MicroRNAs, RNA modifications, and RNA editing

Oncology for Scientists (RPN 530)

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MicroRNAs

- History
- Biogenesis
- Nomenclature
- Tissue specificity
- Mechanisms of action
- MicroRNA targets
- Detection
- Engineering
- Applications

History

1993 -- First microRNA discovered (*lin-4* of *C. elegans*)

2000 -- Second microRNA discovered (*let-7* of *C. elegans*)

2001 -- Discovery of Dicer enzyme (generates microRNAs)

2002 -- Association of microRNAs with cancer identified

Known today:

2,588 human microRNAs

1,915 mouse microRNAs

466 Drosophila microRNAs

434 *C. elegans* microRNAs

427 Arabidopsis microRNAs

MicroRNAs

Ultra-short RNAs of 18-23 nucleotides

mRNA

microRNA

Present in all eukaryotes

Also encoded by genes of viruses

Biogenesis of microRNAs

MicroRNA genes

- Standalone microRNA gene: e.g., *MIR210* for *miR-210* microRNA
- Gene encoding another RNA: e.g., Transcription factor TP63-encoding gene for *miR-944*

MicroRNA genesis pathway

Primary RNA (primary or pri-microRNA)

↓

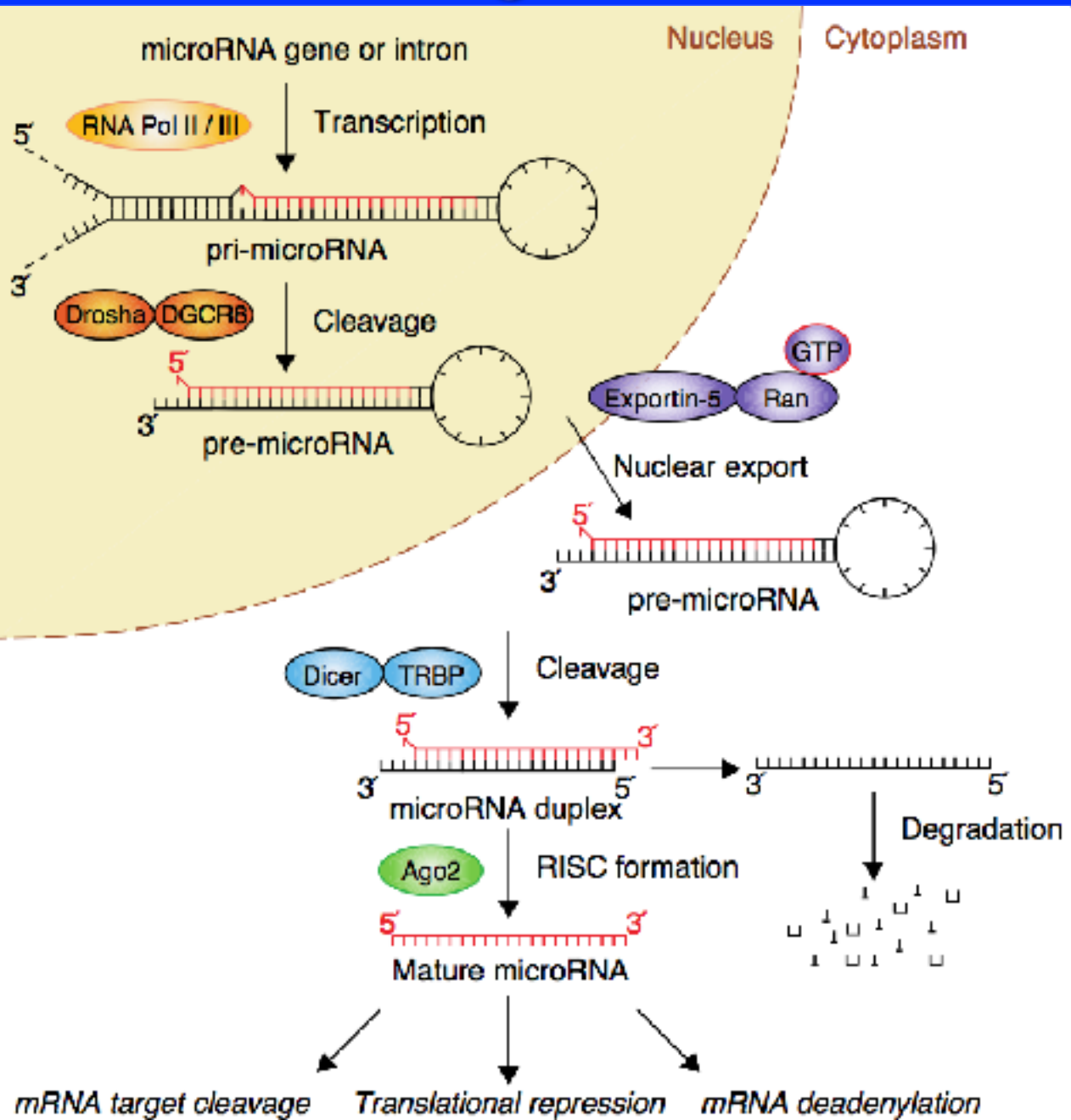
Precursor RNA (pre-microRNA)

↓

Mature microRNA (two produced from a pre-microRNA)

Winter at al. Nature Cell Bio, 2009

Biogenesis of microRNAs



Pre-microRNAs have hairpin stem-loop structure

Winter at al. Nature Cell Bio, 2009

MicroRNA nomenclature

Through miRBase registry of microRNA sequences and annotations (www.mirbase.org)

Prefixes:

All microRNAs are named with a *miR-* prefix (except *let-7*)

A species indicator may be prefixed

miR-210, *miR-375*, *miR-630*, etc.

hsa-miR-210 (human), *mmu-miR-375* (mouse), *ath-miR-630* (Arabidopsis)

MicroRNA nomenclature

Suffixes:

(1) Strand indicator – 5p or 3p

Recommended

(2) Abundance indicator – * (less abundant)

Not recommended

(3) Sequence similarity indicator – a, b, c...

miR-124a-5p and *miR-124b-5p*

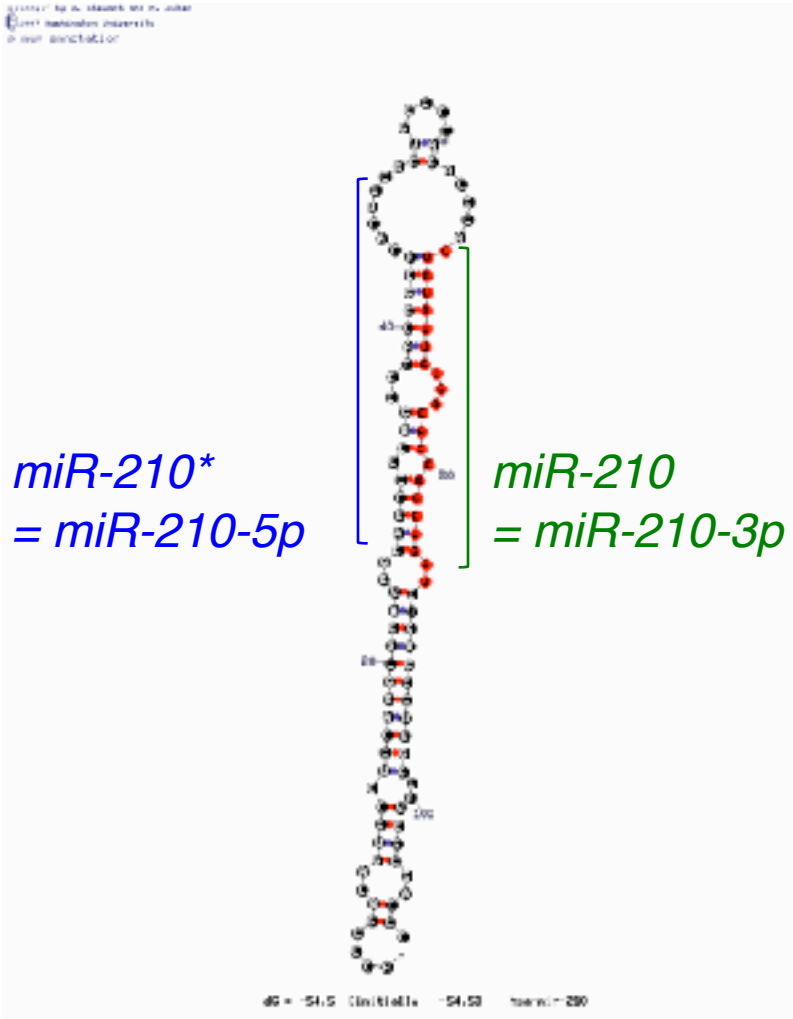
have very similar sequences

(4) Sequence identity indicator – 1, 2, 3...

hsa-miR-194-1-5p and

hsa-miR-194-2-5p

are identical but from different genes



hsa-pre-miR-210

MicroRNA nomenclature

MicroRNA genes: *MIR* prefix. E.g., *MIR375*

Pri-microRNAs: *pri-* prefix. E.g., *pri-miR-210*

Pre-microRNAs: *pre-* prefix. E.g., *pre-miR-210*

miRBase IDs:

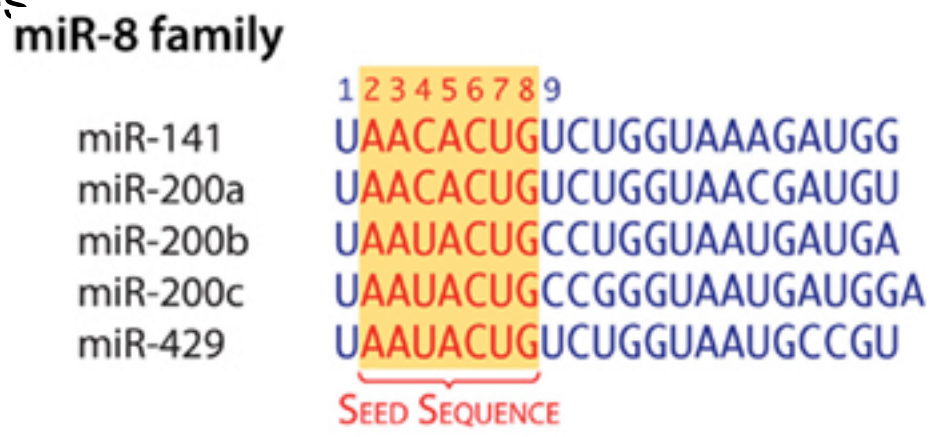
Pre-microRNAs: *MI* ID (e.g., MI0000286 for *hsa-pre-miR-210*)

MicroRNAs: *MIMAT* ID (e.g., MIMAT0026475 for *hsa-miR-210-5p*)

MicroRNA families:

Conserved 'seed' region in sequences

Eipper-Mains at al. Front Gen, 2012

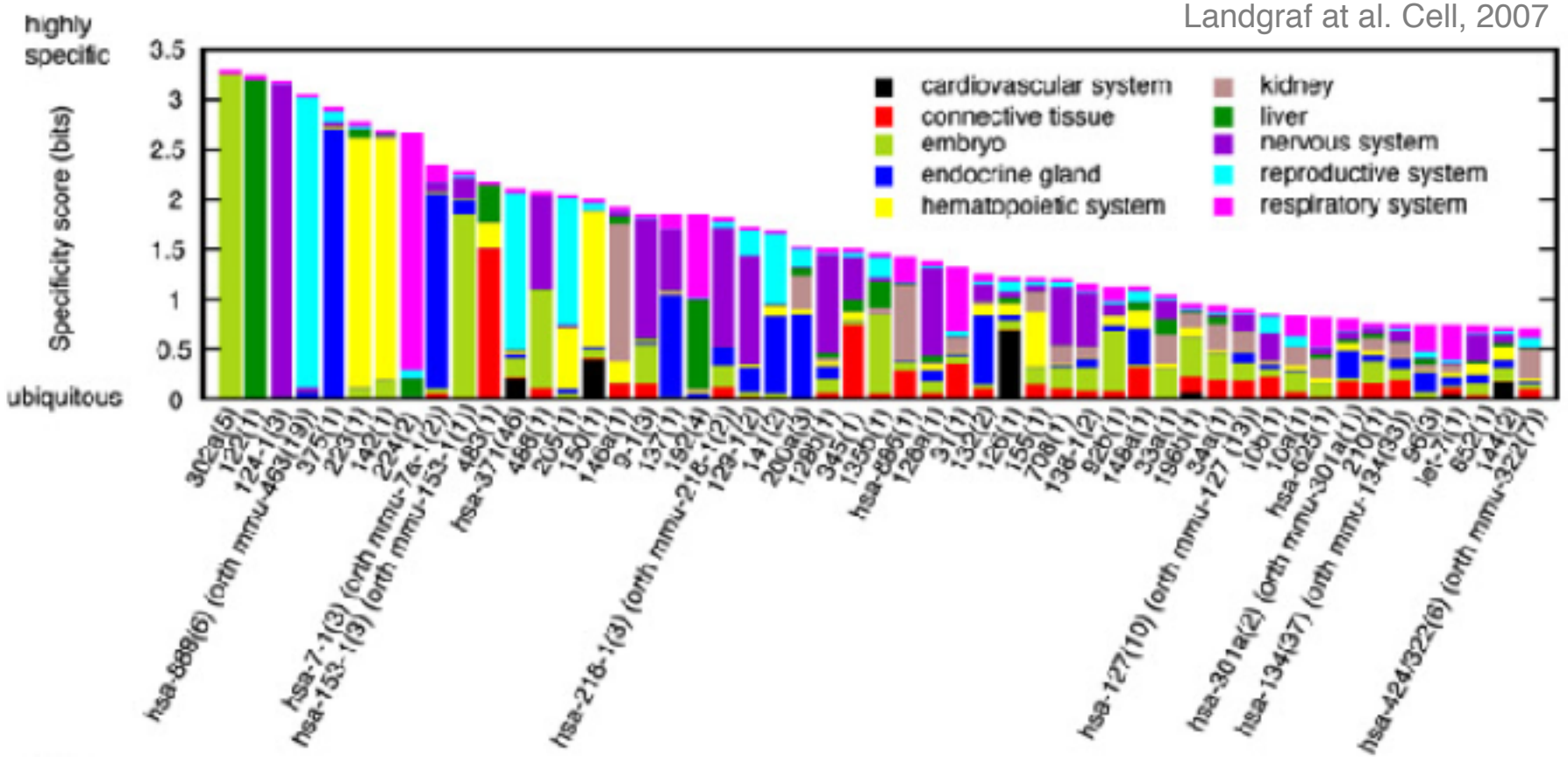


Tissue specificty of microRNA expression

Most microRNAs are not tissue-specific

Examples of tissue-specific microRNAs:

miR-124 (nervous system), *miR-122* (liver), *miR-142* (immune cells)

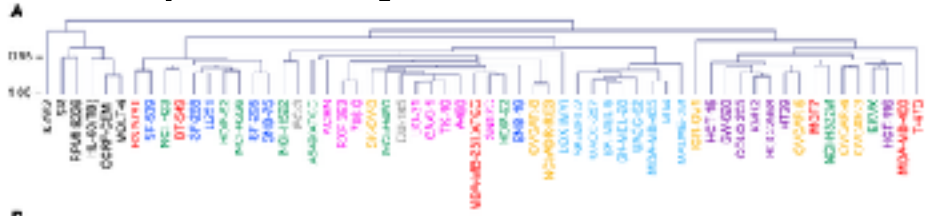


Landgraf at al. Cell, 2007

Tissue specificty of microRNA expression

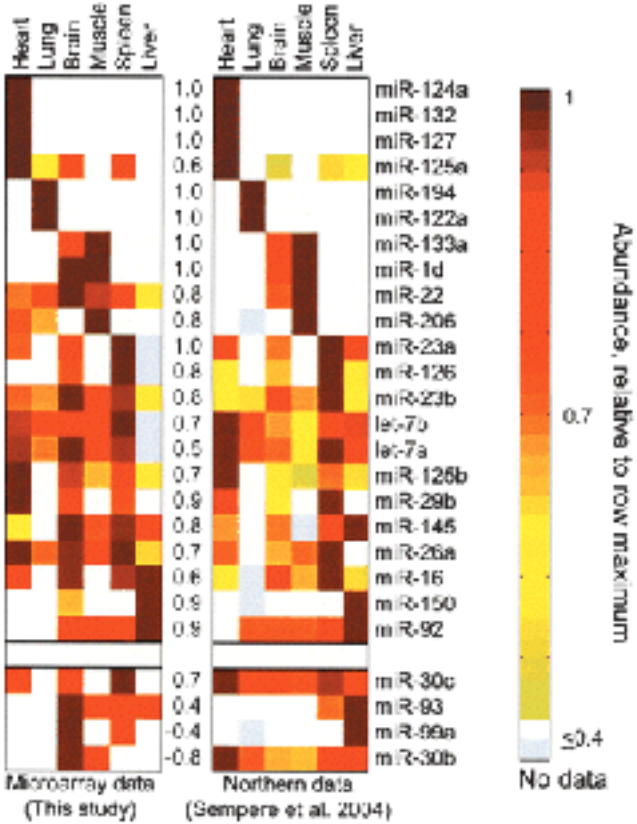
Relative expression of groups of microRNAs (microRNA signatures) has some degree of tissue specificity

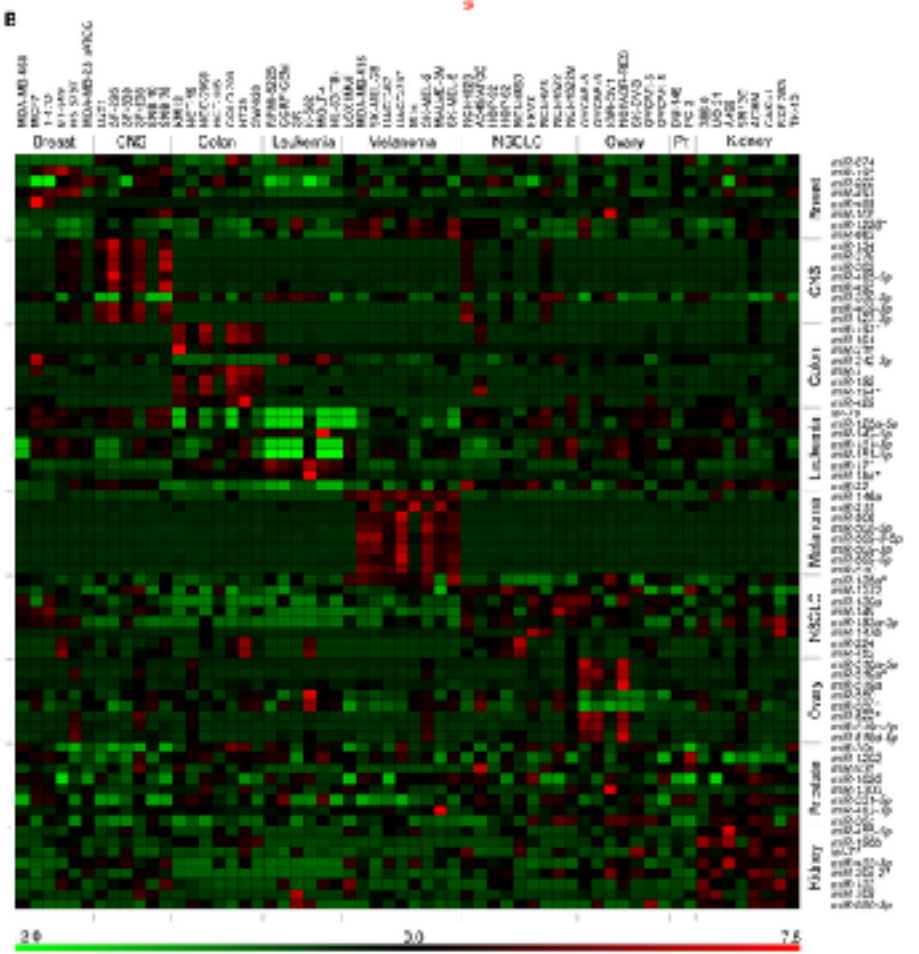
Patnaik et al. PLOS One, 2012



Babak et al. RNA, 2004

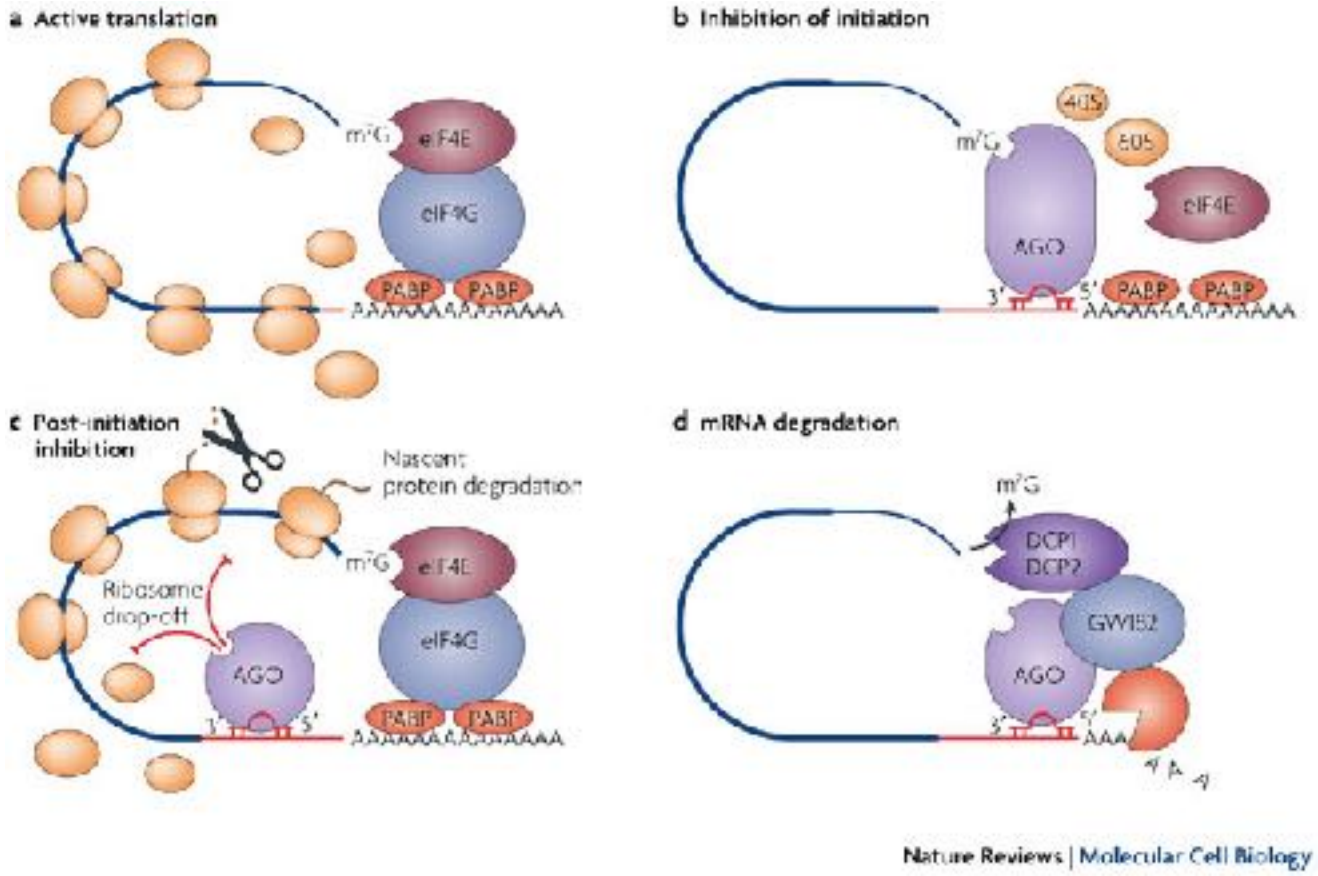
NCI-60 cell-lines of cancers of different tissues >





Mechanism of action of microRNAs

- MicroRNAs are incorporated in RNA-induced silencing complex (RISC) and direct RISC to target mRNAs
- RISC is also used by siRNAs
- Argonaute proteins are the major component of RISC (AGO1-4 in humans)



mRNA targeting by RISC/microRNA leads to translation inhibition, mRNA degradation, etc.

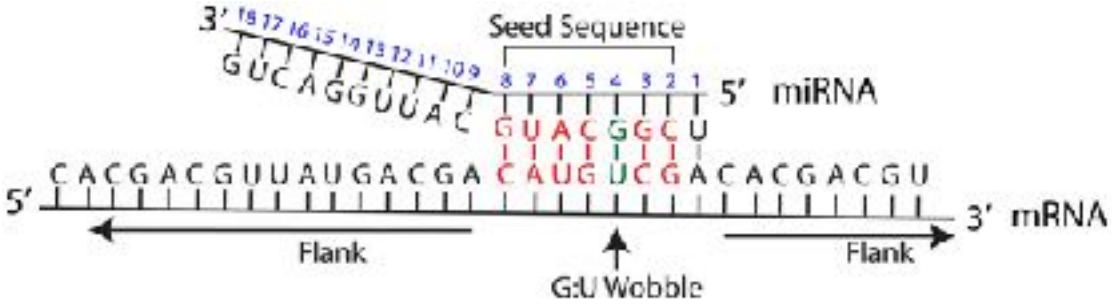
Stefani et al. Nature Rev Mol Cell Bio, 2008

mRNA targeting by microRNAs

MicroRNAs typically target 3'untranslated region of mRNAs

The 'seed' sequence (nts. 2-8) of a microRNA is a major determinant of mRNA targeting specificity

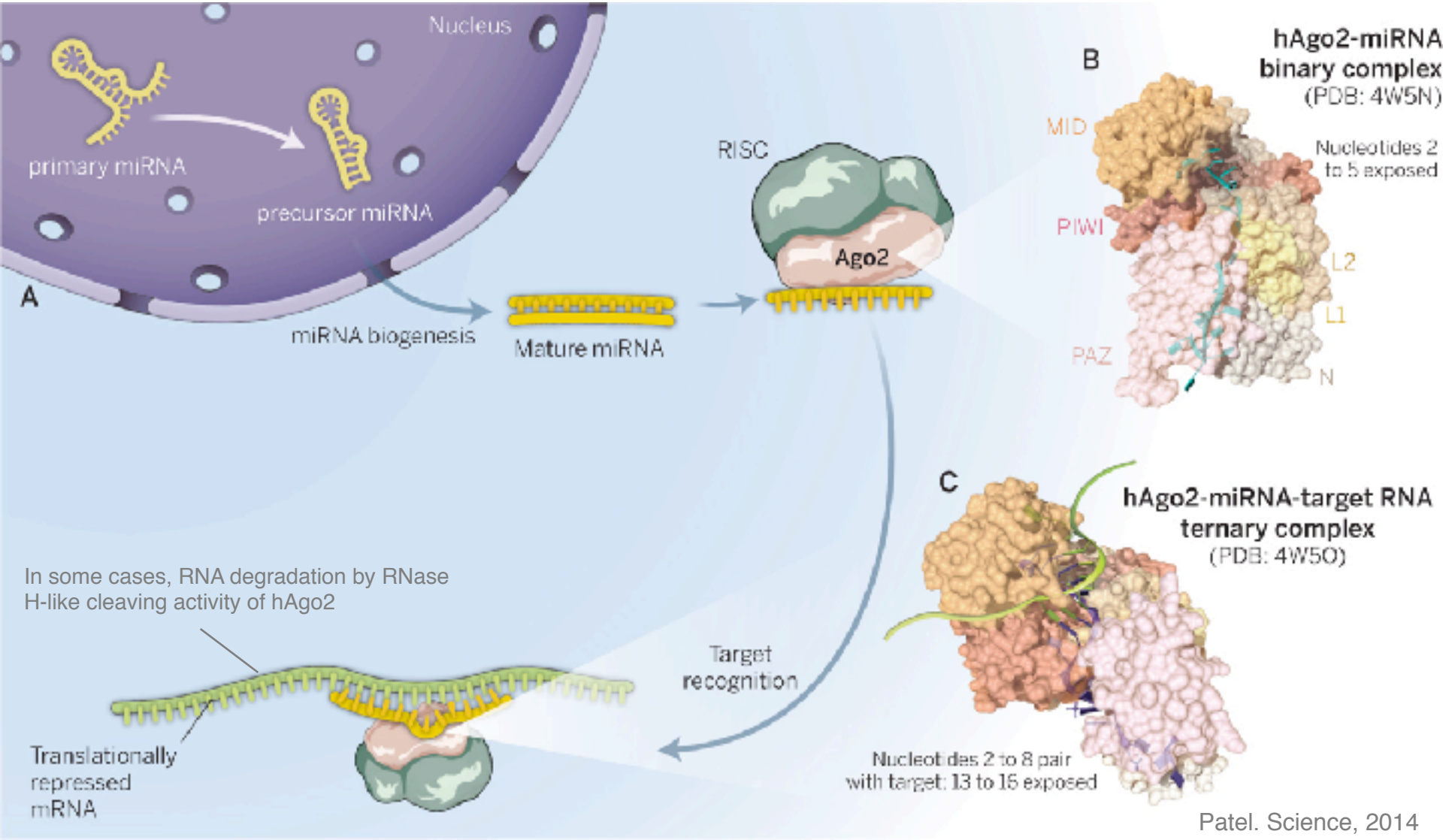
Targeting of an RNA is driven by sequence complementarity, with tolerance for imperfection



Peterson at al. Front Gen, 2014

mRNA targeting by microRNAs

Current model: MicroRNA nts. 2-5 used to scan RNAs for potential targeting; if complementarity present, further interrogation with nts. 6-8 and 13-16.



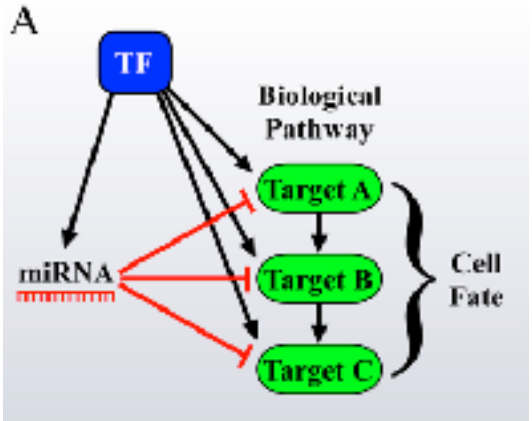
Patel. Science, 2014

‘Pleiotropic’ targeting by microRNAs

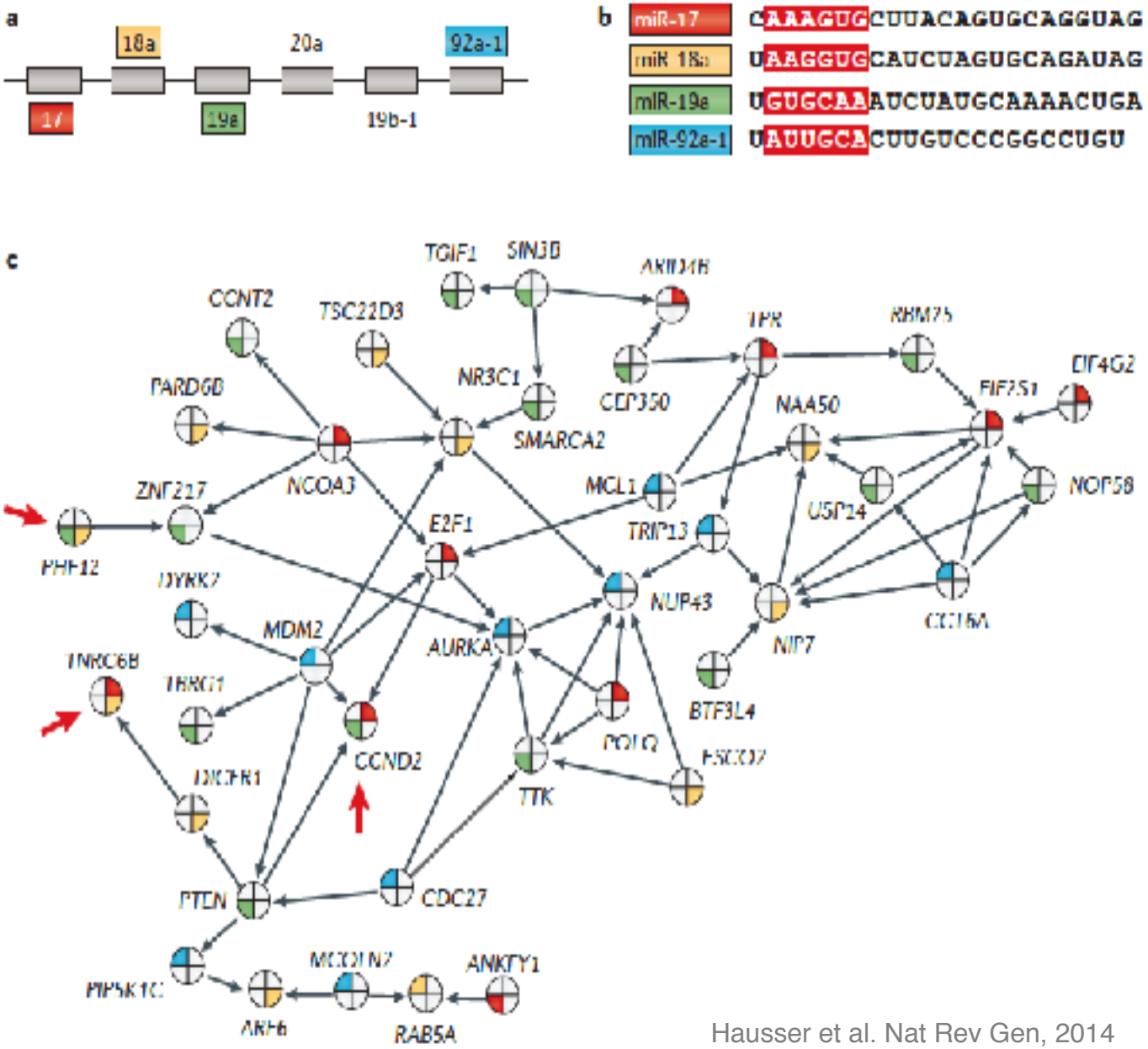
Multiple microRNAs can target the same mRNA

The same microRNA can target multiple mRNAs

Gene	Conservation	3’UTR Position	Target Site
KLF4	H/R/M/D	256-285	5' ...UUACAAAAUGCCAAGGGGGUGACUGGAAG... (UTR) 3' UUCCCUAAGG--ACCCUUUUGACCUG (miR-145)
POU5F1 (OCT4)	H	138-157	3' ...AGGGGAGUUUGGGGCAACUGGUU... (UTR) 5' UCCCUAAGGACCCUUUUGACCUG (miR-145)
SOX2	H/M/R	1-20	5' ...GGCC--GGACAGCGAACUGGAG... (UTR) 3' UUCCCUAAGGACCCU----UUUGACCUG (miR-145)



Co-targeting of molecular pathways



Genes of 4 microRNAs of the miR-17~92 cluster are close to each other.

Targets of the 4 microRNAs functionally interact with each other.

Hausser et al. Nat Rev Gen, 2014

MicroRNA target prediction

Multiple computational tools

Tool name	Seed match	Conservation	Free energy	Site accessibility	Target-site abundance	Machine learning
miRanda	X	X	X			
miRanda-mirSVR	X	X	X	X		X
TargetScan	X	X				
DIANA-microT-CDS	X	X	X	X	X	X
MirTarget2	X	X	X	X		X
RNA22-CUI	X		X			
TargetMiner	X	X	X	X	X	X
SVMicrO	X	X	X	X	X	X
PITA	X	X	X	X	X	
RNAhybrid	X		X		X	

Peterson at al. Front Gen, 2014

MicroRNA target prediction

Target prediction tools have high inaccuracy rates

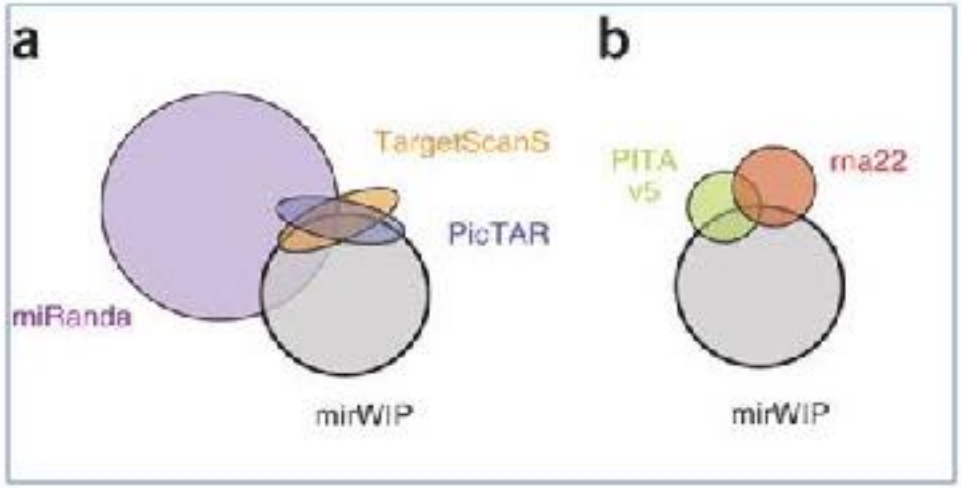


Figure 1. Distribution of miRNA predictions.
(a) Degree of overlap between mirWIP, miRanda, PicTAR and TargetScanS.
(b) That between mirWIP, PITA and ma22.

(Ambros V., et al, Nature Methods, vol.5 No.9 2008)

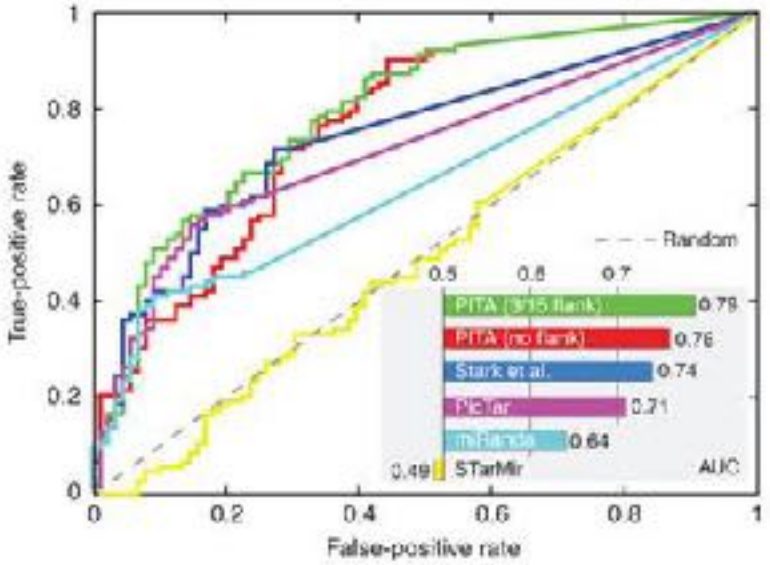
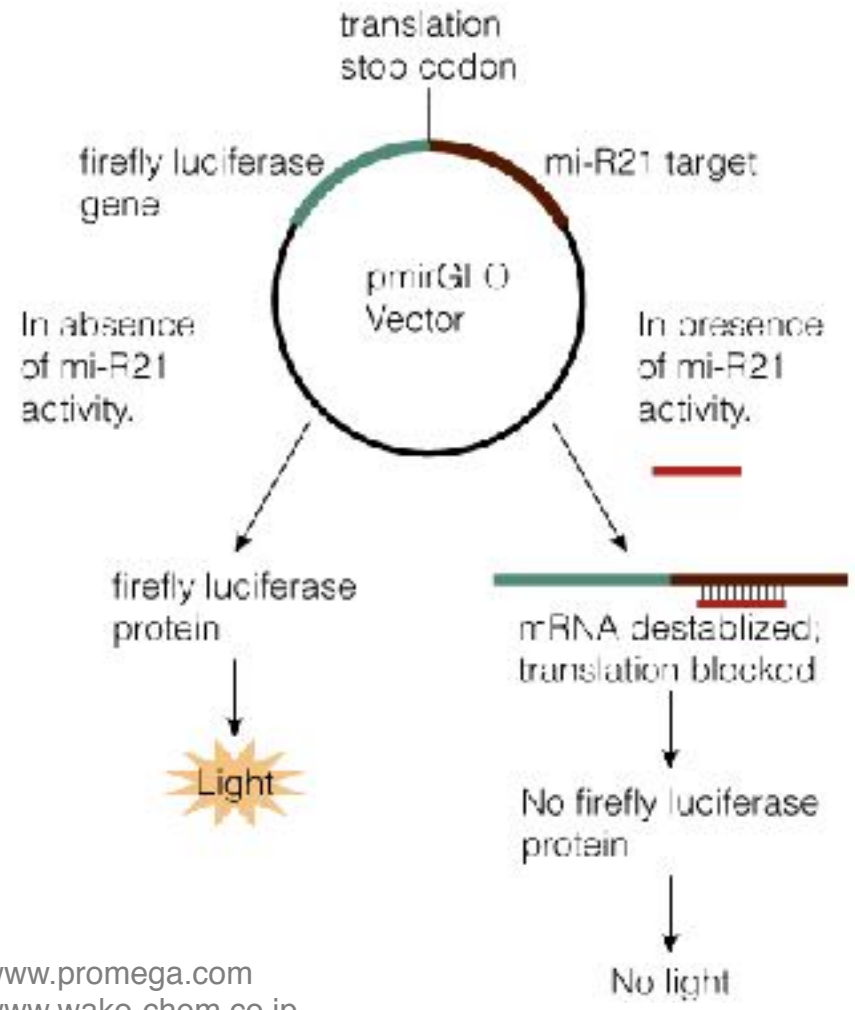


Figure 2.
Comparison of sensitivity and specificity of four existing miRNA-target prediction methods on 190 miRNA-target interaction pairs experimentally tested in flies. (Segal E., et. al, Nature Genetics vol. 39, No.10, 2007)

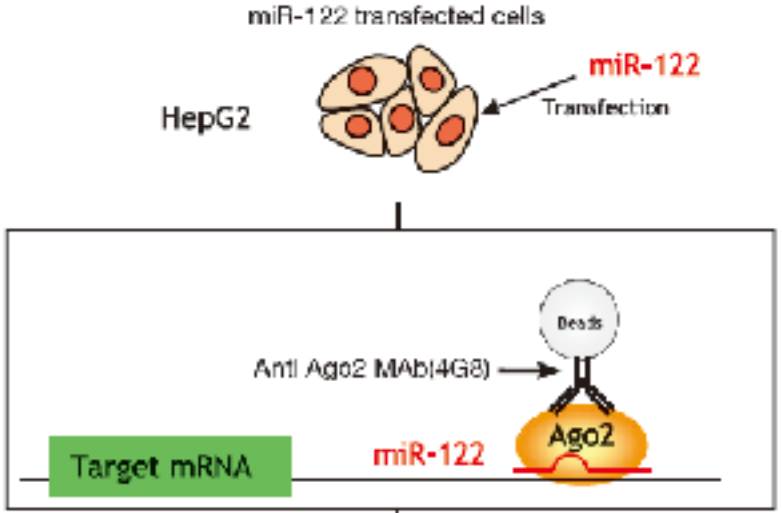
Experimental validation of microRNA targeting of an mRNA

Examine microRNA overexpression/knockdown with target mRNA/protein levels

Reporter assays →

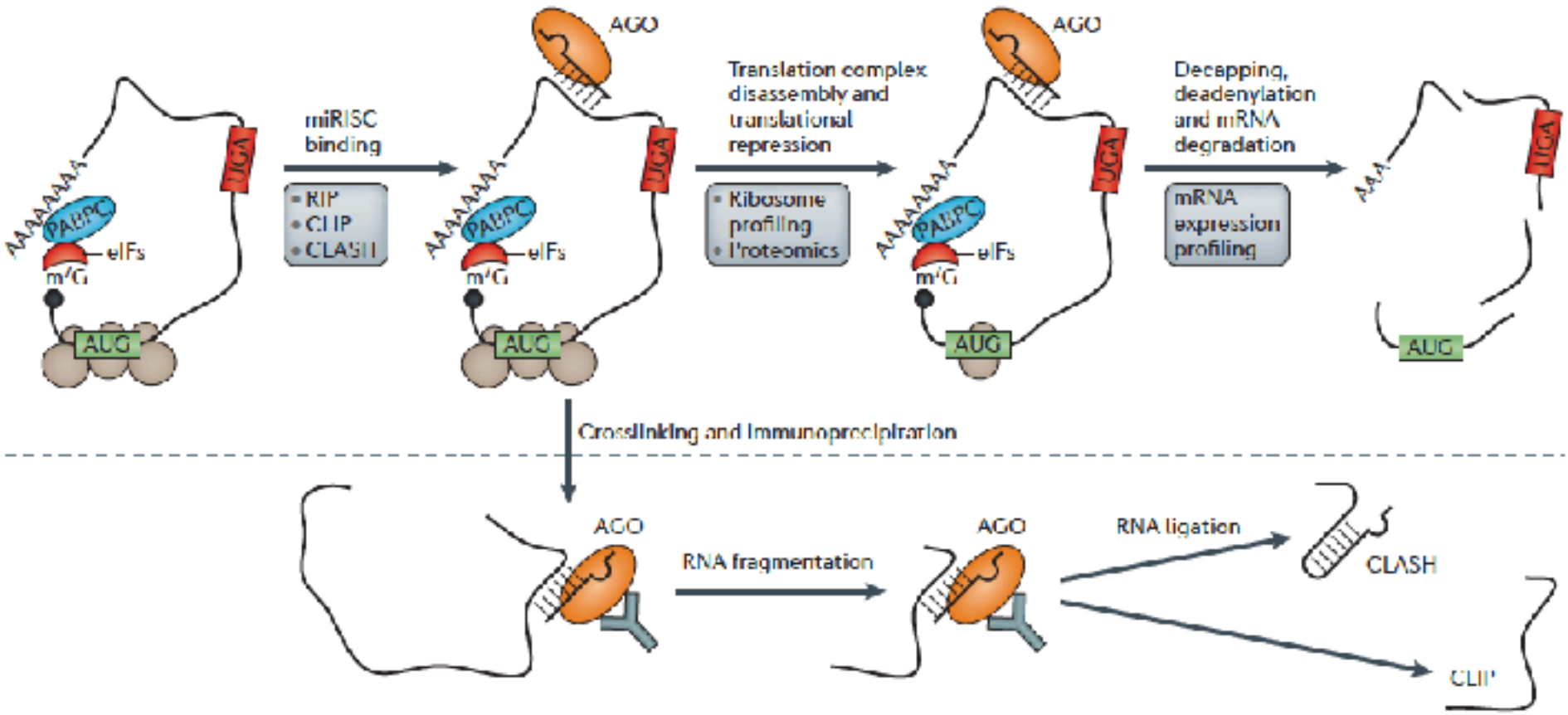


Co-immunoprecipitation of target RNA with Ago protein/RISC pull-down



www.promega.com
www.wako-chem.co.jp

Experimental validation of microRNA targeting of an mRNA



RNA immunoprecipitation (RIP)

crosslinking and immunoprecipitation (CLIP)

crosslinking, immunoprecipitation and sequencing of hybrids (CLASH)

Hausser et al. Nat Rev Gen, 2014

Extraction of microRNAs

Small RNAs, including microRNAs, are purified along with larger RNAs during typical RNA extractions:

- Organic solvent-based methods (e.g., TRIzol™)
- Affinity chromatography with silica matrix (spin-columns): appropriate alcohol and salt conc. in buffers are important for microRNA binding to matrix

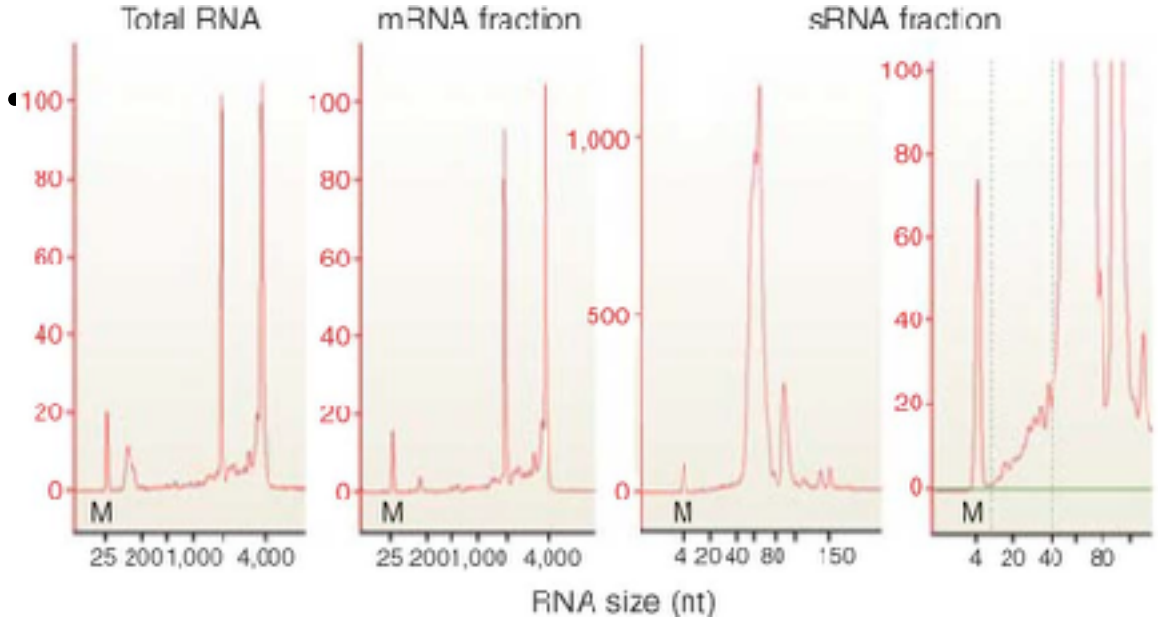
Enriching for small/microRNA fraction:

- Affinity chromatography with different ethanol conc.
- Size-exclusion chromatography
- Capture using hybridization to antisense DNA oligonucleotides

Assessment of total microRNA quality & amount

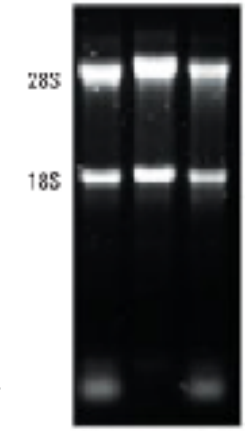
Quality assessment:

•Difficult because of small size (e.g., Bioanalyzer™ assay is not informative)



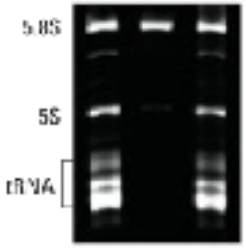
Chomczynski at al. Nature Meth 7, 2010

Agarose gel



small RNA >

Polyacrylamide gel (for small RNAs)



small RNA >

Quantification:

• Absorbance spectrometry – 260 nm; like total RNA; unreliable for low RNA concentration

• Fluorescence – RNA-specific dyes like RiboGreen; very sensitive

Detection and quantification of microRNAs

MicroRNAs are detected like 'regular' RNAs:

- Hybridization-based approaches:
  - Northern blotting
  - RNA/DNA in situ hybridization
  - Microarrays
  - (RT-) PCR
  - Other (mass spectrometry, Raman spectroscopy, etc.)
- RNA sequencing

Considerations:

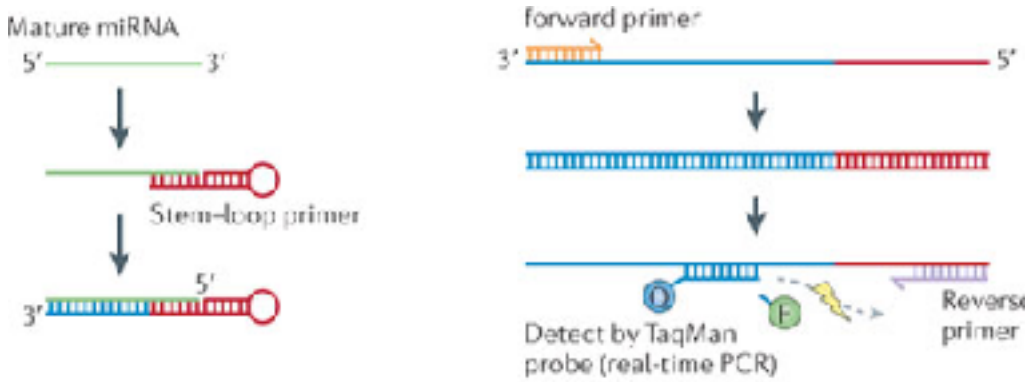
- In situ vs. extracted RNA
- Presence of other RNAs (e.g., small RNA fragments of degraded RNA in formalin-fixed tissue)

RT-PCR of microRNAs

Need strategies to overcome limitations due to the short length of microRNAs

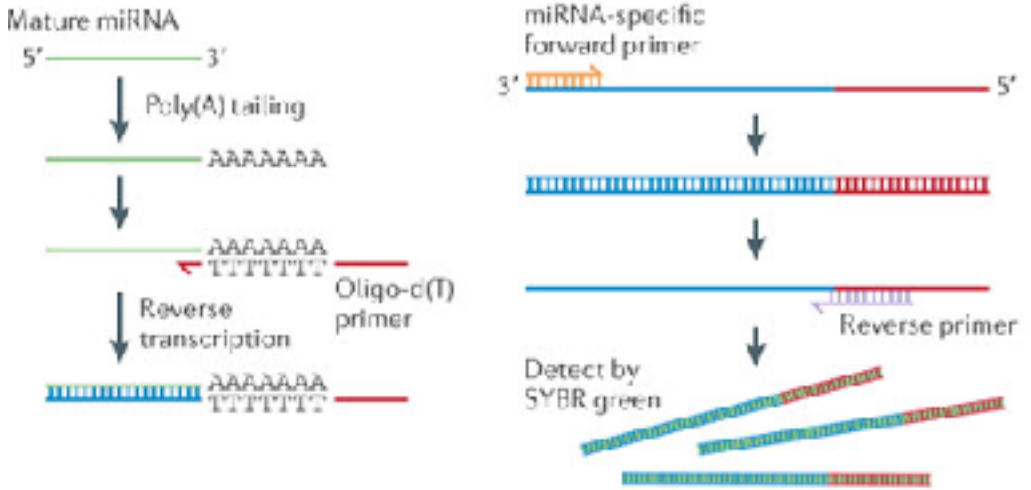
1. Stem-loop RT primer

   Separate RT for separate miRs



2. Poly(A) tailing

   One RT for multiple miRs



Pritchard at al. Nature Rev Gen 13:358-69, 2012

Normalization of microRNA detection values

Normalization: Adjust data to remove non-biological variation across biological samples

1. Endogenous or house-keeping controls

- Typically for RT-PCR of individual microRNAs
- Small nucleolar RNAs like *U6B* (*RNU6-2*/*RNU6B*), *SNORD43*
- Control should be present in biological sample and invariant

2. High throughput data (hundreds of microRNAs quantified)

- Global mean normalization, etc. (RT-PCR)
- Quantile normalization, etc. (microarray)
- Fraction of read counts, etc. (RNA sequencing)

MicroRNA engineering

Overexpression

Principle:

- Overexpress pre-microRNA
- Introduction of synthetic mature microRNA doesn't work

Methods:

- Expression from DNA (plasmid or virus)
- Introduce synthetic pre-microRNA (electroporation, transfection, etc.)
- Physiological stimulus (e.g., hypoxia for *miR-210*, endotoxin for *miR-146a*)

MicroRNA overexpression

Synthetic pre-microRNAs

Modifications:

- Enhance delivery: E.g., 3' cholesterol conjugation
- Increase nuclease resistance: E.g., phophorothioate

Selective overexpression of either 5p or 3p microRNA:

- Change nucleotide sequence for the sister microRNA

MicroRNA knockdown

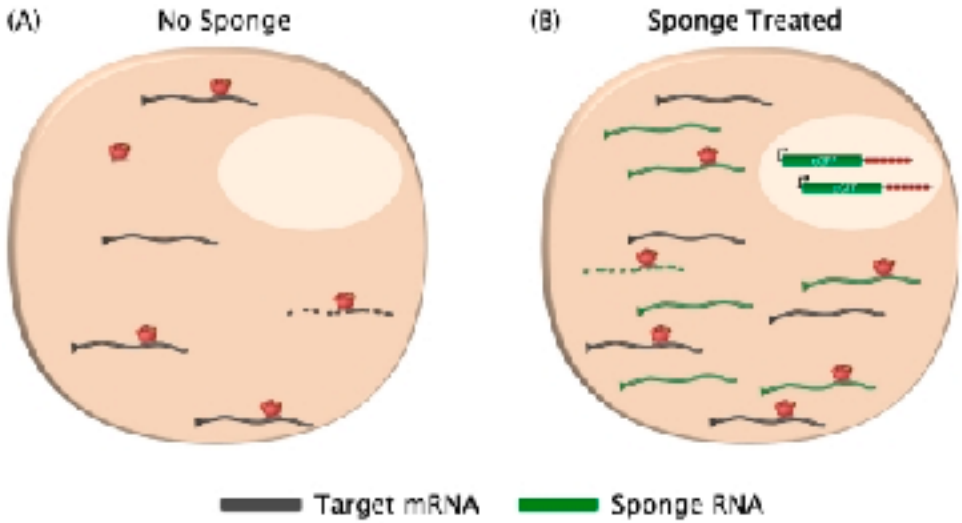
(1) MicroRNA gene knock-out

(2) Antisense DNA oligonucleotides: Can have modifications such as locked nucleic acid, phosphorothioate, etc. to enhance delivery, nuclease resistance, etc.

(3) MicroRNA sponge: Artificial RNA with multiple microRNA targets; transcribed from introduced plasmid/gene/virus

(4) Small molecule inhibitors

Ebert at al. RNA, 2010



MicroRNA applications

(1) Artificial microRNAs

•Short hairpin RNAs (shRNAs) for efficient RNA interference
•Targeting of multiple RNAs

miR-30-based shRNA design

Paddison at al. Nature Meth, 2004

(2) Biomarkers

(3) MicroRNA therapeutics

MicroRNA biomarkers

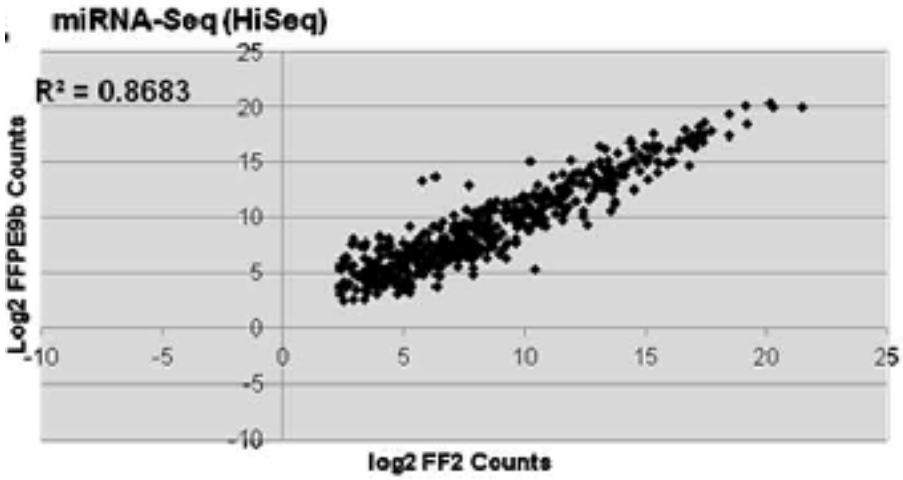
Advantages compared to mRNA biomarkers:

- ~2500 microRNAs vs ~25000 genes (mRNAs) – more robust analysis
- Better preservation in non-frozen samples
- Presence in body fluids

Generally ubiquitous expression
No cancer-specific microRNA, e.g.
Degree of over- or under-expression is important

Preservation of microRNAs

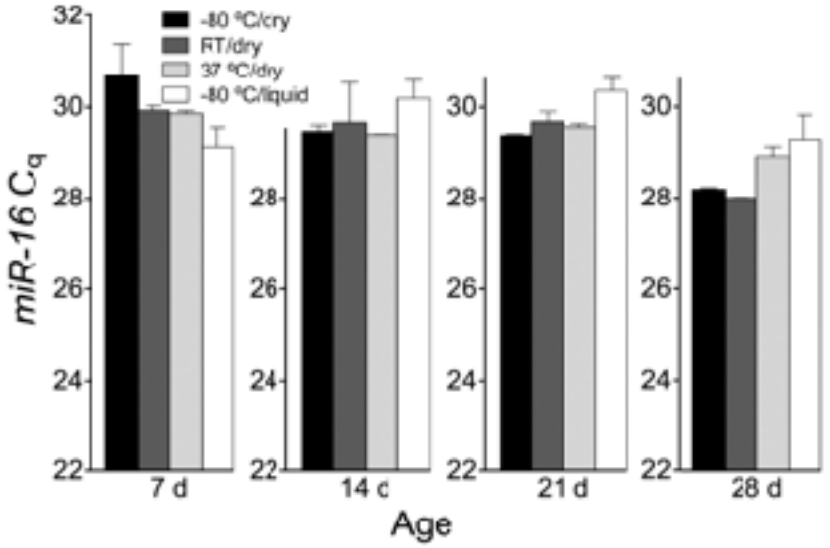
Unlike mRNAs, microRNAs are preserved well in formalin-fixed tissues stored at room temperature (typical clinical specimen storage)



frozen vs. formalin-fixed;
measurements of 500+ microRNAs >

Kolbert et al. PLOS One, 2013

MicroRNAs are preserved in dried body fluids at temperatures as high as 60 deg. C



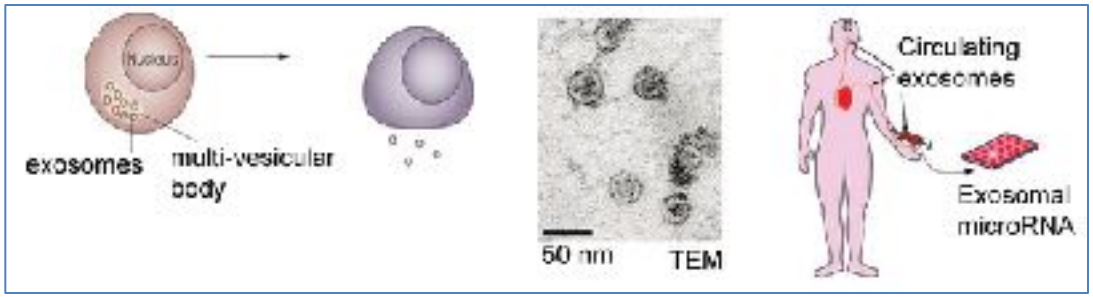
serum >

Patnaik et al. Analytical Biochem, 2010

MicroRNAs in body fluids

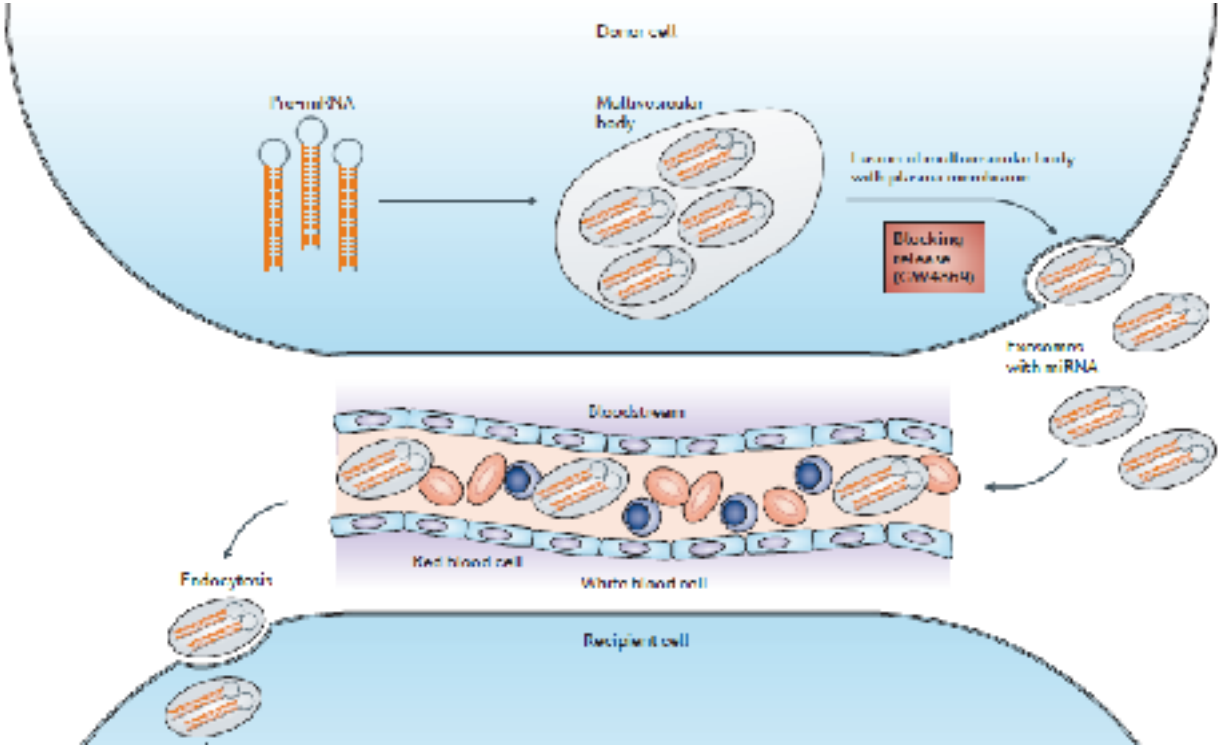
MicroRNAs are secreted by cells

Secreted, extracellular microRNAs are within membrane-bound exosomes



Exosomes enter blood stream and eventually urine, saliva, tear fluid...

Exosomes are picked by other cells

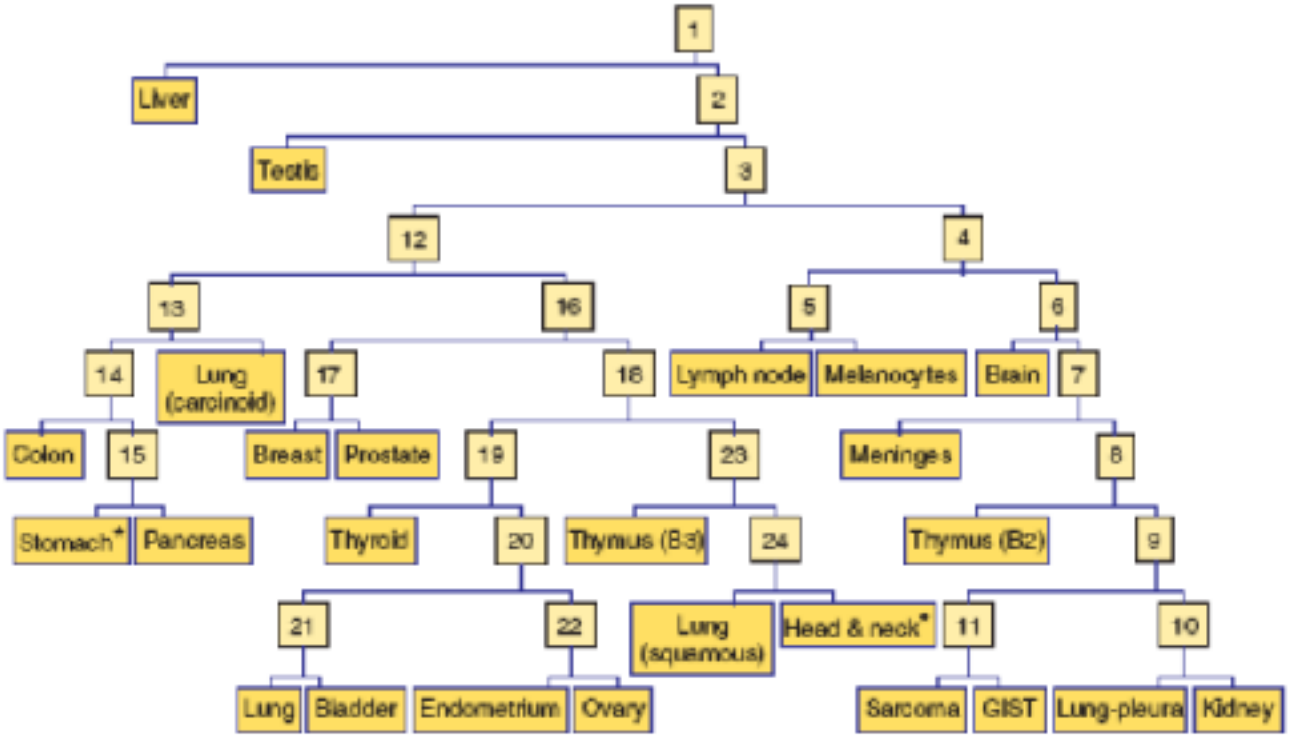


Calin et al.
Nature Rev Drug Disc, 2013

MicroRNA biomarkers / examples

Identification of origin of cancer of unknown origin

Flow chart, using different microRNAs at multiple decision points:

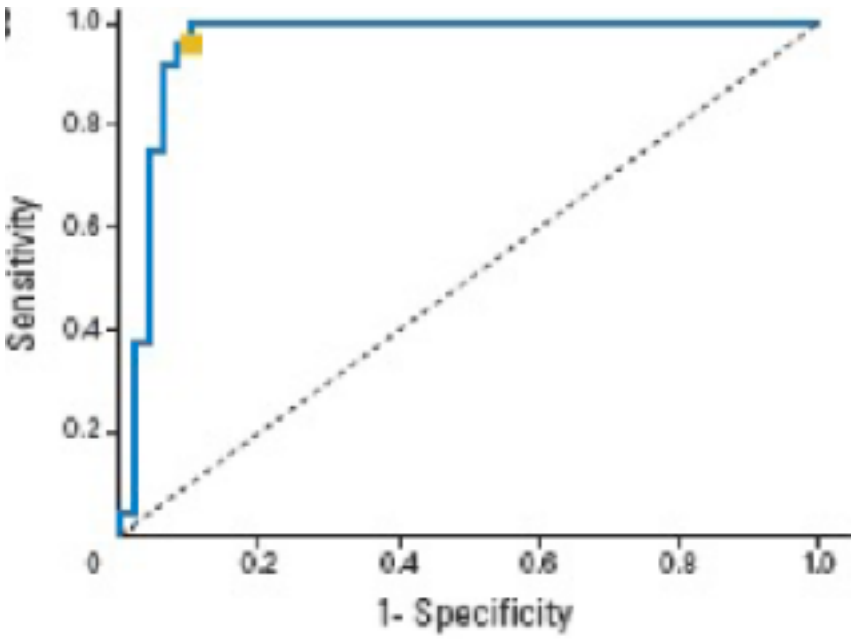


Rosenfeld et al. Nature Biotech, 2008

MicroRNA biomarkers / examples

Distinguishing squamous cell carcinoma sub-type of lung cancer using *miR-205* expression level

Receiver operating curve (ROC) analysis of performance of *miR-205*-based assay:

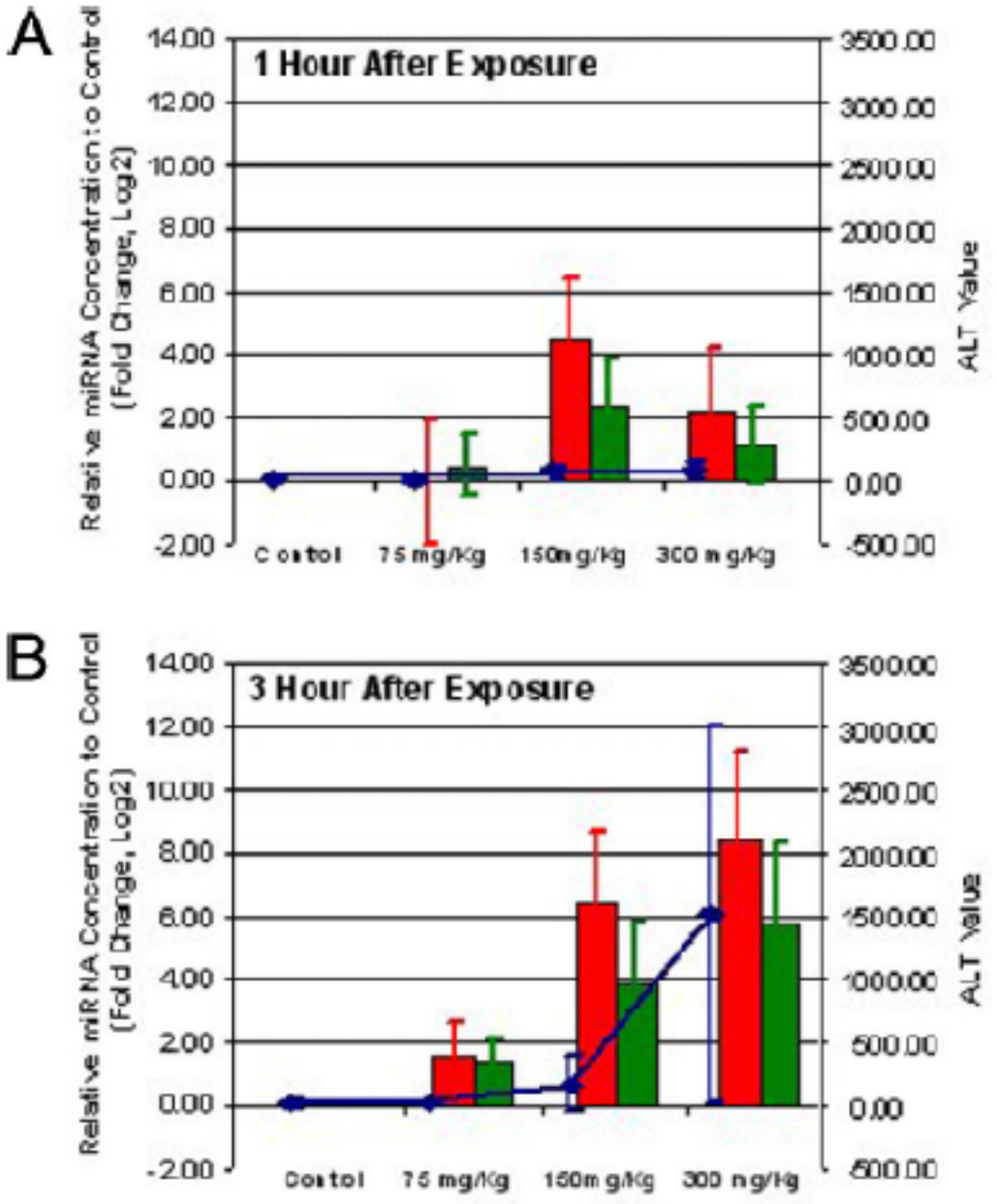


Lebanony et al. J Clin Oncol 2009

MicroRNA biomarkers / examples

Detection of hepatotoxicity resulting from acetaminophen (Tylenol™) overdose

MicroRNA-based test performs better than the standard test (ALT enzyme assay):

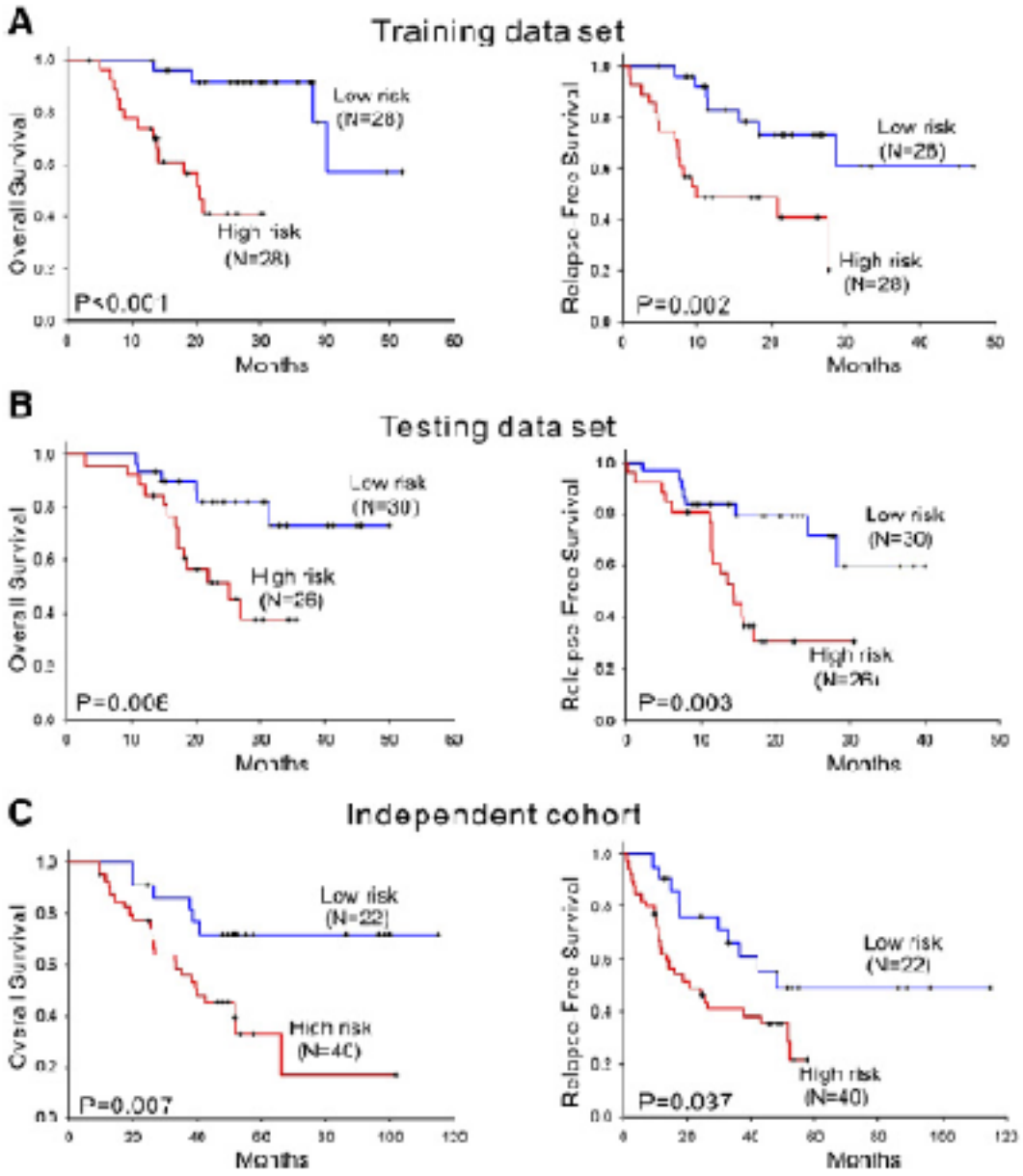


Wang et al. PNAS, 2009

MicroRNA biomarkers / examples

Prediction of recurrence and survival in non-small cell lung cancer

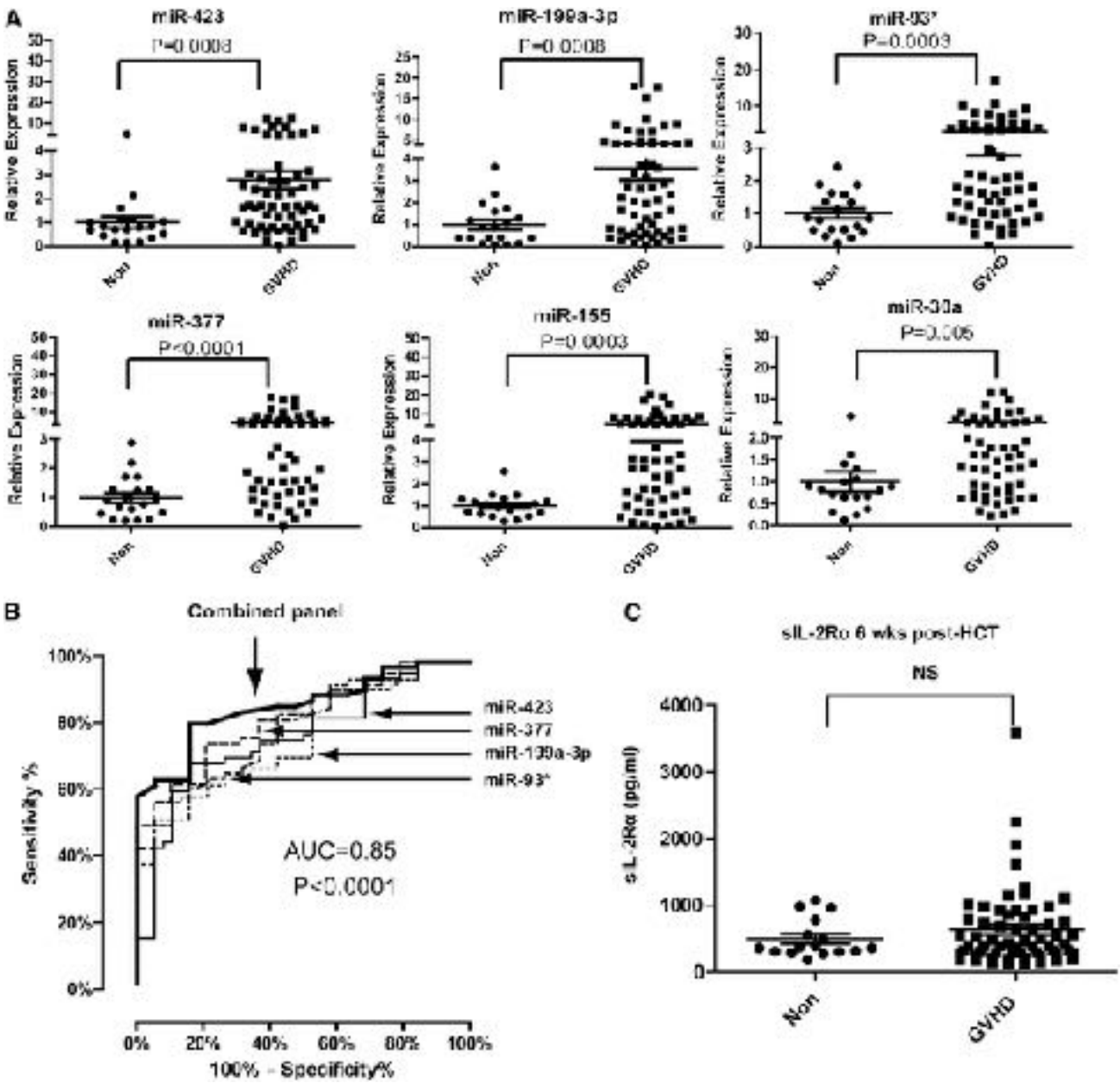
Kaplan-Meier survival curves:



Yu et al. Mol Cell, 2008

MicroRNA biomarkers / examples

Blood (plasma fraction) microRNAs to diagnose transplant rejection (graft vs. host disease)



Xiao et al. Blood, 2013

MicroRNA biomarkers / atypical examples

Exosome-enclosed microRNAs in **exhaled breath** hold potential for biomarker discovery in patients with pulmonary diseases. (J Allergy Clin Immunol, 2013)

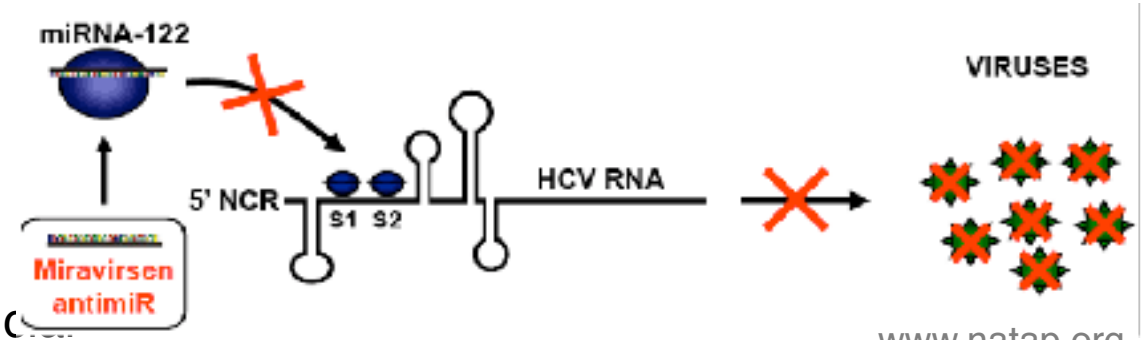
Specific microRNA signatures for the detection of saliva and blood in **forensic body-fluid** identification (J Forensic Sci, 2011)

Detection of miR-92a and miR-21 in **stool samples** as potential screening biomarkers for colorectal cancer and polyps (Gut, 2012)

Chronic **academic stress** Increases a Group of microRNAs in Peripheral Blood (PLOS One, 2013)

MicroRNA-based therapeutics

- Under development
- Most advanced for hepatitis C treatment: *miR-122*/*HCV* RNA complex stabilizes *HCV*
- Delivery system and pharmacodynamics/kinetics crucial



www.natap.org

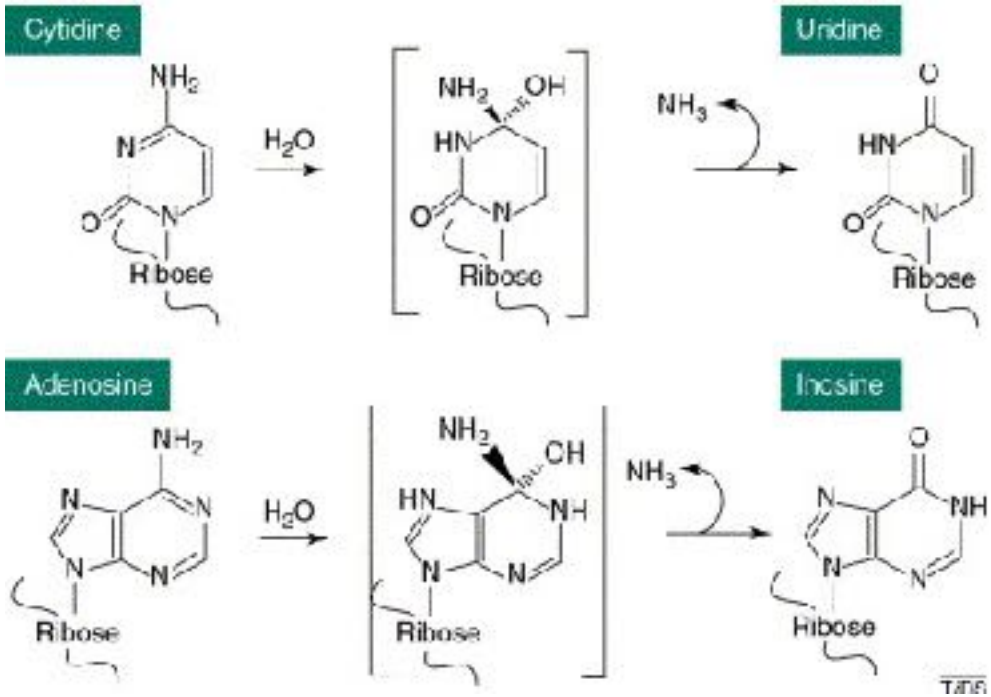
Company	miRNA target	Mode of action	Indication	Status
Santaris Pharma	miR-122	antimiR	HCV	Clinical Phase II
Mirna Therapeutics	miR-34	mimic	Unresectable primary liver cancer	Clinical Phase
	let-7	mimic	Cancer	Preclinical
Regulus Therapeutics	miR-122	antimiR	HCV	Clinical Phase
	miR-221	antimiR	Hepatocellular carcinoma	Preclinical
	miR-10b	antimiR	Glioblastoma	Preclinical
	miR-21	antimiR	Hepatocellular carcinoma	Preclinical
	miR-21	antimiR	Kidney fibrosis	Preclinical
	miR-33	antimiR	Atherosclerosis	Preclinical
miRagen Therapeutics	miR-208	antimiR	Heart failure	Preclinical
	miR-15/195	antimiR	Post-MI remodeling	Preclinical
	miR-145	antimiR	Vascular disease	Preclinical
	miR-451	antimiR	Myeloproliferative disease	Preclinical
	miR-29	mimic	Fibrosis	Preclinical
	miR-208	antimiR	Cardiometabolic disease	Preclinical
	miR-92	antimiR	Peripheral artery disease	Preclinical

van Rooij at al. EMBO Mol Med, 2014

RNA editing

RNA editing through base deamination

Editing is not non-specific; edited base typically in certain RNA sequence context



Gerberet al. Trends Biochem Sci, 2001

C-to-U (C>U) editing

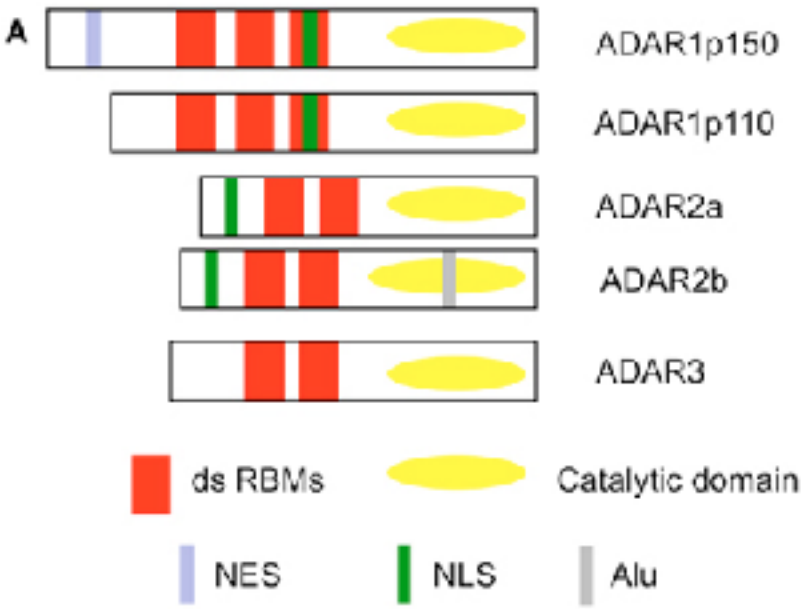
- Trypanosome parasites (mitochondrial tRNA)
- Mammalian small intestine (apolipoprotein B)

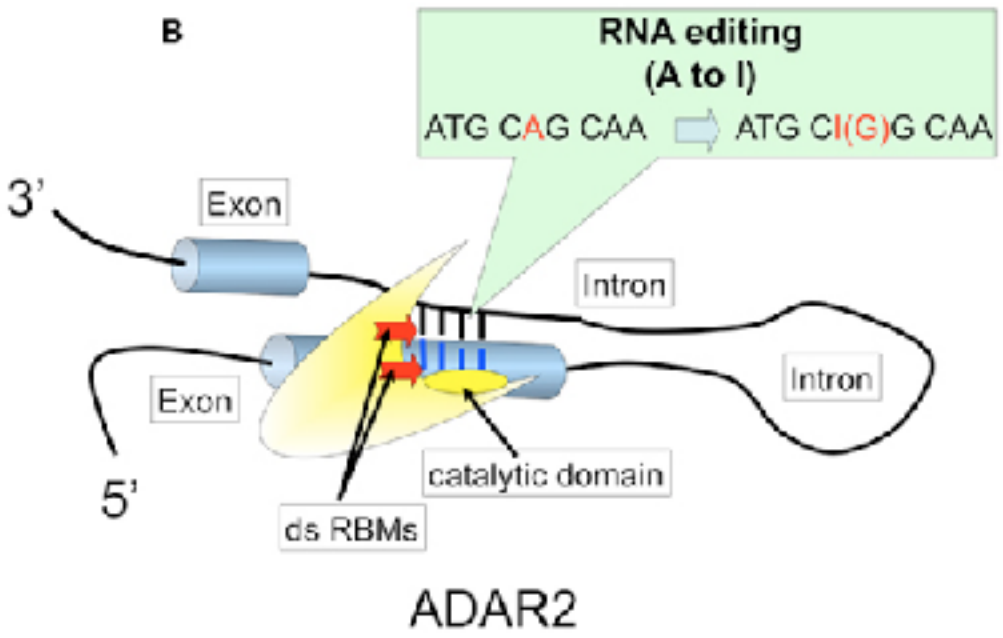
A-to-I (A>I) == A>G editing

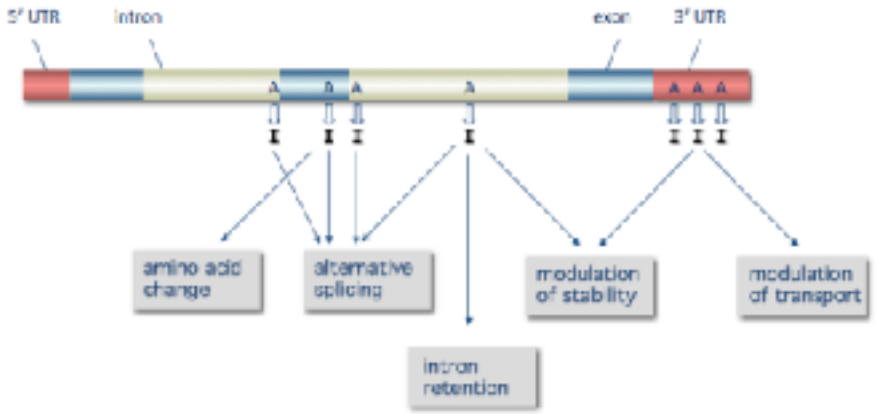
- Hepatitis D virus (anti-genomic RNA)
- Octopus potassium channel mRNA
- Eukaryotic/prokaryotic tRNAs
- Mammalian serotonin receptor mRNAs

Aminotransferases for U>C or I>A editing?

A>I RNA editing by ADAR enzymes







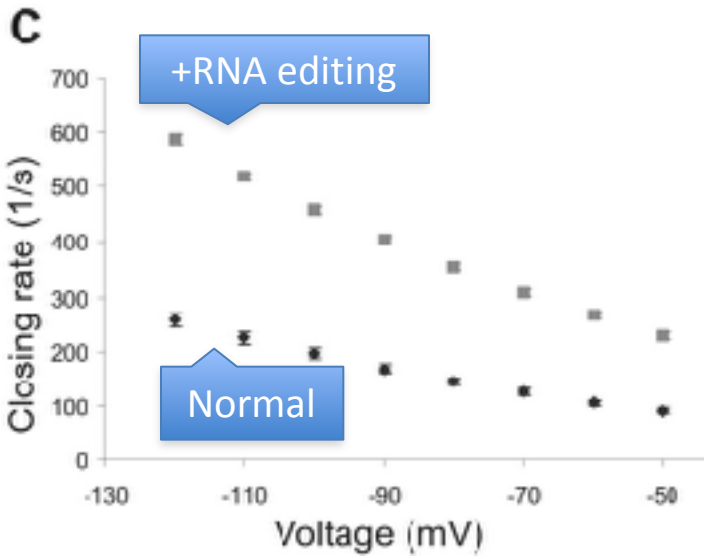
* ADARs acts on double-stranded RNA
* RNA binding motif (RBM)
  + catalytic deaminase domain
* Mammals: *ADAR1-3* genes
  Dosophila: *ADAR*
  C. elegans: *ADR1-2*
* Present in nucleus

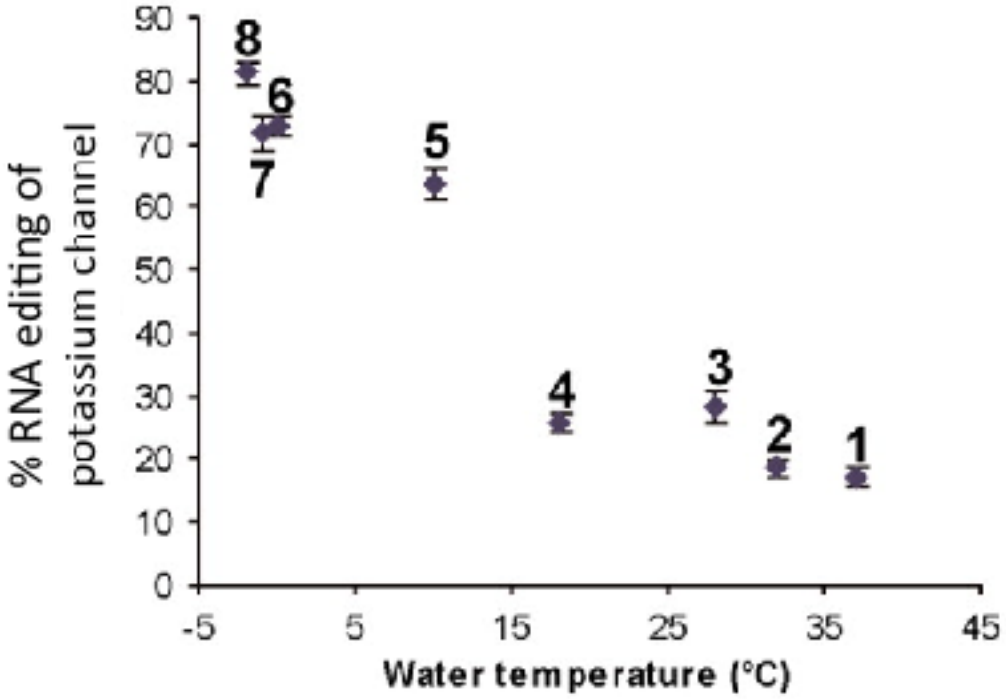
Hideyama et al. Front Mol Neurosci, 2011

A>I RNA editing of a nueronal potassium channel

Facilitating survival of octopuses in cold temperatures

Garrett et al. Science, 2012





C>U RNA editing by APOBEC proteins

APOBEC1 enzyme edits
apolipoprotein B mRNA
in small intestine

Liver:
Longer protein is made
Incorporated in low density lipoproteins (LDL)

Small intestine:
Shorter protein because of RNA editing
Incorporated in chylomicrons and not LDLs

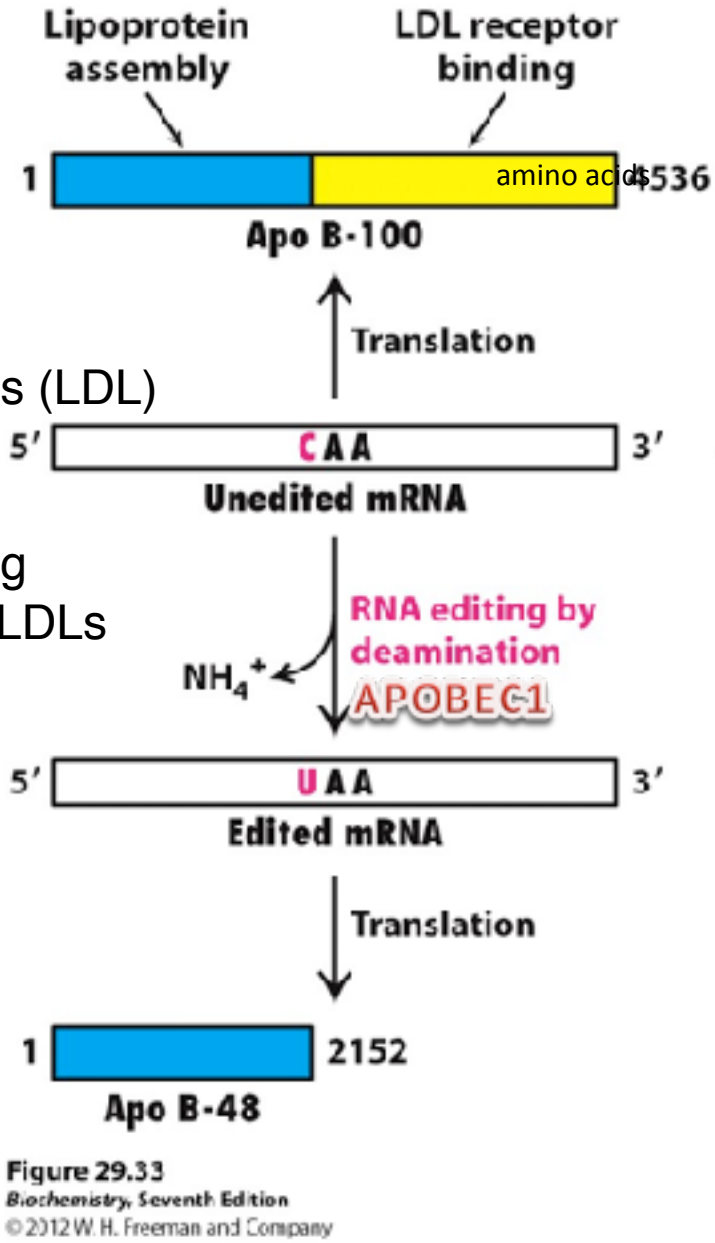
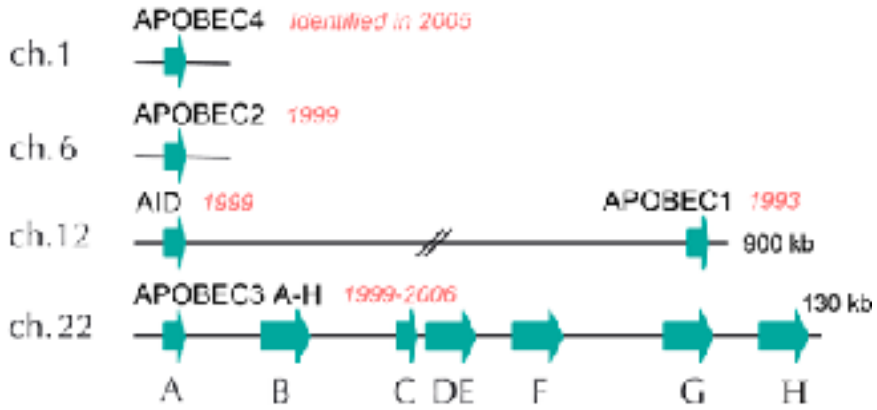
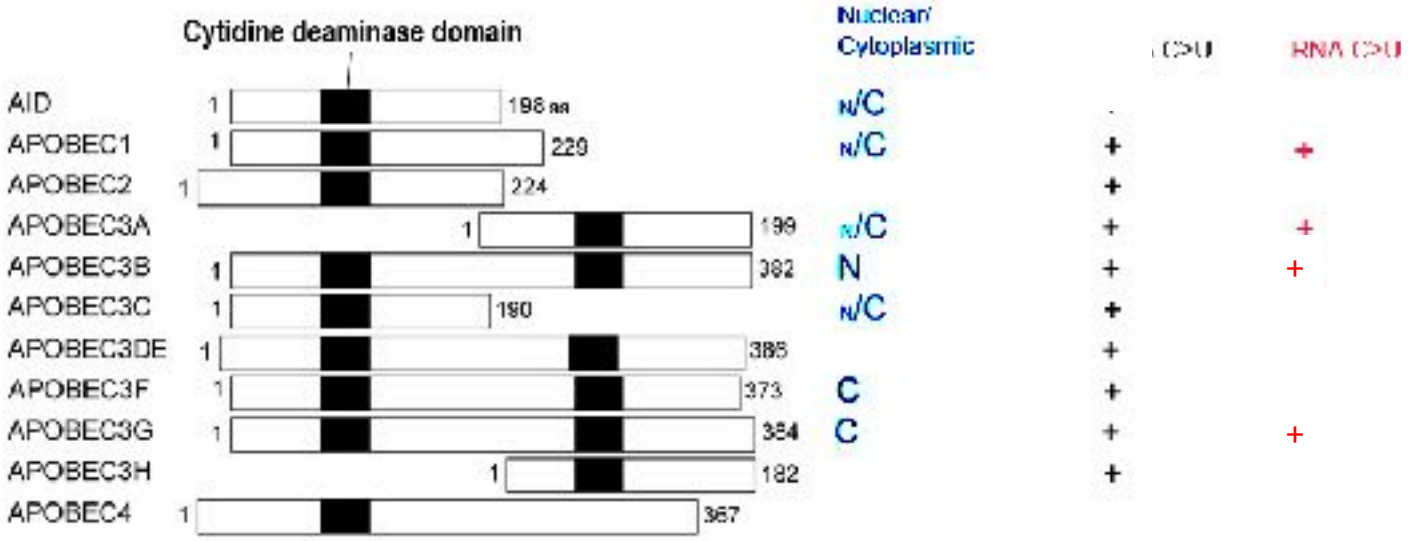


Figure 29.33
Biochemistry, Seventh Edition

Human APOBEC genes and proteins





APOBEC3A, APOBEC3B and APOBEC3G proteins are also C>U RNA editors

APOBEC proteins:

- Also deaminate C residues to U in DNA
- Possess 1-2 cytidine deaminase domains
- Important roles in viral immunity, retrotransposition, cancer mutagenesis

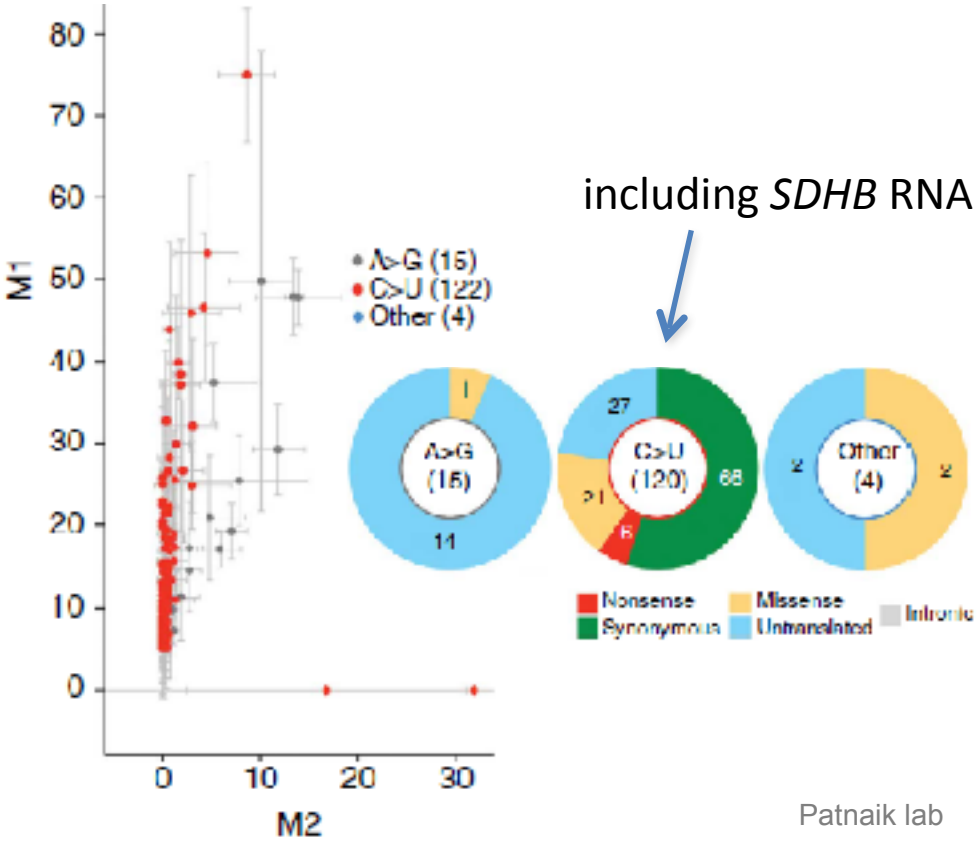
Discovery of APOBEC3A as a C>U RNA editor

Monocytes → Basal (M0) macrophages → M1 (pro-inflammatory)

Basal (M0) macrophages → M2 (anti-inflammatory)

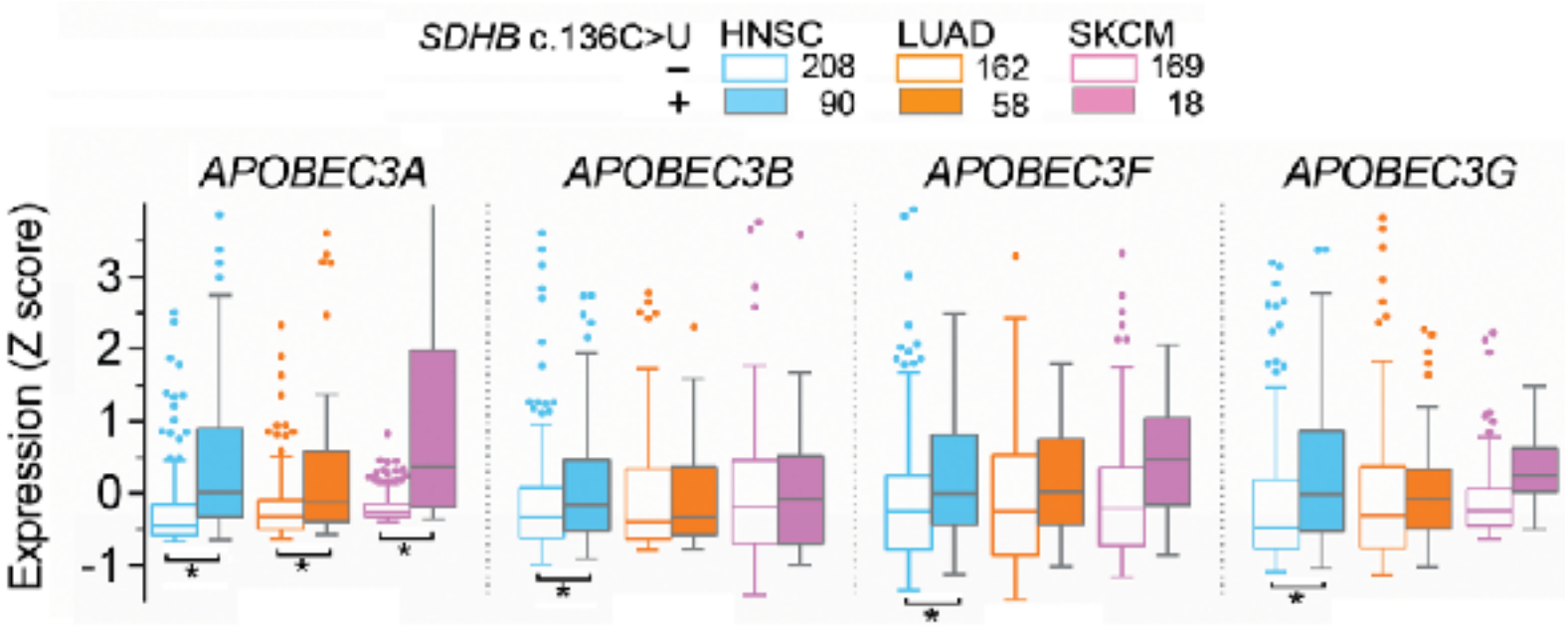
M1 vs. M2 transcriptome-wide editing levels

mean & range; n = 3 pairs



SDHB RNA editing is associated with *APOBEC3A* expression

Examination of the Cancer Genome Atlas (TCGA)



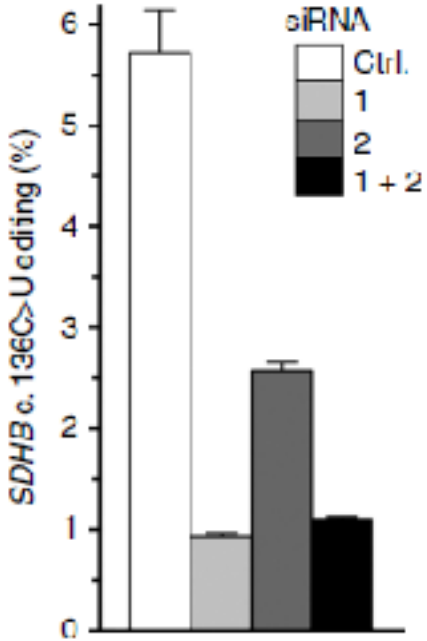
Patnaik lab

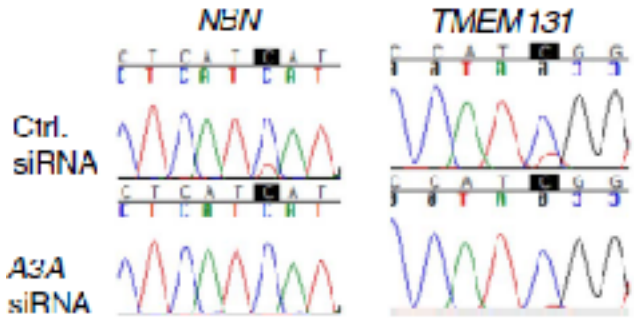
HNSC = head & neck sq. cell ca.; LUAD = lung adenoca., SKCM = skin melanoma

APOBEC3A edits *SDHB* RNA

APOBEC3A knockdown in M1 macrophages

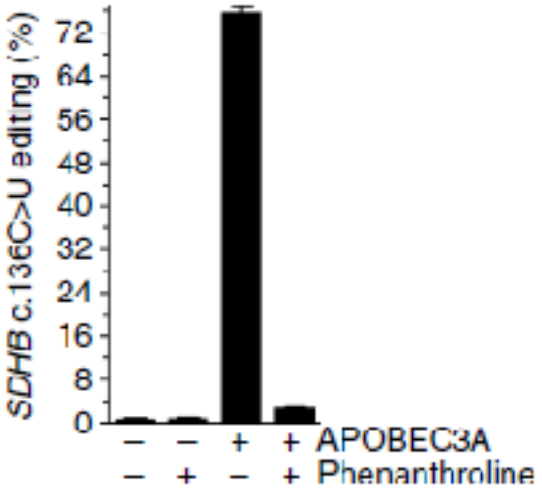
mean & range; n = 3

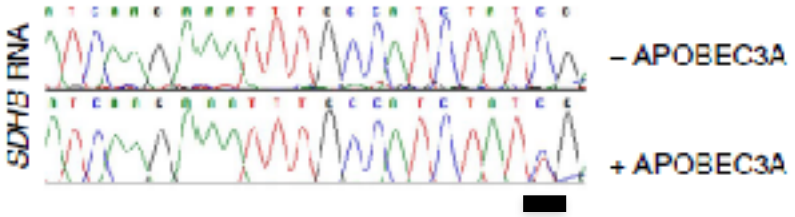




In vitro *SDHB* RNA editing by APOBEC3A protein

mean & range; n = 3

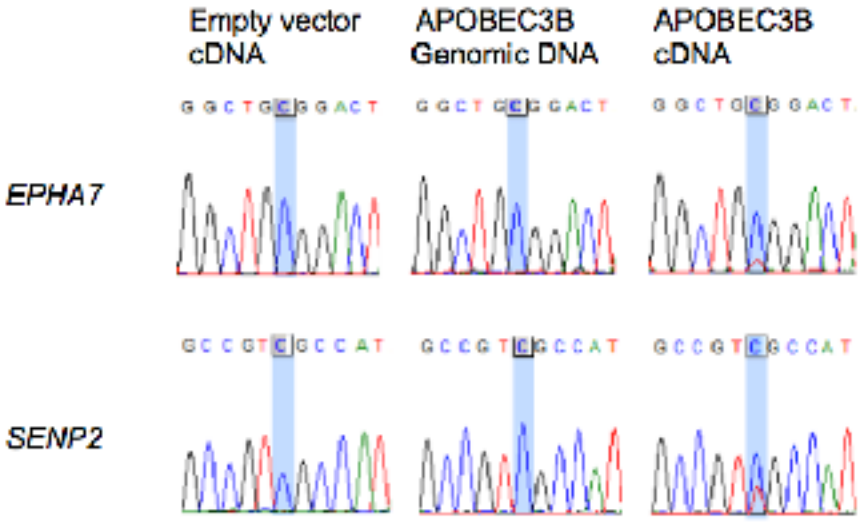




Assays for RNA editing

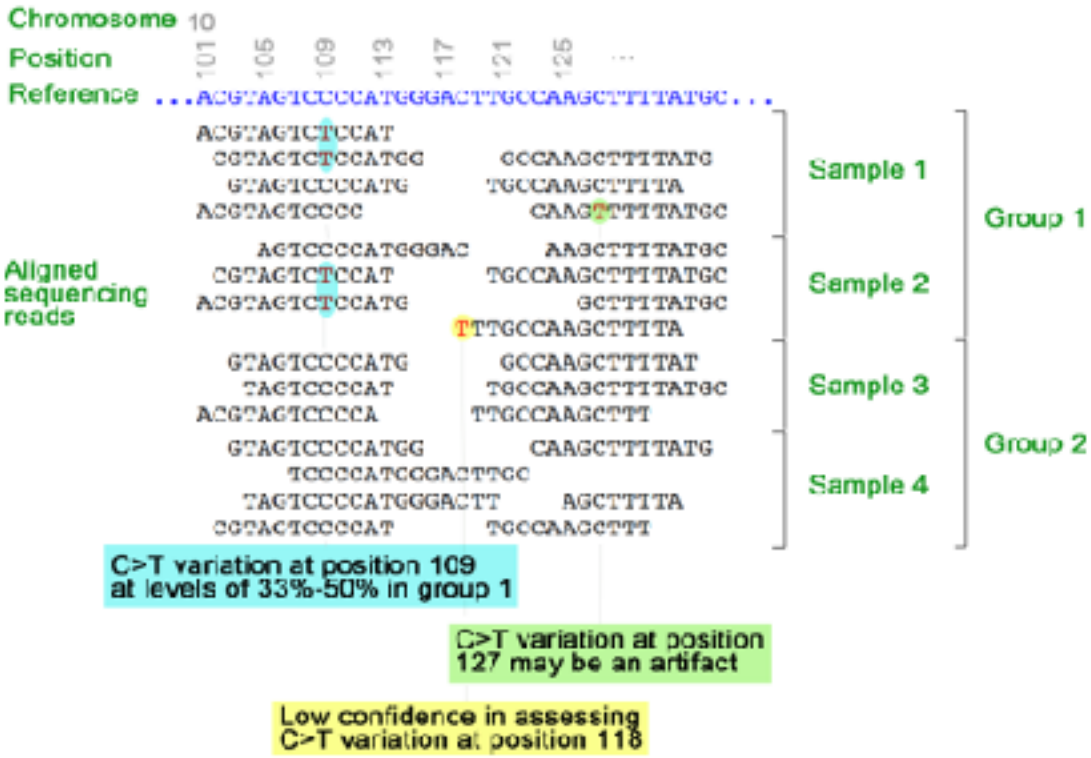
1. Sanger sequencing of RNA (cDNA)

Example: Sequencing of cDNA and genomic DNA
of control and APOBEC3B transfectants
for 2 genes whose RNAs are edited by APOBEC3B



2. "Next generation" RNA sequencing

Uses computational/bioinformatics workflows



Assays for RNA editing

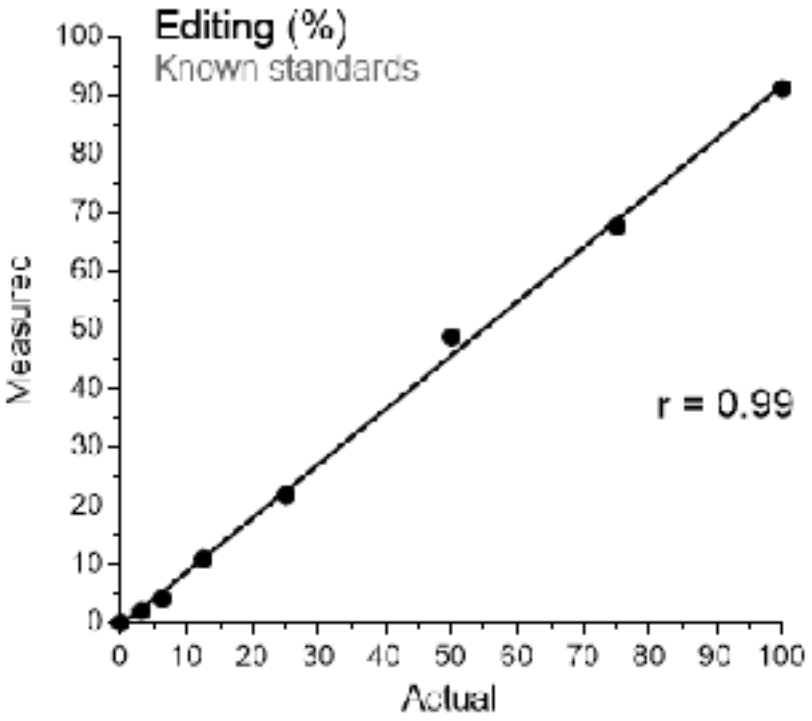
3. Allele-specific RT-PCR

```
Target RNA          xxxxx ..... Uxxxxx

PCR 1
Forward primer      xxxxx
Reverse primer 1                xxxxx

PCR 2
Forward primer      xxxxx
Reverse primer 2                Axxxxx
```

Editing α PCR 1 – PCR 2 signal



Summary

RNAs of **thousands of genes** are edited

Editing is **not non-specific**
– edited nucleotides have a sequence context

Editing can have **high impact at protein level**
– a 'new' angle to examine cellular phenomena

Editing can be **physiologically affected**
– examples: increased editing in M1 macrophages (vs. M2), in hypoxic monocytes (vs. normoxic)

A>G (I) and C>U types of editing are typical

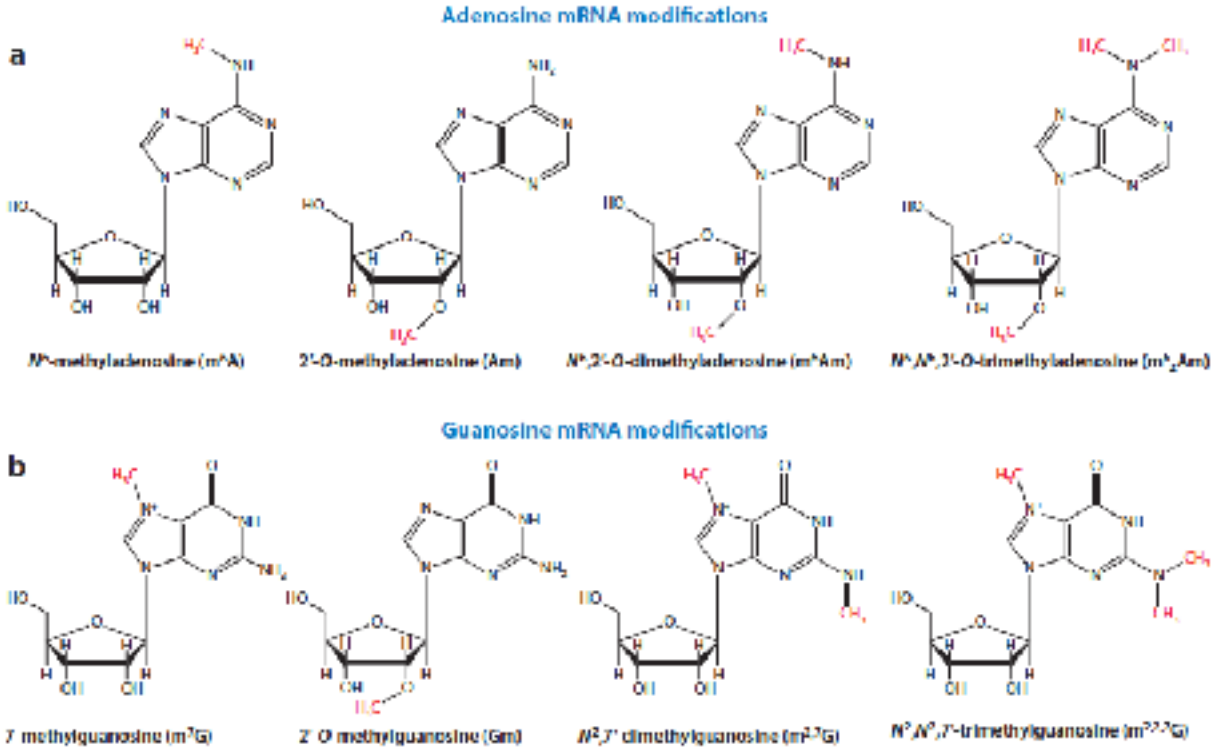
Much to discover (fertile field)
•Prevalence across cell types, diseases
•Mechanisms and their modulation
•Functional relevance
•Non-A>G/C>U editing

RNA modifications

Types of RNA modifications

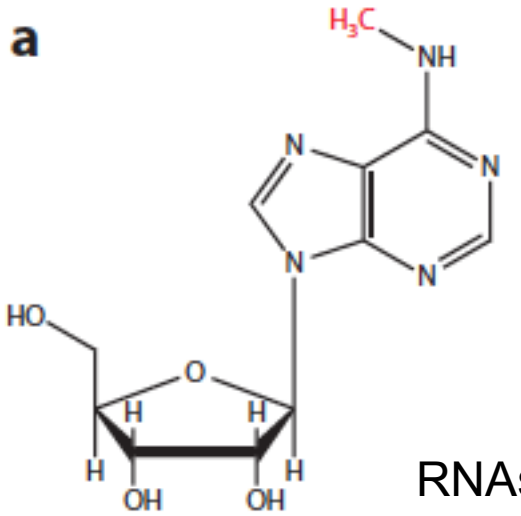
Not including:
RNA editing, splicing, mRNA 5' capping or poyladenylation, etc.
Transfer RNA (tRNA) modifications (pseudouridylation, methylation, etc.)

(messenger) RNA modifications:
~110 types – majority methylations – may not be reversible (unidirectional)
DNA methylation discovered in 1925; RNA,1968
Examples:



Li et al. Ann Rev Genom Hum Gen, 2014

m^{6}A modification



Like protein phosphorylation

Noticed in 1970s (0.1%-0.4% of all A residues of cellular RNA)

Recent discoveries facilitated by RNA sequencing technology (capture with anti-m^{6}A antibody)

RNAs of majority (>15,000) of human genes are m^{6}A-methylated

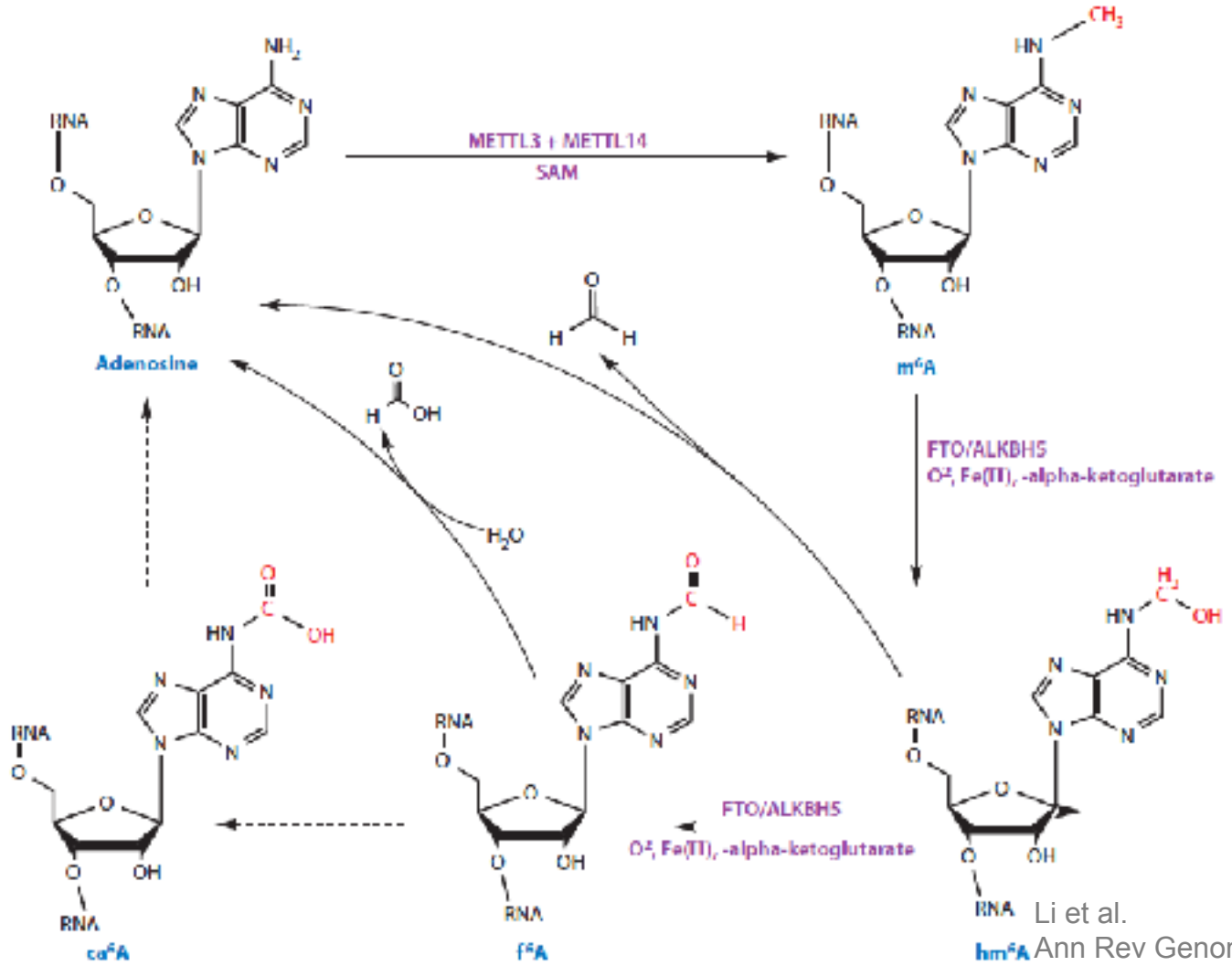
Sequence context: G [G/A] m^{6}A C U

Reversible (=> "dynamic epitranscriptome")

Widespread across eukaryota

m^{6}A modification – writers & erasers

METTL (methyltransferase-like) 3 and 14 **methylases** catalyze m^{6}A modification
FTO and ALKBH5 **demethylases** reverse the modification

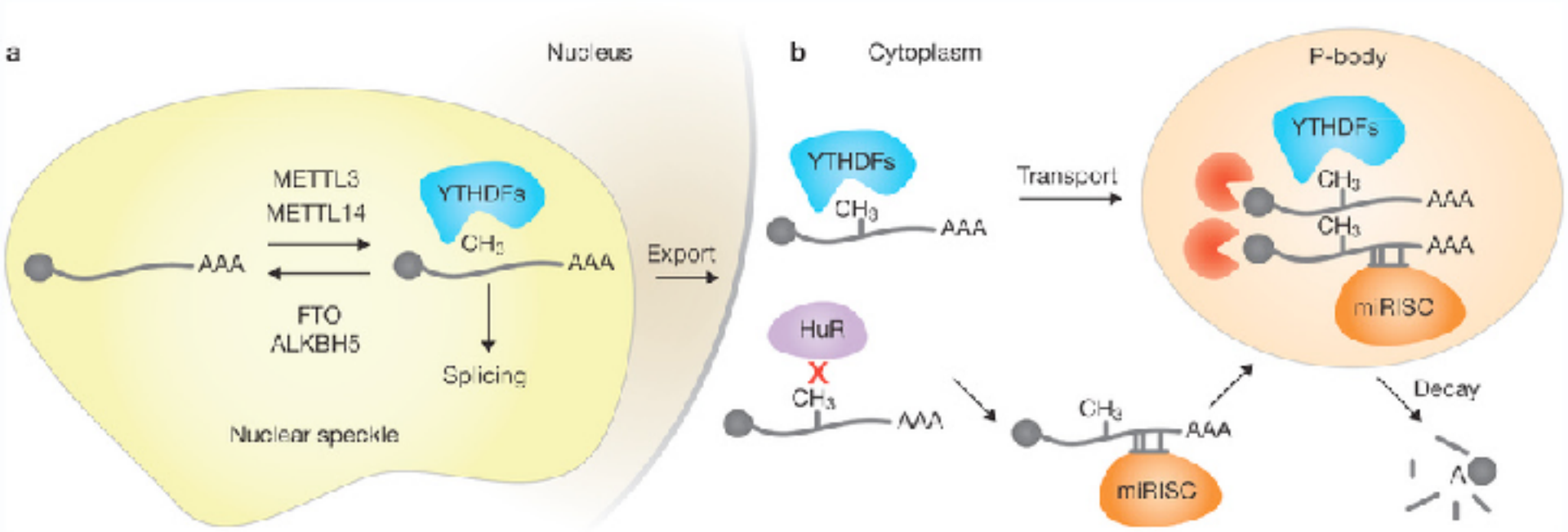


Li et al.
Ann Rev Genom Hum Gen, 2014

m^{6}A modification – readers

m^{6}A modification affects binding of various proteins

* reduced binding to RNAs of HuR (human antigen R, an RNA stabilizing protein)
* enhanced binding of YTHDF1-3 (YTH domain family proteins)
* enhanced binding of RBM15/15B, HNRNP and other proteins

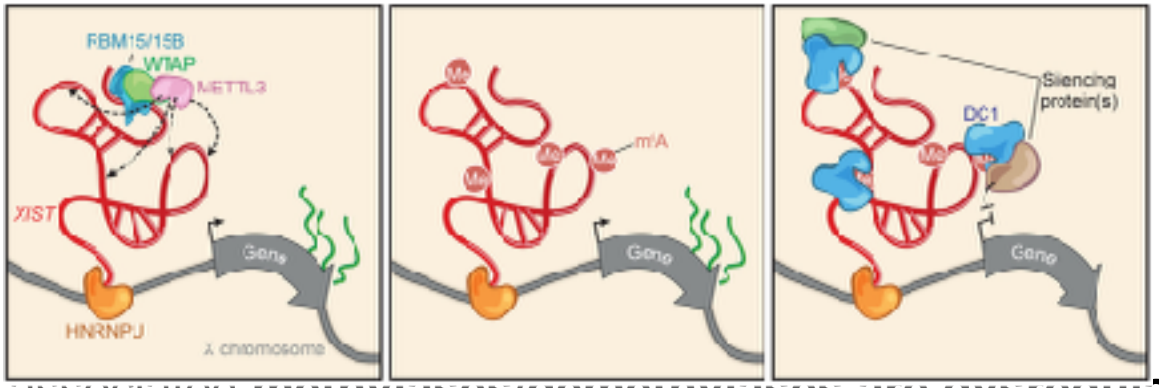


Lin e al. Nat Cell Bio, 2014

m^{6}A modification – relevance

Under active investigations
Effects of modification vary by gene/cellular context

1. enhanced **nuclear exit of RNA**
   Ex.: circadian clock gene transcripts => regulation of circadian rhythm
2. reduced **RNA stability**
   Ex.: transcripts of pluripotency-associated proteins => initiation of cellular differentiation
3. enhanced **RNA translation**
   Ex.: heat shock transcripts => protection from stress
4. reduced **gene transcription**
   Ex.: modification of XIST => inactivation of X chromosome genes



Patil et al. Nature, 2016

m6A associated **methylases/demethylases are dysregulated in cancer**

Suggested reading

Classics:

- Identification of microRNAs: Lee et al. Cell 75, 843–854 (1993)
- Identification of multiple microRNA genes: Lagos-Quintana et al. Science, Vol. 294 no. 5543 pp. 853-858 (2001)
- Discovery of Dicer: Bernstein et al. Nature 409, 363-366 (2001)
- Discovery of association of microRNAs with cancer: Calin et al. Proc Natl Acad Sci U S A, 99(24): 15524–15529 (2002)
- Discovery of APOBEC1 RNA editing enzyme: Teng et al. Science 260, 1816-1819 (1993)
- Demonstration that m^6A RNA modification is widespread: Meyer et al. Cell 149, 1635-1646 (2012)

Reviews:

- MicroRNA biogenesis: Ha et al. Nature Reviews Molecular Cell Biology 15, 509–524(2014)
- MicroRNA targeting: Hausser et al. Nature Reviews Genetics 15, 599-612 (2014)
- MicroRNA assays: Pritchard et al. Nature Reviews Genetics 13, 358-369 (2012)
- MicroRNAs and cancer: Hayes et al. Trends in Molecular Medicine, 20(8):460-9 (2014)
- RNA editing and RNA modifications: Licht et al. Journal of Cell Biology, 213:15-22 (2016)

Exam

Multiple choice questions
Question itself a learning material
Answer key with explanations

Example:

Question

Exosomes are lipid bilayer-bound vesicles of about 30-150 nm diameter that are secreted by cells, and they can be detected in bodily fluids such as blood and urine. These vesicles contain microRNAs, mRNAs and proteins, and the uptake of exosomes by other cells can affect their cellular processes. In a project to study exosomal microRNA-mediated cell-cell communication, you wish to remove pre-existing exosomal microRNAs from the serum that is to be used for in vitro cell culture. Which one of the following techniques may be suitable for this purpose?

A. Addition of a mild detergent to serum

B. Addition of a generic ribonuclease such as RNAse A to serum

C. Ultracentrifugation of serum

D. Heat treatment of serum

Answer

C

RNAses will not enter the lipid bilayer-bound exosomes. A detergent will disrupt the exosome but unless it is removed from the serum, it will affect cell culture as well. Heat treatment at a high enough temperature might damage exosomes but it will likely degrade proteins and other factors in serum that are necessary for cell growth. Overnight ultracentrifugation is the currently accepted standard for depleting serum of exosomes .

Questions?

santosh.patnaik@, x8364, MRC 207