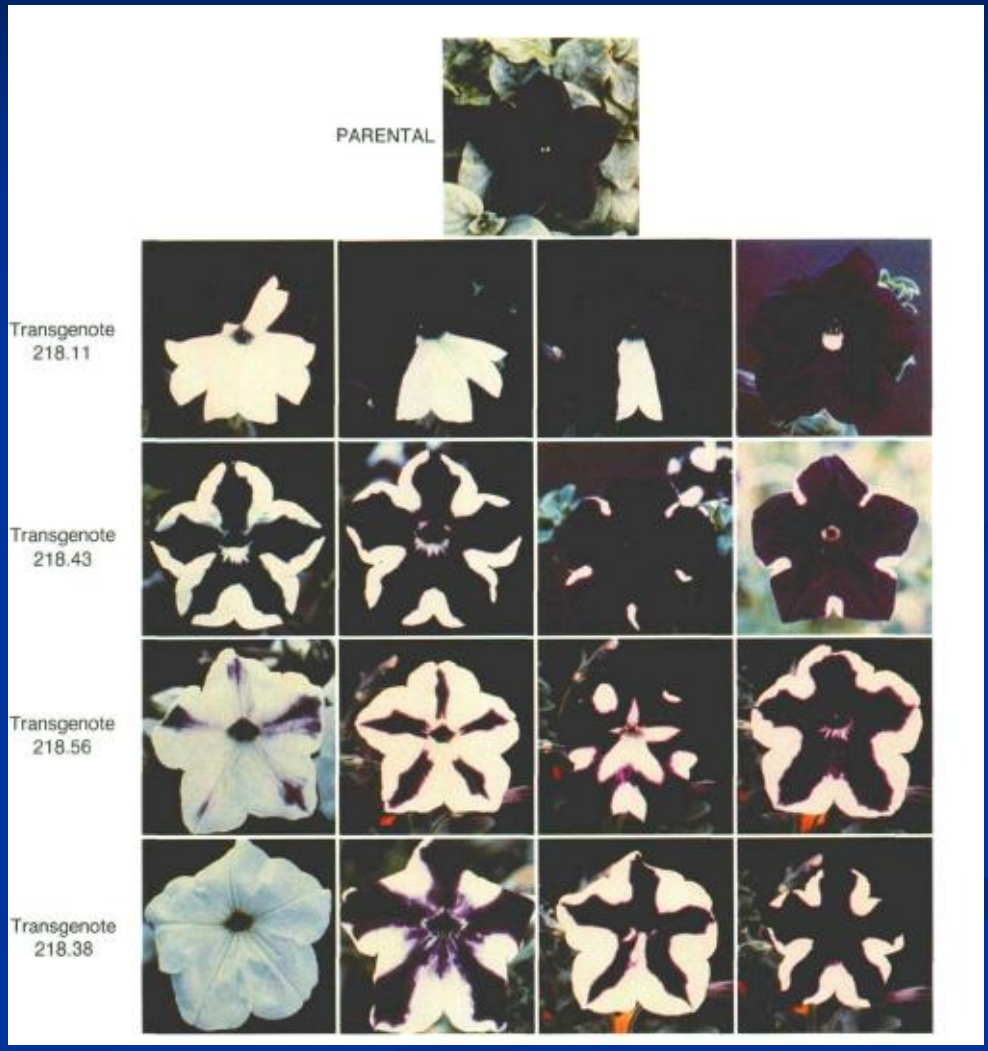
An unexpected result

- 1990: the introduction of transgenic copies of a gene led to the down-regulation of those transgenic copies as well as the endogenous gene

Phenotypes of chimeric chalcone synthase transgenotes:

Top: parental flower (control)

Bottom lines: four different transgenotes, four representative flowers are shown in a row for each of the transgenotes.



Napoli et al. Plant Cell. ***2*** (1990)

Transgene co-suppression

Observations:

- the expression of transgene often resulted in the silencing of the transgene, plus the silencing of the corresponding endogenous gene.

- In *C. elegans*, the injection of antisense-RNA caused the silencing of the endogenous gene, however, the sense RNA strand (negative control) has the same effect (Guo and Kemphues, 1995).

  Similar results have been obtained in other species.

The discovery of RNA-mediated interference

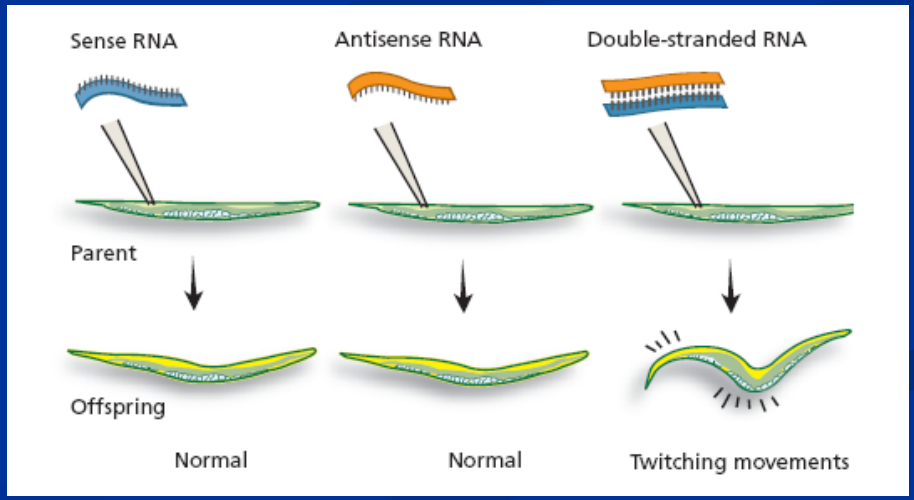
Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

Working hypothesis: the silencing effect by antisense or sense RNA might be due to low-level contaminations of **double-stranded RNA**.

- 4-6 hours after injection, eggs collected.
- Screened for phenotypic changes
  - twiching

Unc-22 phenotype



Fire et al., Nature **391**. (1998)

The discovery of RNA-mediated interference

Gene	segment	Size (kilobases)	Injected RNA	F_1 phenotype
unc-22				*unc-22*-null mutants: strong twitchers[7,8]
*unc22A**	Exon 21–22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22B	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
unc22C	Exon 21–22†	785	Sense + antisense	Strong twitchers (100%)

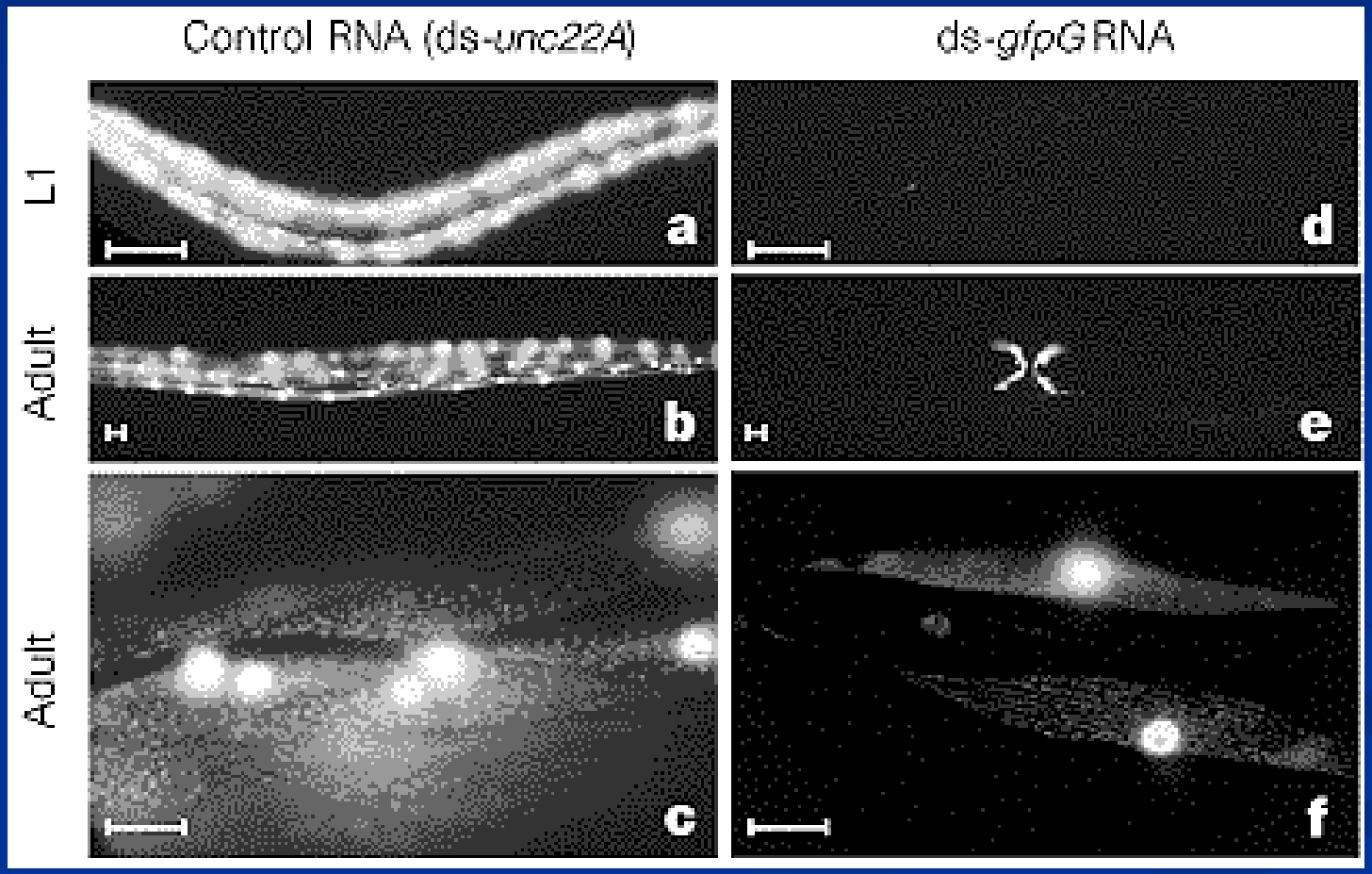
Results:

- double-stranded RNA is far more effective than single-stranded RNA.
- The sense or antisense RNAs lose their silencing effect if they are purified from the contaminating double-stranded RNA (dsRNA).
- only a few molecules of dsRNA are required per cell ® non-stochiometric effect that implies an amplification component.

Fire et al., Nature **391**. (1998)

The reporter transgene drives nuclear and mitochondrial expression of green fluorescent protein (GFP)

+ DS RNA against GFP



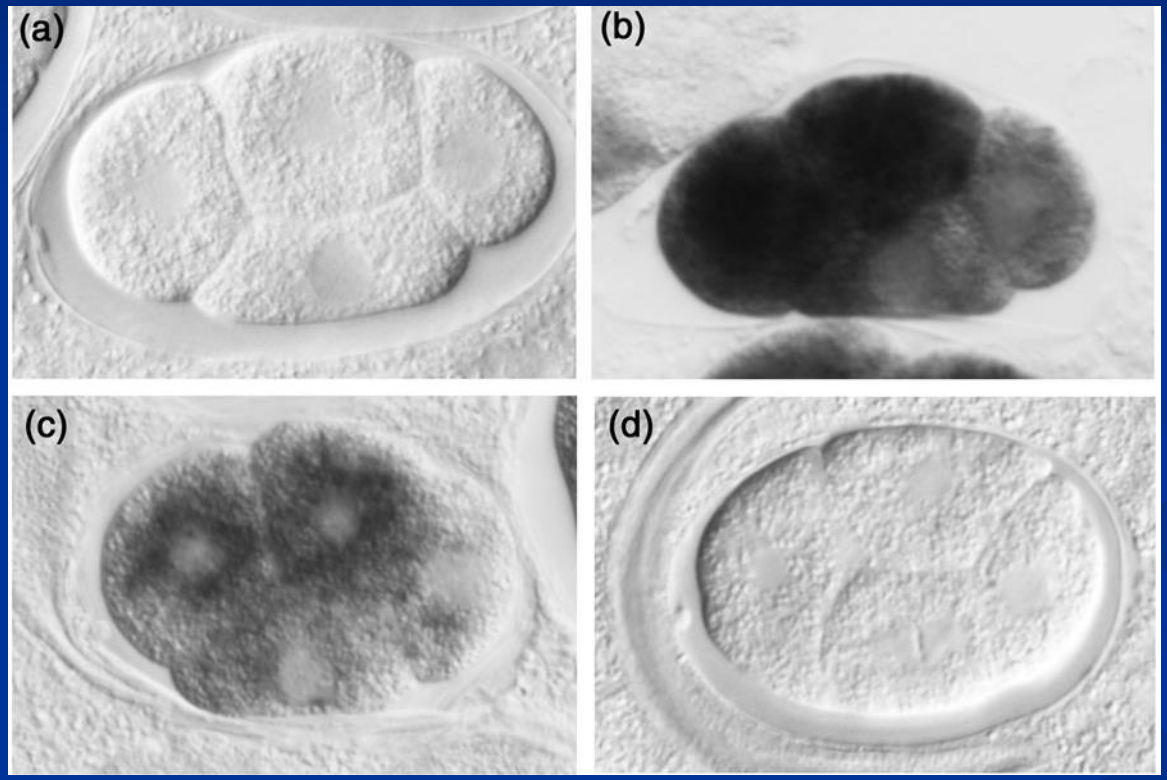
- dsRNA against the *gfp* gene causes the silencing of the *gfp:* reporter construct in almost all cells.
- This effect is specific for the gene that is targeted.

Fire at al, Nature V391 pp 806-811 (1998)

Double-stranded RNA-induced RNA interference causes destruction of a specific mRNA in *C. elegans*

uninjected, no probe

uninjected, mex-3 probe



antisense mex-3 RNA, mex-3 probe

double-stranded mex-3 RNA injected, mex-3 probe

Guo, S. and Kemphues, K. J. *Cell* 81, 611-620 (1995)
Fire, A. et al. *Nature* 391, 809 (1998)

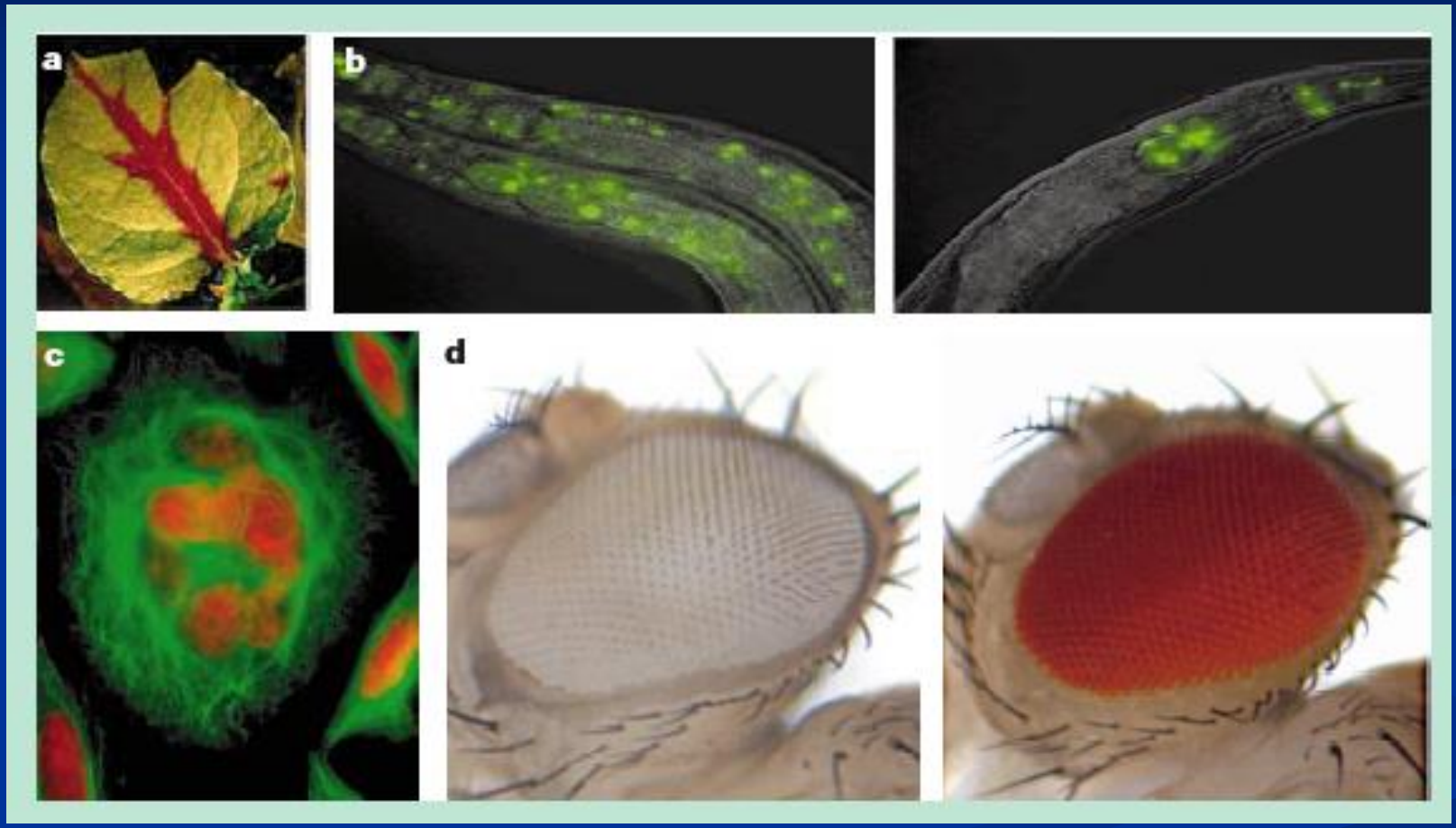
Key points of *C. elegans* experiment

- sub-stoichiometric amounts of dsRNA relative to the targeted mRNA are required to completely eliminate the mRNA (i.e. the dsRNA is catalytic)
- dsRNA is 10-100X better than anti-sense or sense RNA
- doesn't work if introns or promoters are targeted by the dsRNA
- doesn't interfere with transcription initiation or elongation (it is possible to target a single gene in an operon) (i.e. RNAi is a post-transcriptional phenomena)
- the targeted mRNA is degraded (i.e. it can't be detected by probes)
- dsRNA can cross cellular boundaries (i.e. there is a transport mechanism)

RNAi works in other organisms

silencing of GFP in leaf veins

silencing of GFP in *C. elegans* nuclei



depletion of ORC6 results in multinucleated HeLa cells

depletion of White results in unpigmented Drosophila eyes

Hannon, G. J. *Nature* 418, 244-251 (2002)

Nobel Prize in Physiology or Medicine 2006

Andrew Fire, born in 1959, is a US citizen. Since 2003 he has been professor of Pathology and Genetics at Stanford University School of Medicine, Stanford, California, USA.

In 1983 he took his PhD in Biology at the Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. He began his research on the nematode *C. elegans* during his time as visiting scientist in Cambridge, England, at the laboratory of Sydney Brenner (Nobel Laureate 2002). When Fire and Mello made their key discoveries about RNA interference, Fire was working at the Carnegie Institution of Washington.

L. CICERO/STANFORD

R.CARLINUMMAS

Craig Mello, born in 1960, is a US citizen and a professor of Molecular Medicine. Since 1994 he has worked within the Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA. He is also a Howard Hughes Medical Institute Investigator.

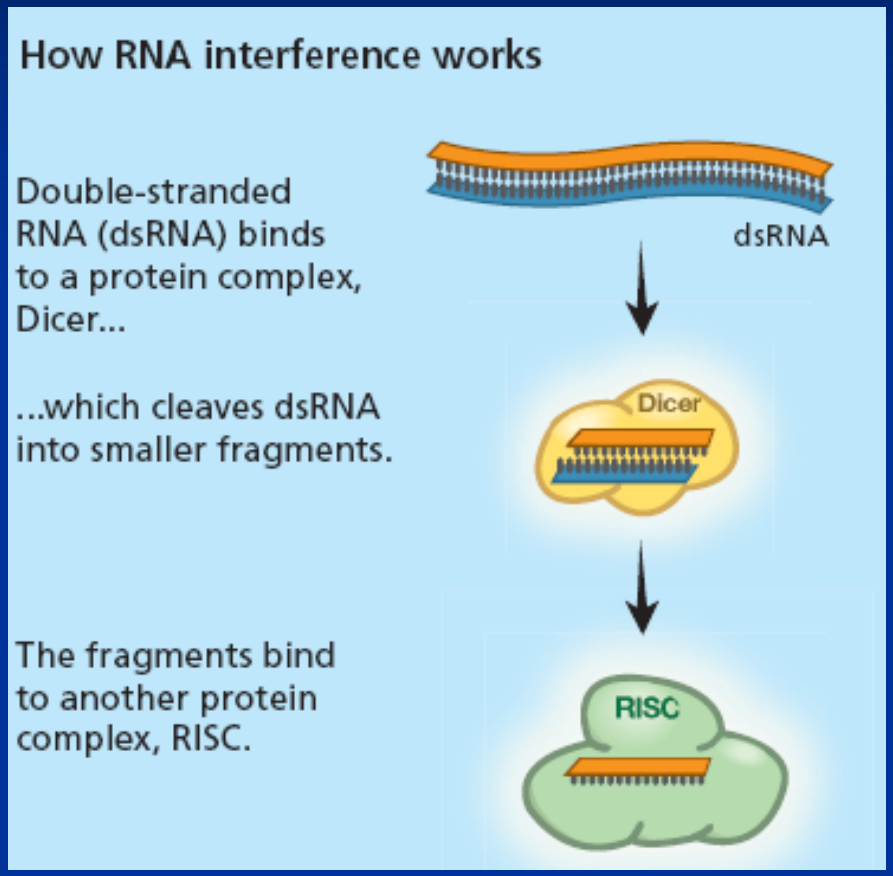
In 1990 he took his PhD in Cellular and Developmental Biology at Harvard University, Boston, Massachusetts. Before he moved to the University of Massachusetts Medical School in Worcester, he worked at the Fred Hutchinson Cancer Research Center.

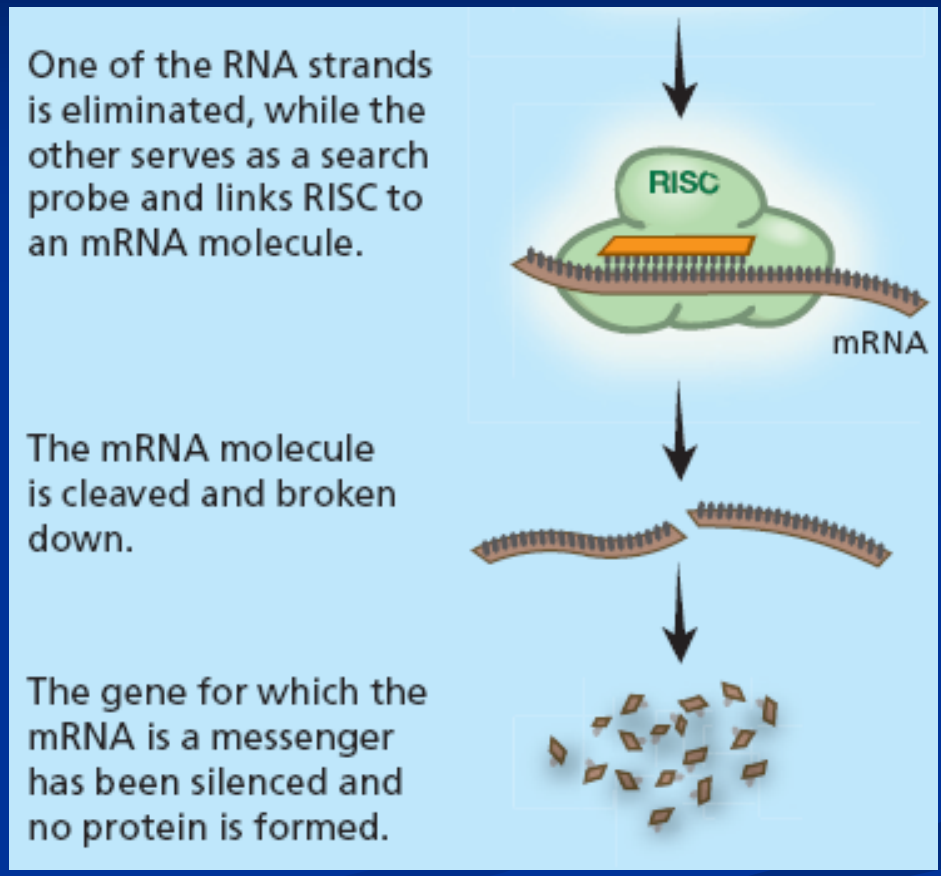
RNA interference (RNAi)

- A phenomenon in which the introduction of double stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA.

- a.k.a. post-transcriptional gene silencing

How does RNAi work?





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Why is RNAi important?

- Most widely held view is that RNAi evolved to protect the genome from viruses (or other invading DNAs or RNAs)
- Importantly, very small (micro) RNAs have been discovered in several eukaryotes that regulate developmentally other large RNAs
  - May be a new use for the RNAi mechanism besides defense

siRNA and miRNA

MicroRNAs. Genomics, Biogenesis, Mechanism, and Function.

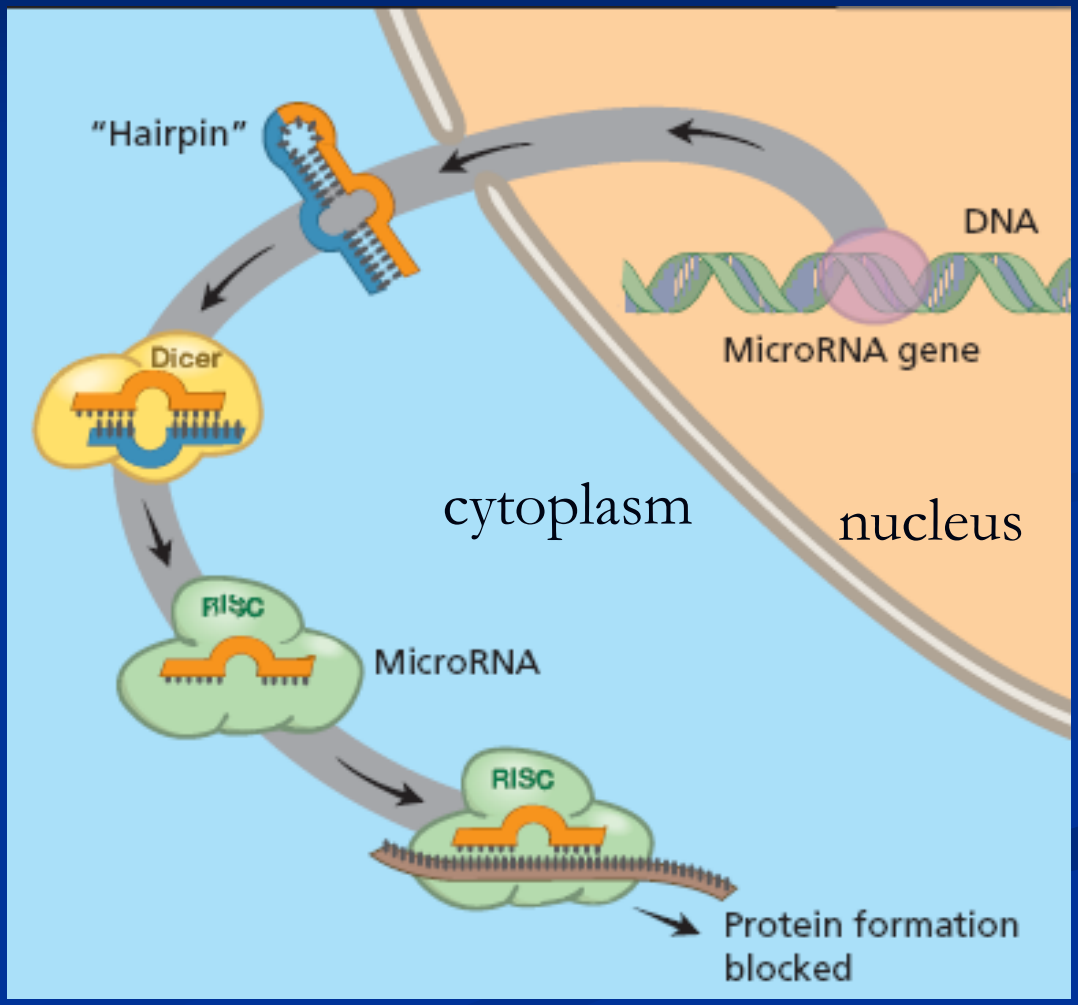
Bartel DP.

MicroRNAs (miRNAs) are endogenous non-coding RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression.
miRNAs comprise one of the more abundant classes of gene regulatory molecules in multicellular organisms and likely influence the output of many protein-coding genes.

Cell, v116, pp 281-297 (2004) (review)

Endogenous RNAi-miRNA

- We have hundreds of miRNA genes whose precursors can form double-stranded RNA. These can activate the RNA interference process and thus switch off the activity of various genes with matching segments.
- First miRNA is lin-4



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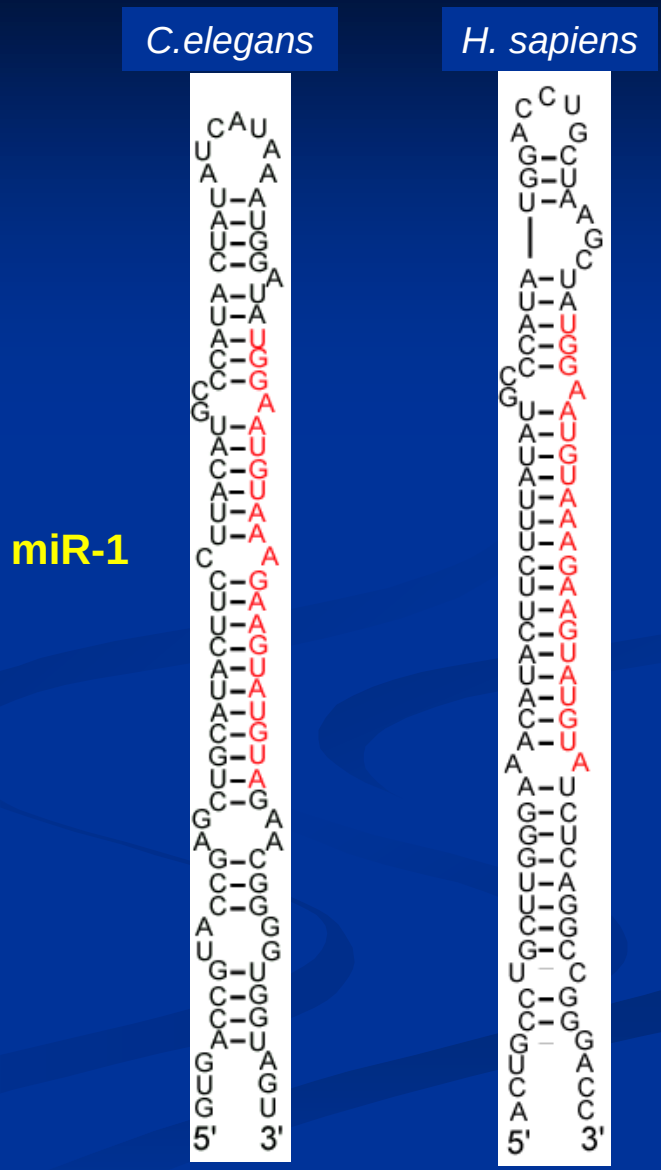
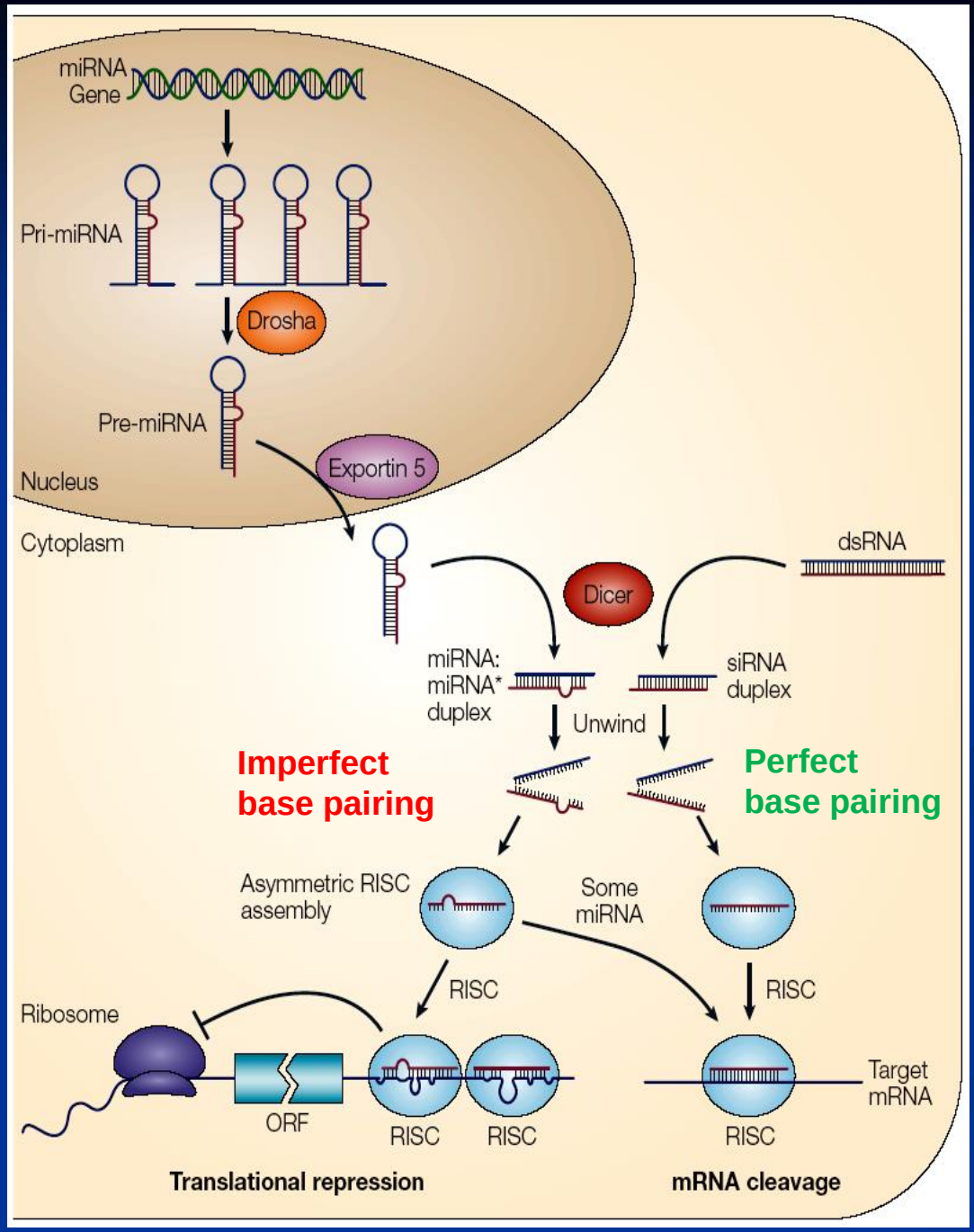
miRNA Genes

- Conserved across species
- Some appear in introns
- Possibly transcribed by pol II
- Some clustered and co-transcribed in one transcript
- Expressed in particular cell types
- Some abundant (50K molecules / cell)

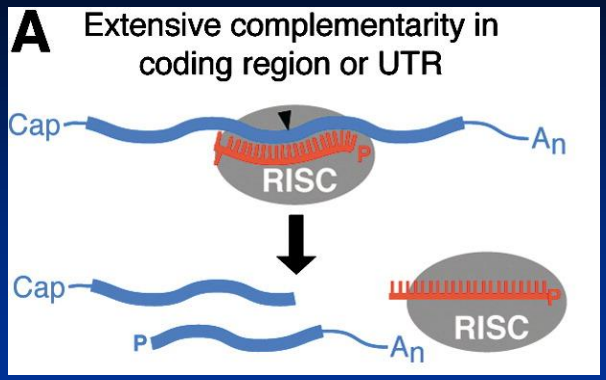




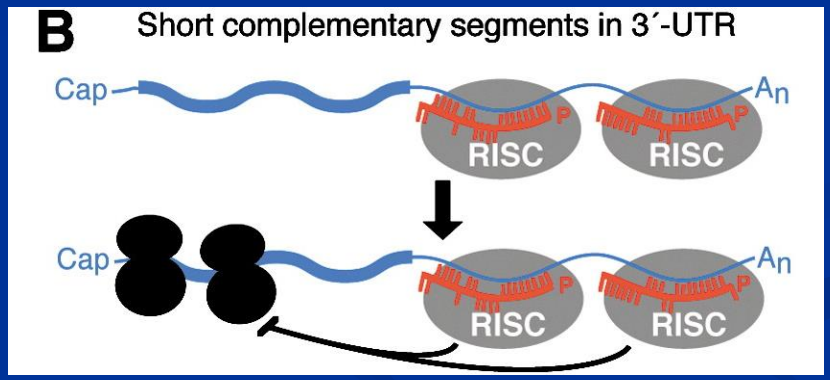
Biogenesis

- RNAse III enzymes
  - Drosha (nucleus)
  - Dicer (cytoplasm)
- Both enzymes involved in the generation of siRNA
- RISC = RNA-induced silencing complex (contains Argonaute family proteins)
- RISC = Degradation/Silencing?

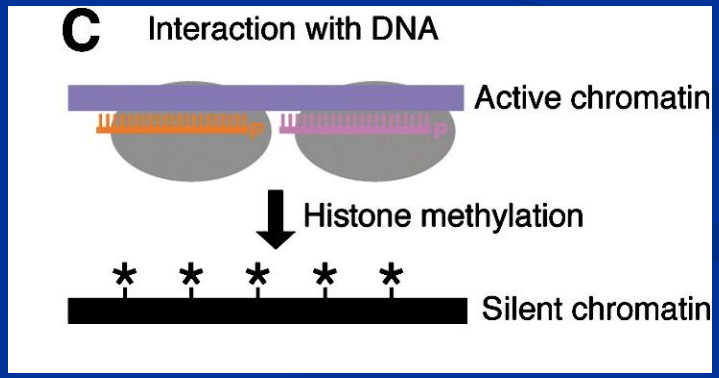
Post-transcriptional Cleavage of sequence-Complementary mRNA



Translational repression of the partially Complementary mRNA

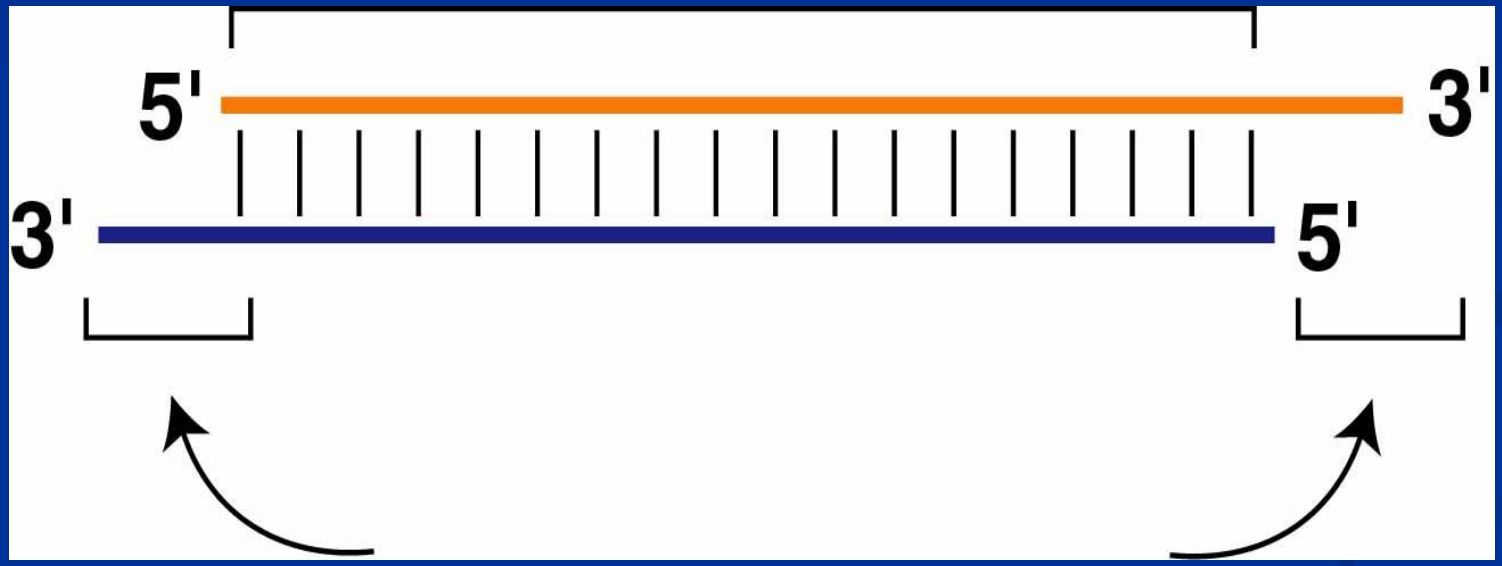


Transcriptional silencing



siRNAs have a defined structure

19 nt duplex



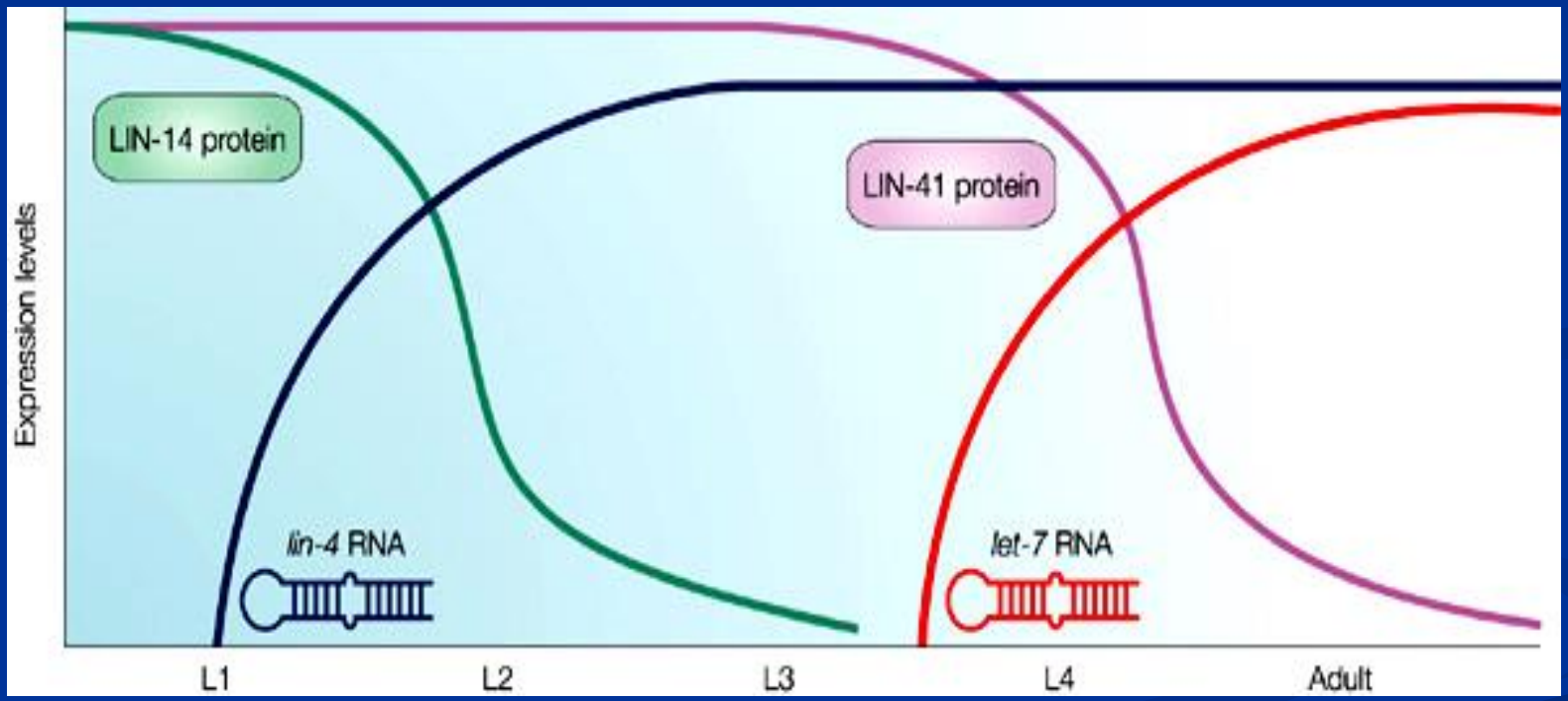
2 nt 3′ overhangs

RNA Induced Silencing Complex (RISC)

- RNAi effector complex
- Preferentially incorporates one strand of unwound RNA [Khvorova et al., 2003]
  - Antisense
- How does it know which is which?
  - The strand with less 5' stability usually incorporated into RISC [Schwarz et al., 2003]

The first described miRNA (2000)

lin-4= 22 nucleotides miRNA

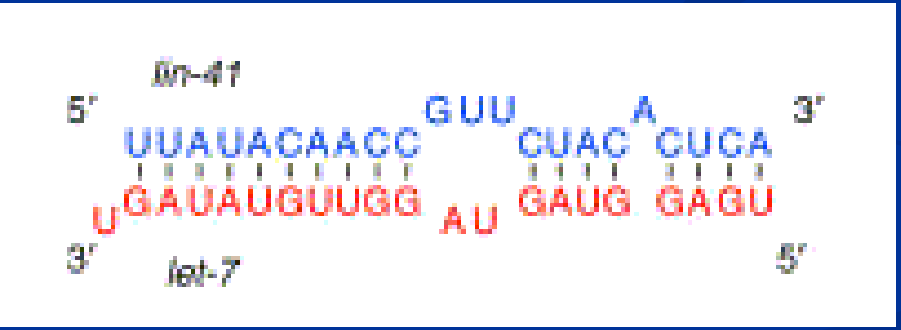


represses accumulation of LIN-14 protein

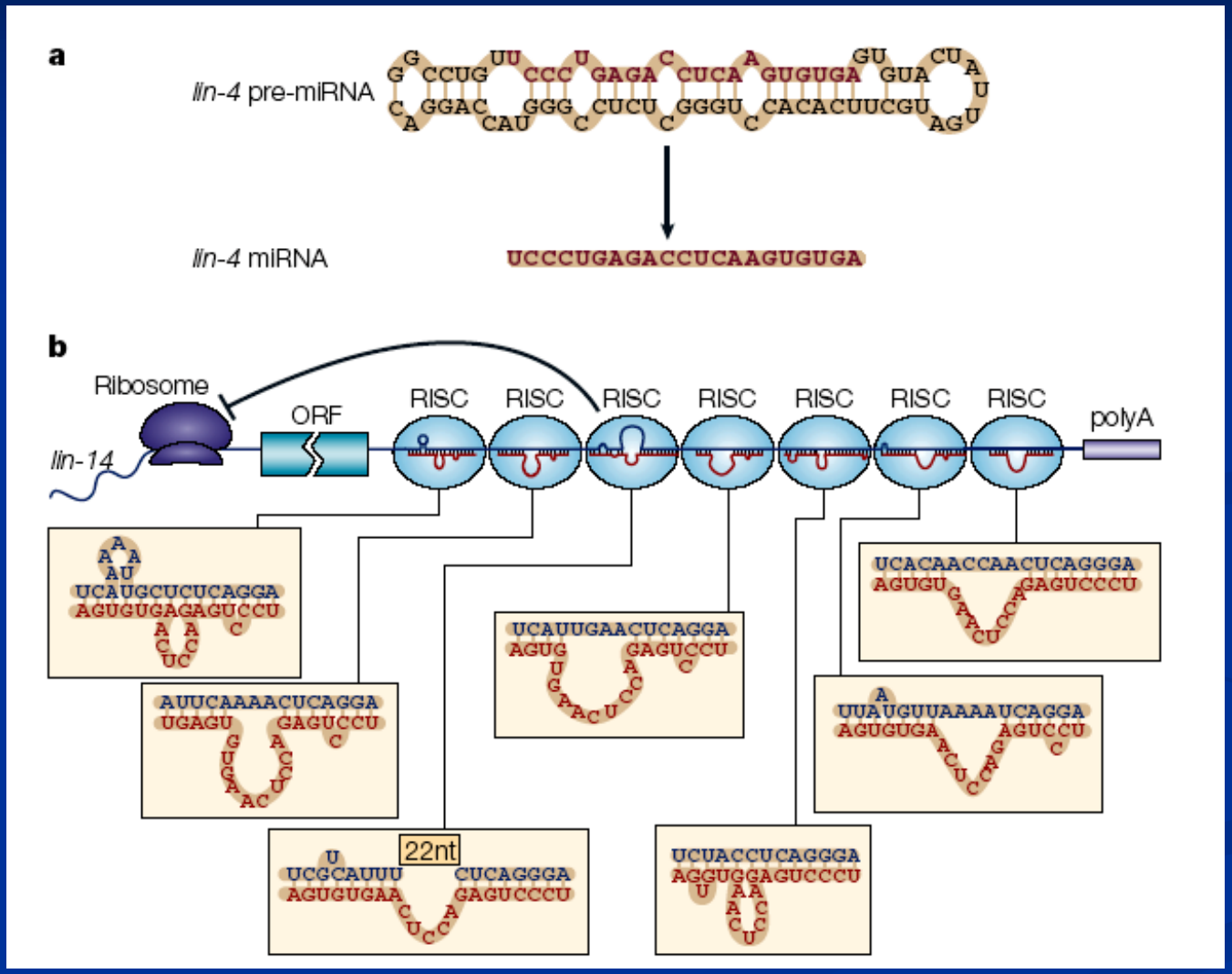
•Alex Eccleston

Ex: *C. elegans* development

- *lin-4* and *let-7* anti-sense or miRNAs
- Regulate larval development in *C. elegans*
- One of the two binding sites for *lin-41* and *let-7* interaction:



Molecular Hallmarks: Imperfect base pairing



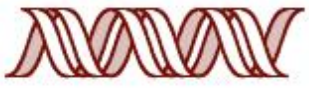
~ 70 nucleotides

~ 22 nucleotides

- Only part of miRNA is complementary to its target, thus its specificity is more limited.
- Therefore one miRNA can have different targets

miRNAs Correlate with Complexity

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



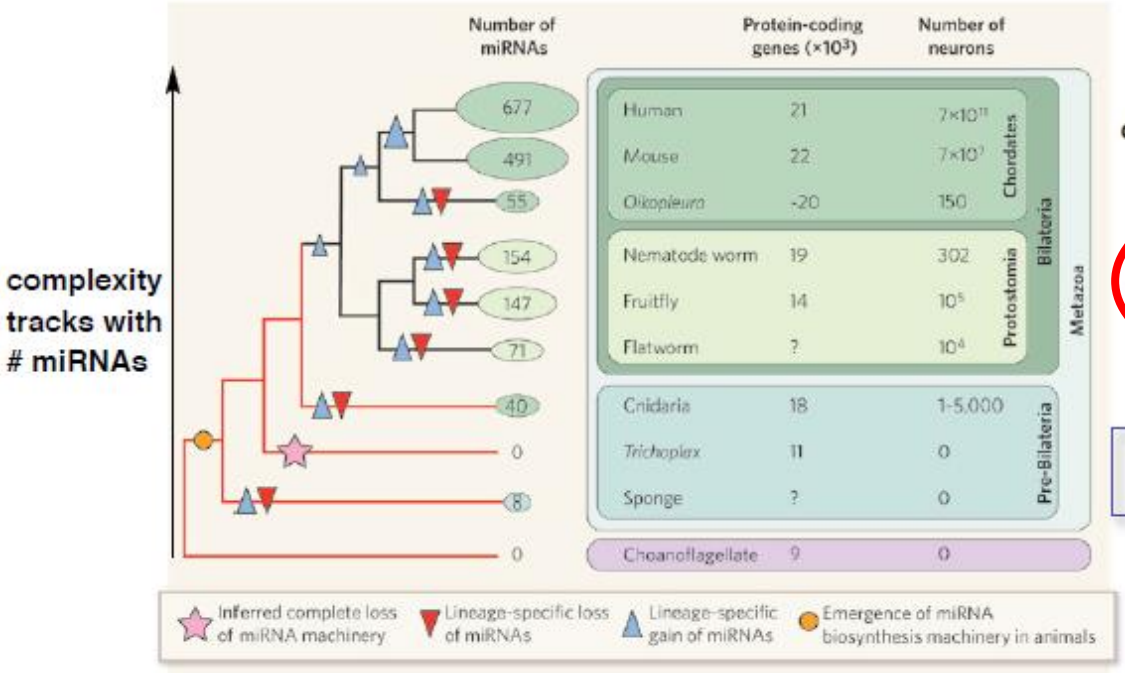
human genome

cDNA sequencing

forward genetics

reverse genetics (knockdown)

>500 miRNA encoding genes
(>1000 postulated)



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

Table 1 | **Expression studies on mammalian microRNAs**

Expression pattern	microRNA	References
Tissue-specific expression patterns of mammalian microRNAs		
ES-cell specific	*miR-296*	86
Expressed in ES cells, but upregulated on differentiation	*miR-21* and *miR-22*	86
Expressed in both ES cells and various adult tissues	*miR-15a*, *miR-16*, *miR-19,b*, *miR-92*, *miR-93*, *miR-96*, *miR-130* and *miR-130b*	86
Enriched during mouse brain development	*miR-128*, *miR-19b*, *miR-9*, *miR-125b*, *miR-131*, *miR-178*, *miR-124a*, *miR-266* and *miR-103*	26,90
Enriched in adult brain	*miR-9**, *miR-125a*, *miR-125b*, *miR-128*, *miR-132*, *miR-137*, *miR-139*, *miR-7*, *miR-9*, *miR-124a*, *miR-124b*, *miR-135*, *miR-153*, *miR-149*, *miR-183*, *miR-190* and *miR-219*	26
Enriched in lung	*miR-18*, *miR-19a*, *miR-24*, *miR-32*, *miR-130*, *miR-213*, *miR-20*, *miR -141*, *miR-193* and *miR-200b*	26
Enriched in spleen	*miR-99a*, *miR-127*, *miR-142-a*, *miR-142-s*, *miR-151*, *miR-189* and *miR-212*	26
Haemetopoietic tissues	*miR-181*, *miR-223* and *miR-142*	26
Enriched in liver	*miR-122a*, *miR-152*, *miR-194*, *miR-199* and *miR-215*	26
Enriched in heart	*miR-1b*, *miR-1d*, *miR-133*, *miR-206*, *miR-208* and *miR-143*	26
Enriched in kidney	*miR-30b*, *miR-30c*, *miR-18*, *miR-20*, *miR-24*, *miR-32*, *miR-141*, *miR-193* and *miR-200b*	26
Ubiquitously expressed	*miR-16*, *miR-26a*, *miR-27a*, *miR143a*, *miR-21*, *let-7a*, *miR-7b*, *miR-30b* and *miR-30c*	26
Abnormal microRNA expression during tumorigenesis		
Downregulated in chronic lymphocytic leukaemias	*miR-15* and *miR-16*	102
Downregulated in lung cancer cell lines	*miR-26a* and *miR-99a*	89
Downregulated in colon cancers	*miR143/miR-145* cluster	103
Upregulated in Burkitt lymphoma	*miR-155*	88

ES cells, embryonic stem cells.

Functions ??

Table 2 | **microRNAs and their targets: examples of microRNAs with experimentally validated functions/targets**

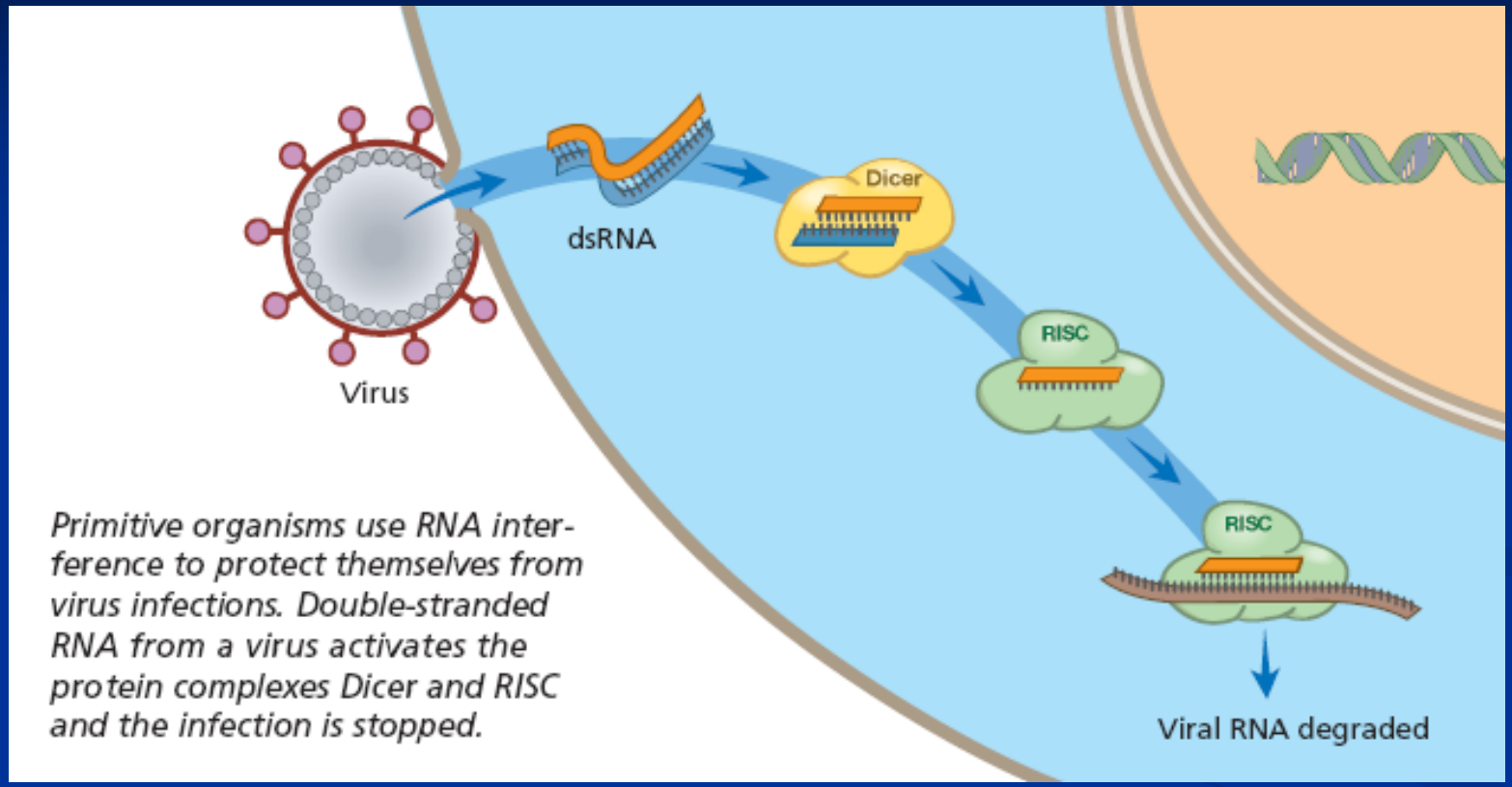
microRNA	Target gene(s)	Function	Mode of repression	References
Caenorhabditis elegans				
lin-4	*lin-14, lin-28*	Regulation of developmental transition between the first two larval stages, L1 and L2	Translational repression	13,18,19
let-7	*lin-41, hbl-1*	Regulation of developmental transition between the last larval stage (L4) and the adult stage	Translational repression	20–23
lsy-6	*cog-1*	Determination of left/right asymmetry of neuronal development	Unknown	9
Drosophila melanogaster				
Bantam	*hid*	Promotion of cell proliferation and suppression of apoptosis	Translational repression	73
miR-14	Unknown	Suppression of apoptosis and regulation of fat metabolism	Unknown	97
Mus musculus				
miR-181	Unknown	Promotion of haematopoietic differentiation towards the B-cell lineage	Unknown	99
miR-196	*Hoxb8*	Unknown	PTGS	44
Arabidopsis thaliana				
miR-165, miR-166	*PHB*, *PHV* and *REV*	Regulation of leaf morphogenesis	PTGS	104,105
miR-172	*AP2*	Regulation of flowering time and floral-organ identity	Translational repression	41
miR-JAW	*TCP* transcription factors	Regulation of leaf development and embryogenesis	PTGS	98
miR-39	*SCL* family proteins	Unknown	PTGS	42
miR-159	*MYB33* family transcription factors	Regulation of leaf morphogenesis	PTGS	43,63,98
Zea mays				
miR-166	*rld1*	Regulation of leaf morphogenesis	PTGS	106

AP2, *APETALA 2*; *Hid*, head involution defective; Hoxb8, homeobox B8; *PHB*, *PHABULOSA*; *PHV*, *PHAVOLUTA*; PTGS, post-transcriptional gene silencing; *REV*, REVOLUTA; *rld1*, rolled leaf1; *SCL*, SCARECROW-LIKE; *TCP*, teosinte branched 1-cycloidea-PCF.

RNAi in Plants

- Defense mechanism against pathogens
- Post-transcriptional Cleavage of sequence-complementary mRNA
- Developed anti-viral RNAi pathway

Defense Against Viruses



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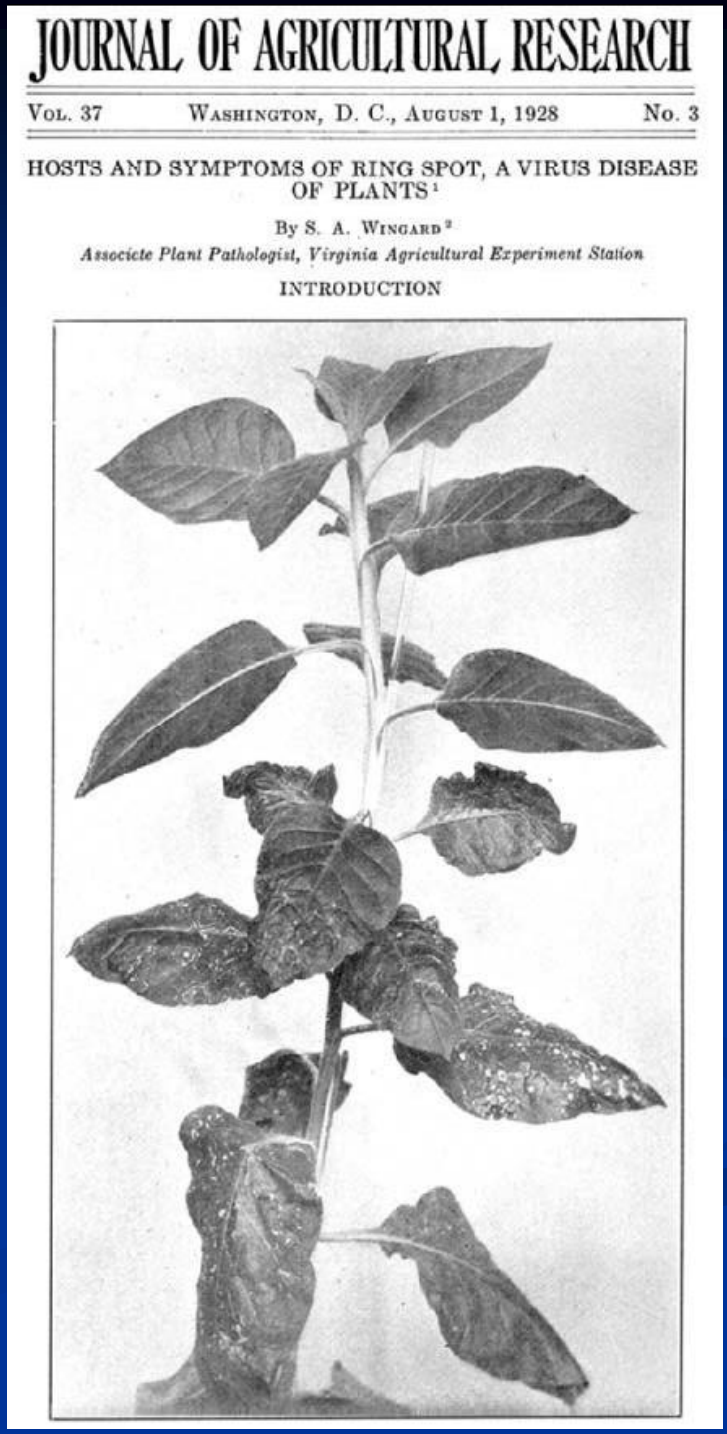
- In contrast, in the continuing evolutionary war to survive and reproduce, plant viruses have evolved genes that enable them to suppress silencing.

RNAi movement

1928 report of development of viral (tobacco ringspot virus) resistance in tobacco, now known to be due to endogenous RNAi.

- dsRNA can cross cellular boundaries (i.e. there is a transport mechanism)

- Amplification of RNAi



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HOSTS AND SYMPTOMS OF RING SPOT, A VIRUS DISEASE OF PLANTS[1]

By S. A. WINGARD[2]

Associate Plant Pathologist, Virginia Agricultural Experiment Station

INTRODUCTION

RNAi in Higher Mammals

- Translational repression of the mRNA

  (partially sequence-complementary)
- Transcriptional silencing (chromatin)?
- Evidence for anti-viral RNAi pathway,

  but not movement or amplification

Vol 449|18 October 2007|doi:10.1038/nature06205

nature

LETTERS

Interferon modulation of cellular microRNAs as an antiviral mechanism

Irene M. Pedersen[1], Guofeng Cheng[3], Stefan Wieland[3], Stefano Volinia[4], Carlo M. Croce[4], Francis V. Chisari[3] & Michael David[1,2]

RNA interference through non-coding microRNAs (miRNAs) represents a vital component of the innate antiviral immune response in plants and invertebrate animals; however, a role for cellular miRNAs in the defence against viral infection in mammalian organisms has thus far remained elusive[1]. Here we show anti-miRNAs reduces the antiviral effects of IFNβ against HCV. In addition, we demonstrate that IFNβ treatment leads to a significant reduction in the expression of the liver-specific miR-122, an miRNA that has been previously shown to be essential for HCV replication[2]. Therefore, our findings strongly support the notion that mammalian organisms too, through the interferon system, use cellular miRNAs to combat viral infections.

replication[2]. Therefore, our findings strongly support the notion that mammalian organisms too, through the interferon system, use cellular miRNAs to combat viral infections.

Mx GTPases are important contributors to the antiviral properties of these cytokines[8,9]. However, the possibility that IFNα/β might induce cellular miRNAs that target viral transcripts and thereby use RNAi as part of their arsenal against invading viruses has been left unexplored. To test whether IFNα/β could alter the expression of transcripts or viral genomic RNAs with an initial focus on the crucial seed sequence. This approach revealed promising matches among several viruses, most of which harbour an RNA-based genome. Specifically, eight of the IFNβ-induced miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448) displayed nearly perfect complementarity in their seed sequences with hepatitis C virus (HCV) RNA genomes. This finding was rather

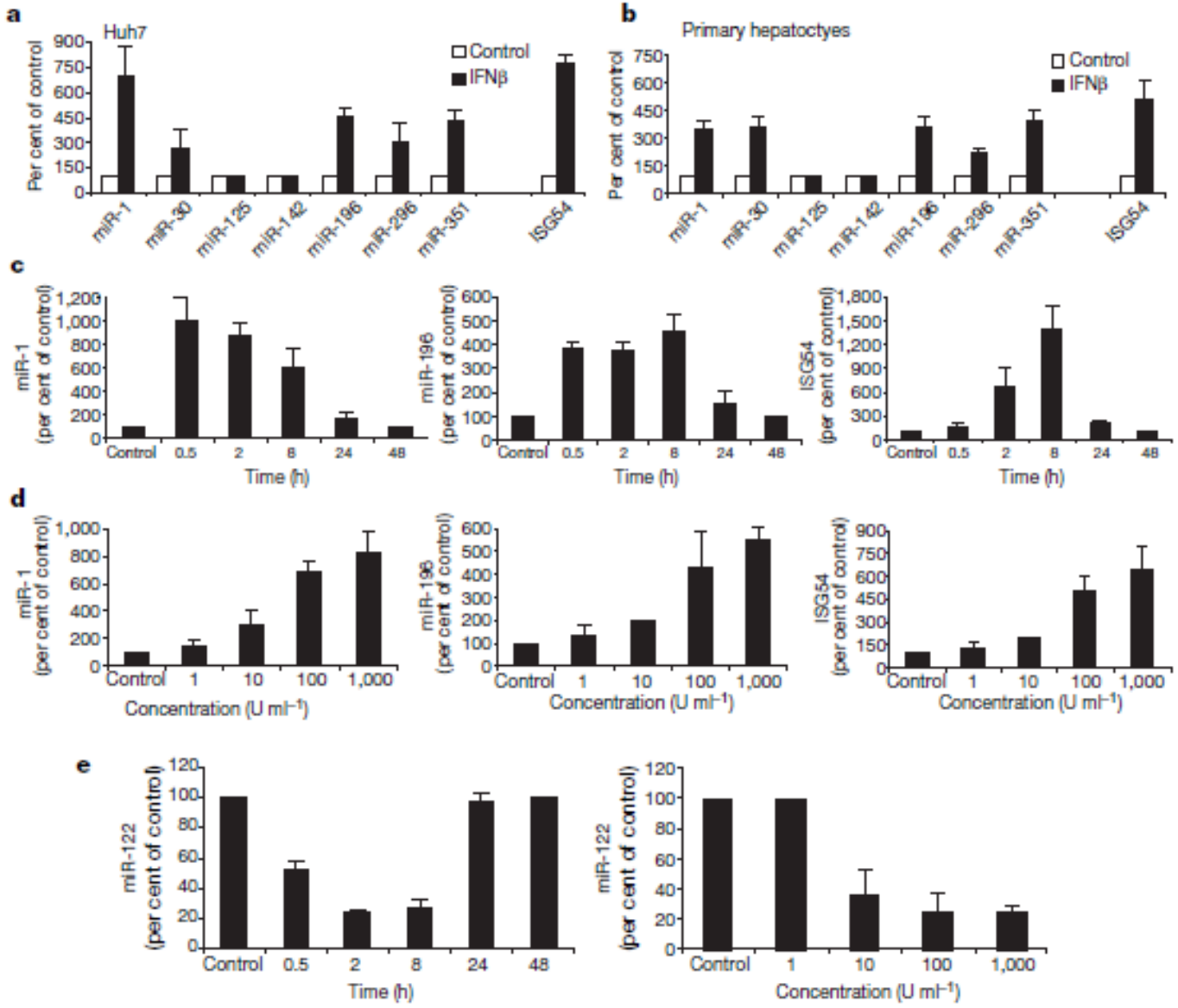
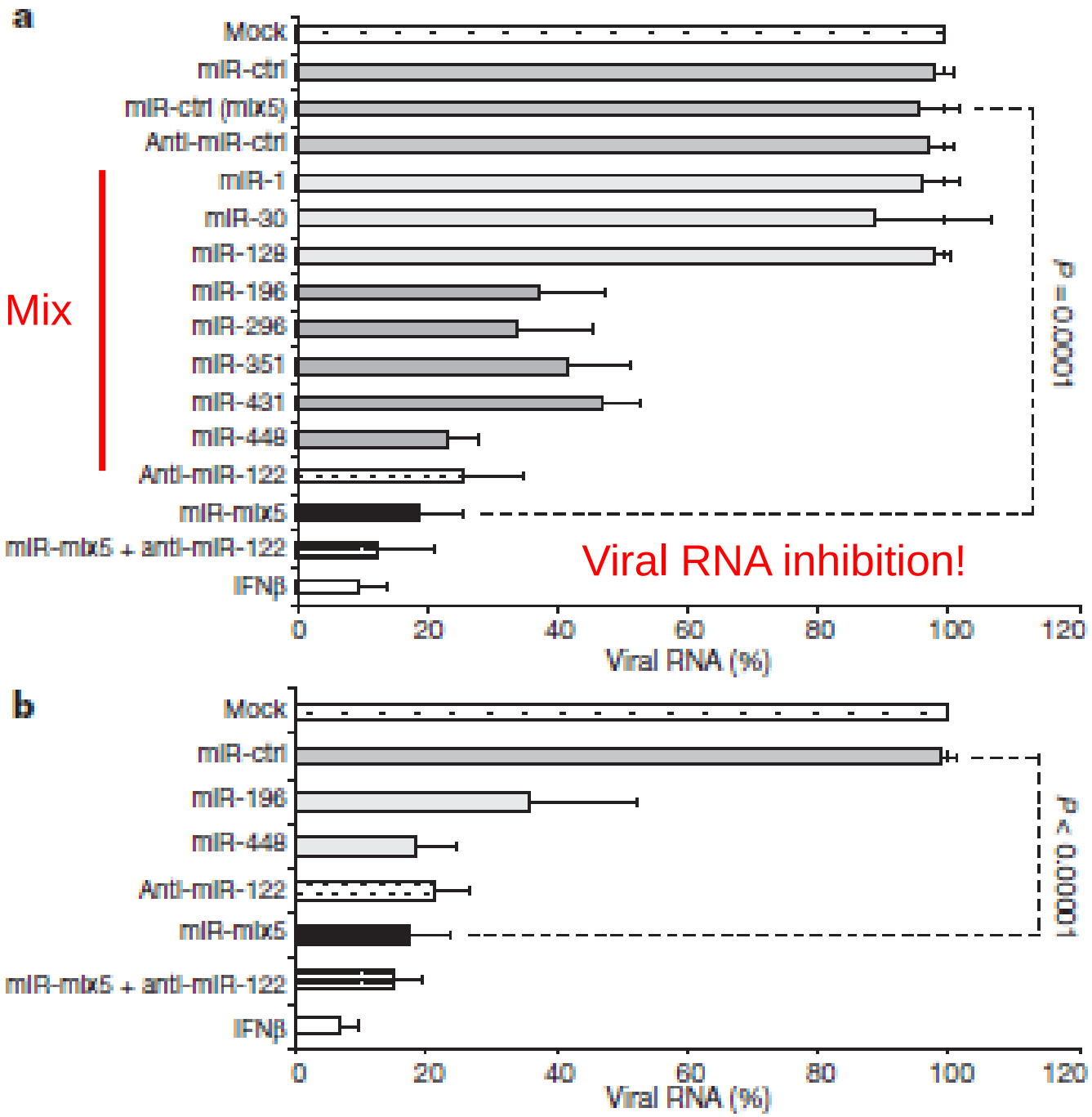


Figure 1 | Regulation of miRNA expression by IFNβ in Huh7 cells and primary hepatocytes. **a**, **b**, Huh7 cells (**a**) or primary hepatocytes (**b**) were stimulated with 100 U ml^{-1} IFNβ for 2 h, and the indicated miRNAs were quantified by qPCR. ISG54 induction is shown for comparison. **c**, Time course of miRNA induction by IFNβ: Huh7 cells were stimulated with 100 U ml^{-1} IFNβ for the indicated times, and miR-1, miR-196 or ISG54 expression was quantified by qPCR. **d**, Dose–response analysis of miRNA induction by IFNβ: Huh7 cells were stimulated with the indicated doses of IFNβ for 2 h, and miR-1, miR-196 or ISG54 expression was quantified by qPCR. **e**, Time course and dose–response analysis of miR-122 downregulation by IFNβ: Huh7 cells were stimulated as described in **c** and **d**, and miR-122 was quantified by qPCR. Error bars, means ± s.d. of at least four independent experiments.

miR-1, miR122
miR-196


a
Mock
miR-ctrl
miR-ctrl (mix5)
Anti-miR-ctrl
miR-1
miR-30
miR-128
miR-196
miR-296
miR-351
miR-431
miR-448
Anti-miR-122
miR-mix5
miR-mix5 + anti-miR-122
IFNβ
Mix
P = 0.0001
Viral RNA inhibition!
0
20
40
60
80
100
120
Viral RNA (%)
b
Mock
miR-ctrl
miR-196
miR-448
Anti-miR-122
miR-mix5
miR-mix5 + anti-miR-122
IFNβ
P < 0.0001
0
20
40
60
80
100
120
Viral RNA (%)

MicroRNAs & Disease

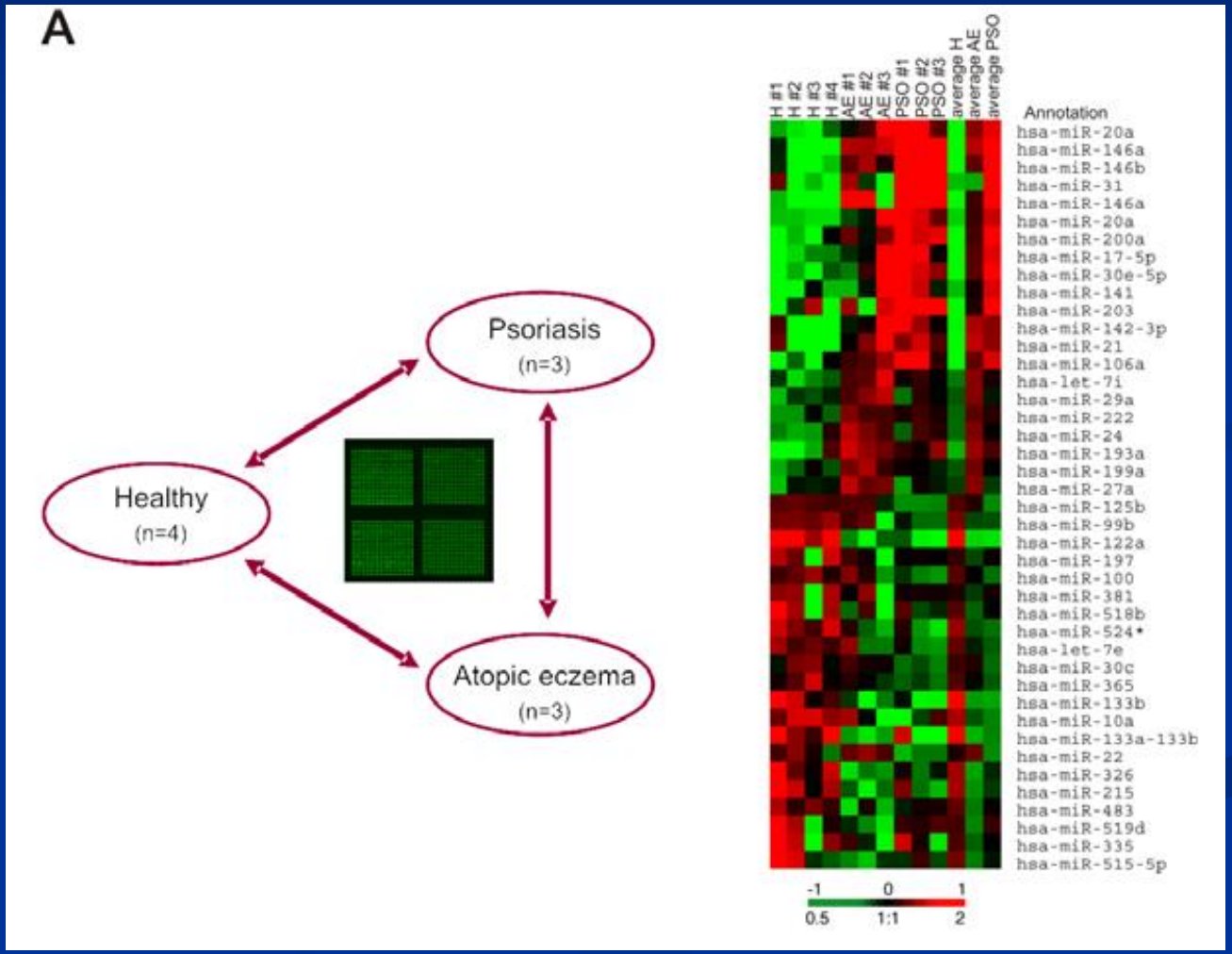
MicroRNAs: Novel Regulators Involved in the Pathogenesis of Psoriasis

Sonkoly E. et al., 2007

- To determine whether miRNAs are involved in the pathogenesis of Psoriasis
- Performed comprehensive analysis of all human miRNAs registered in mirBase 8.0 (342 known human miRNAs on microarray) in skin lesions of:
  -psoriasis (n=3)
  -healthy (n=4)
  -atopic eczema (n=3)

MicroRNA expression profiling

>40 genes differentially expressed between psoriasis and healthy skin



Up- and Down-regulated miRNAs

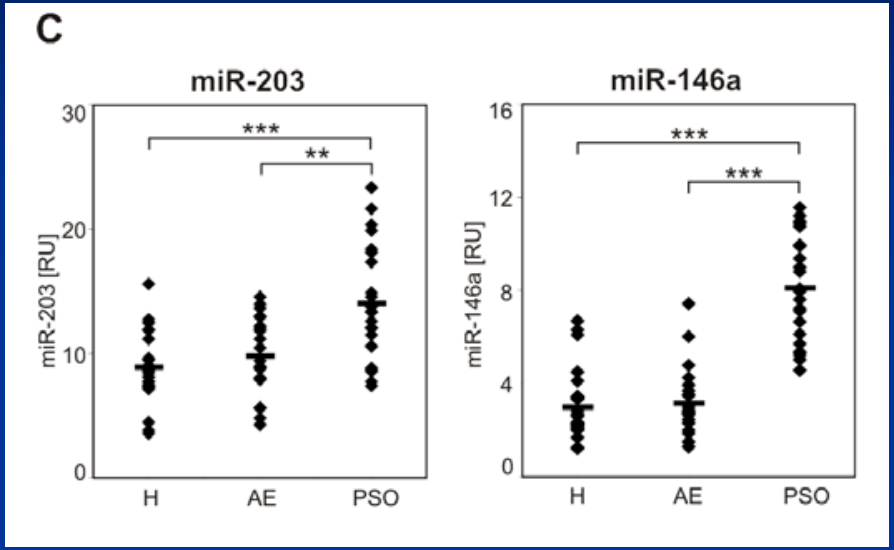
Microarray:

29 genes that were consistently differentially expressed between psoriasis and healthy skin

B

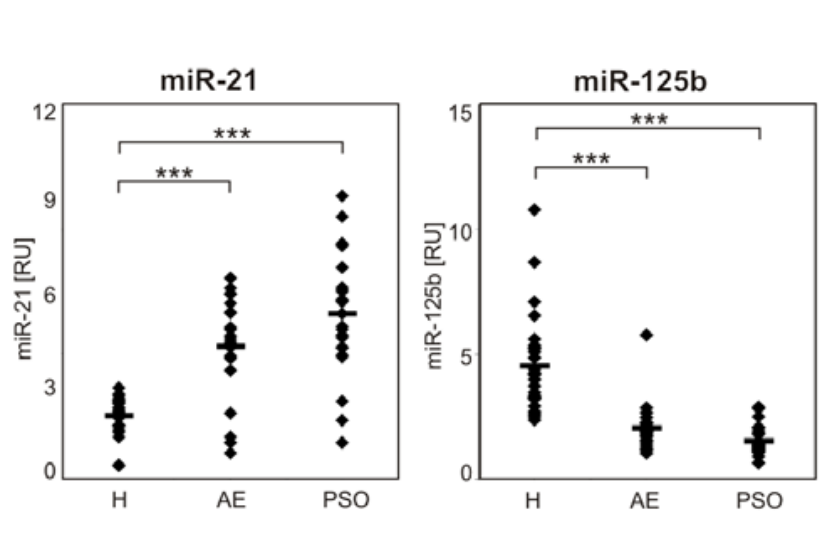
Psoriasis			Atopic eczema			
miRNA	Score	Fold change	miRNA	Score	Fold change	
miR-146b	4.68	3.31	let-7i	2.50	2.04	UP-REGULATED
miR-20a	4.08	2.90	miR-29a	2.39	1.83	
miR-146a	4.05	3.30	miR-146a	2.35	2.22	
miR-31	3.47	4.69	miR-222	2.22	1.67	
miR-200a	2.85	2.75	miR-24	2.20	1.85	
miR-17-5p	2.67	3.77	miR-193a	2.14	2.27	
miR-30e-5p	2.61	3.61	miR-199a	2.13	1.72	
miR-141	2.26	3.45	miR-27a	2.04	1.72	
miR-203	2.23	5.86	miR-21	2.04	3.26	
miR-142-3p	2.22	2.55	miR-20a	2.02	2.35	
miR-21	2.21	2.51	miR-17-5p	1.82	2.58	
miR-106a	2.16	2.37	miR-106b	1.78	1.72	
miR-125b	-5.31	0.55	miR-122a	-2.75	0.19	DOWN-REGULATED
miR-99b	-3.32	0.58	miR-133a-133b	-2.73	0.28	
miR-122a	-3.09	0.18	miR-326	-2.50	0.39	
miR-197	-2.69	0.64	miR-215	-2.48	0.42	
miR-100	-2.62	0.59	miR-483	-1.89	0.57	
miR-381	-2.60	0.71	miR-519d	-1.86	0.48	
miR-518b	-2.55	0.56	miR-335	-1.78	0.63	
miR-524*	-2.40	0.50	miR-133b	-1.68	0.23	
let-7e	-2.25	0.60	miR-515-5p	-1.57	0.43	
miR-30c	-1.98	0.63				
miR-365	-1.87	0.62				
miR-133b	-1.78	0.22				
miR-10a	-1.78	0.67				
miR-133a-133b	-1.76	0.40				
miR-22	-1.68	0.61				
miR-326	-1.61	0.56				
miR-215	-1.59	0.56				

Up- and Down-regulated miRNAs

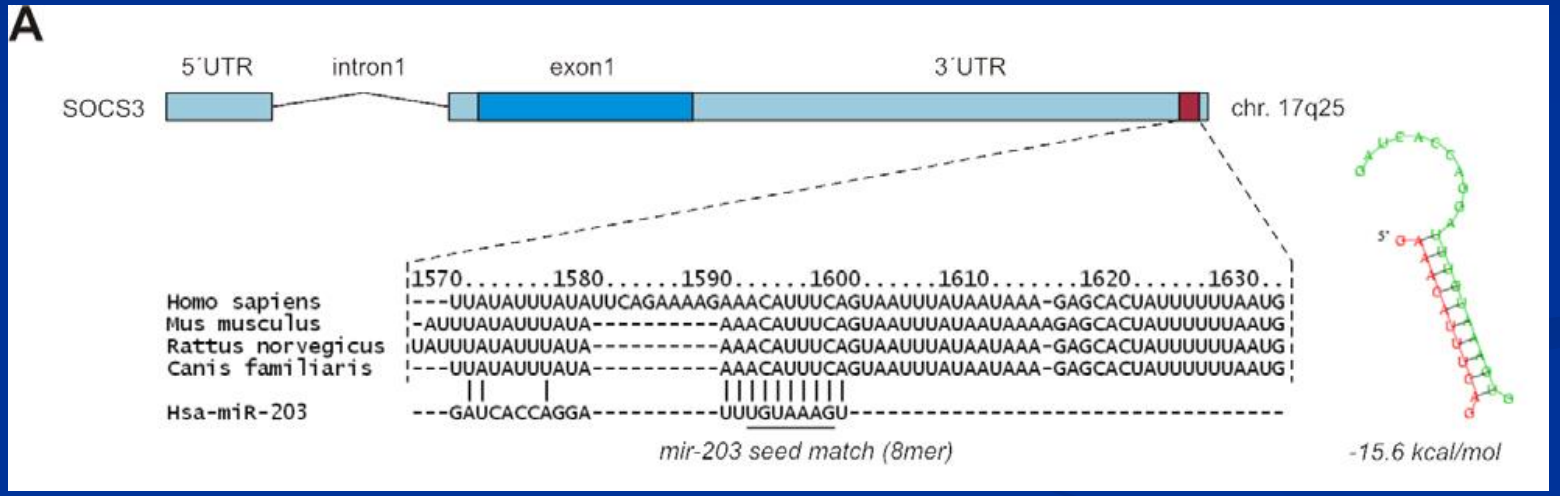


Confirmation
Real-Time PCR

Healthy: n=26
AE: n=20
PSO: n=25



SOCS3: Molecular target of miR-203
Post-transcriptional repression



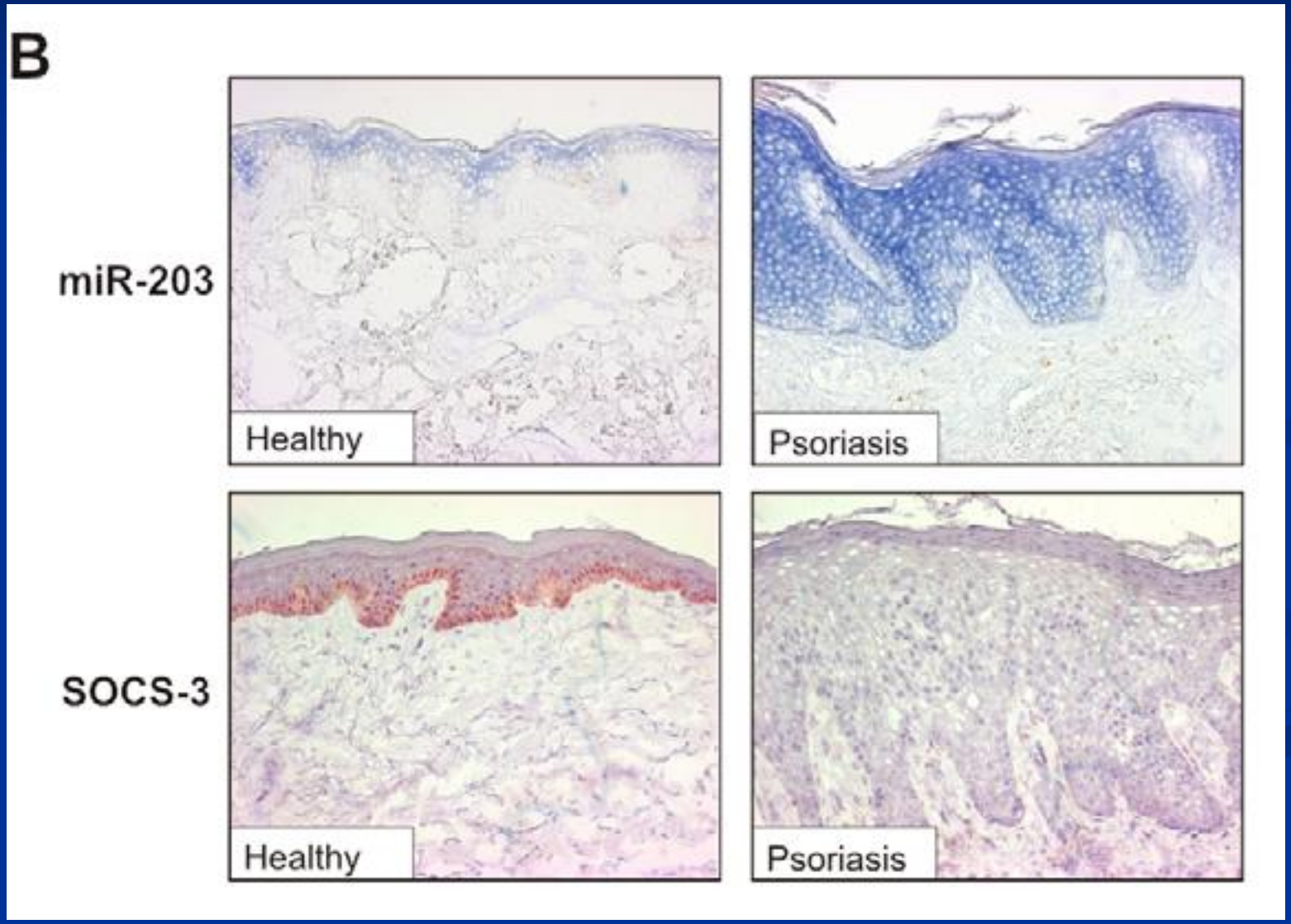
Psoriatic keratinocytes:

IL-6 → STAT3-p ⇑

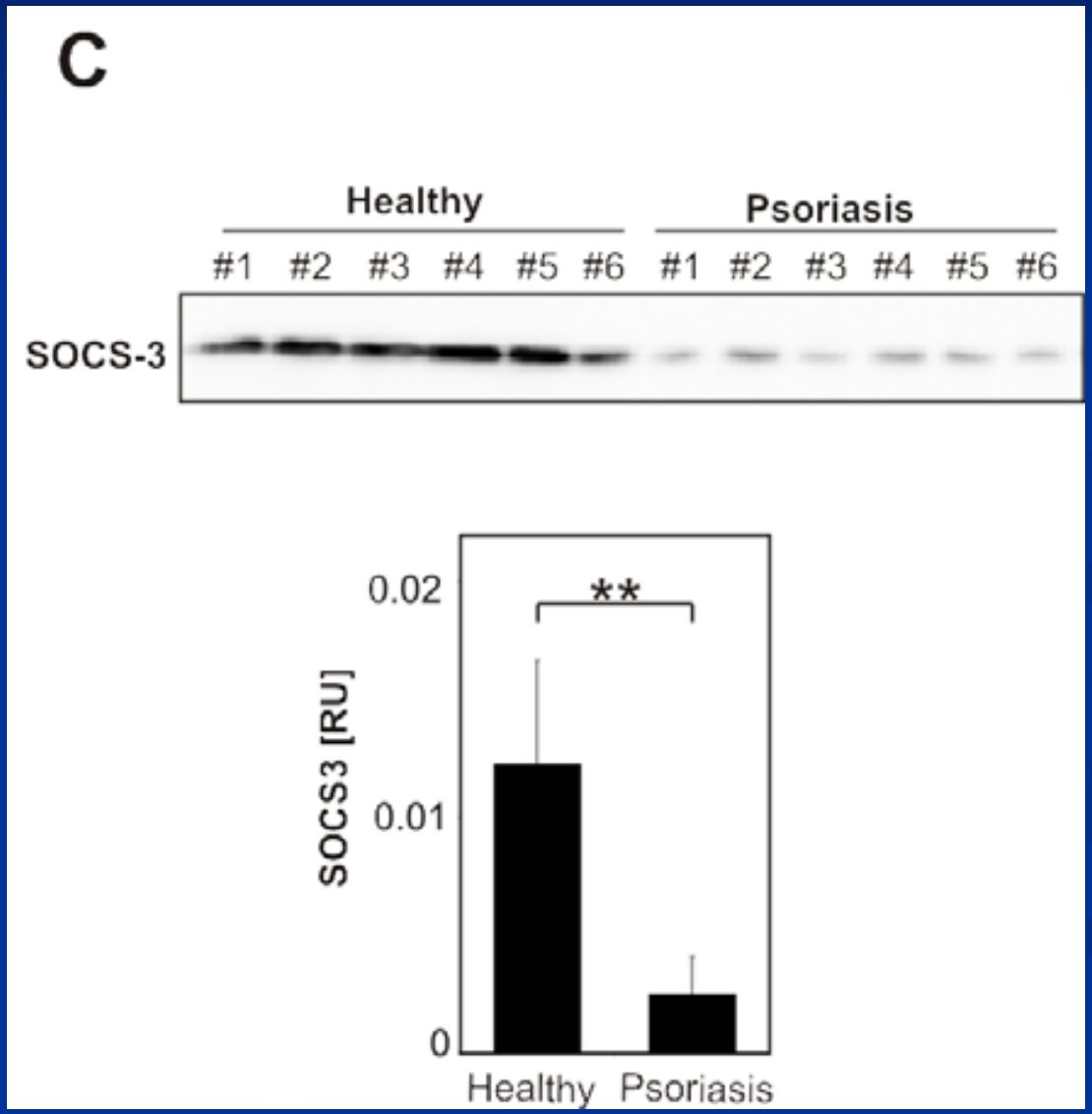
T

SOCS3 ✗

SOCS3 vs. miR-203 expression in Skin sections



SOCS3 vs. miR-203 expression in Skin sections

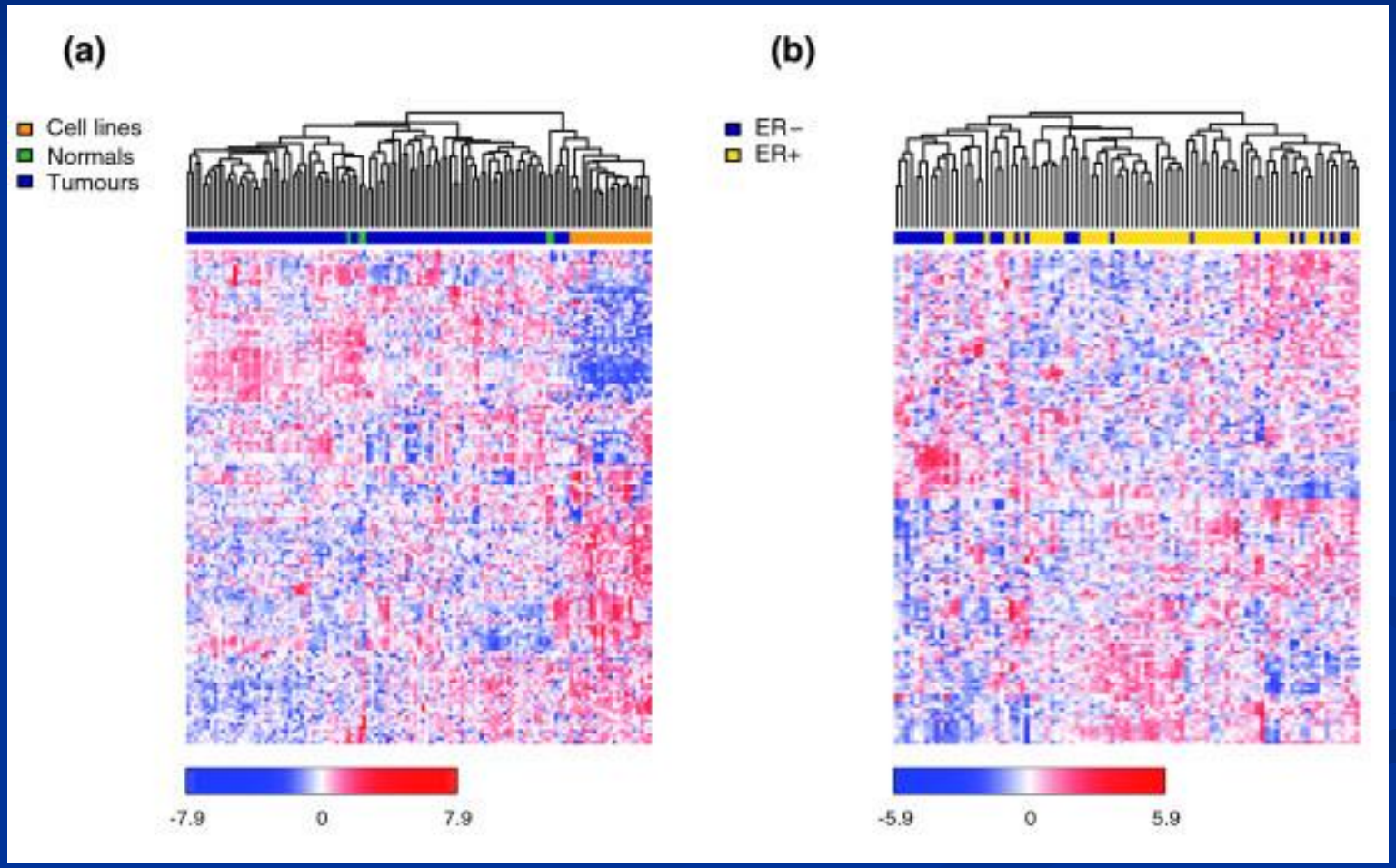


MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype

Cherie Blenkiron et al., 2007

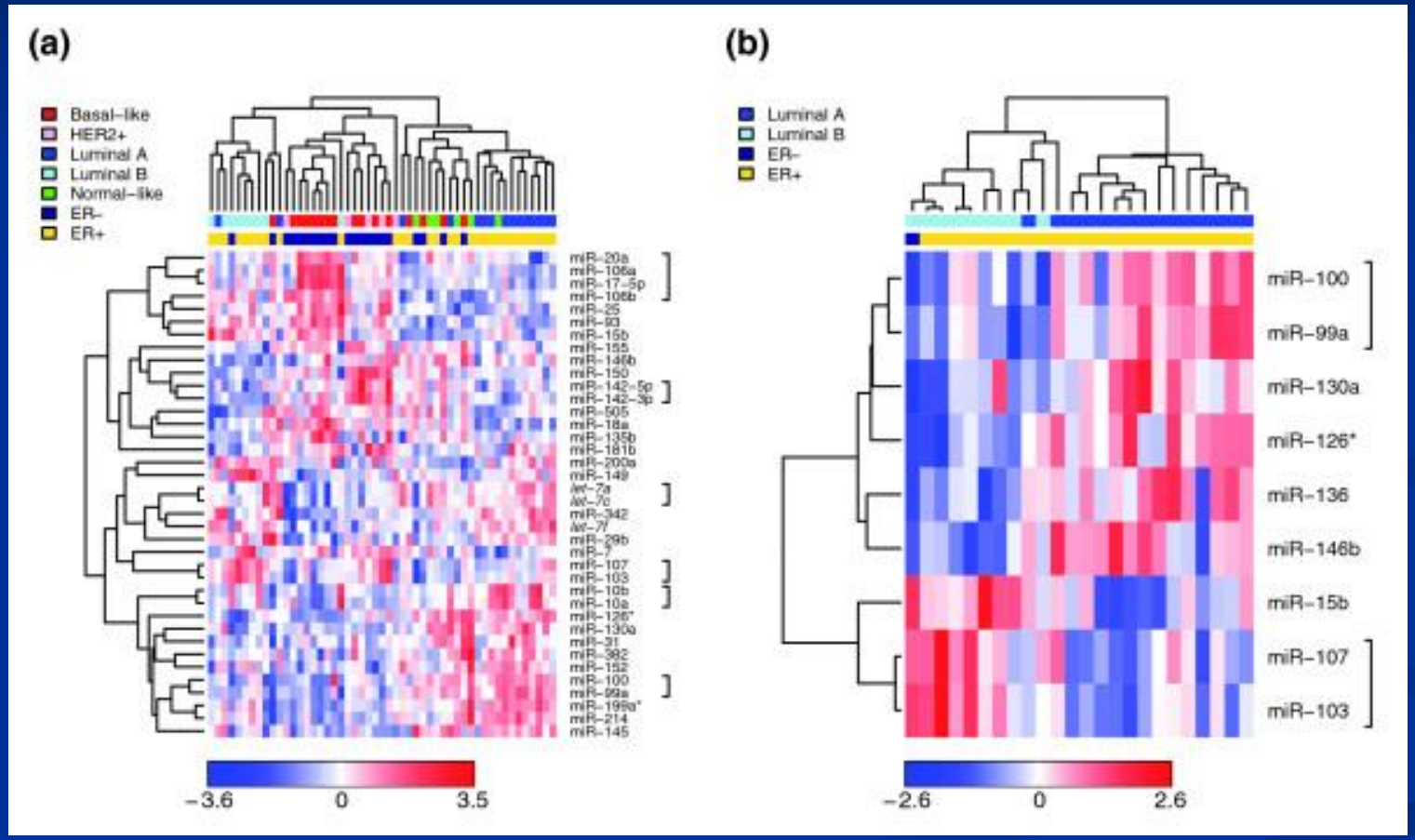
- Integrated analysis of miRNA expression, mRNA expression and genomic changes in human breast cancer
- Analysis of miRNA expression in 93 primary human breast tumors, using a bead-based flow cytometric miRNA expression profiling method
- Breast tumor classification??

99 primary human tumors, 5 normal breast samples and 33 breast cancer cell lines vs. 309 miRNAs



Unsupervised hierarchical clustering over 137 detected miRNAs

Clustering of tumor samples in different classes



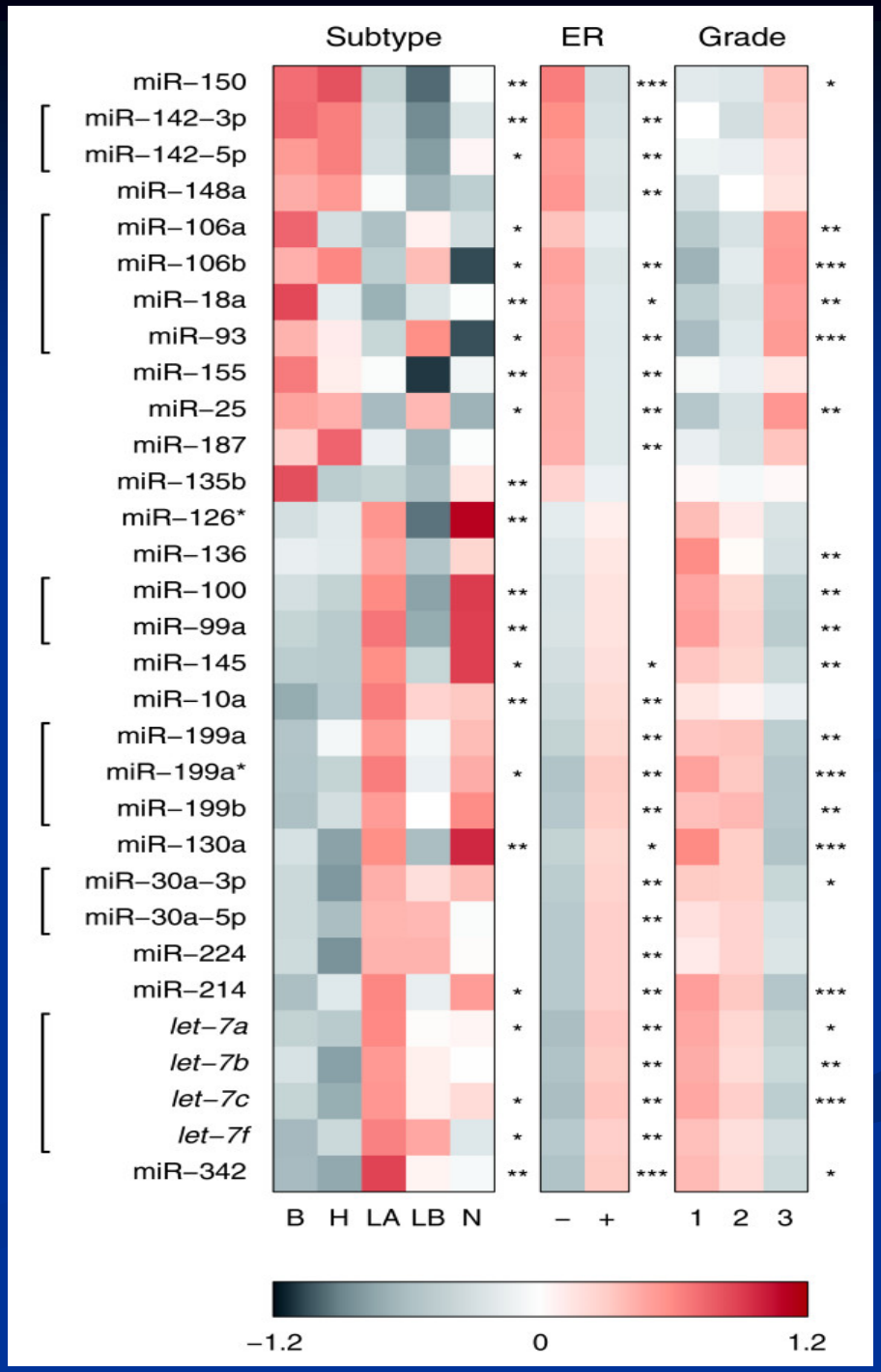
Supervised hierarchical clustering over selected miRNAs:

51 tumor samples over 38 miRNAs ----- 24 tumor samples over 9 miRNAs

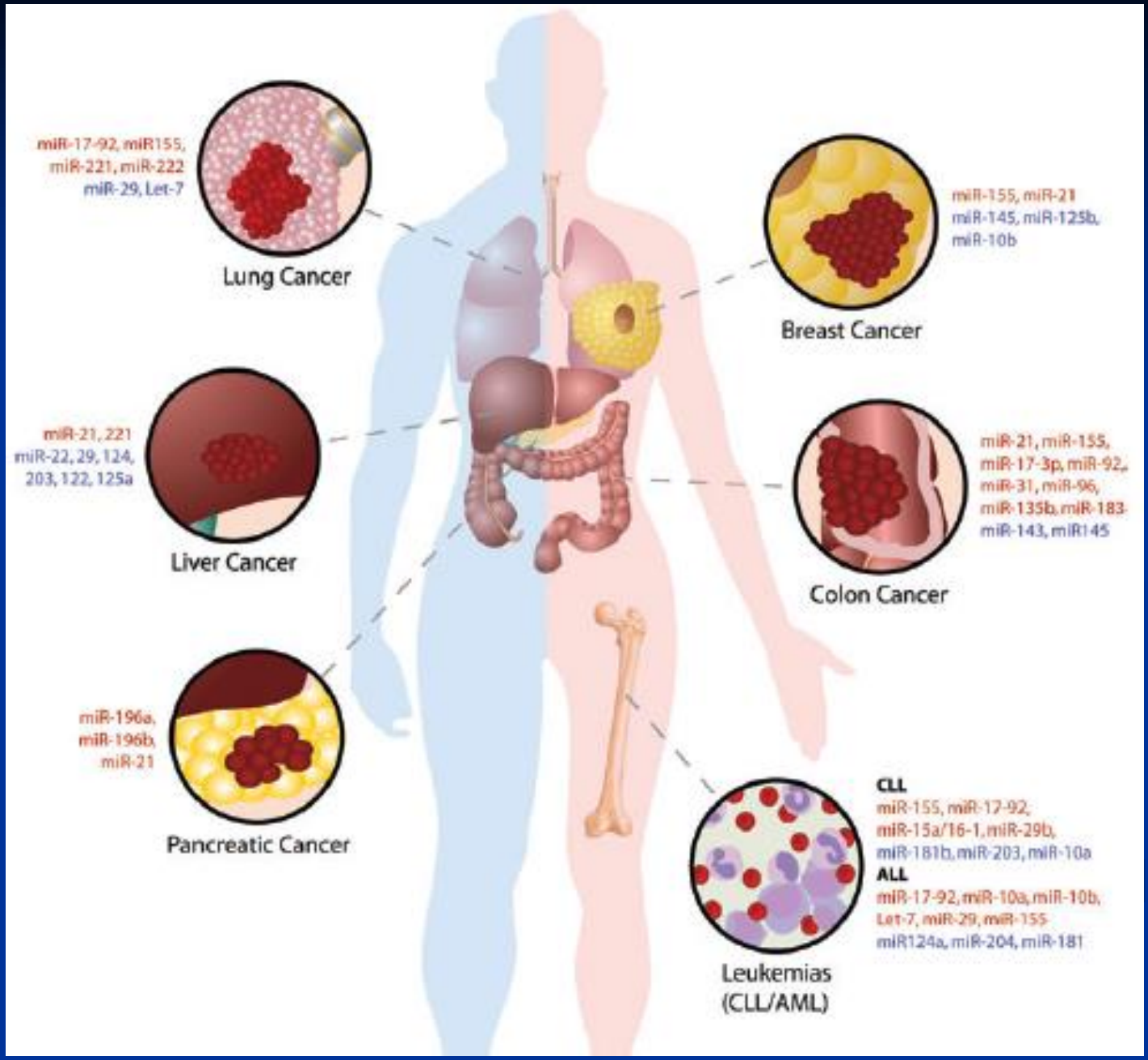
Association of individual miRNAs and tumor subtype or clinico-pathological factors

31 miRNAs and three factors with at least one association at adjusted $p <$ 0.01

bead-based flow cytometric miRNA expression profiling might be a suitable platform to classify breast cancer into prognostic molecular subtypes

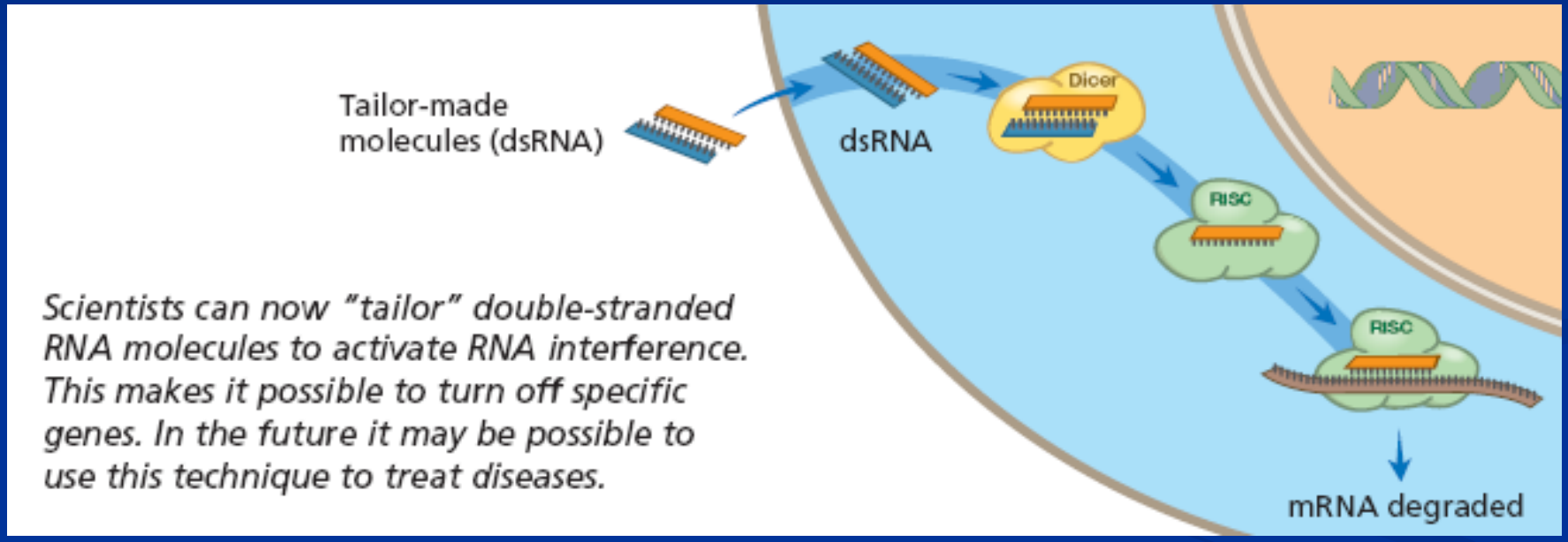


De-regulated miRNAs in Cancer



RNAi as a tool for knock down in mammalian cells

Custom-made siRNAs



Scientists can now "tailor" double-stranded RNA molecules to activate RNA interference. This makes it possible to turn off specific genes. In the future it may be possible to use this technique to treat diseases.

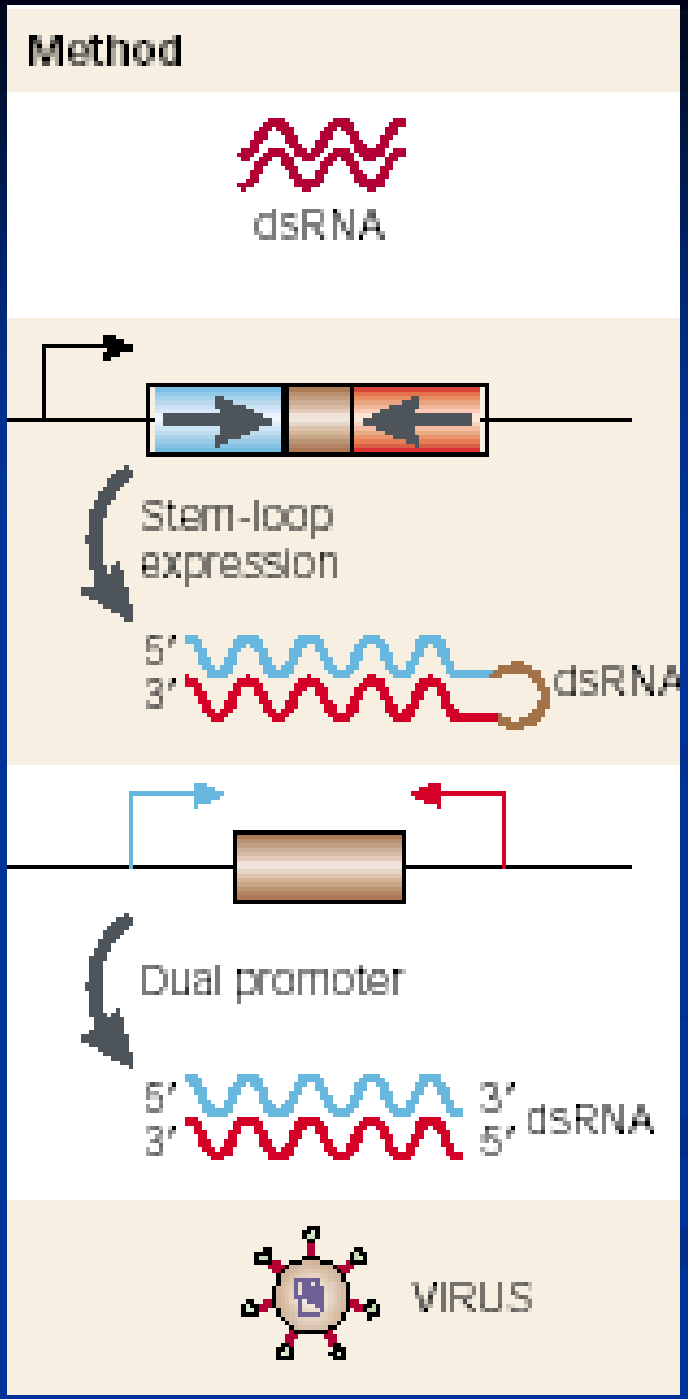
Critical Factors for RNA Interference

- Delivery: efficient (close to 100%) to any cell type
- Stability: Long Term siRNA expression
- Silencing Efficiency: siRNA design & validation
- Specificity: non-specific response, toxicity, non-target knockdown

RNAi

- Double-stranded RNAs are introduced into the cell
  - Complementary to mRNA for a gene
  - Directly introduced to the cell, or
  - Produced by the cell itself

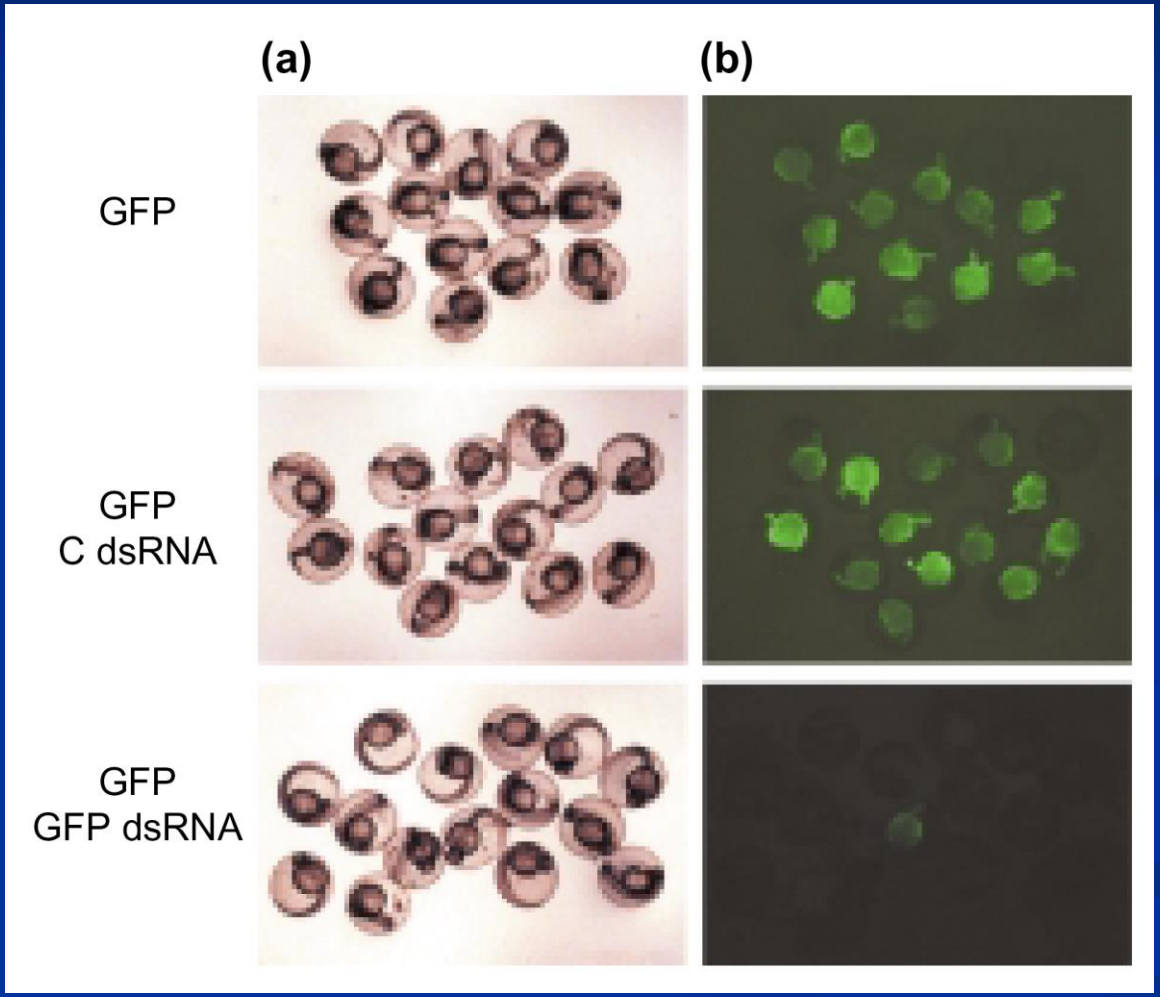
Summary


Method
dsRNA
Stem-loop
expression
5′
3′
dsRNA
Dual promoter
5′
3′
3′
5′
dsRNA
VIRUS

Methods of siRNA delivery

- Introduction of dsRNA or siRNA is dependent upon cell type or organism
- *C.elegans* -- injection, soaking, or feeding
- *Drosophila* cells -- exposure through culture medium
- Mammalian cells -- transfection or electroporation

siRNA gene silencing example



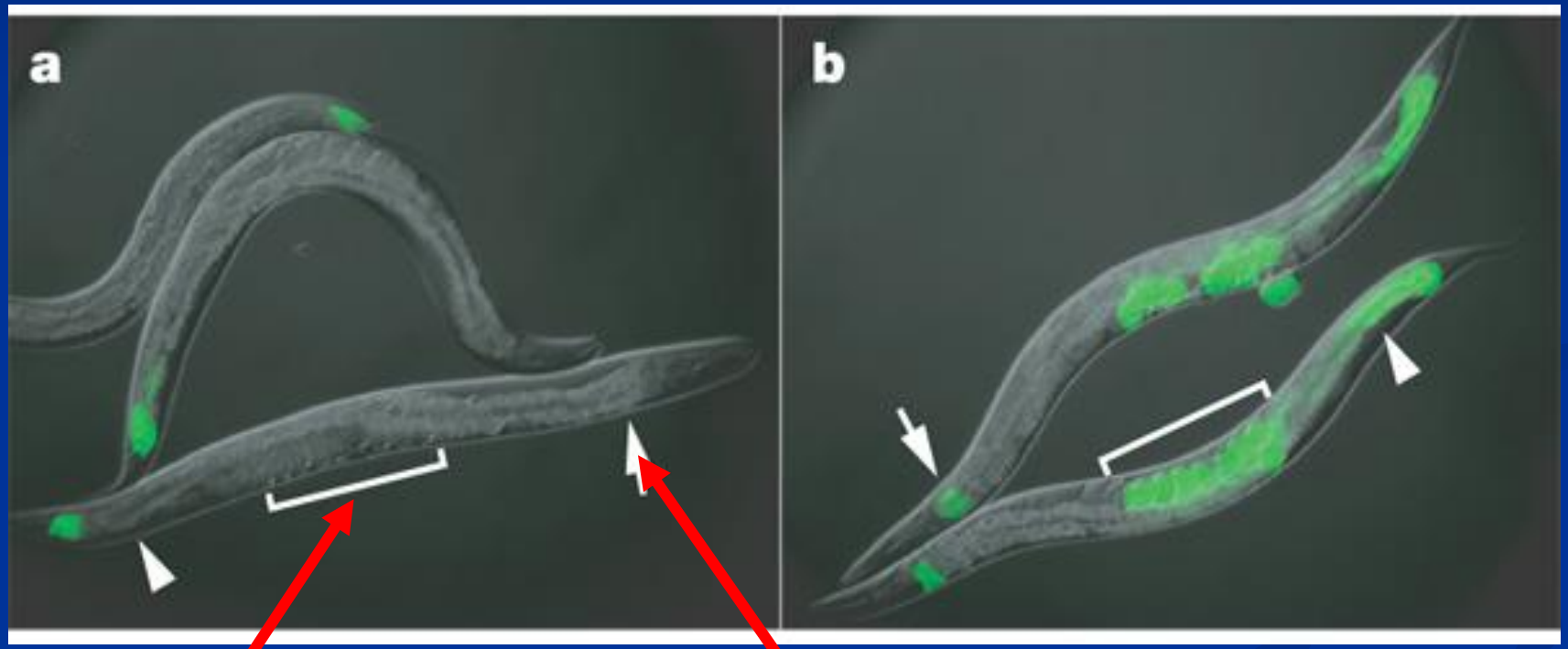
Drosophila S2 cells with or without GFP RNAi

2002 Drug Discovery Today 7(20):1040-1046

Worms Eating Bacteria Expressing dsRNA to a GFP Reporter Gene Activate RNAi

wild type worms

RNAi-defective mutants



from Mello and Conte, Nature 431, 338-342.

GFP lost in embryos

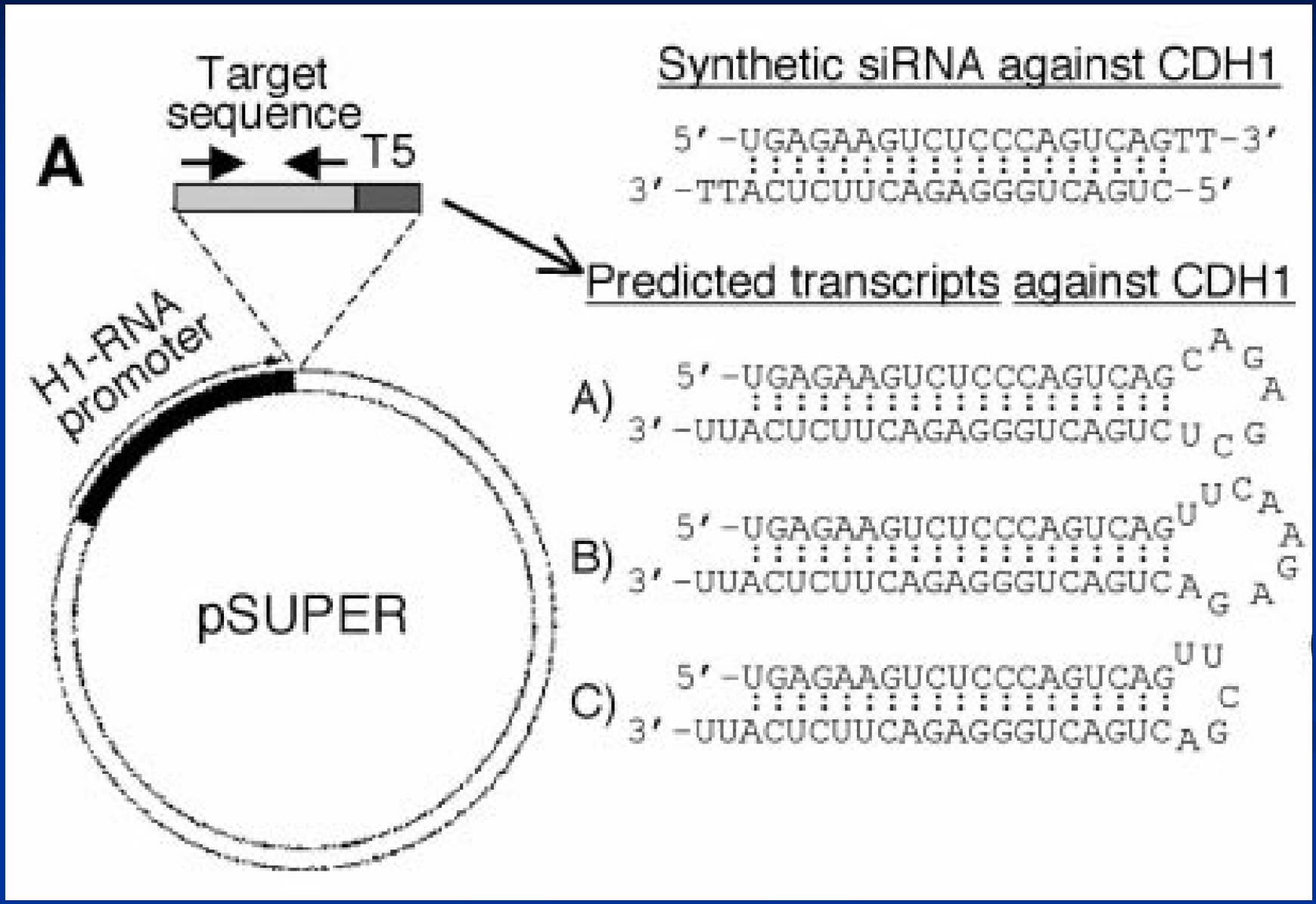
GFP lost in the gut

A System for Stable Expression of Short Interfering RNAs in Mammalian Cells

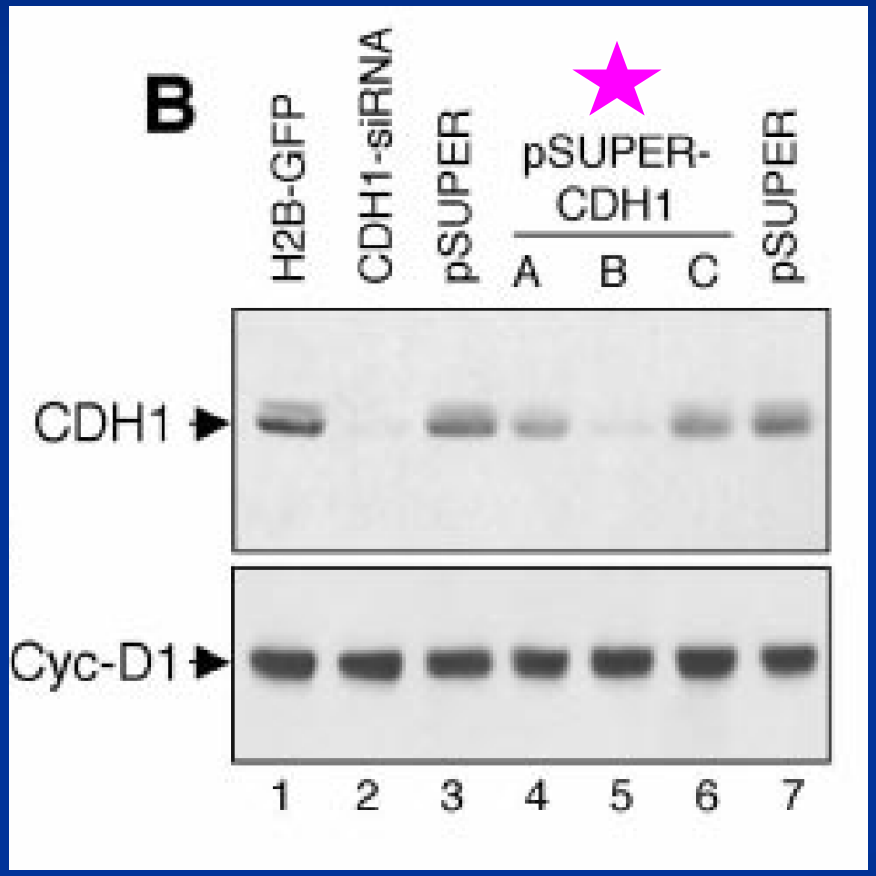
Thijn R. Brummelkamp,[1] René Bernards,[1,3] Reuven Agami[1,2,3]*

19 APRIL 2002 VOL 296 SCIENCE

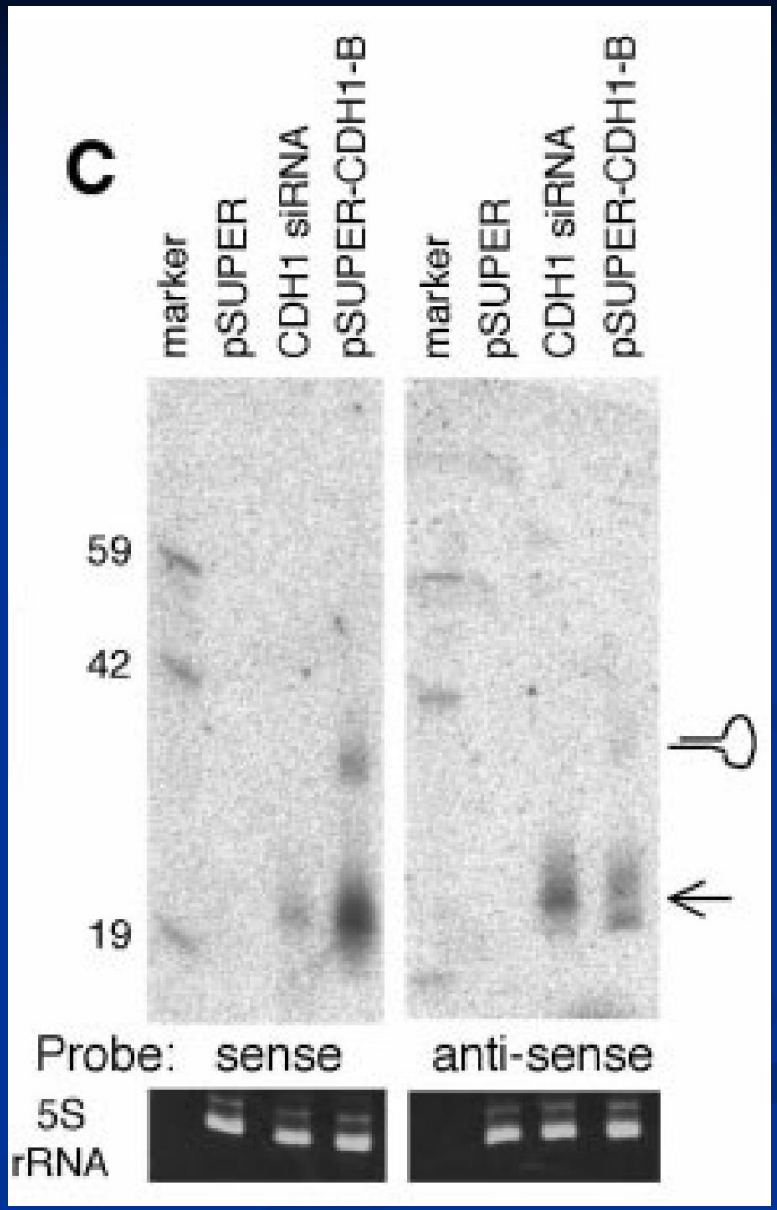
pSUPER-CDH1 constructs



Size Matters!
(9-nt loop active, others not)

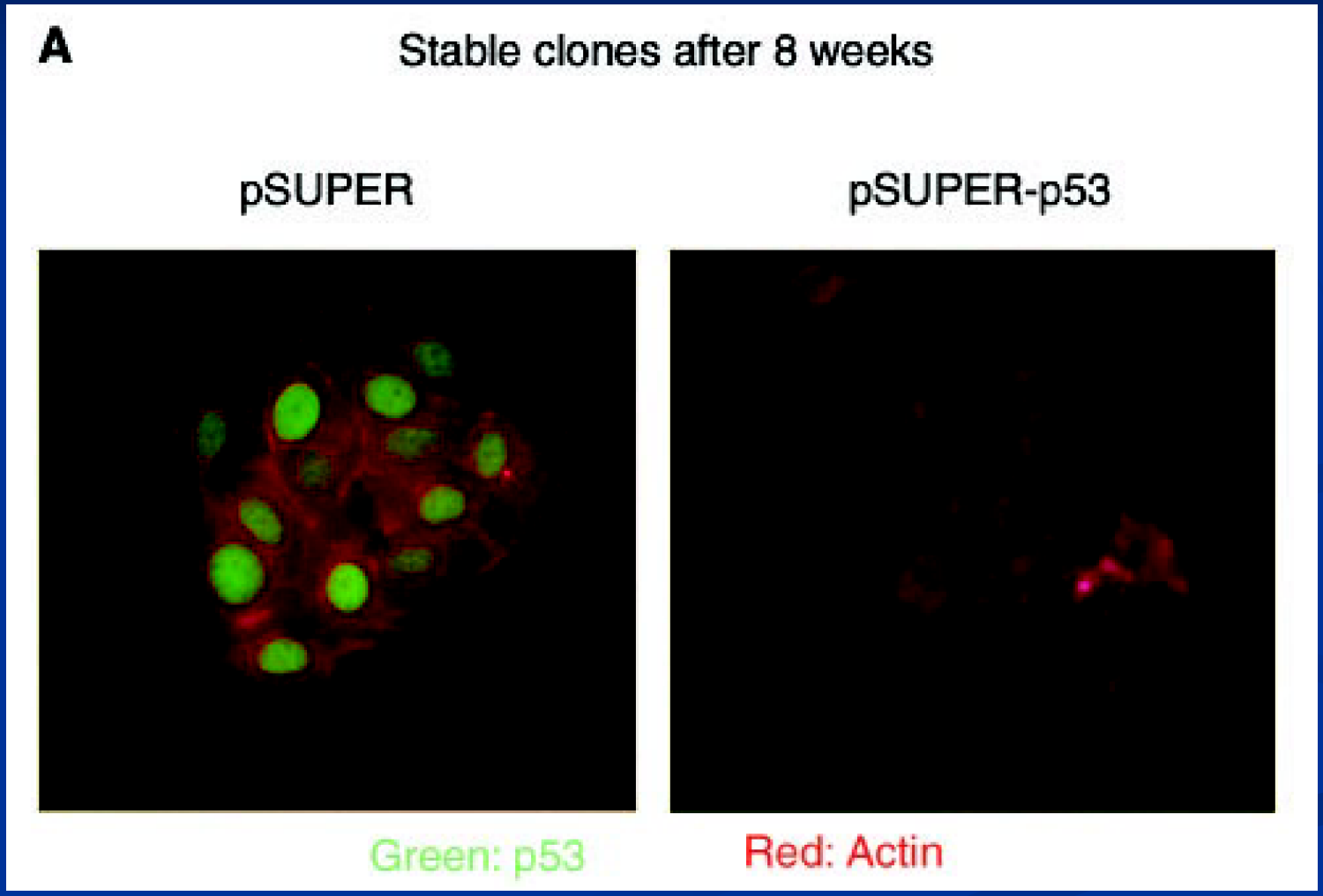


Western Blot (anti-CDH1)

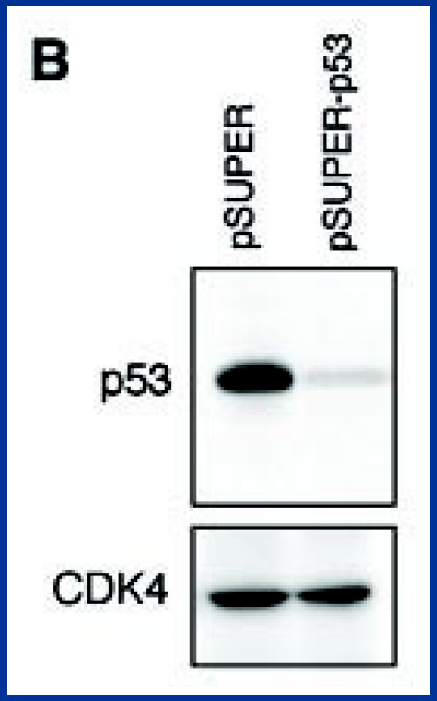


Northern Blot

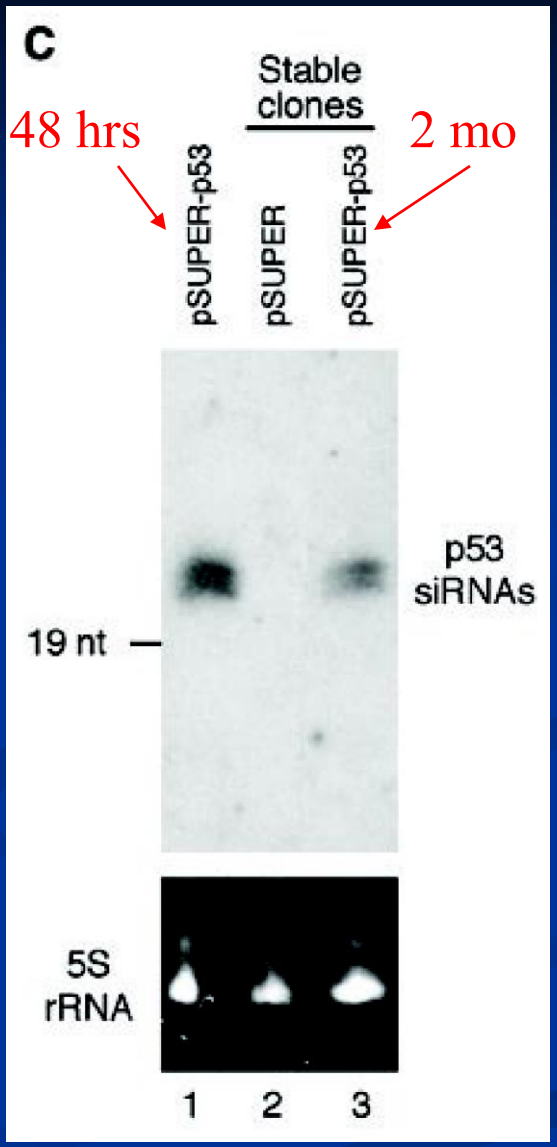
pSUPER & Stable suppression



More evidence for stable suppression

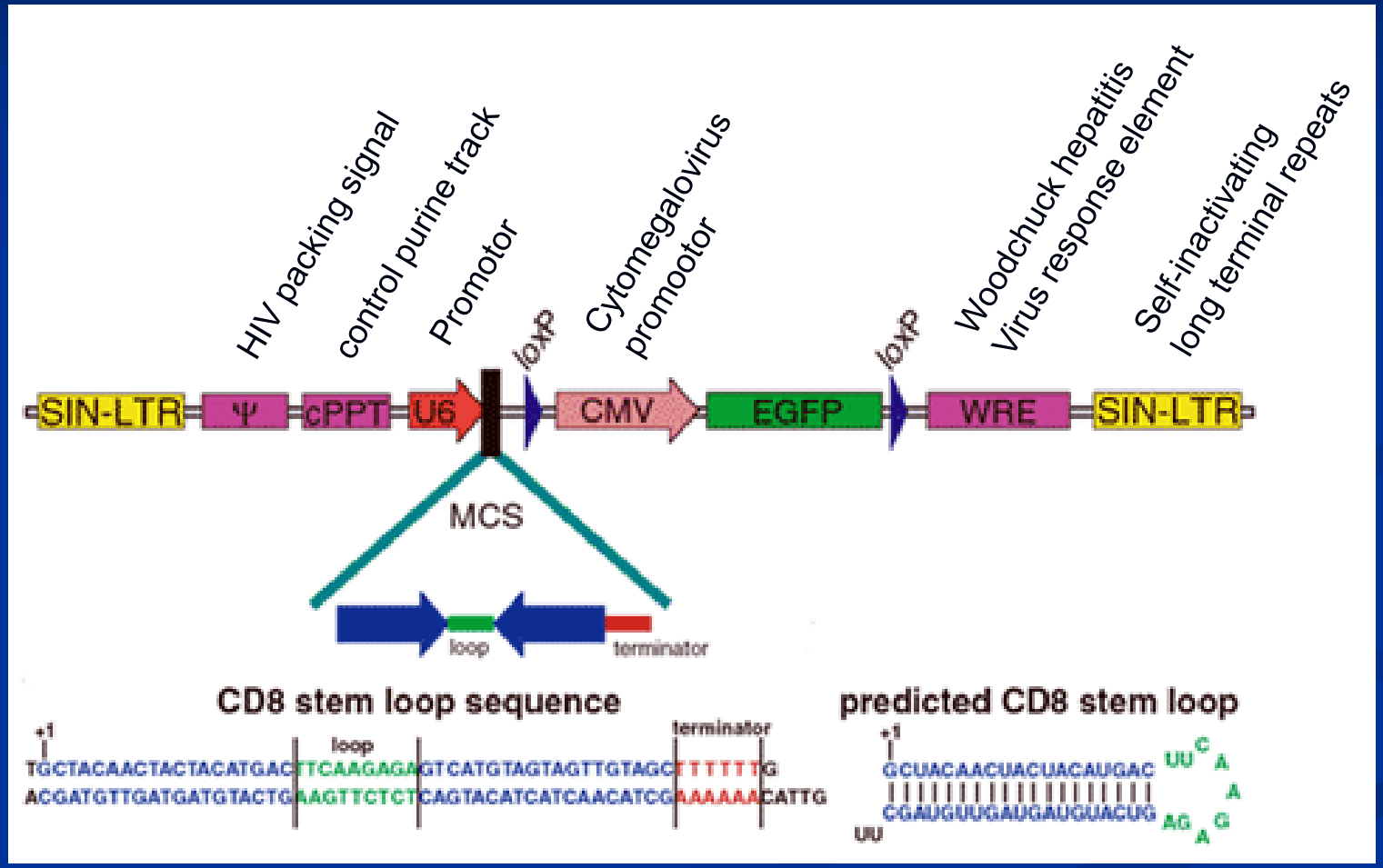


Western Blot



Northern Blot

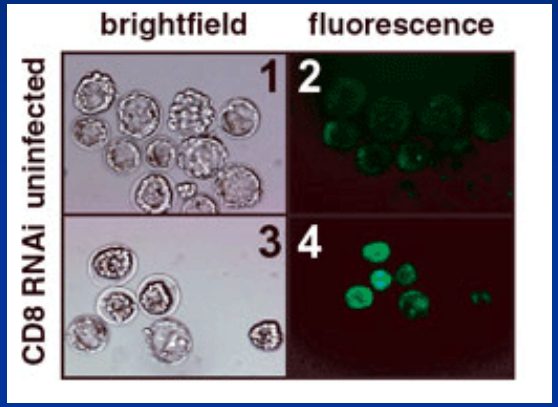
Lentivirus-Based Approach: shRNA-expressing vector



Rubinson, Nat Genet. 2003 Mar;33(3):401-6

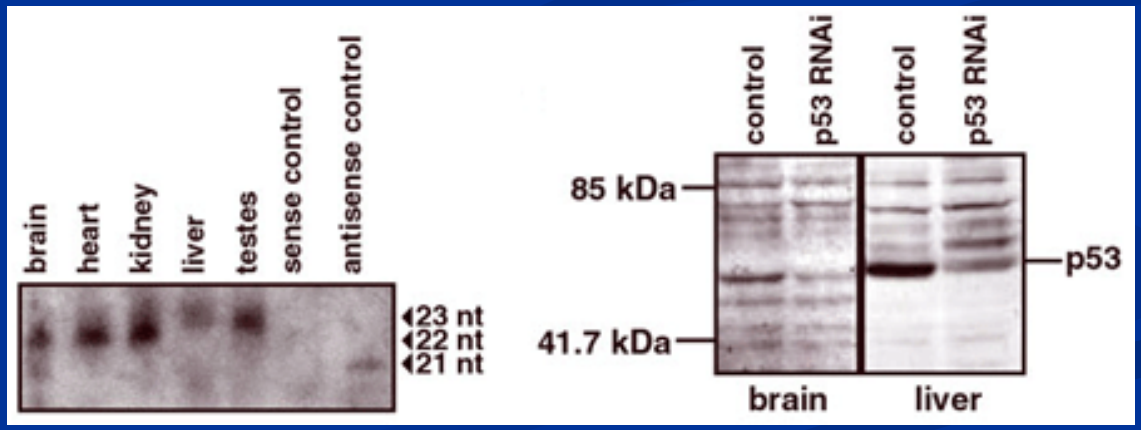
Functional silencing of genes in mice by Lentivirus-infection

Generation of lentivirus infected zygotes

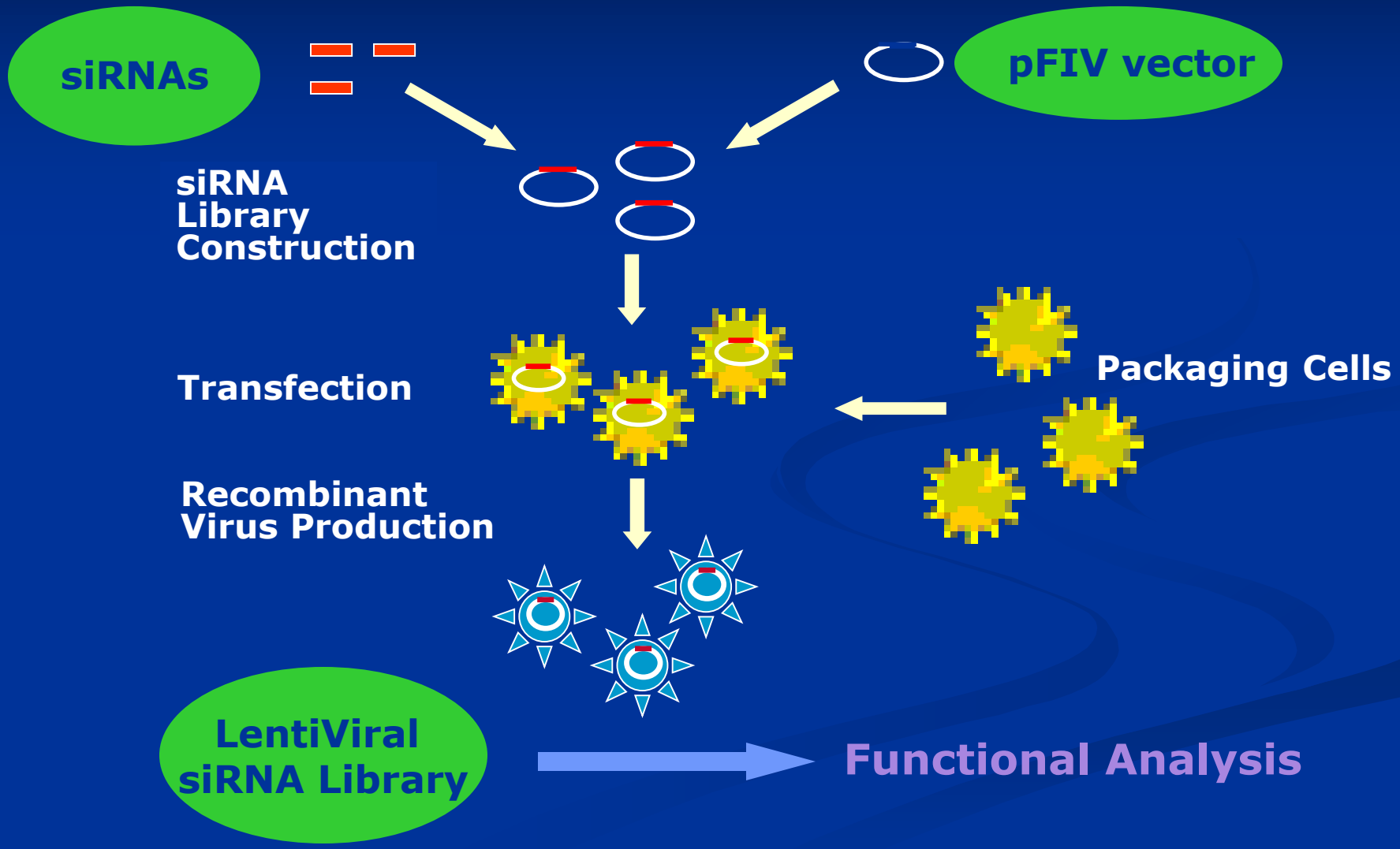


Silencing of p53:

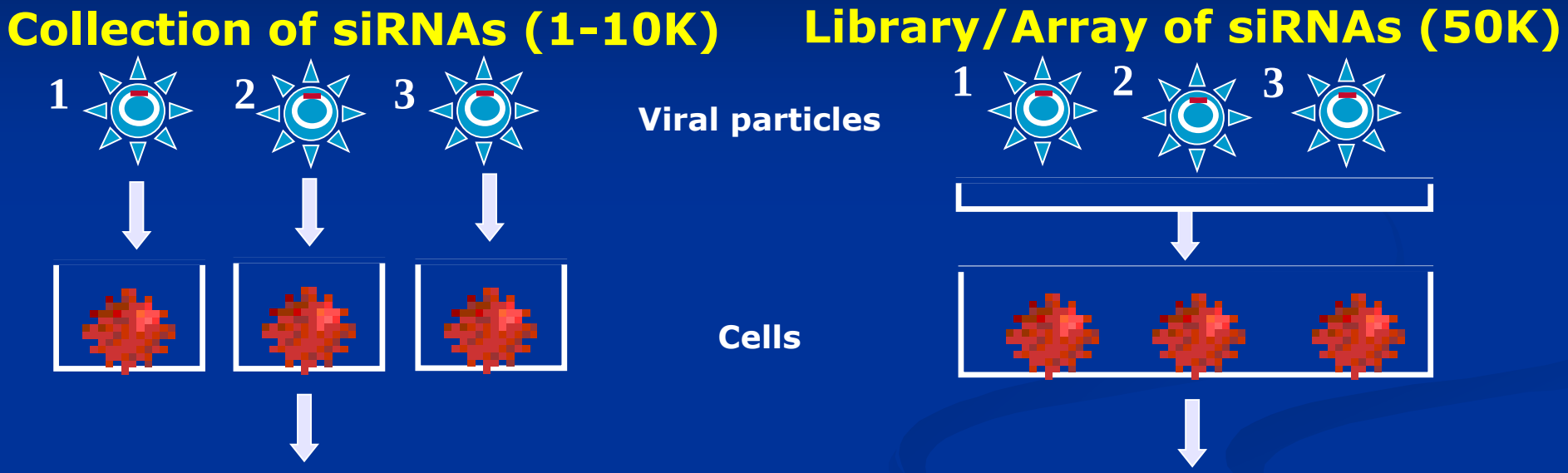
Tissue was harvested from 8-wk-old mice



Construction of Lentiviral siRNA Libraries



High-Throughput Functional Analysis



Gene-Phenotype Association Studies

Specific Gene(s) Analysis — Novel Gene Discovery

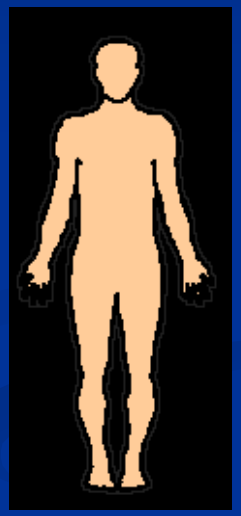
Multiple samples — All genes

RNAi & Therapeutic Suppression

Delivery Requirements

Achieving RNAi as Therapy

- Introducing "drug-like" properties into siRNAs
  - » Potency
  - » Selectivity
  - » Stability
- Achieving delivery to target tissues/cells
  - » PK/PD/Biodistribution
  - » Cellular uptake

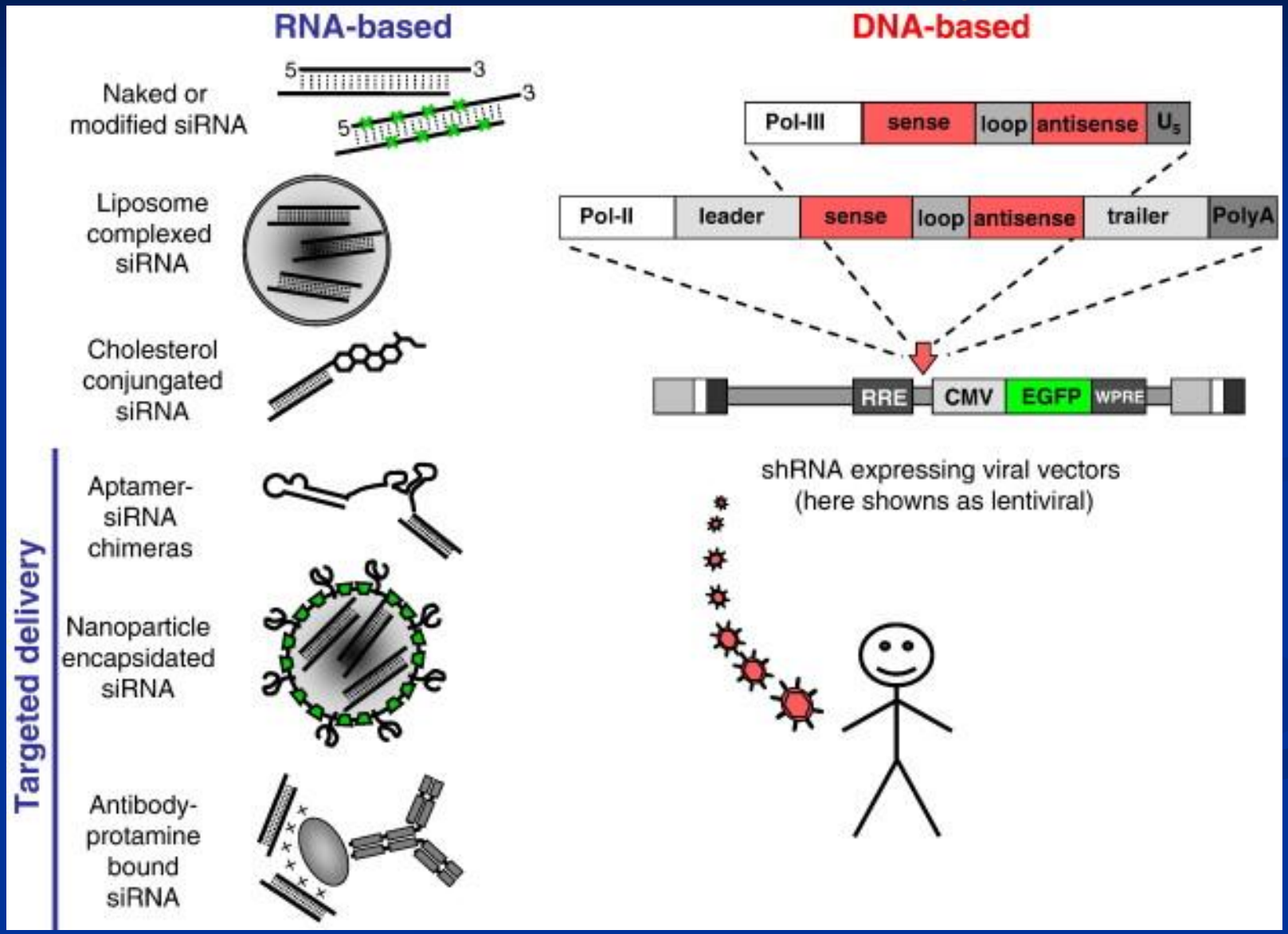


In vivo

Delivery Approaches

- Conjugates
- Liposomal NPs
- Peptides
- Antibodies

Delivery strategies for RNA- and DNA-based siRNA drugs



Proof of concept:

Efficient delivery of siRNA for inhibition of gene expression in postnatal mice

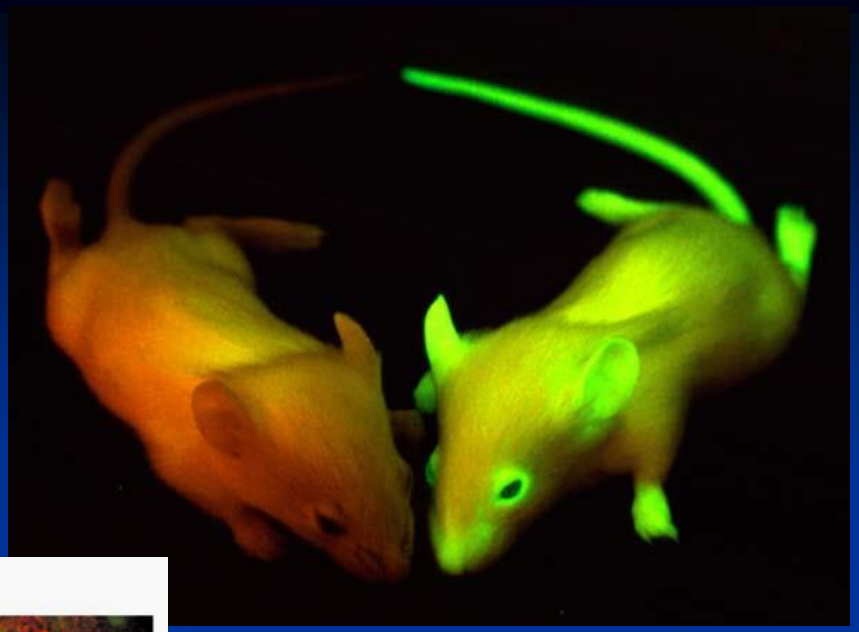
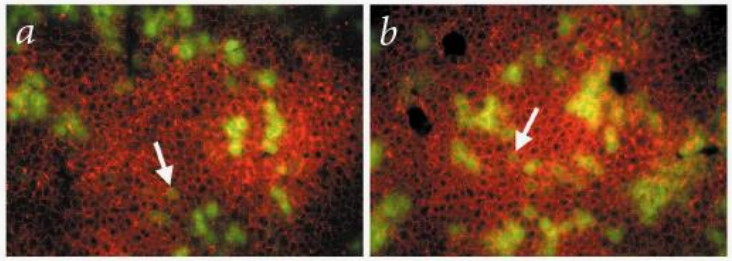
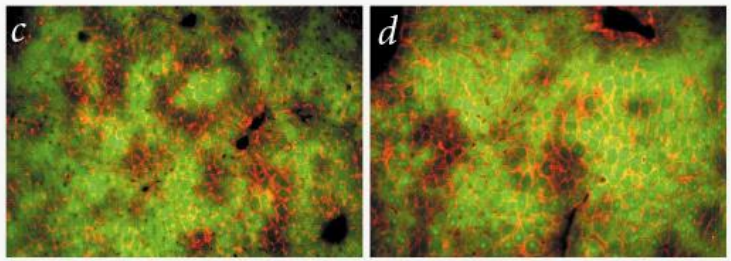


Fig. 2 Inhibition of EGFP expression in transgenic mice after delivery of siRNA. Mice (strain C57Bl/6-TgN(ACTbEGFP)10sb, Jackson Laboratories, 10 wk old) were injected with siRNA-*EGFP* (**a**, **b**) or a control siRNA, siRNA-*HBsAg* (**c**, **d**). Livers were collected 48 h after injection. Frozen sections were fixed and then counterstained with Alexa 568 phalloidin (red) to visualize cell outlines. Images were acquired using a Zeiss Axioplan 2 fluorescence microscope outfitted with a Zeiss AxioCam digital camera. There was some variability in the degree of EGFP expression across the liver. The images show areas that are representative of the level and distribution of EGFP expression across all sections examined. Examples of cells in mice treated with siRNA-*EGFP*, containing decreased but detectable amounts of EGFP, are indicated by arrows.



siRNA-*EGFP*



siRNA-*HBsAg*

Injection of 50µg siRNA into tailvein of EGFP-transgenic mice downregulates GFP

in various organs,

however not in all cells in the organ

RNAi can be used to suppress unwanted alternatively spliced transcripts

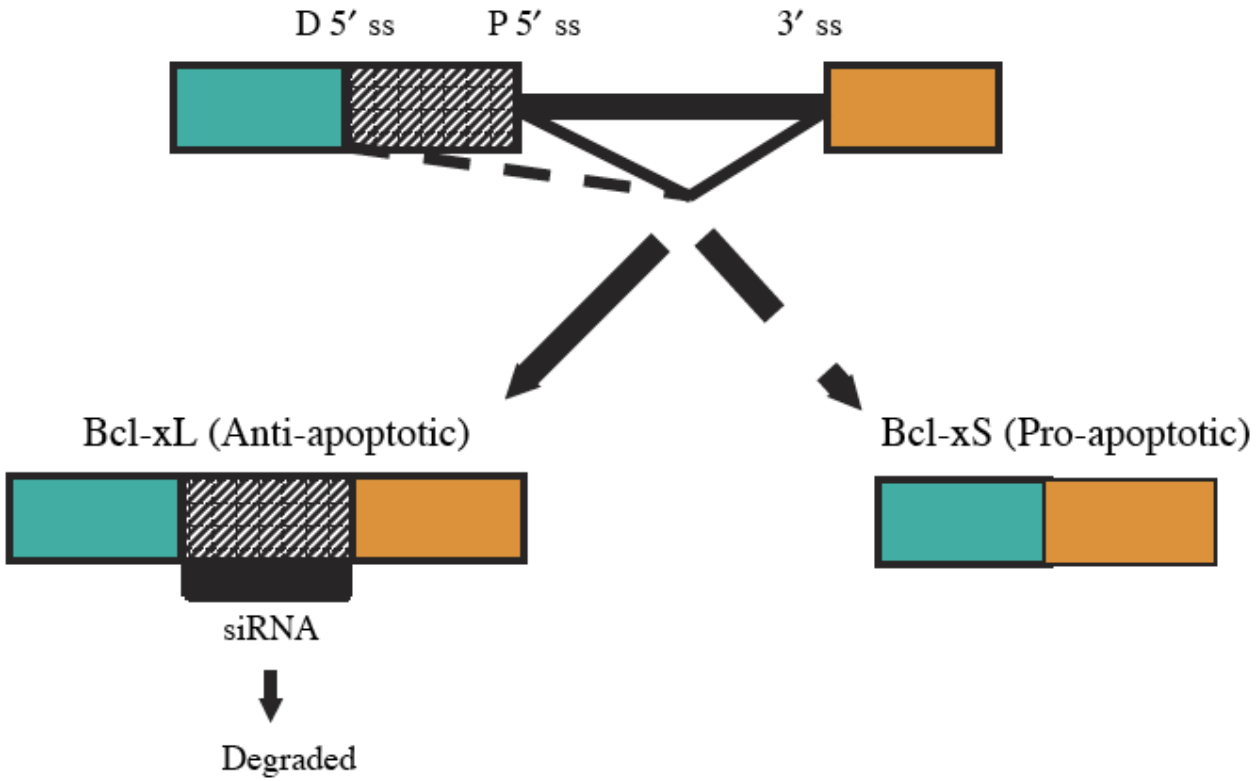


Figure 2. RNA interference (RNAi)-mediated down-regulation of a splicing isoform. Bcl-xL-specific small interfering RNA (siRNA) down-regulates Bcl-xL protein and inhibit the proliferation of 5-fluorouracil and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant cells. D, distal 5′ splice site; P, proximal 5′ splice site.

RNAi can be used to suppress exon skipping

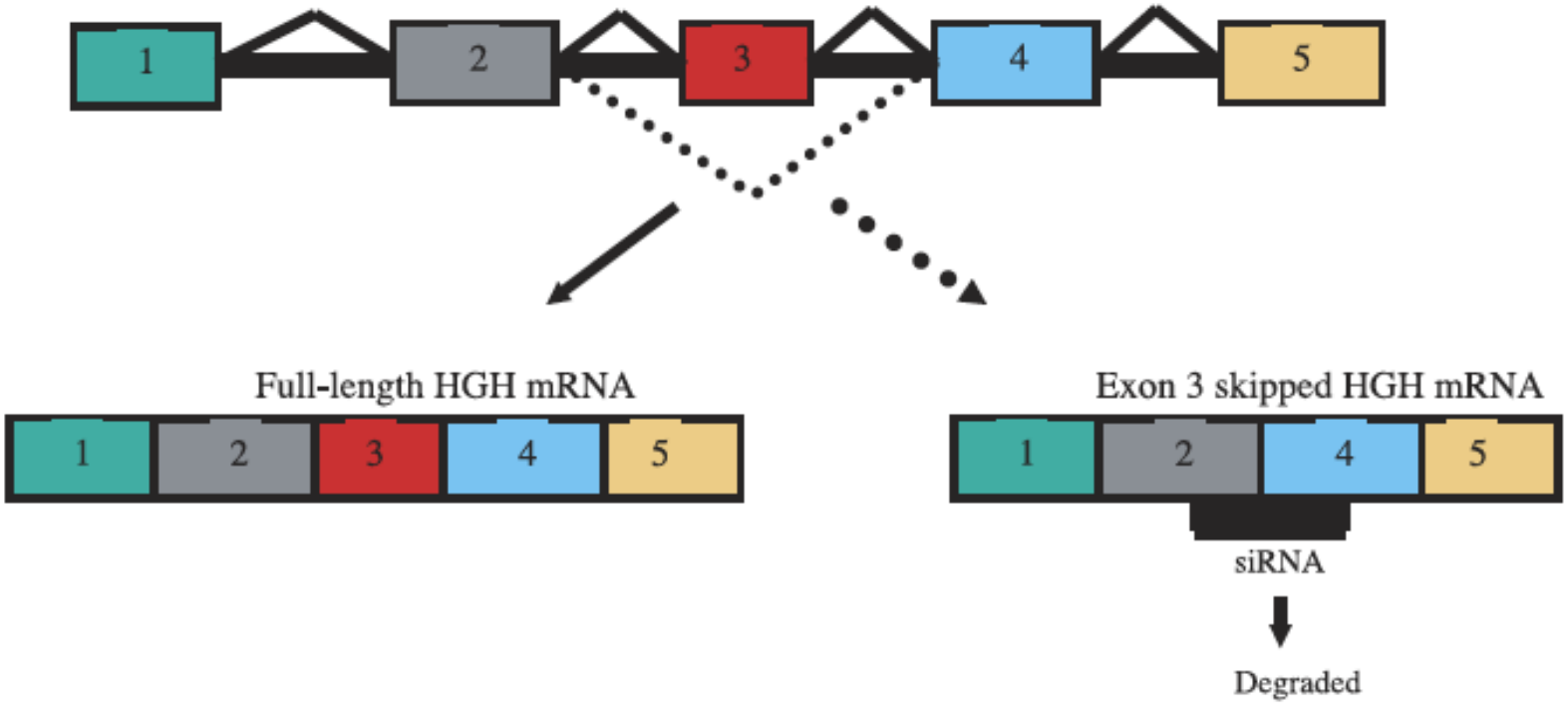


Figure 3. RNA interference (RNAi)-mediated suppression of exon 3 skipped human growth hormone (HGH) isoform. A small interfering RNA (siRNA) designed to target the exon2-exon 4 junction specifically degrade exon 3-skipped transcripts (65). mRNA, messenger RNA.

RNAi can be used to suppress transcripts with point mutations

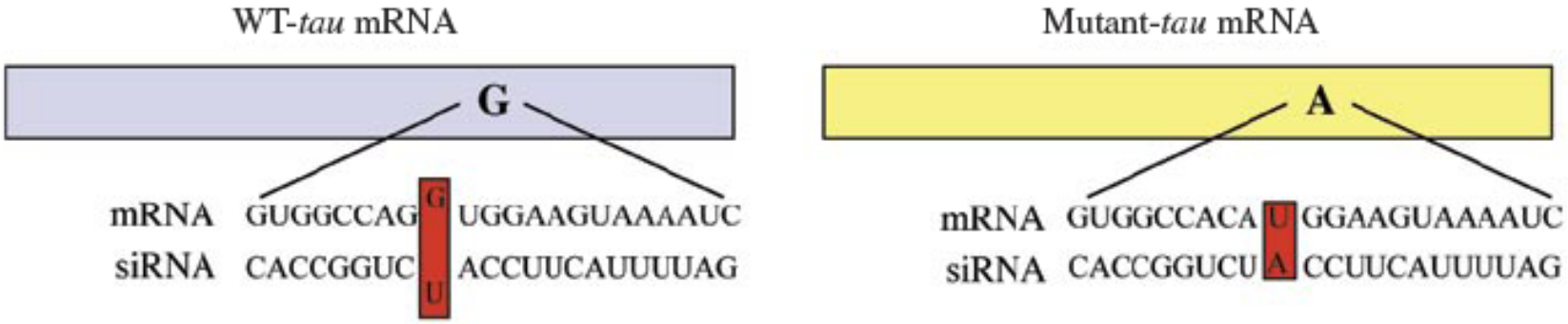


Figure 4. Allele-specific small interfering RNA (siRNA) for suppression of a missense *tau* mutation. The siRNA designed to target the mutant allele forms a perfect duplex, but contain mismatches with the normal allele. This leads to the preferential degradation of the mutant transcript (97,98). WT, wild-type; mRNA, messenger RNA.

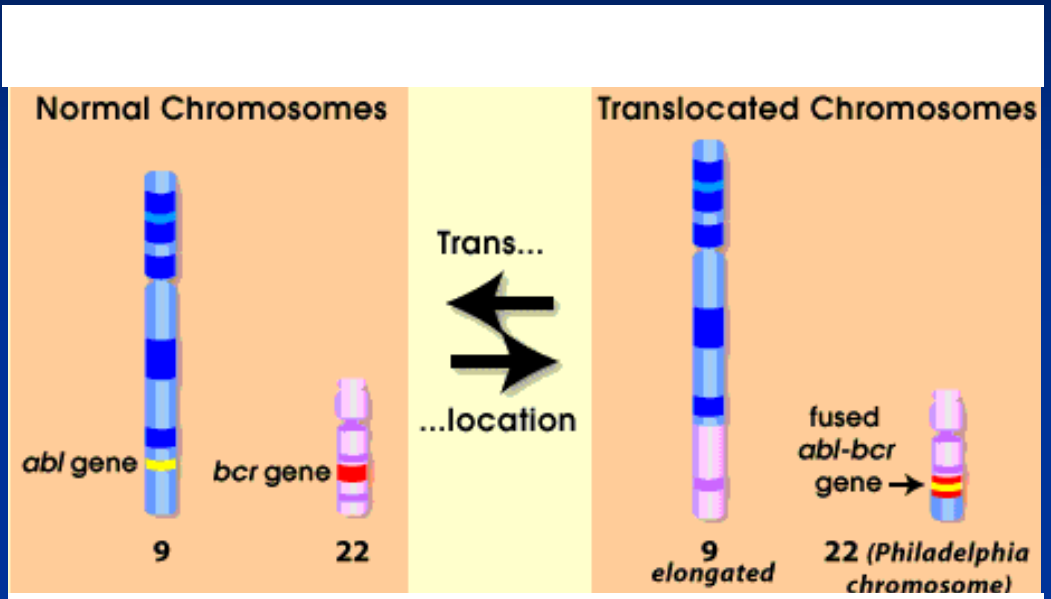
Targeting translocations associated with Leukemia

BCR-ABL1 (9;22), TEL-AML1, t(12;21) and Sil-Tal1, (del(1p))

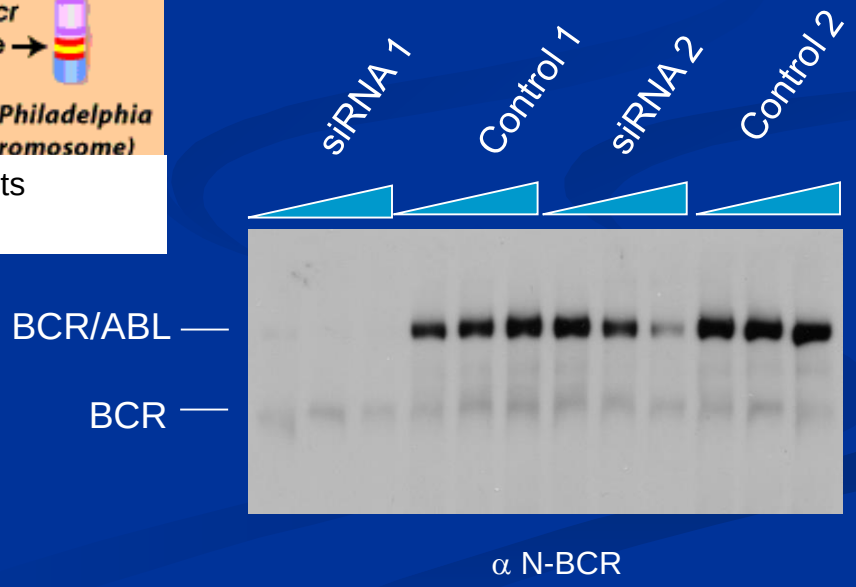
Knock-down of gene expression in cultured cells

BCR/ABL1 to be tested in transgenic mouse model supplied by Professor Daniel Tenen, Harvard Medical School, Boston, USA.

Knockdown of BCR/ABL oncogene

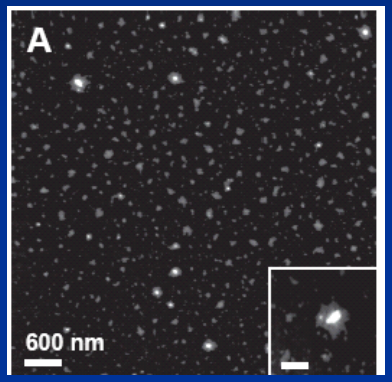


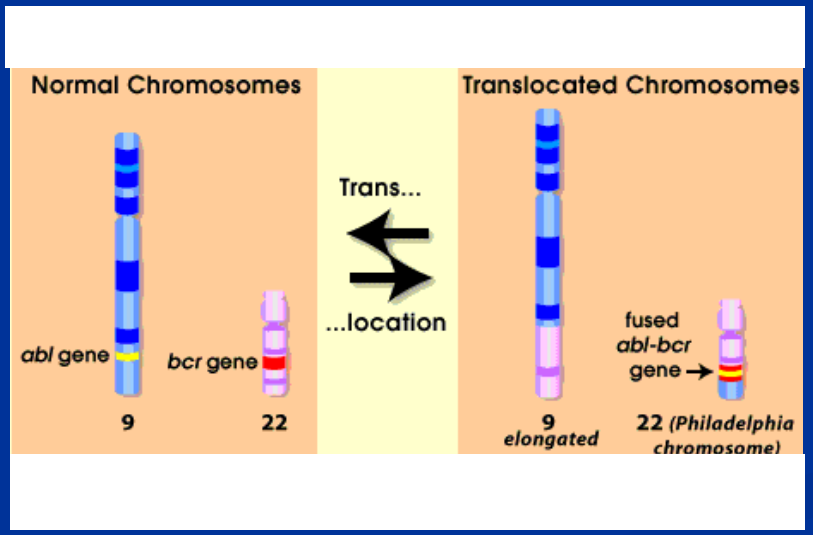
90% af Chronic Myelogenous Leukemia (CML) patients

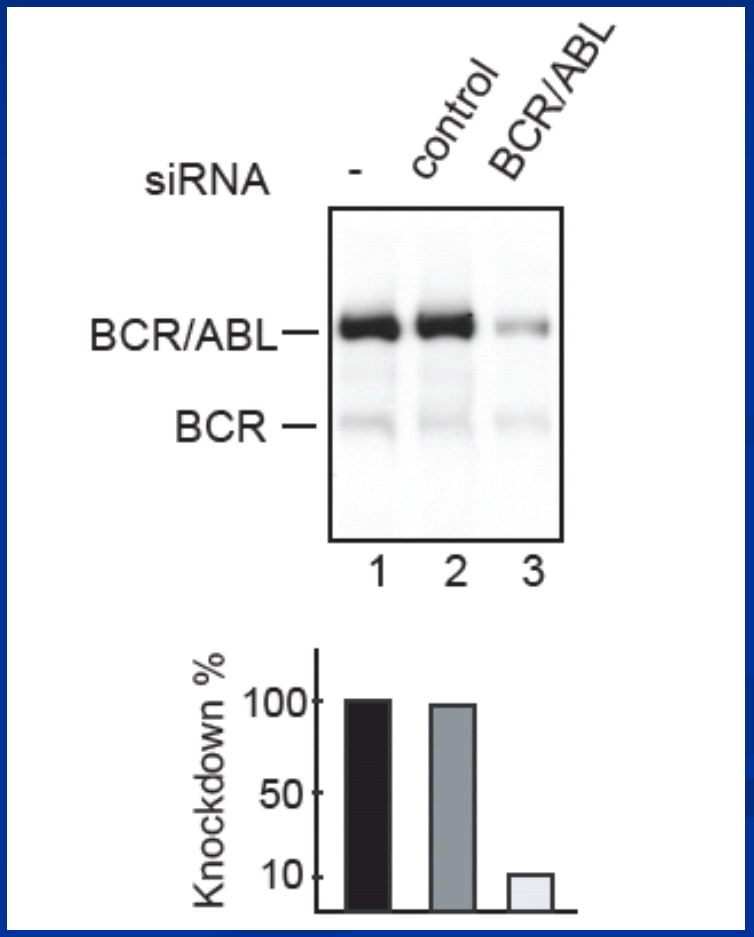


Chitosan-based Systems

Nasal delivery to intravascular cells or CNS?



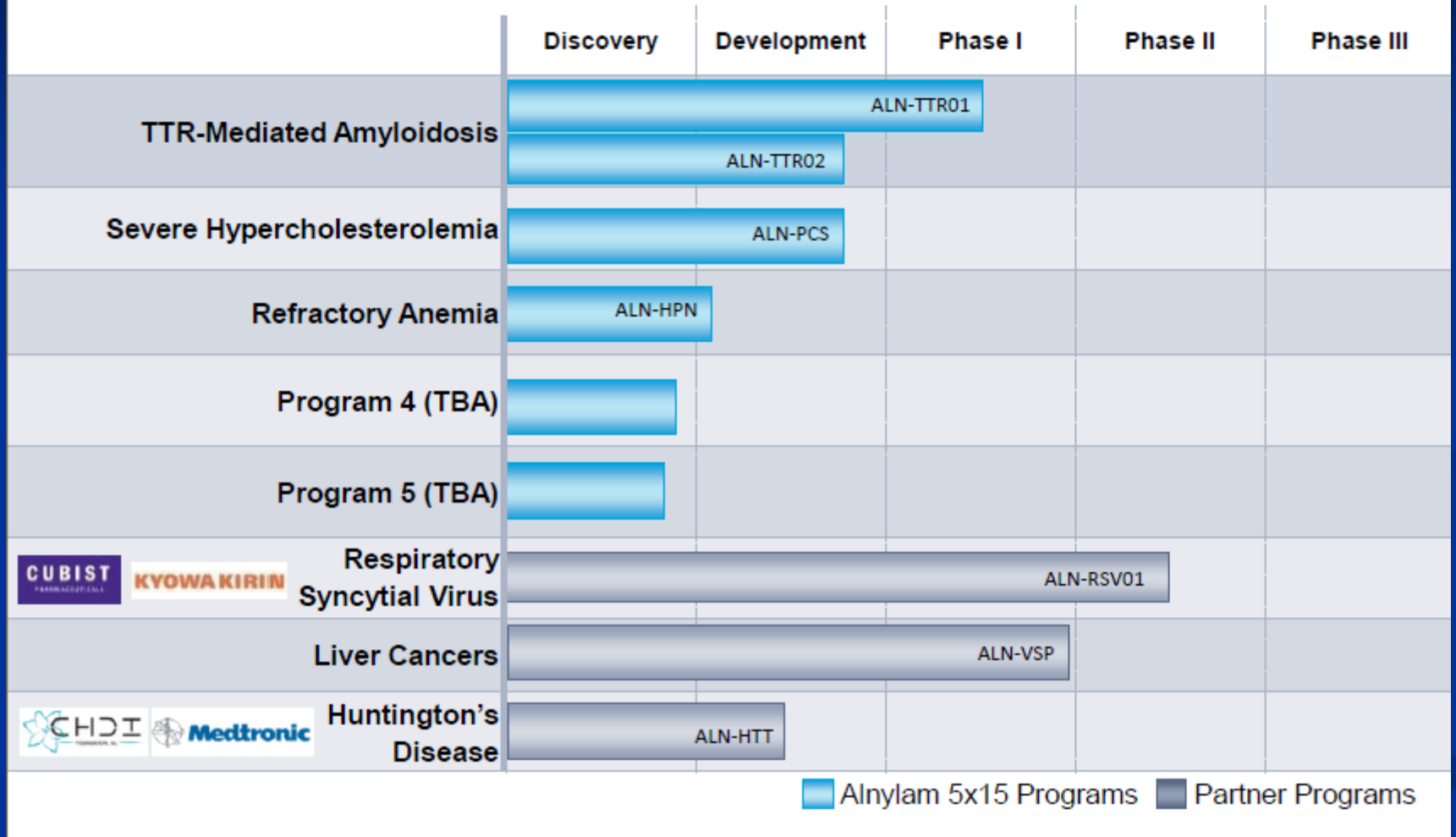




RNAi to Treat Primary and Secondary Liver Cancers

November 12, 2012

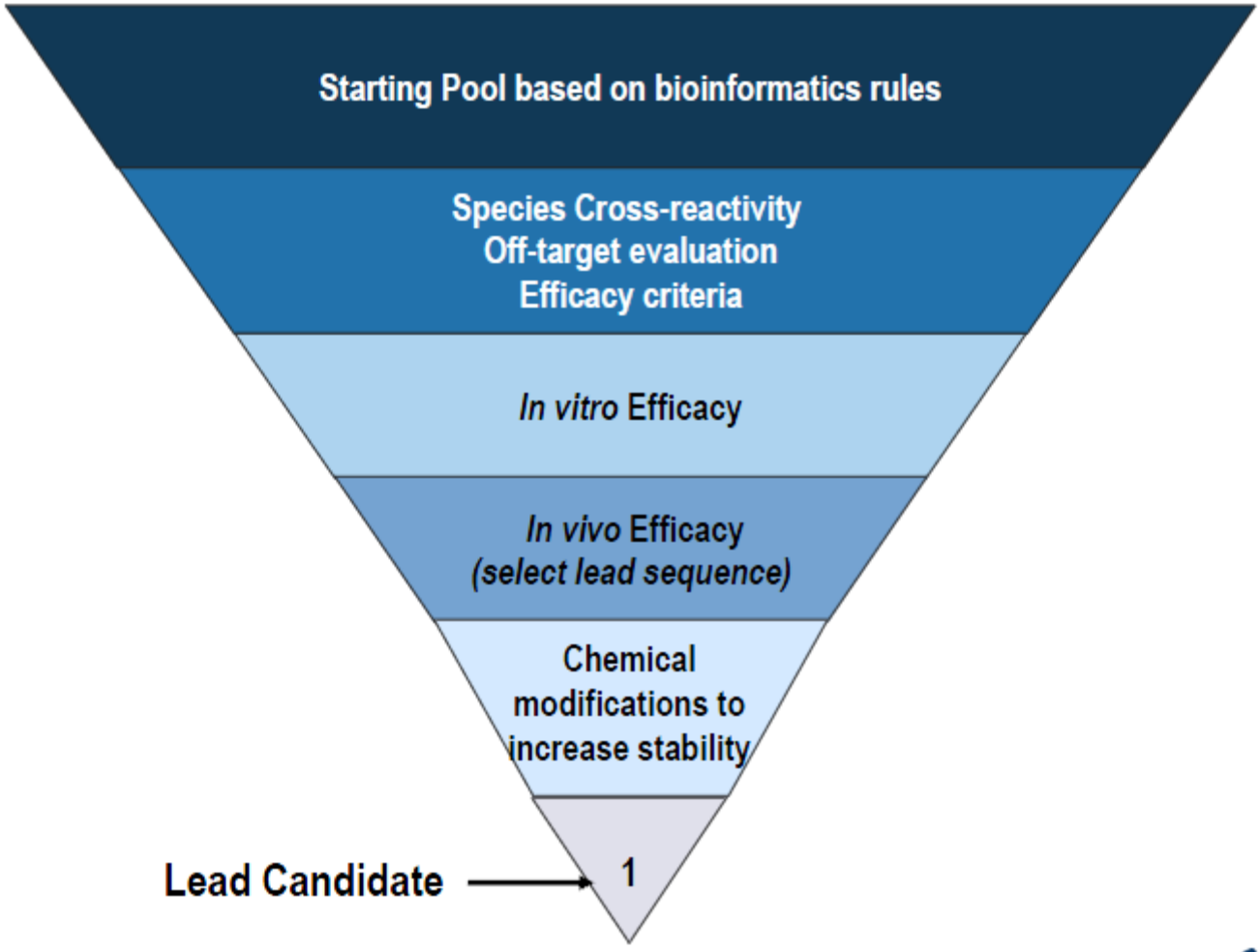
Alnylam Development Pipeline



Alnylam PHARMACEUTICALS

RNAi Lead Discovery

Work-flow



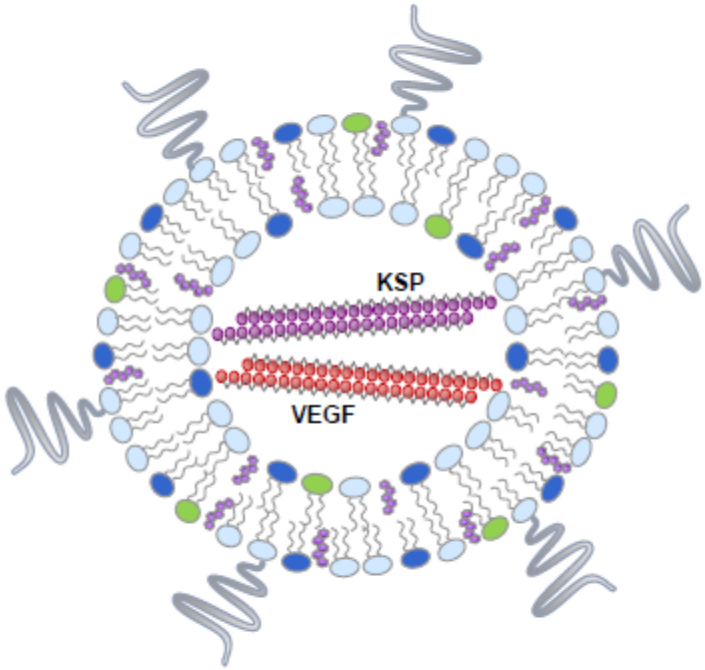
Alnylam PHARMACEUTICALS

Liver Cancer Program

ALN-VSP

RNAi to treat primary and secondary liver cancers

- Prevalent solid tumor and common site of metastatic disease
  - ~700,000/yr: Incidence of HCC worldwide
  - ~500,000/yr: Patients with liver metastasis
- ALN-VSP is first dual-targeted RNAi drug
  - Targets two distinct genes involved in cancer pathways
    - Proliferation: Kinesin Spindle Protein (KSP)
    - Angiogenesis: VEGF
  - Lipid nanoparticle (LNP) formulation
    - From Tekmira Pharmaceuticals
- Phase I clinical trial for liver cancer
  - Encouraging initial data and RNAi POM
  - Enrollment completed
    - >40 patients
    - Doses range: 0.1-1.5 mg/kg
    - Multiple patients continuing therapy
  - Data to be presented at ASCO, June 3-7, 2011
    - Poster Session: Developmental Therapeutics – Experimental Therapeutics; June 4, 2pm-6pm CDT
    - Poster Discussion; June 4, 5pm-6pm CDT



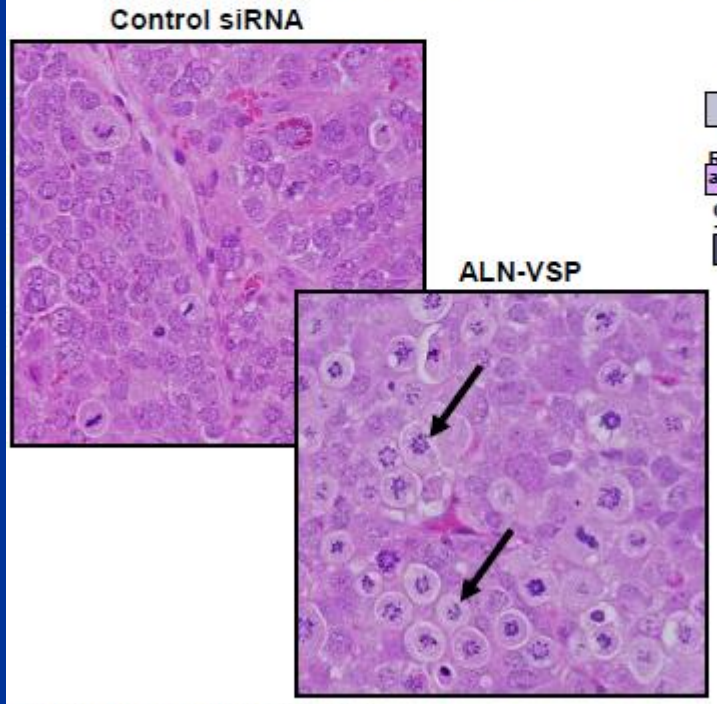
ALN-VSP

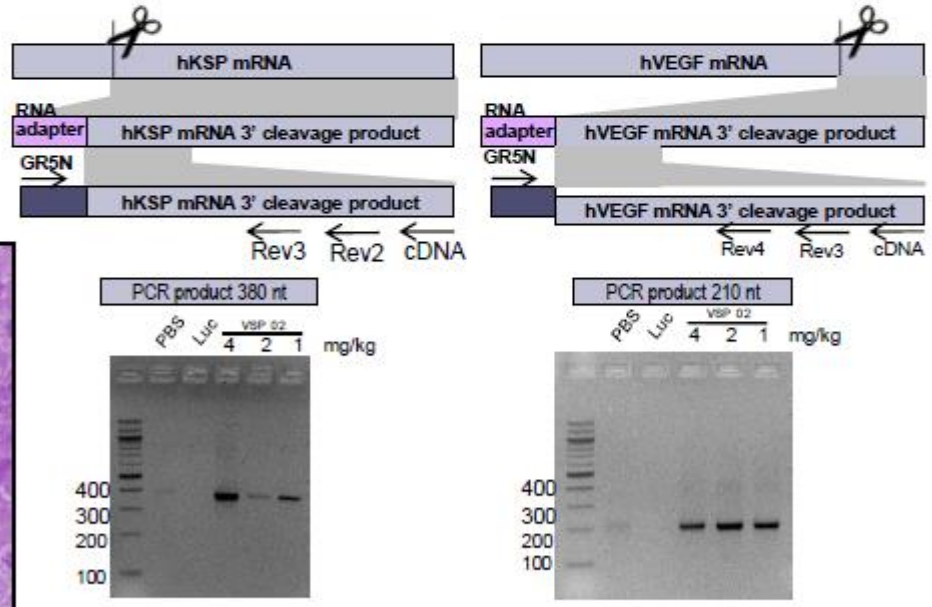
Alnylam PHARMACEUTICALS

Tumor Targeting
Murine Liver Cancer Model

Orthotopic tumor model with intrahepatic Hep3B seeding in SCID mice

- Single IV bolus injection of ALN-VSP or control siRNA
- Mitotic arrest (monoasters) clearly detected in VSP-treated animals
- KSP and VEGF target mRNAs cleaved in tumors confirming RNAi mechanism





Keystone: RNAi, Feb 2009

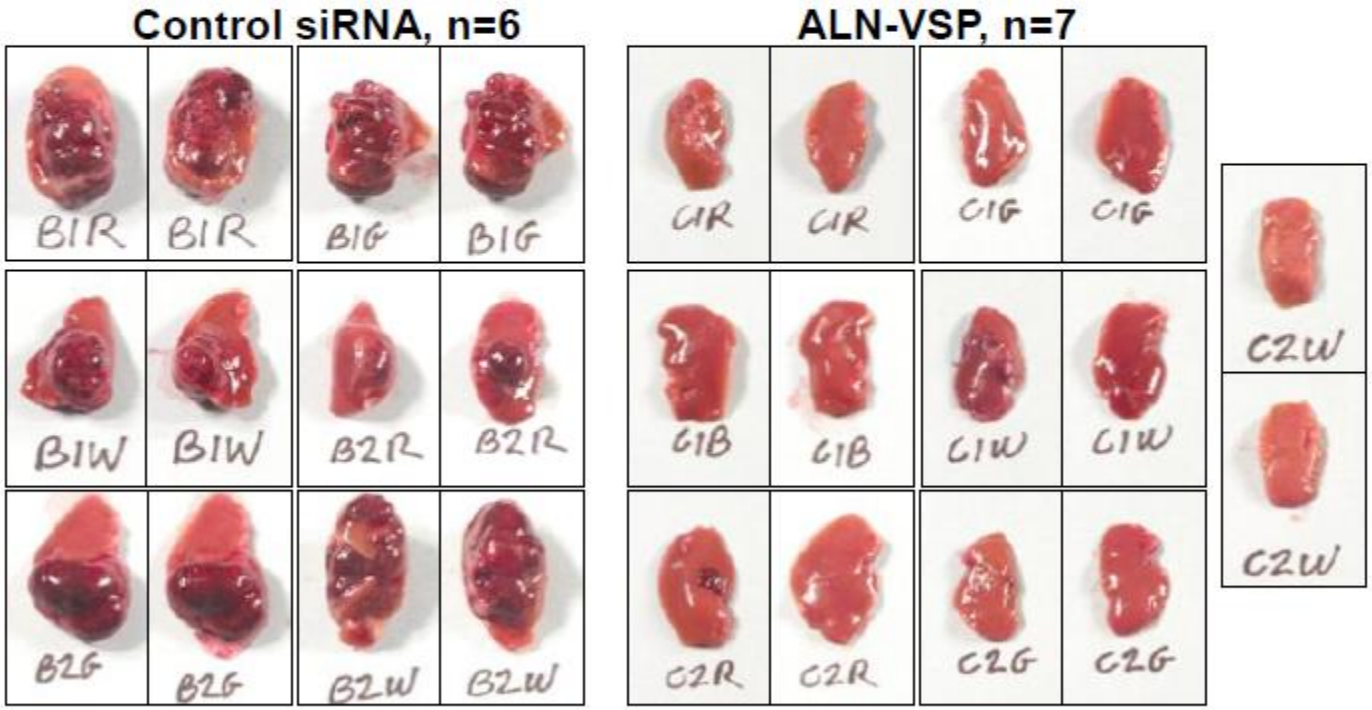
Alnylam PHARMACEUTICALS

Systemic Delivery to Liver Tumors

Efficacy in Pre-clinical Orthotopic Liver Cancer Model

Orthotopic tumor model with intrahepatic Hep3B seeding in pre-clinical studies

- ALN-VSP demonstrates clear anti-tumor activity compared with controls

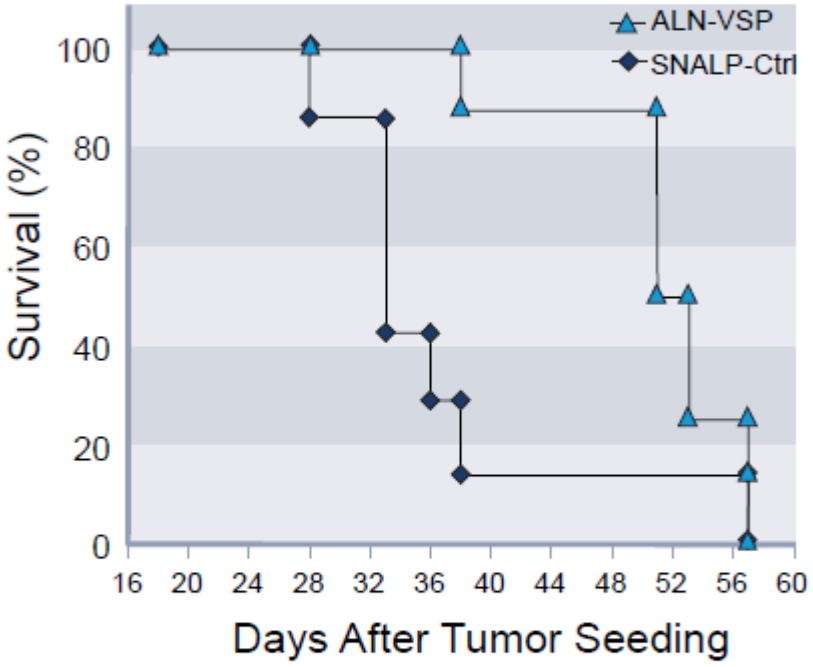


Keystone: RNAi, Feb 2009

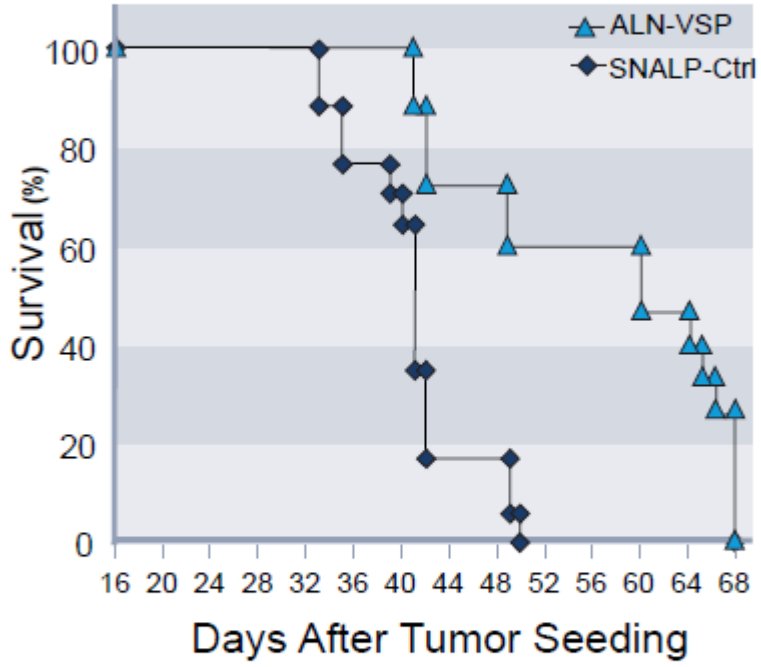
Alnylam PHARMACEUTICALS

Prolonged Survival With ALN-VSP Treatment

Study 1



Study 2



Orthotopic Tumor Model (Hep3B) – Treated 18 days post seeding;
IV bolus injections of 4 mg/kg VSP or control siRNA 2x/wk for 3 wks

Keystone Symp: Ther. Mod. of RNA Using Oligos, Feb 2009

Alnylam PHARMACEUTICALS

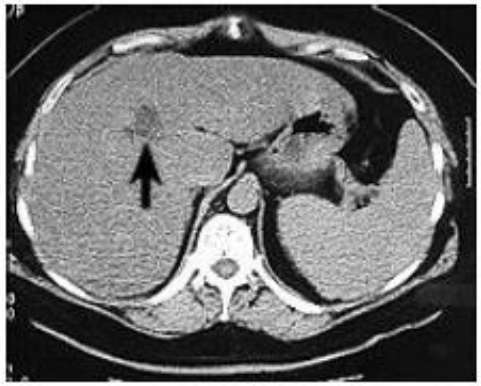
ALN-VSP Phase I Study Status

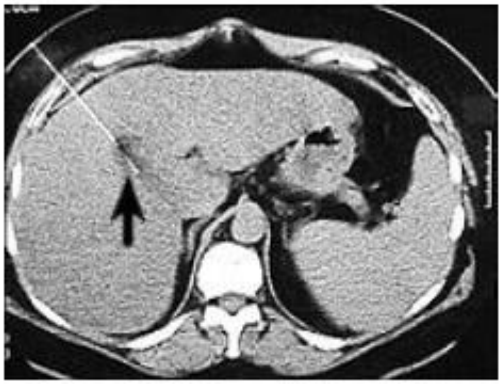
- Enrollment completed
  - >40 patients at doses ranging from 0.1 to 1.5 mg/kg
- Tumor types include:
  - Colorectal cancer
  - Pancreatic neuroendocrine tumor
  - Papillary renal cell cancer
  - Squamous cell cancer of head and neck
  - Pancreatic cancer
  - Esophageal cancer
  - Endometrial cancer
  - Angiosarcoma
  - Ovarian cancer
  - Synovial sarcoma
  - Mullerian stromal tumor
- All patients treated with multiple prior anti-angiogenic and/or chemotherapy regimens

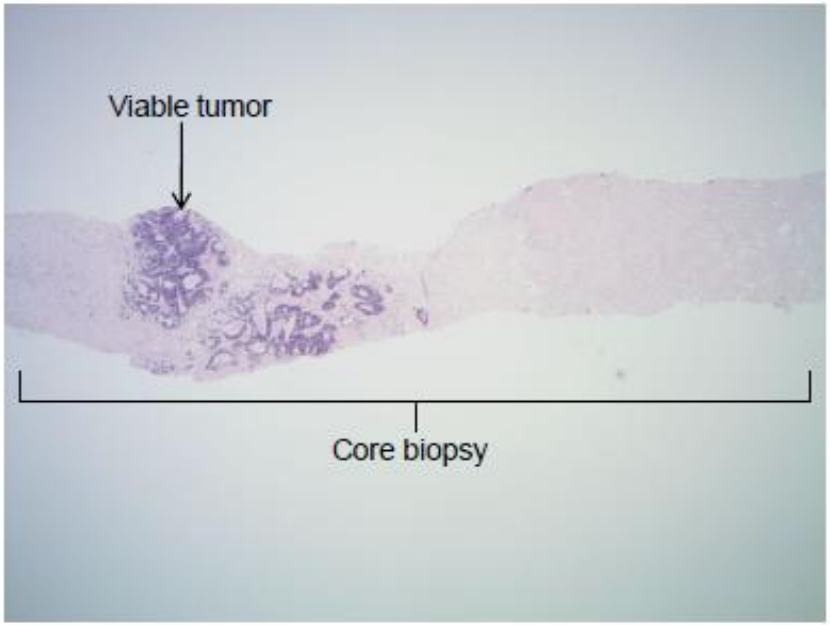
Secondary liver cancer!

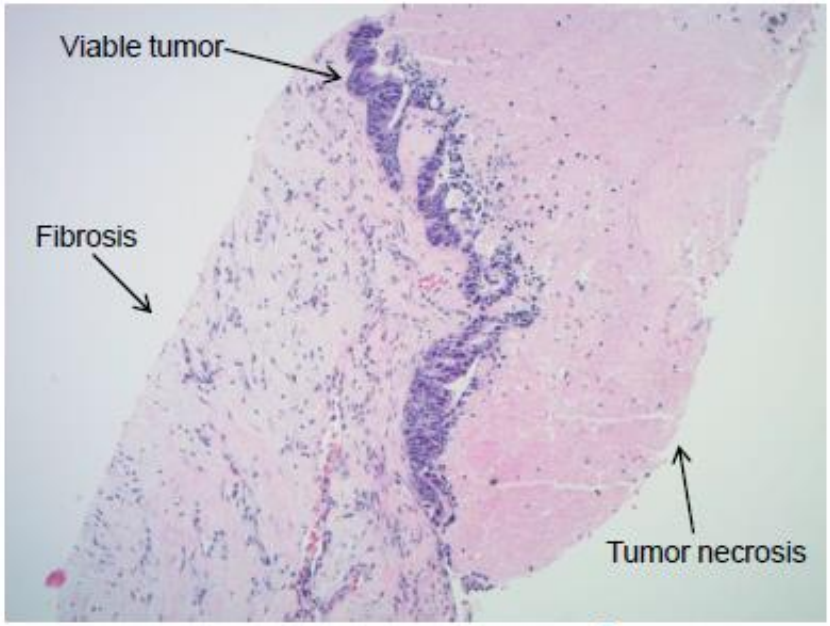
Tumor Core Biopsies

CT-guided Core Needle Biopsy



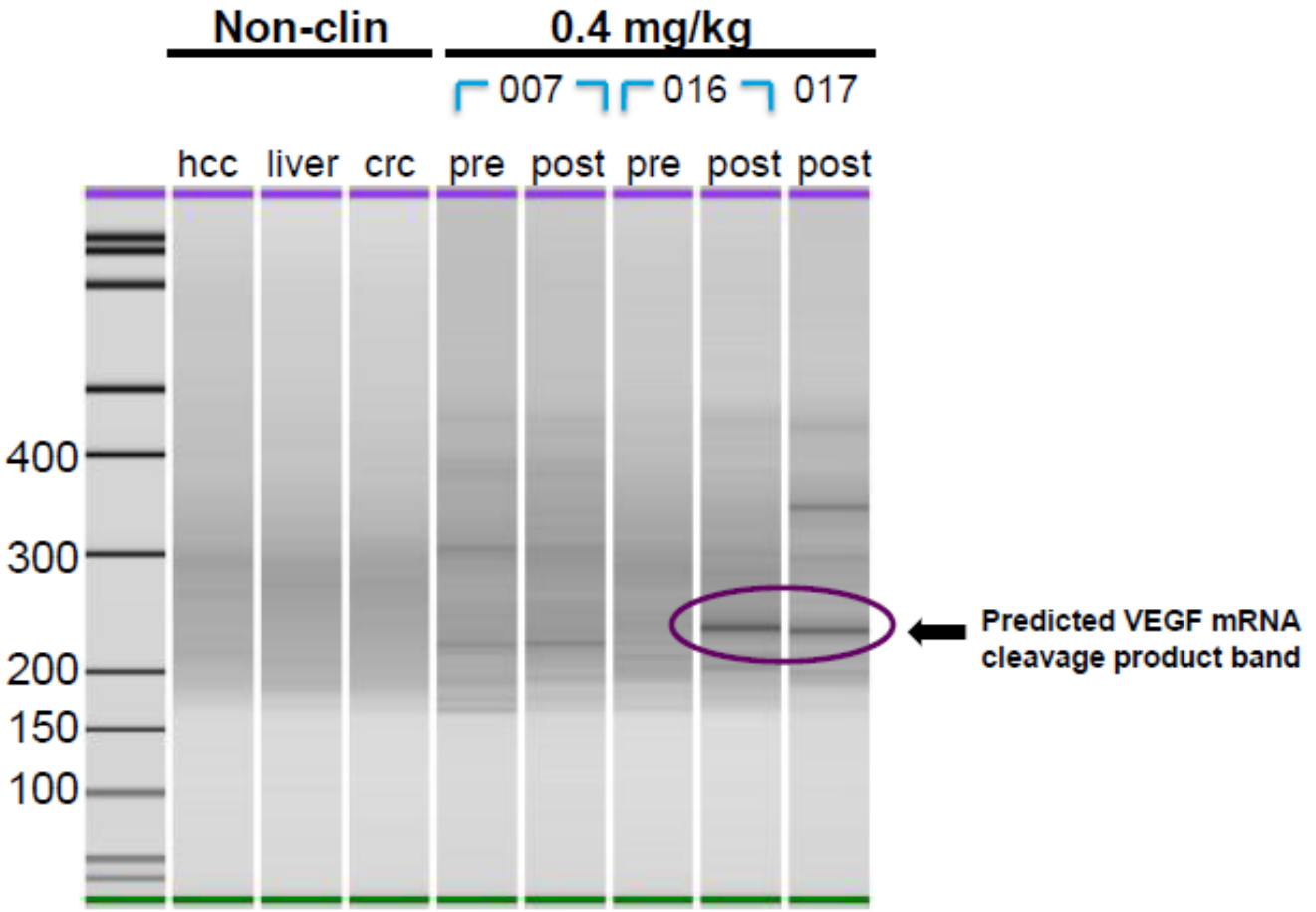






Dominant Band Seen in 5' RACE for VEGF

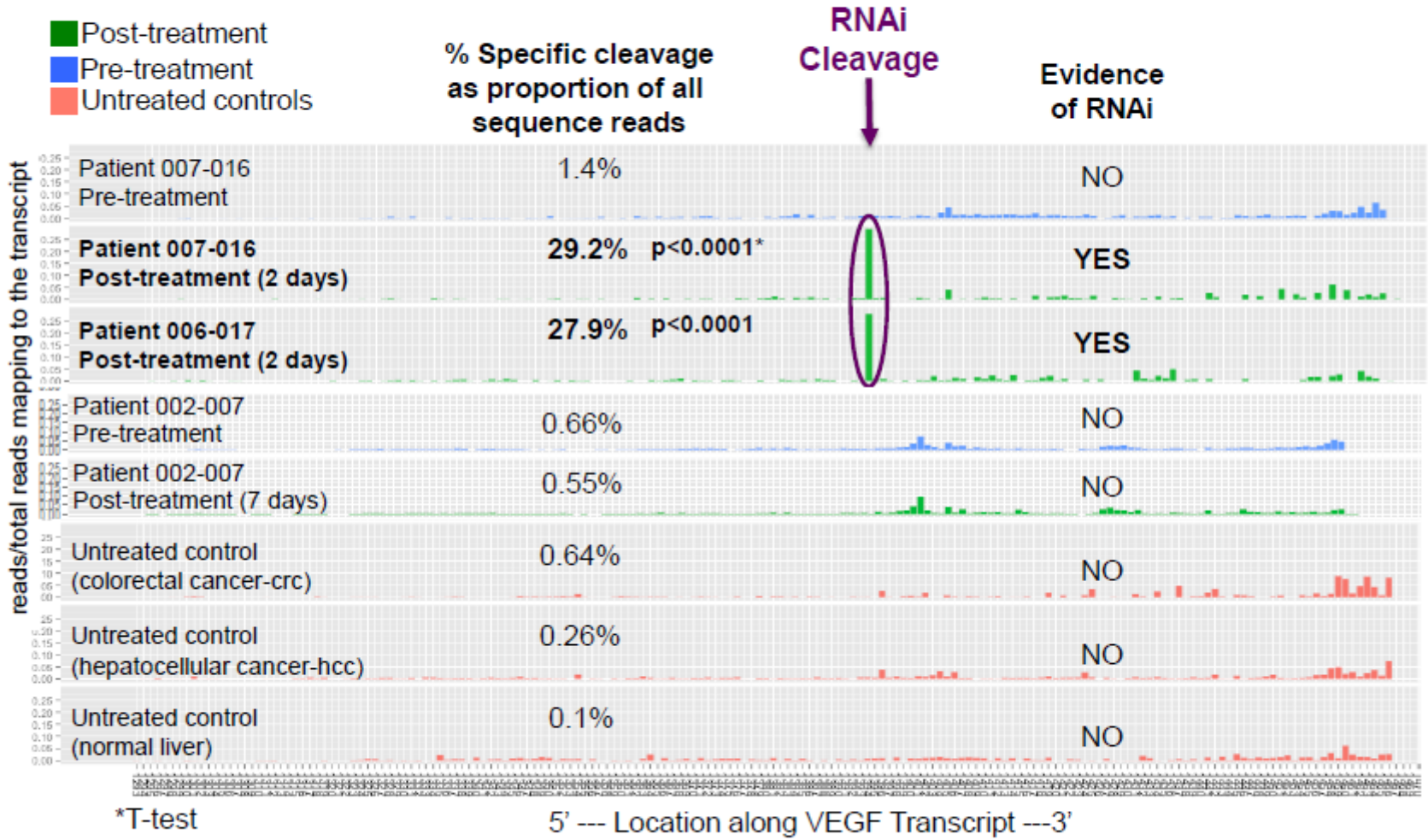
in Two Post-Dose Clinical Samples



Alnylam PHARMACEUTICALS

Human RNAi Proof of Mechanism

Results from Blinded Molecular Analysis of Human Biopsy Samples



Dana Farber Cancer Institute, January 2011

Alnylam PHARMACEUTICALS

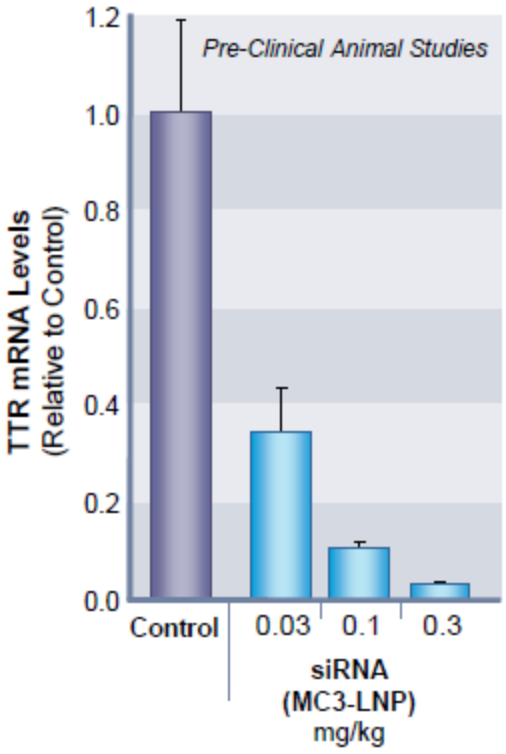
5' RACE Tumor Biopsy Data Conclusions

- In first 5 patients analyzed, 3 had abundant normal liver/total mRNA that permitted VEGF 5' RACE analysis
- Predicted VEGF mRNA cleavage product seen post-treatment in livers of 2/3 patients
  - » $p<0.0001$
  - » Biopsy from negative patient was obtained 7 days post treatment
- First demonstration of RNAi in man with LNP-formulated siRNA

Alnylam PHARMACEUTICALS

RNAi Therapeutics
The Time is Now: 3 Reasons

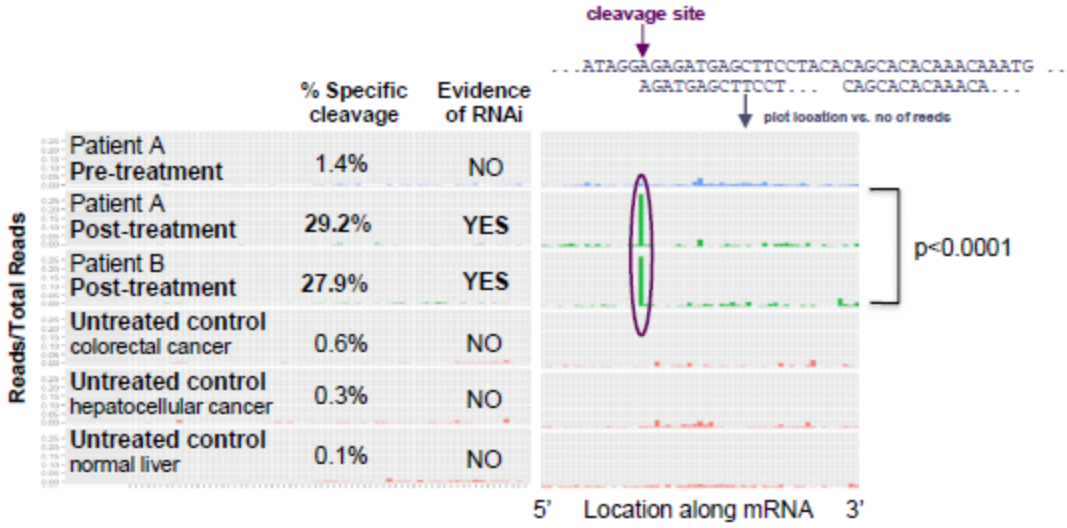
1. Delivery breakthroughs enable clinical translation



2. Growing human experience: safety and predictable PK

- >500 Subjects/patients enrolled overall
- Systemic delivery in human trials
  - » > 40 Patients dosed
  - » Over 6 months of dosing
- RNAi therapeutics generally well tolerated
- Pharmacologically relevant human tissue levels achieved

3. Human RNAi proof of mechanism established



Alnylam PHARMACEUTICALS

Clinical pipelines in RNAi therapeutics

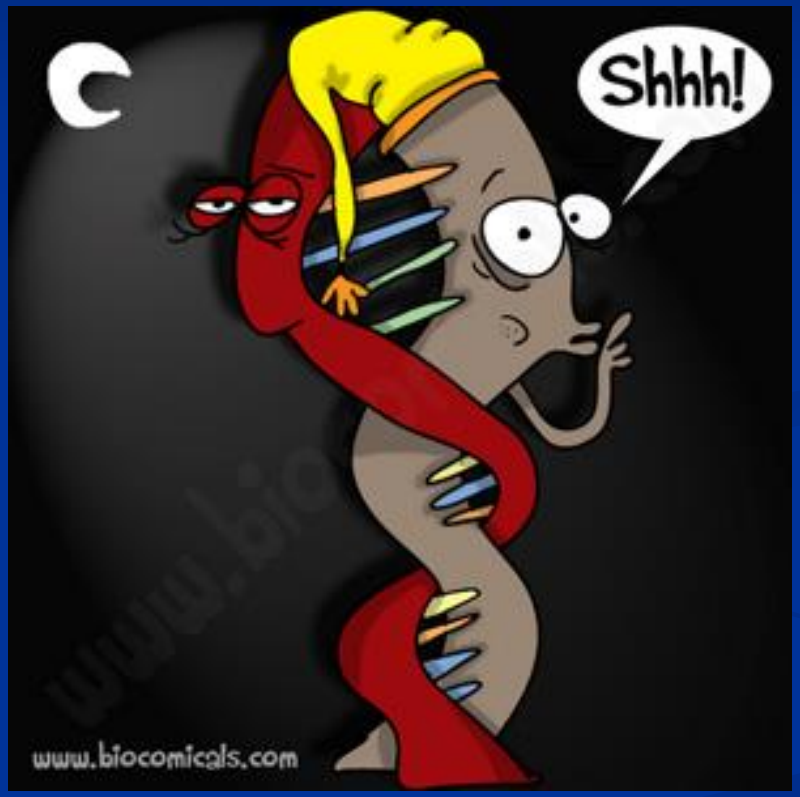
Sponsor	Program	Phase	Target	Indication	Number enrolled
Alnylam/Cubist/ Kyowa Kirin	ALN-RSV	Phase IIb (ongoing)	RSV nucleocapsid	Adult RSV infection	354
Pfizer/Quark	PF-04523655	Phase II (ongoing)	RTP801	AMD, diabetic macular edema	244
Quark	QPI 1002	Phase II (ongoing)	p53	Acute kidney injury, delayed graft function	56
Zabecor	Excellair	Phase II (ongoing)	Syk kinase	Asthma	?
Alnylam	ALN-VSP	Phase I (ongoing)	VEGF, KSP	Primary and secondary liver cancer	55
Calando	CALAA-01	Phase I (ongoing)	RRM2	Cancer	36
Silence	Atu-027	Phase I (ongoing)	PKN3	Cancer (GI, lung, other)	33
Sylentis	SYL040012	Phase I (ongoing)	β2-Adrenergic receptor	Glaucoma	?
Alnylam	ALN-TTR	Phase I (ongoing)	TTR	TTR amyloidosis	Enrollment in 2010
Opko	Bevasiranib	Phase III (terminated)	VEGF-A	AMD	522
Allergan/SIRNA	AGN211745	Phase II (terminated)	VEGFRI	AMD	164
Tekmira	ApoB SNALP	Phase I (completed)	ApoB	Hypercholesterolemia	23
Transderm	TD101	Phase I (completed)	Mutant K6a	Pachyonychia congenita	1
Univ. Duisburg-Essen	Bcr-abl	Phase I (completed)	Bcr-abl oncogene	CML	1

Reprinted from ref. 9 (Table 2). All data are from corporate websites, press releases, and ClinicalTrials.gov.

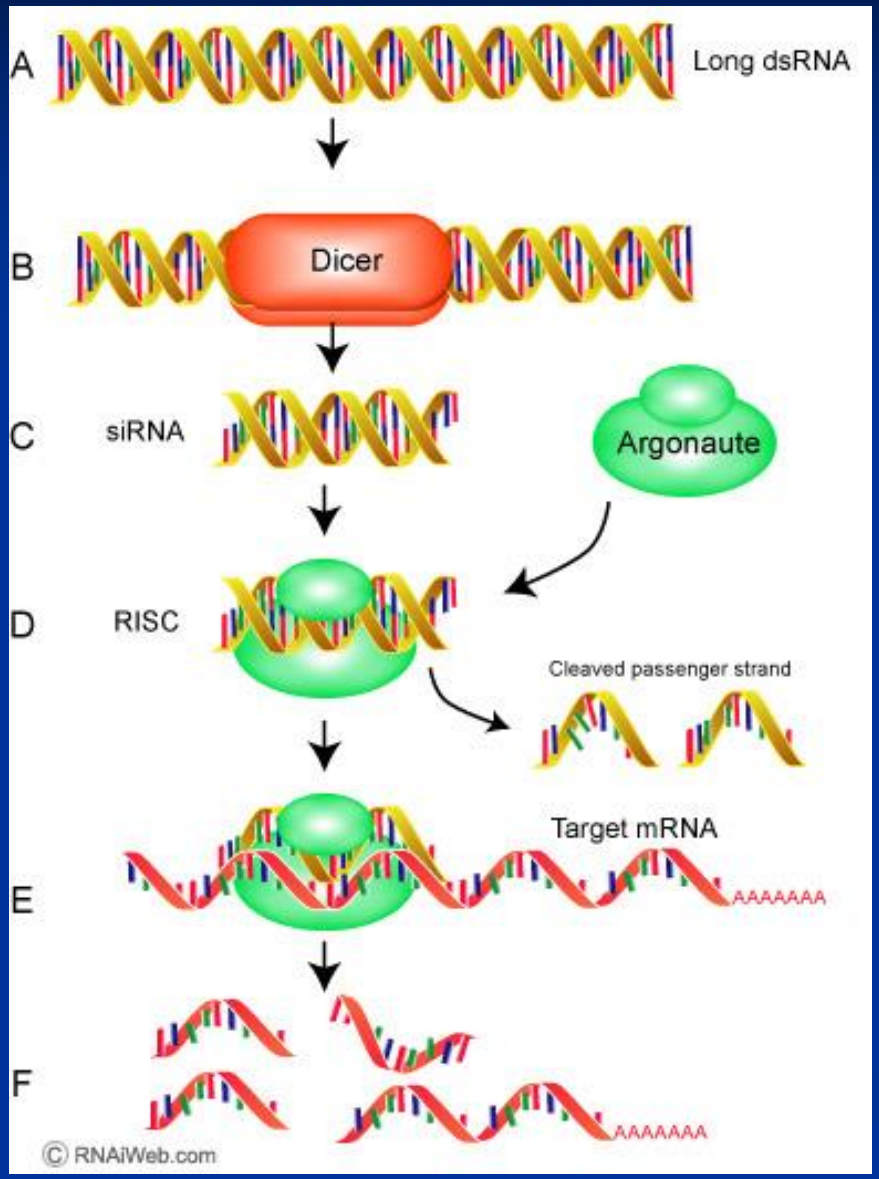
AMD, age-related macular degeneration; CML, chronic myeloid leukemia; GI, gastrointestinal; KSP, kinesin spindle protein; PKN3, protein kinase N3; RRM2, ribonucleotide reductase M2; RSV, respiratory syncytial virus; TTR, transthyretin; VEGF-A, vascular endothelial growth factor A; VEGFRI, vascular endothelial growth factor receptor I.

	graft function		tubule cells; systemic)
PF-4523655	Wet AMD, diabetic macular edema	RTP801/REDD1	Intravitreal needle injection (retina; local)
rHIV-shI-TAR-CCR5RZ	HIV infection	Viral RNA and host factors	Lentiviral (hematopoietic stem cells; *ex vivo*)
NucB1000	Hepatitis B viral infection	HBV RNAs	Liposomal plasmid (hepatocytes; systemic)
TD101	Pachyonychia congenita	Mutant keratin	Intradermal needle injection (skin; local)
Therapeutic vaccine	Metastatic melanoma	Immunoproteasome	Electroporation (autologous monocytes; *ex vivo*)
Excellair	Asthma	Syk kinase	Inhalation of unformulated siRNAs (lung epithelium; local)
CALAA-01	Nonresectable or metastatic solid tumors	M2 subunit of ribonucleotide reductase	RONDEL (solid tumor cells; systemic)
ALN-VSP02	Liver cancer, cancer with liver involvement	VEGF, KSP	SNALP liposome (hepatocytes; systemic)
Atu027	Advanced solid tumors	PKN3	AtuPLEX lipoplex (vascular endothelial cells; systemic)
QPI-1007	Chronic nerve atrophy, nonarteritic ischemic optic neuropathy	Caspase 2	Intravitreal needle injection
SYL040012	Intraocular pressure and glaucoma	β-Adrenergic receptor 2	Eye drop (ciliary epithelial cells; local)
TKM-ApoB	Hypercholesterolemia	Apolipoprotein B	SNALP liposome (hepatocytes; systemic)
bi-shRNAfurin/ GMCSF	Ovarian cancer, advanced melanoma	Furin	Electroporation plasmid (autologous tumor samples; *ex vivo*)
ALN-TTR01	Transthyretin amyloidosis	Transthyretin	SNALP liposome (hepatocytes; systemic)
siG12D LODER	Operable pancreatic ductal adenocarcinoma	Mutated KRAS	LODER local drug elution
TKM-PLK1	Solid cancers and lymphoma	Polo-like kinase 1	SNALP liposomal (solid tumor cells; systemic)
CEQ508	Familial adenomatous polyposis/ colon cancer prevention	-Catenin	Bacterial (mucosal layer of small and large intestine; oral)
ALN-PCS02	Hypercholesterolemia	PCSK9	SNALP liposome (hepatocytes; systemic)
TKM-EBOLA	Ebola infection (biodefense)	Viral RNA	SNALP liposome (hepatocytes and

RNA Silencing

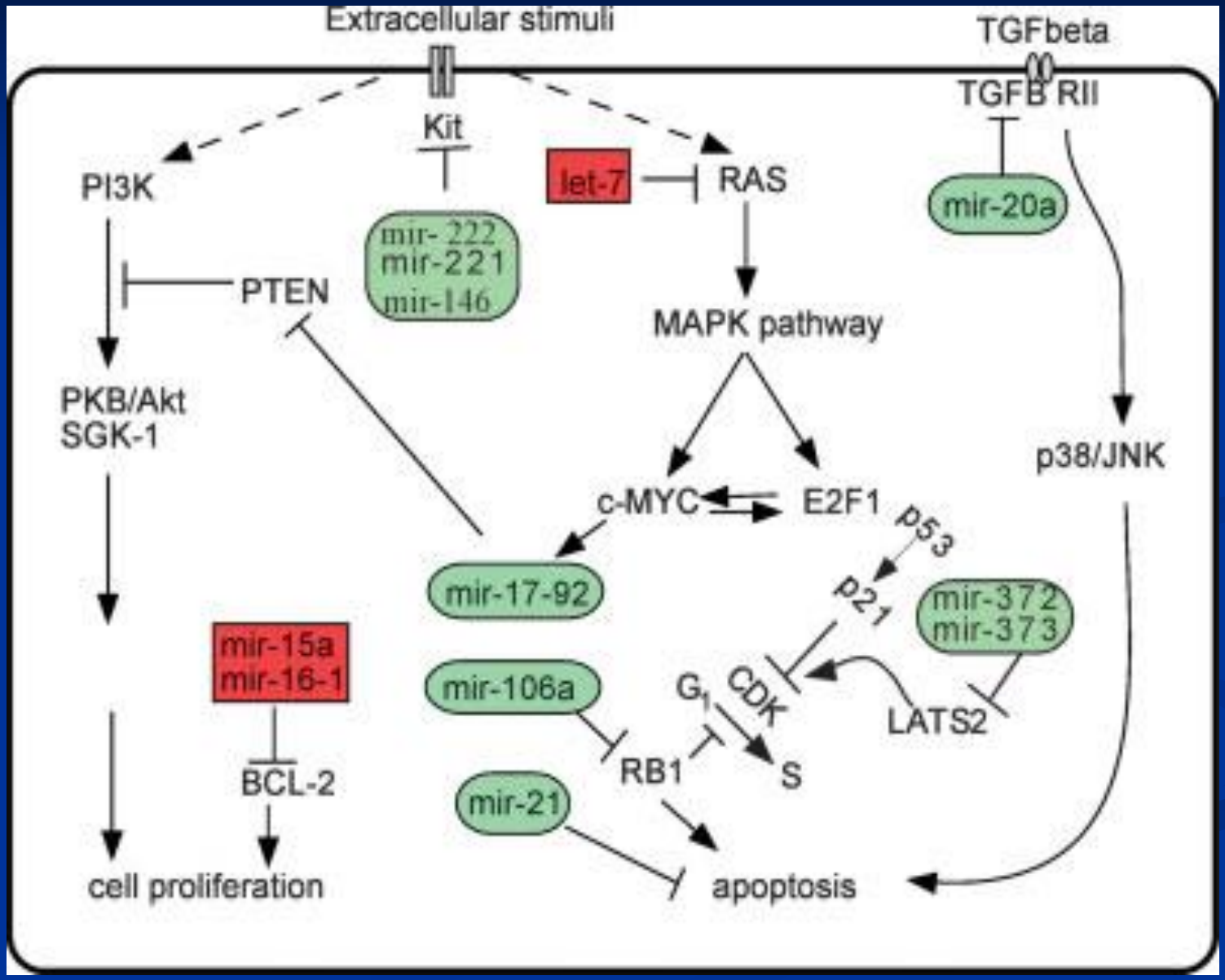


RNAi: innate antiviral mechanism



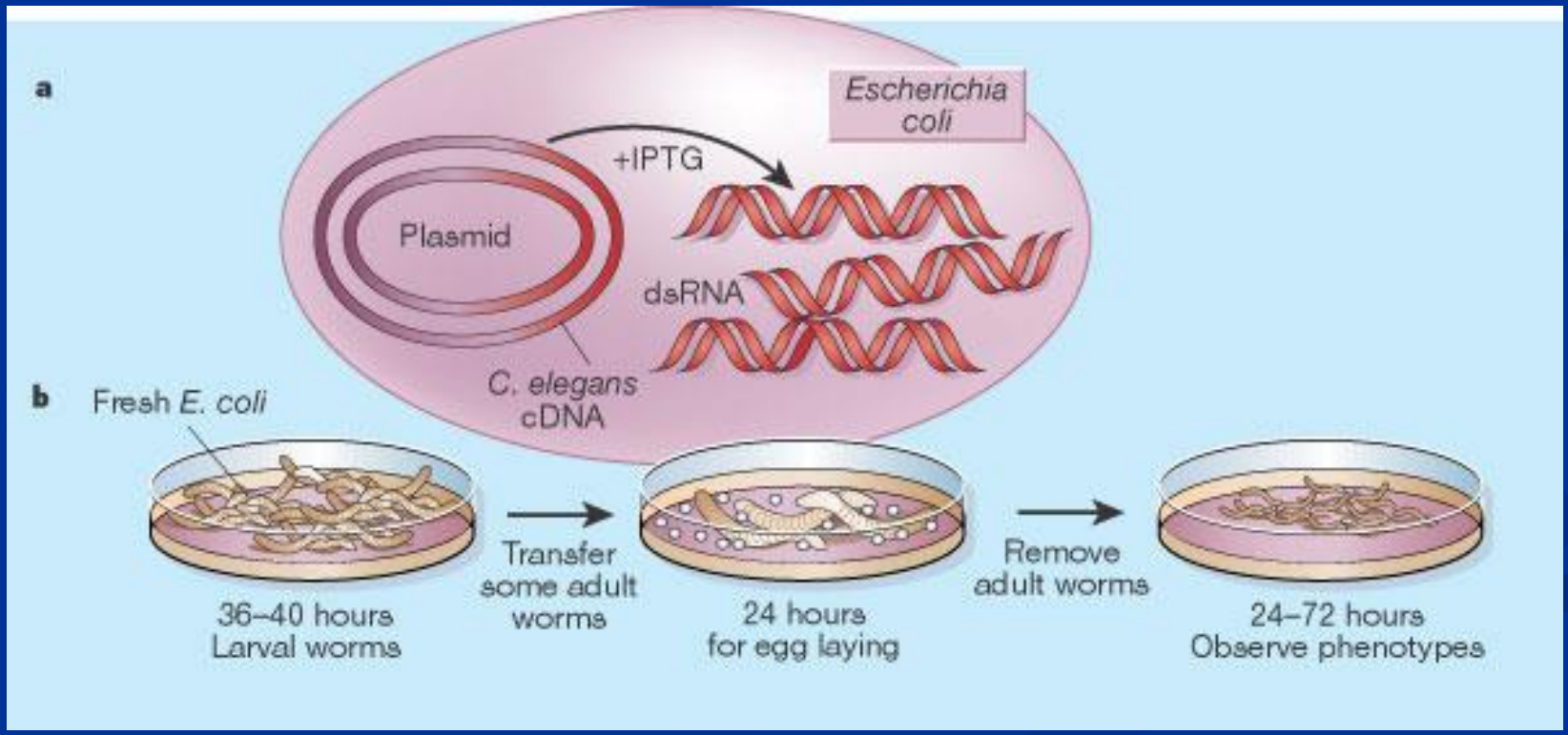
- Infection with virus leads to development of dsRNA during virus life cycle
- Viral dsRNA is generally long and perfectly complementary -> outcome: cleavage by Dicer ->siRNA duplexes are generated
- One strand of siRNA duplex is loaded into RISC -> RISC is guided to complementary viral mRNAs -> RISC binding leads to cleavage and degradation-> inhibiting virus replication
- In plants: 2. wave of siRNA generated by RNA-dependent RNA polymerases (RdRPs): more siRNA available to RISC

miRNAs and Cancer



siRNA libraries

- Generation of a feeding clone



Tuschl, 2003

Genome-wide Screens

Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*

B. Sönnichsen[1], L. B. Koski[1*], A. Walsh[1], P. Marschall[1*], B. Neumann[1*], M. Brehm[1], A.-M. Alleaume[1*], J. Artelt[1*], P. Bettencourt[1*], E. Cassin[2*], M. Hewitson[1], C. Holz[1], M. Khan[1], S. Lazik[1], C. Martin[1], B. Nitzsche[1*], M. Ruer[2], J. Stamford[2], M. Winzi[1], R. Heinkel[1*], M. Röder[1*], J. Finell[1*], H. Häntsch[1], S. J. M. Jones[3], M. Jones[4*], F. Piano[5], K. C. Gunsalus[5], K. Oegema[2*], P. Gönczy[2*], A. Coulson[4*], A. A. Hyman[2] & C. J. Echeverri[1]

[1]*Cenix BioScience GmbH, Tatzberg 47-51, D-01307 Dresden, Germany*
[2]*Max-Planck-Institute for Cell Biology and Genetics (MPI-CBG), Pfotenhauerstrasse 108, D-01307 Dresden, Germany*
[3]*Genome Sciences Centre, British Columbia Cancer Research Centre, 570 West 7th Avenue, Vancouver, British Columbia V5Z 4E6, Canada*
[4]*The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK*
[5]*Department of Biology, Centre for Comparative Functional Genomics, New York University, 1009 Silver Centre, 100 Washington Square East, New York, New York 10003, USA*

NATURE | VOL 434 | 24 MARCH 2005 | www.nature.com/nature

siRNA libraries

- Result: 16 757 bacterial strains
- 86.3% of predicted genes with RNAi phenotypes assigned

Assayed Phenotypes: Examples

- Emb – embryonic lethal
- Ste – sterile
- Gro – slow growth
- Adl – adult lethal
- Lvl – larval lethality
- Lva – larval arrest
- Bmd – body morphological defects
- Unc – uncoordinated
- Clr – clear
- Prz – paralyzed
- Lon – long
- Mlt – moulting defects
- Egl – egg laying defects
- Him – high incidence of males

nature Vol 435|9 June 2005|doi:10.1038/nature03702

LETTERS

MicroRNA expression profiles classify human cancers

Jun Lu[1,4]*, Gad Getz[1]*, Eric A. Miska[2]*†, Ezequiel Alvarez-Saavedra[2], Justin Lamb[1], David Peck[1], Alejandro Sweet-Cordero[3,4], Benjamin L. Ebert[1,4], Raymond H. Mak[1,4], Adolfo A. Ferrando[4], James R. Downing[5], Tyler Jacks[2,3], H. Robert Horvitz[2] & Todd R. Golub[1,4,6]

Recent work has revealed the existence of a class of small non-coding RNA species, known as microRNAs (miRNAs), which have critical functions across various biological processes[1,2]. Here we use a new, bead-based flow cytometric miRNA expression profiling method to present a systematic expression analysis of 217 mammalian miRNAs from 334 samples, including multiple human cancers. The miRNA profiles are surprisingly informative, reflecting the developmental lineage and differentiation state of the tumours. We observe a general downregulation of miRNAs in tumours compared with normal tissues. Furthermore, we were able to successfully classify poorly differentiated tumours using miRNA expression profiles, whereas messenger RNA profiles were highly inaccurate when applied to the same samples. These findings highlight the potential of miRNA profiling in cancer diagnosis.

Much progress has been made over the last d
molecular taxonomy of cancer (see ref. 3).
become clear that among the ~22,000 protei
are mRNAs that can be used to classify a wi
cancers[4]. Recently, hundreds of small, non-c
been discovered (see ref. 1). The first iden
products of the *C. elegans* genes *lin-4* and *l*
roles in controlling developmental timing a
regulating mRNA translation[5–7]. When *lin-4* o
specific epithelial cells undergo additional cell
their normal differentiation. Because abnormal
hallmark of human cancers, it seems possible th
patterns might denote the malignant state. Inde
of a few miRNAs has been found in some tumou
the potential for miRNA expression to inform
not been systematically explored.

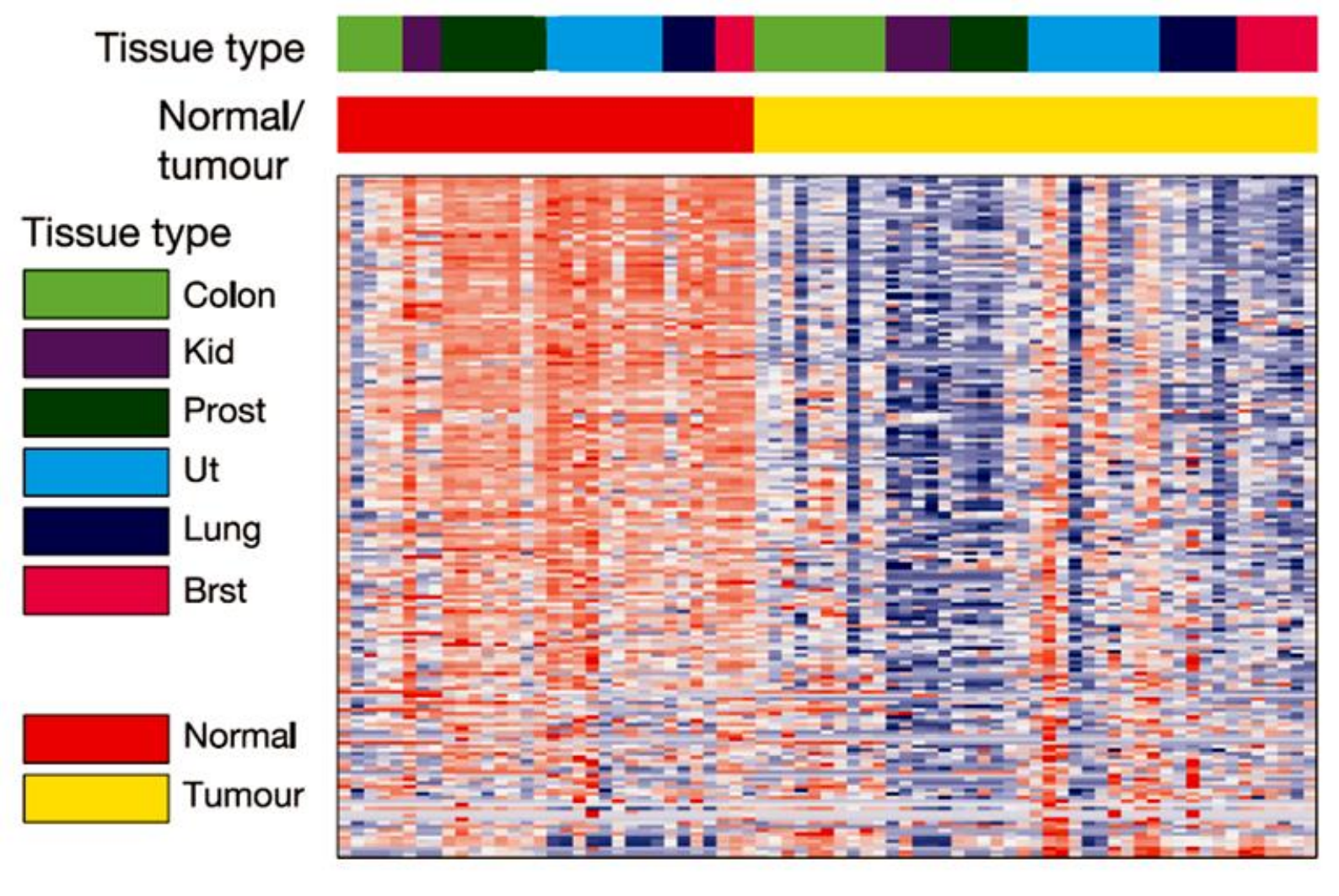
To determine the expression pattern of all kn
needed to develop an accurate and inexpensi
This goal is challenging, because of the sh
(about 21 nucleotides) and the sequence
miRNA family members. Glass-slide microar
for miRNA profiling[12–18], but cross-hybridizatio
has been problematic. We therefore developed a
method. Oligonucleotide-capture probes
miRNAs of interest were coupled to carboxyl
styrene beads impregnated with variable mixtu
dyes (that can yield up to 100 colours),
single miRNA. Following adaptor ligations

5′-phosphate and the 3′-hydroxyl groups of miRNAs[13], reverse-transcribed miRNAs were (1) amplified by polymerase chain reaction (PCR) using a common biotinylated primer, (2) hybridized to the capture beads, and (3) stained with streptavidin-phycoerythrin. The beads were then analysed using a flow cytometer capable of measuring bead colour (denoting miRNA identity) and phycoerythrin intensity (denoting miRNA abundance) (see Supplementary Fig. 1).

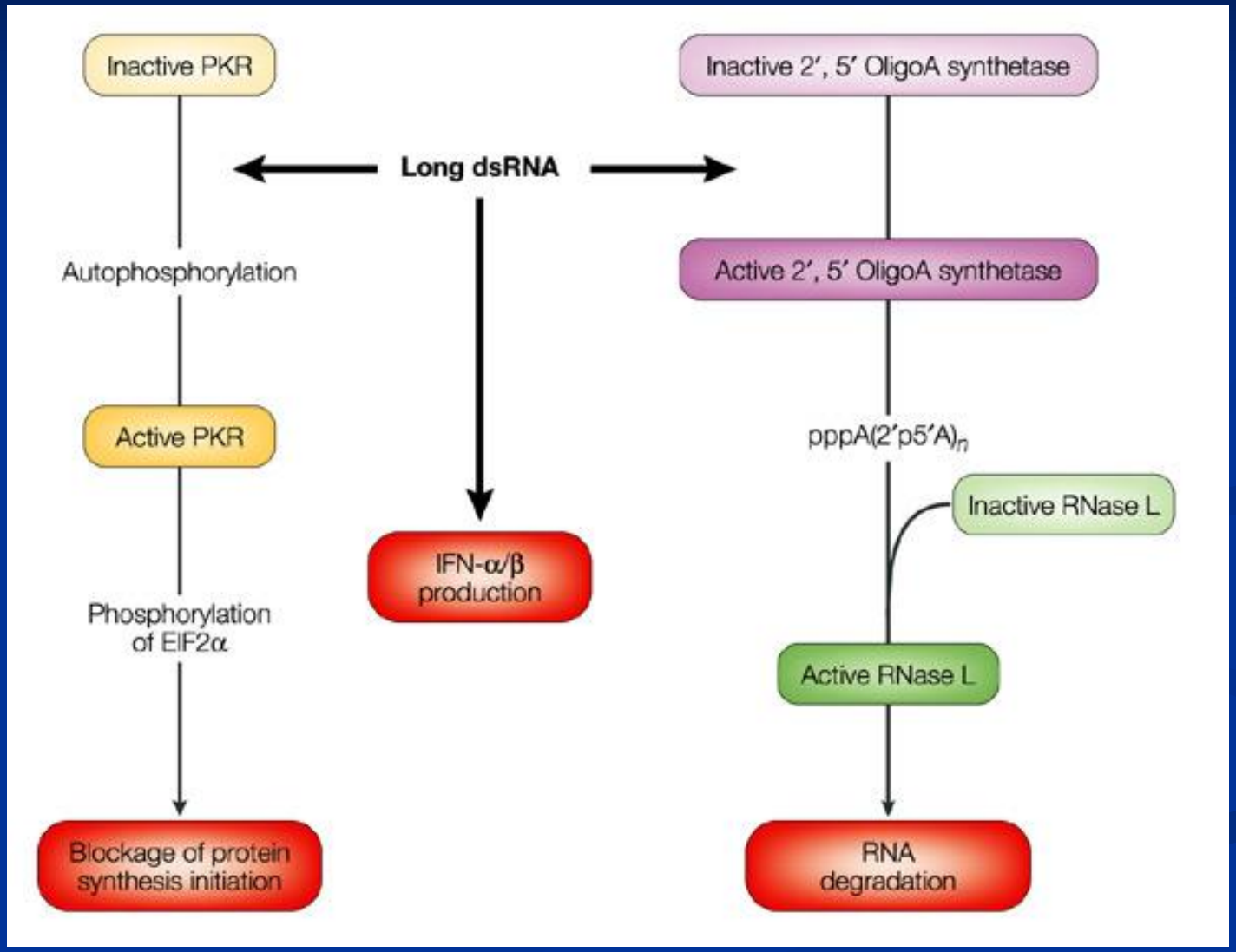
Bead-based hybridization has the theoretical advantage that it

[1]Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02141, USA. [2]Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, and [3]MIT Center for Cancer Research, Cambridge, Massachusetts 02139, USA. [4]Department of Pediatric Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115, USA. [5]Department of Pathology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. [6]Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115, USA. †Present address: Wellcome Trust/Cancer Research UK, Gurdon Institute, University of Cambridge, Cambridge CB2 1QN, UK.
*These authors contributed equally to this work.

Most miRNAs have a lower expression level in tumors compared with normal tissue



Mammalian RNAi

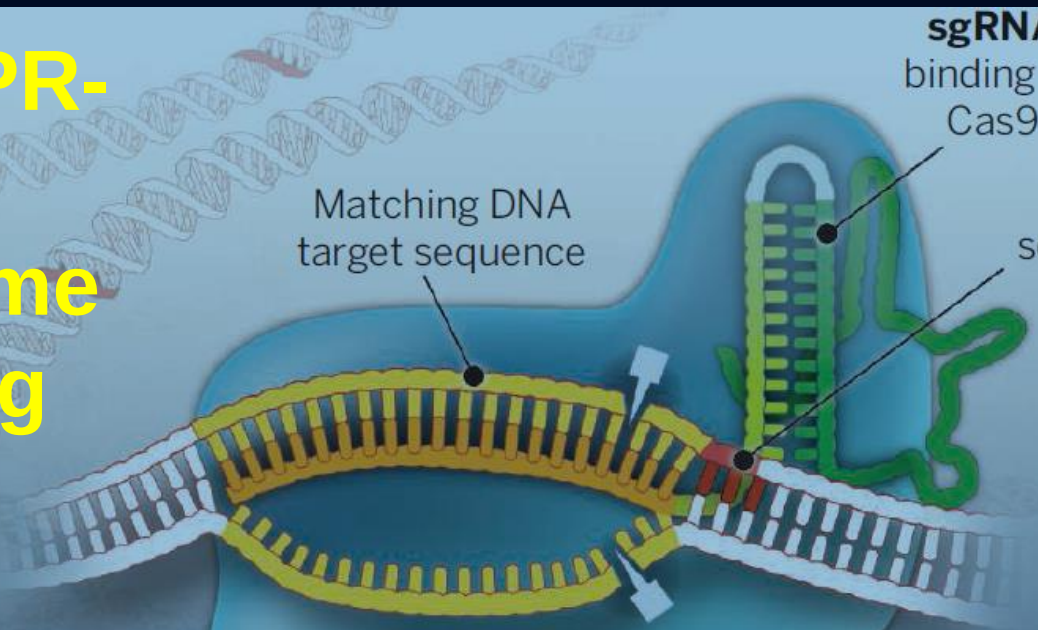


•McManus and Sharp, 2002

Getting Around the Problem

- siRNA (21-22nt) mediate mammalian RNAi
  - Introducing siRNA instead of dsRNA prevents non-specific effects

CRISPR-CAS9 Genome Editing



- https://youtu.be/2pp17E4E-O8
- https://youtu.be/SuAxDVBt7kQ

CRISPR-Cas9 development

- DNA deletion
- DNA insertion
- DNA replacement
- DNA modification
- DNA labeling
- Transcription modulation
- RNA targeting
- . . .

CRISPR-Cas9 applications

- Biological research
- Research and development
- Human medicine
- Biotechnology
- Agriculture
- . . .

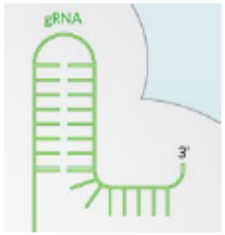
CRISPR loci and Cas nuclease nomenclature

CRISPR: **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats
Loci in 40% of bacteria and 90% of archaea

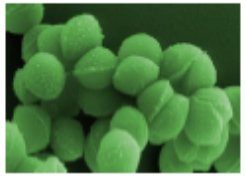
Cas9: CRISPR associated protein 9
a nuclease, an enzyme specialized for cutting DNA
Cas1..Cas10 exist

CRISPR/CAS: type I, type II and type III

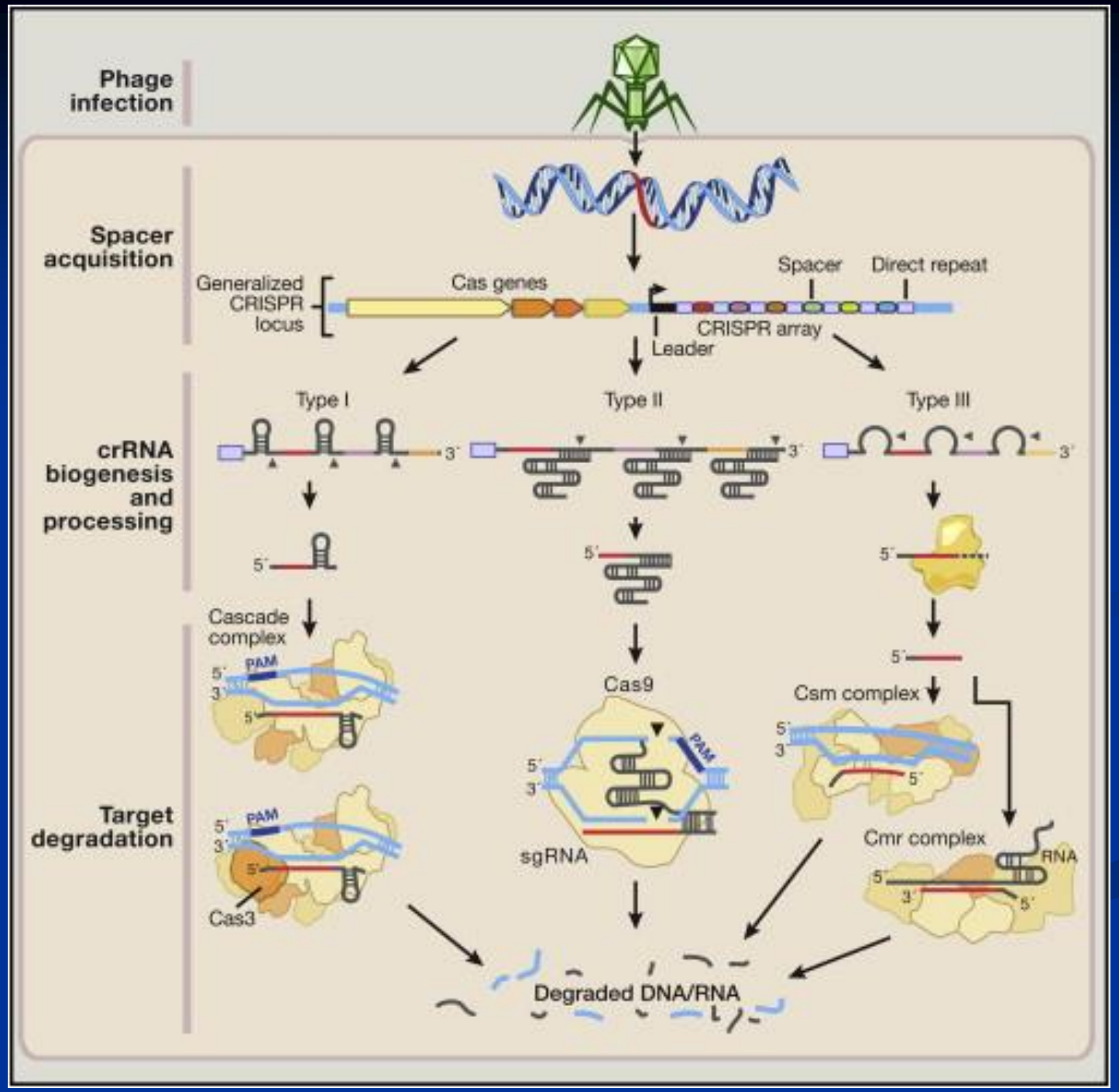
gRNA: guide RNA – a construct/chimera of CRISPR RNA (crRNA)
and trans-activating CRISPR RNA (tracrRNA)



PAM: protospacer adjacent motif with sequence
NGG (any, guanine, guanine) specific to *Streptococcus pyogenes*
and 5′-NAG (any, adenine, guanine) PAM tolerated in human cells



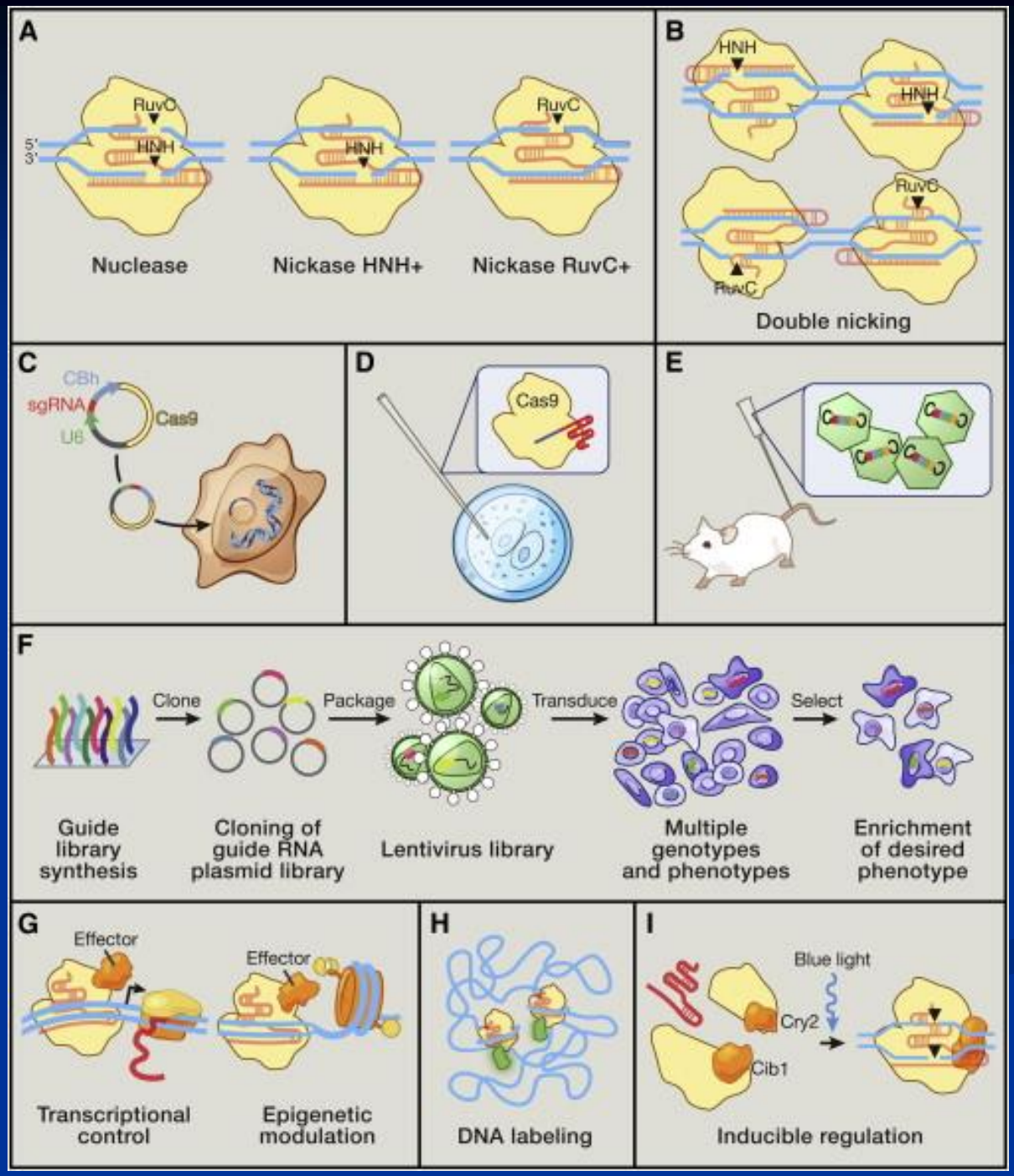
Bacterial Adaptive Immune System



Hsu P. et al., Cell 157, 2014

CRISPR-CAS9 Genome Editing Tool

“2012”



Hsu P. et al.,
Cell 157, 2014

Examples of cell types and organisms that have been engineered using Cas9

Biology

Cell lines
HEK293
U2OS
K562

Model organisms
Mice
Rats
Fruit flies
Nematodes
Arabidopsis
Salamanders
Frogs
Monkeys

Biotechnology

Crop plants
Rice
Wheat
Sorghum
Tobacco

Fungi
Kluyveromyces
Chlamydomonas

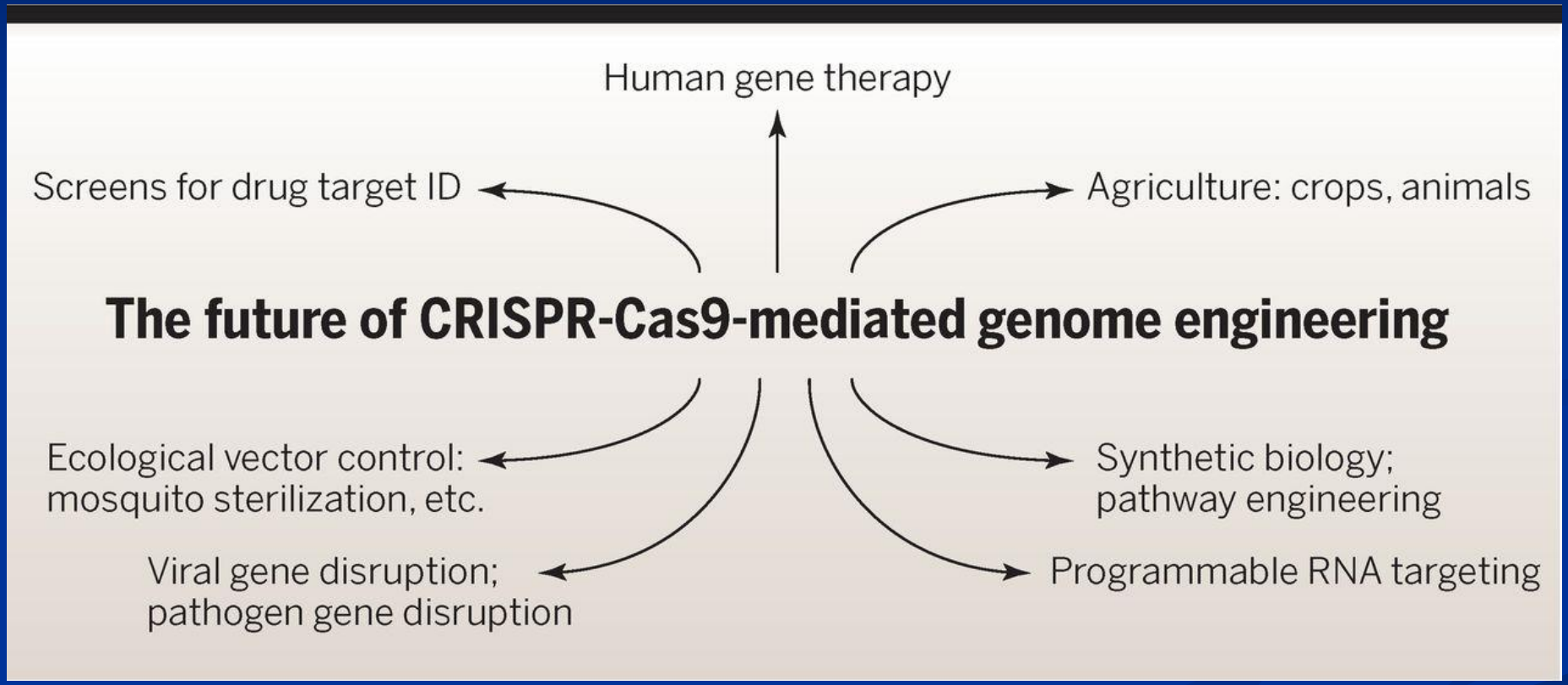
Biomedicine

Organoids
hESCs
iPSCs

Jennifer A. Doudna, and Emmanuelle Charpentier Science 2014;346:1258096

Science
AAAS

Future applications in biomedicine and biotechnology



Jennifer A. Doudna, and Emmanuelle Charpentier Science 2014;346:1258096

Science
AAAS

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CARIBOU BIOSCIENCES

editas MEDICINE

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CRISPR THERAPEUTICS

ToolGen

ERS GENOMICS

TaKaRa Clontech

Broad LIC

life technologies

horizon precision genome editing

Harvard, Broad, ERS LIC

GE Healthcare Dharmacon RNAi

Broad LIC

SIGMA-ALDRICH

Broad LIC

SAGE LABS

Broad, Caribou LIC

TACONIC

Broad LIC

Software & Databases

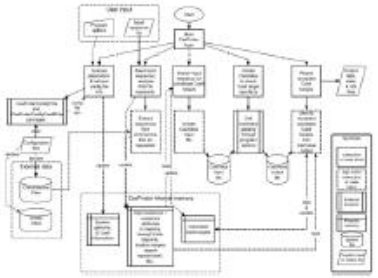
addgene

The nonprofit plasmid repository

GeCKO

Genome-scale CRISPR Knock-Out

CasFinder



TOP

CRISPR direct

Rational design of CRISPR/Cas target.

E-CRISP

Design of CRISPR constructs

ZiFiT Targeter

Figures: Software websites and publications