

# Small RNAs and how to analyze them using sequencing

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Enabler for Life Sciences



# Small RNAs

- Small RNAs are species of short non-coding RNAs, typically <100 nucleotides
  - micro RNAs (miRNAs)
  - short interfering RNAs (siRNAs)
  - piwi associated RNAs (piRNAs)
  - clustered regularly interspaced short palindromic repeats (CRISPRs)
  - mirtrons, cis-natRNAs, TSS-miRNAs, enhancer RNAs and other strange things



# 1. Background on regulatory small RNAs



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UPPSALA  
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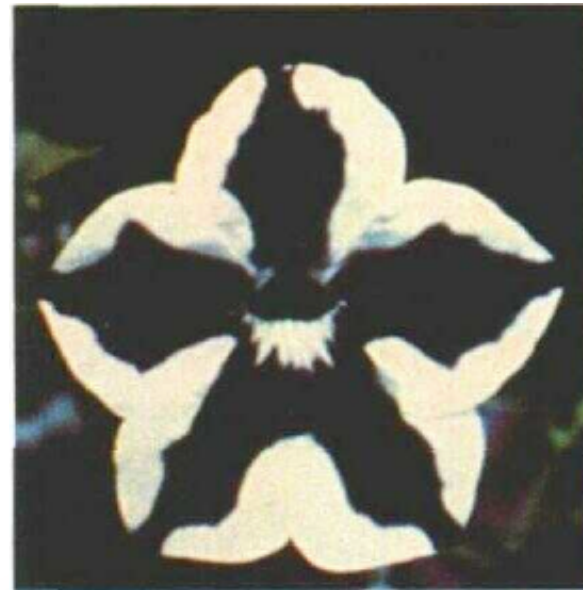
SciLifeLab



# 1991: RNA can repress gene expression



wt



Overexpression of pigment gene CHS

(Napoli et al. Plant Cell, 1991.)

“ The mechanism responsible for the reversible co-suppression of homologous genes *in trans* is unclear ..”



# 1993: The first microRNA is discovered in the worm genome

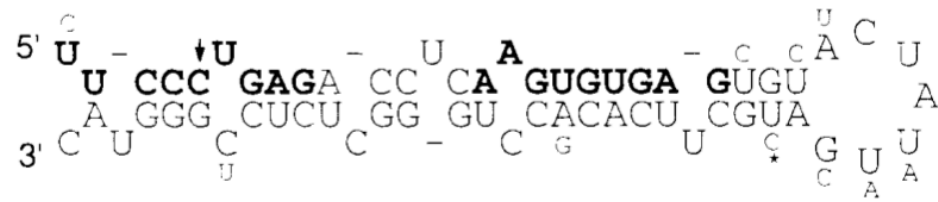
Cell, Vol. 75, 843–854, December 3, 1993, Copyright ©1993 by Cell Press

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

Rosalind C. Lee,\*† Rhonda L. Feinbaum,\*‡ and Victor Ambros†

1. A mutation in the *lin-4* locus disrupts worm development.
2. The *lin-4* locus encodes a non-coding RNA that forms a hairpin structure and produces two small transcripts, 61 and 22 nt.
3. Part of this RNA is complementary to the 3'UTR of a developmental gene, *lin-14*

*lin-4L*



5' GUUCCCUGAGACCUC AAGUG . UGAG	<i>lin-4</i>
3' CAAG . GACUC . . . . . UCGU-ACUC	
UAAG . GACUC . - . . . . . ACUU	
CAAGGGACUC . - . . . . . UUUAC-GCUC	
UAAG . GACUC . - . . . . . U . ACUC	
CAAGGGACUC . . . . . CAU . . CUU	

*lin-14*  
3'UTR

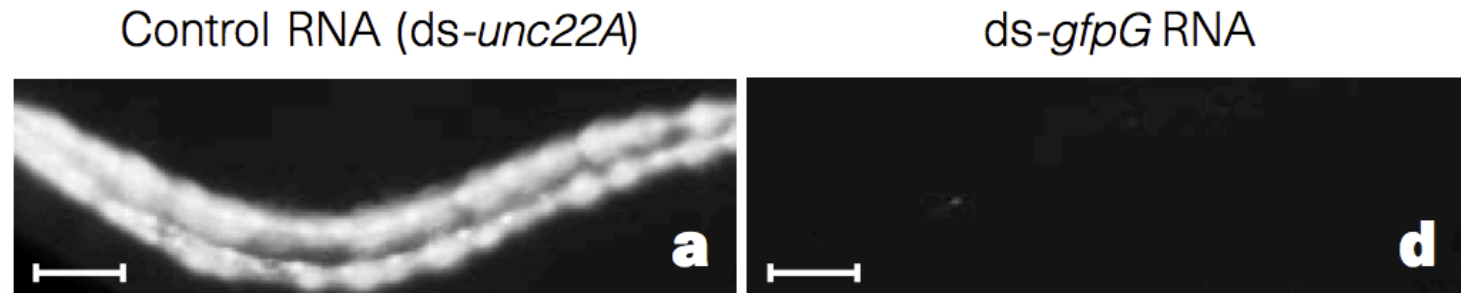


# 1998: double stranded RNA can efficiently repress gene expression

## 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‡

“To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually.”



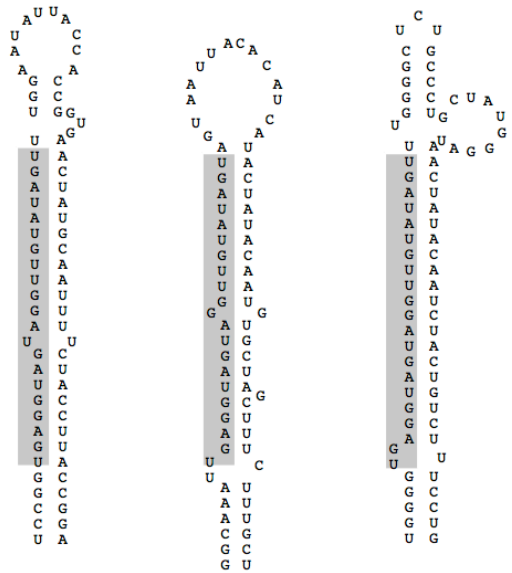
RNAi = RNA interference



# 2000: a second, conserved, microRNA is found

## Conservation of the sequence and temporal expression of *let-7* heterochronic regulatory RNA

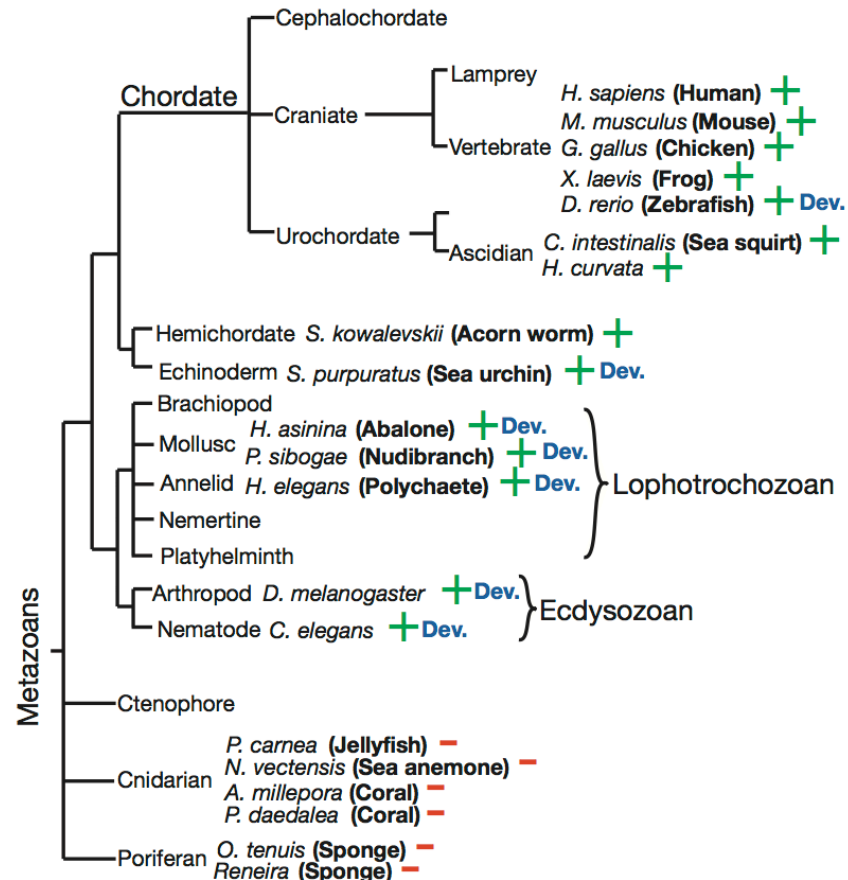
Amy E. Pasquinelli<sup>††</sup>, Brenda J. Reinhart<sup>\*†</sup>, Frank Slack<sup>‡</sup>, Mark Q. Martindale<sup>§</sup>, Mitzi I. Kuroda<sup>||</sup>, Betsy Maller<sup>‡</sup>, David C. Hayward<sup>¶</sup>, Eldon E. Ball<sup>¶</sup>, Bernard Degnan<sup>#</sup>, Peter Müller<sup>\*</sup>, Jürg Spring<sup>\*</sup>, Ashok Srinivasan<sup>\*\*</sup>, Mark Fishman<sup>\*\*</sup>, John Finnerty<sup>††</sup>, Joseph Corbo<sup>‡‡</sup>, Michael Levine<sup>‡‡</sup>, Patrick Leahy<sup>§§</sup>, Eric Davidson<sup>§§</sup> & Gary Ruvkun<sup>\*</sup>



*C. elegans*

*D. melanogaster*

*H. sapiens* chr22





# 2001: many microRNAs are found in various animals

## **An Extensive Class of Small RNAs in *Caenorhabditis elegans***

Rosalind C. Lee and Victor Ambros\*

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

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

Using:

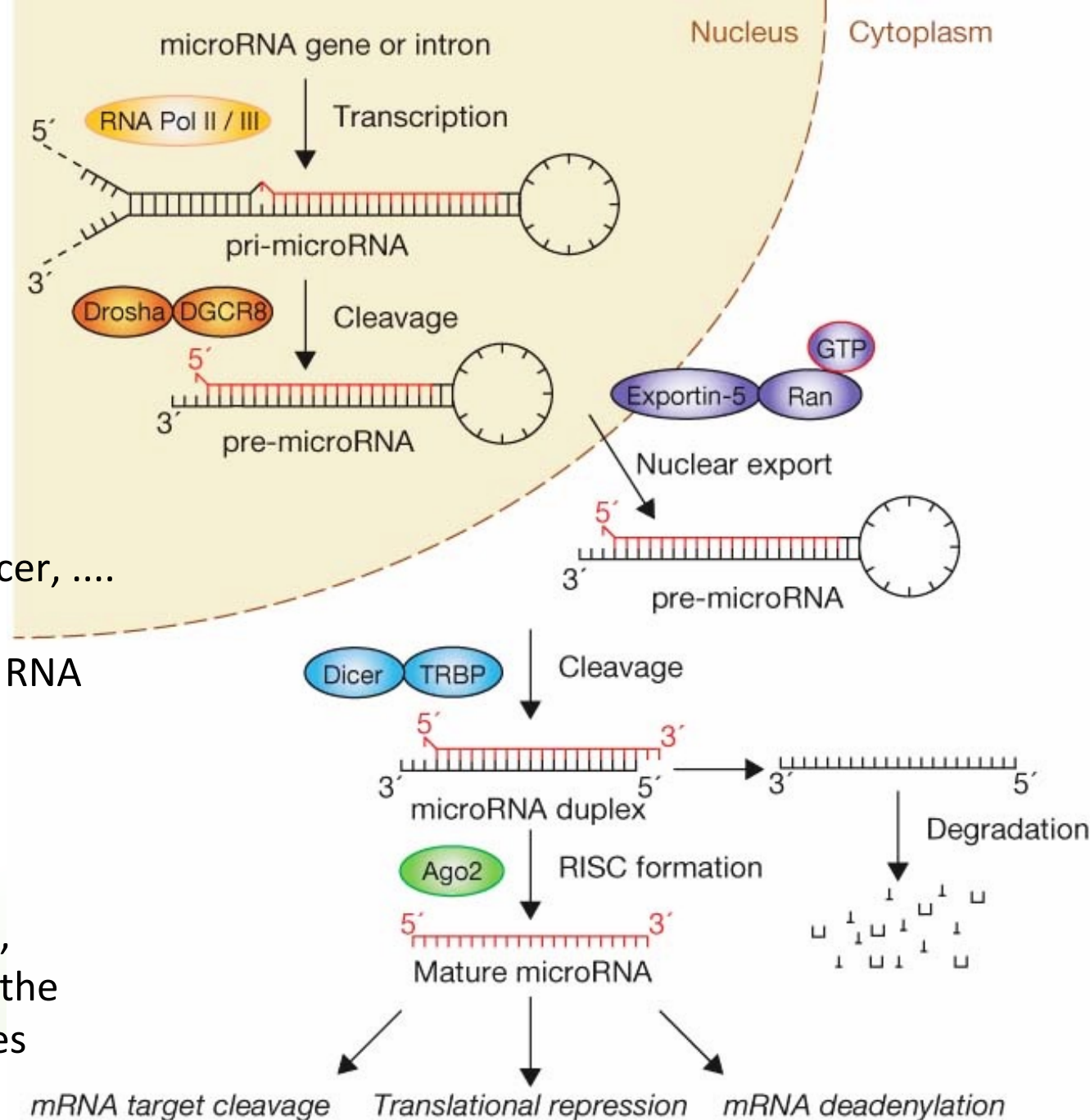
- RNA structure prediction
- Comparative genomics
- (low throughput) sequencing

## **Identification of Novel Genes Coding for Small Expressed RNAs**

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



# microRNA biogenesis



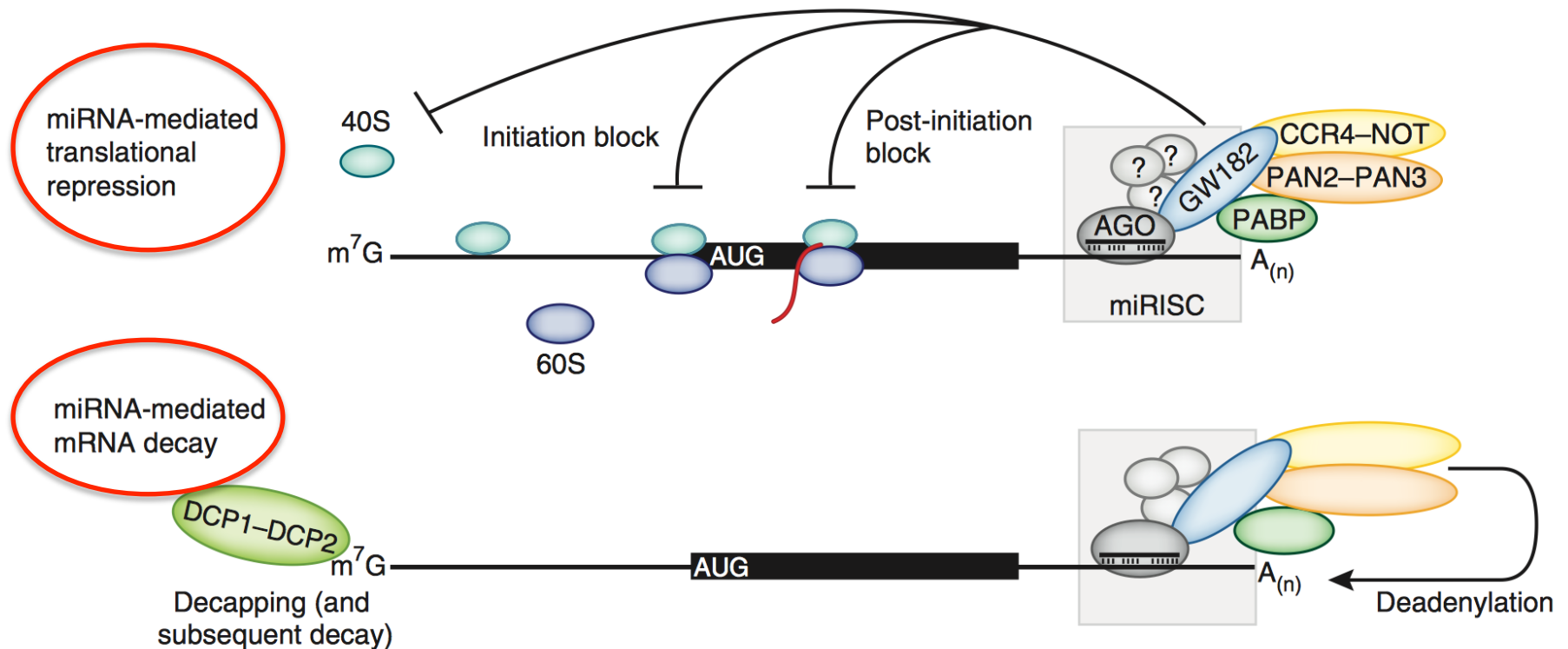
- Many enzymes etc. are involved: Drosha, Exp5, Dicer, ....

- The end result is a ~22nt RNA loaded into an Argonaute complex.

- The microRNA directs Argonaute to target genes, through base pairing with the 3'UTR (pos 2-8). This causes repression.



# Target repression by microRNAs



(This is in animals. microRNAs in plants work differently.)

(Fabian, NSMB, 2012)







# MicroRNA target prediction

- Besides seed pairing, other features are used in the target predictions:
  - Conservation (conserved target sites are more likely to be functional)
  - mRNA structure (it's hard for a microRNA to interact with a highly structured target mRNA)
  - Sequences around the target site (AU rich sequences around targets?)
- Many programs exist for microRNA target prediction (targetScan, mirSVR, PicTar, ..)
- These are not perfect. Target prediction is hard, and a lot of details about the mechanism are still not known.



# MicroRNAs in animal genomes

- There are typically hundreds or thousands microRNAs in animal genomes:
  - Fly: ~300 microRNA loci
  - Mouse: ~1200 microRNA loci
  - Human: ~1900 microRNA loci
- A single locus can produce multiple microRNA forms (called isomirs).
- In a given tissue, their expression can range over 6 orders of magnitude (a few to a few million reads)



# microRNAs regulate many biological processes and are involved in disease

- Development
- Stress response
- Cancer
- Cardiovascular disease
- Inflammatory disease
- Autoimmune disease

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[< Prev](#) | [Table of Contents](#)

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## REPORT

### MicroRNA control of protein expression noise

Jörn M. Schmiedel<sup>1,2,3</sup>, Sandy L. Klemm<sup>4</sup>, Yannan Zheng<sup>3</sup>, Apratim Sahay<sup>3</sup>, Nils Blüthgen<sup>1,2,\*†</sup>,  
Debora S. Marks<sup>5,\*†</sup>, Alexander van Oudenaarden<sup>3,6,7,\*†</sup>

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ABSTRACT

EDITOR'S SUMMARY

MicroRNAs (miRNAs) repress the expression of many genes in metazoans by accelerating messenger RNA degradation and inhibiting translation, thereby reducing the level of protein. However, miRNAs



## 2. Small RNA sequencing



# Sequencing

- Small RNA sequencing is similar to mRNA sequencing, but:
  - There is no poly-A selection. Instead RNA fragments are size selected (typically less than 35 nucleotides, to avoid contamination by ribosomal RNA).
  - Low complexity libraries → more sequencing problems
  - FastQC results will look strange:
    - Length
    - Nucleotide content
    - Sequence duplication



# Pre-processing of small RNA data I

- Since we are sequencing short RNA fragments, adaptor sequences end up in the reads too.
- Many programs available to remove adaptor sequences (cutadapt, fastx\_clipper, Btrim..)
- We only want to keep the reads that had adaptors in them.

GTTTCTGCATTT**TCGTATGCCGTCTTCTGCTTGAA**  
GTGGGTAGAACTTTGATTAAT**TCGTATGCCGTCTT**  
GTTTGTAATTTCTGA**TCGTATGCCGTCTTCTGCTT**  
GAATATATATAGATATATACATACTTATCGT  
GCTGACTTAGCTTGAAGCATAAATGG**TCGTATGCC**  
GACGATCTAGACGGTTTTTCGCAGAATTCTGTTTAT

Adapter missing



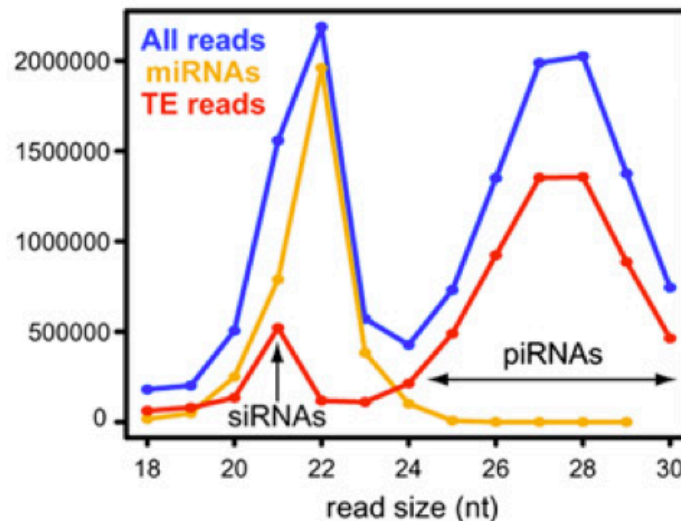
# Pre-processing of small RNA data II

- microRNAs are expected to be 20-25 nt.
  - Short reads are probably not microRNAs, and are hard to map uniquely

GTTTCTGCATTT**TCGTATGCCGTCTTCTGCTTGAA**  
GTGGGTAGAACTTTGATTAAT**TCGTATGCCGTCTT**  
GTTTGTAATTTCTGA**TCGTATGCCGTCTTCTGCTT**  
GCTGACTTAGCTTGAAGCATAAATGG**TCGTATGCC**

← To short

- Long reads are probably not microRNAs

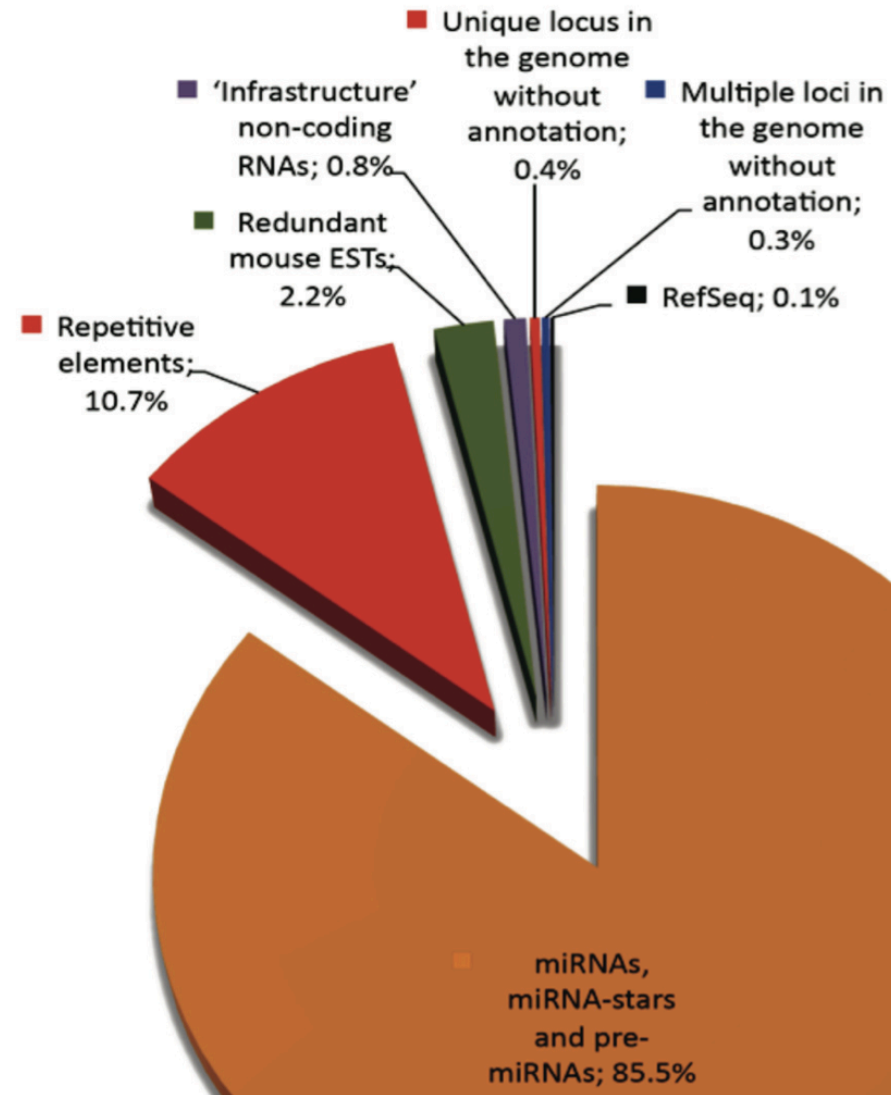




# Pre-processing of small RNA data III

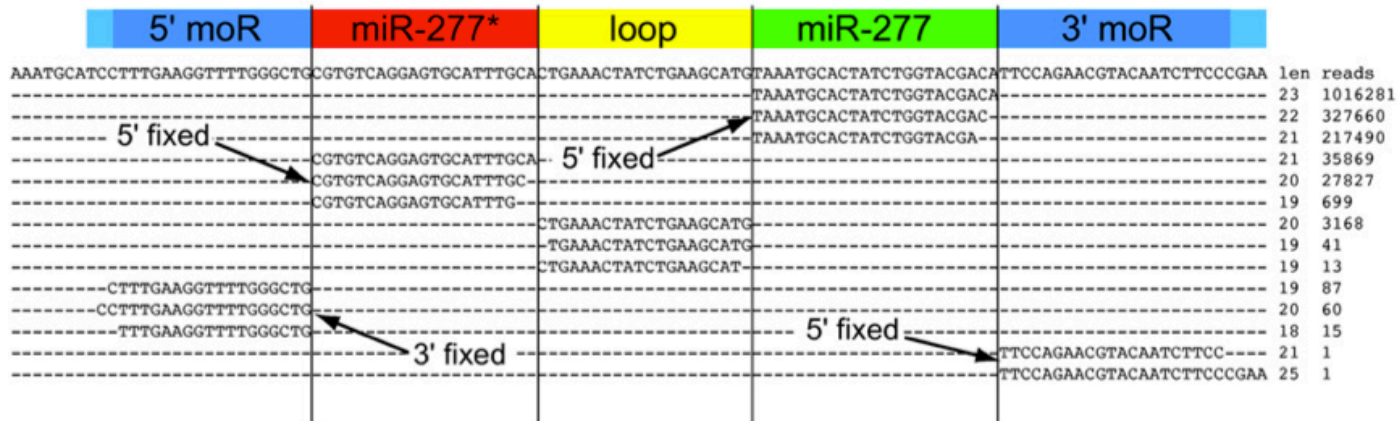
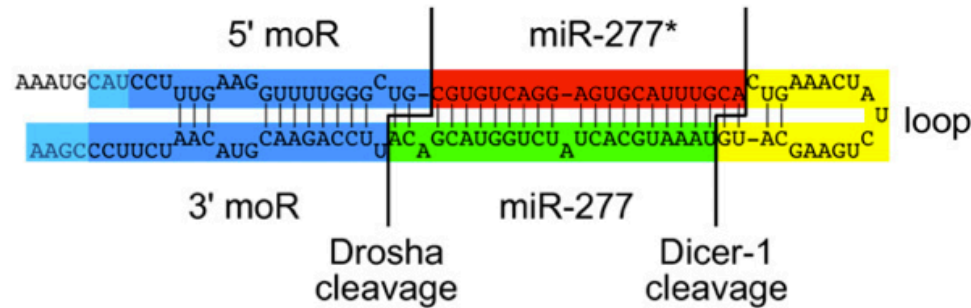
Another useful QC step is to check which loci the reads map to:

(Ling, BMC Genomics, 2011)





# Example of reads mapping to a microRNA locus



(Berezikov et al. Genome Research, 2011.)



# Quantifying small RNA expression I



A

B

C

CGTGT CAGGAGTGCATTGCA

TAAATGCACTATCTGGTACGACA

- Count all reads mapping to a locus? - The simplest option, usually good for profiling.
- Count reads from each hairpin arm? - Useful if we want to correlate this with expression of target genes, or do more careful profiling.
- Only count reads that exactly match the mature microRNA? - Not a good idea, because we will miss variants



# Quantifying small RNA expression II

- microRNAs from the same family can be very similar (or identical)
  - How treat this:
    - Keep in mind that some microRNAs are hard to separate.
    - If a read maps to several N loci, count  $1/N$  read at each locus.
    - ...



# Error sources

- Different chemistries and protocols can have different effects on expression measurements.
  - We only get a few different sequences from each microRNA, so any biases can have big effects. (In normal RNA-seq each gene generates many different reads, so this is not a big problem)
  - Normalization doesn't fix these problems → it's hard to compare data from different platforms etc.
- The amount of starting material can influence the results:

Molecular Cell

## Letter to the Editor

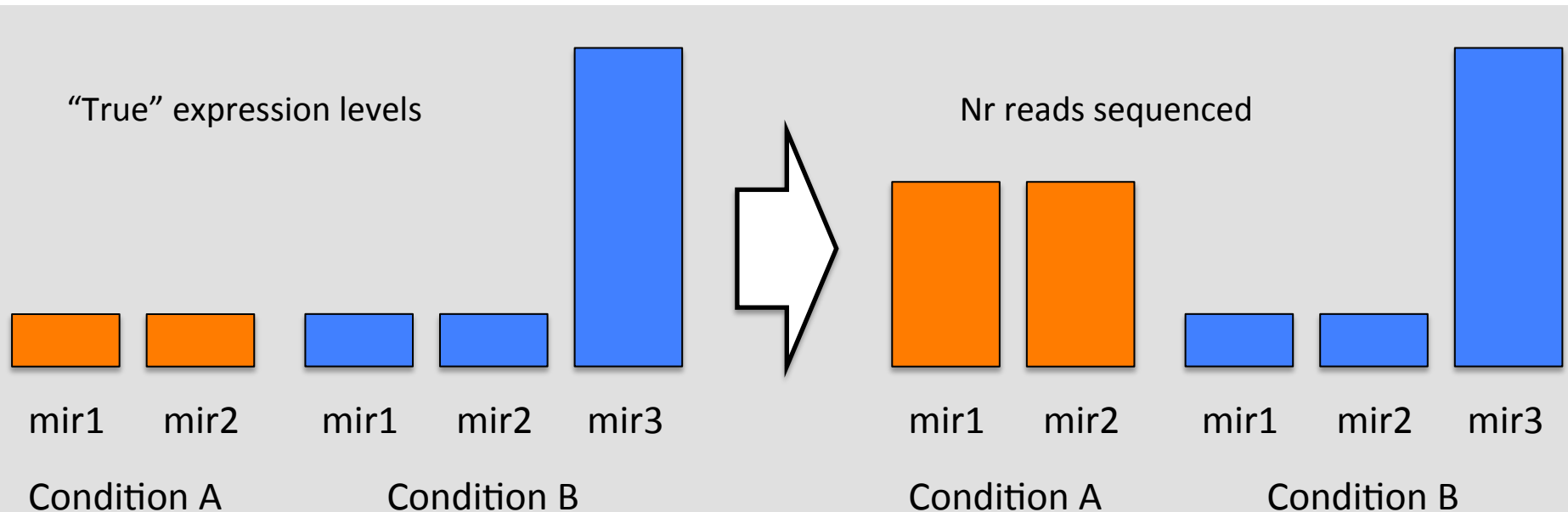
### **Short Structured RNAs with Low GC Content Are Selectively Lost during Extraction from a Small Number of Cells**

In our recent paper (Kim et al., 2011), we reported that a subset of microRNAs certain miRNAs may be lost during RNA preparation depending on the protocol



# Normalizing small RNA expression levels I

- Only a few loci, and huge differences in expression levels → a few miRNAs can account for the majority of all reads, and skew expression levels of all microRNAs.



- Since many reads are used to sequence mir3 in condition B, fewer are available for mir1 and mir2.
- Normalization needs to deal with this situation. Simply scaling read counts by the total number mapped reads will not solve this problem.
- (Spike-in are always useful for normalization.)



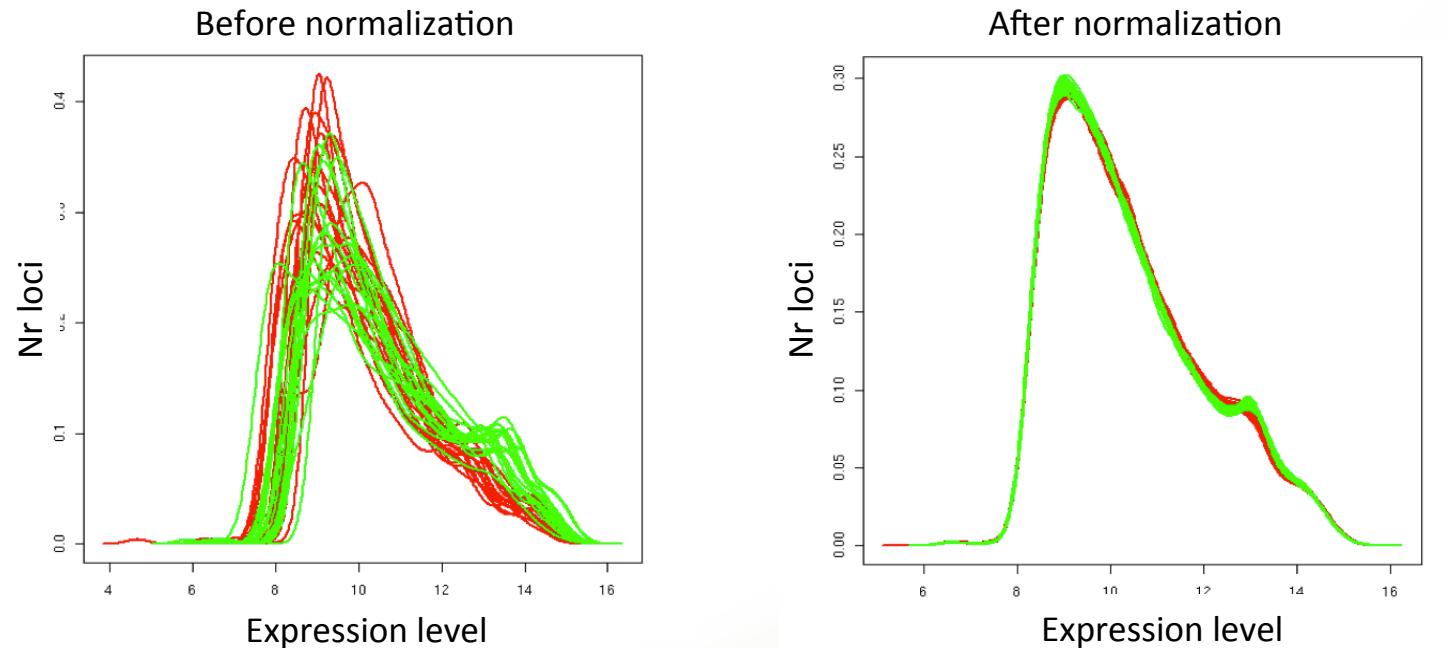
# Normalizing small RNA expression levels II

- Different methods for normalization
  - TMM (“trimmed mean of M-values”) normalization (Robinson et al. 2010, Genome Biology, McCormick et al. 2011, Silence)
  - In short, TMM normalization works like this:
    - Compute log ratios of all microRNA (“M-values”)
    - Remove (“trim”) the highest and the lowest log-ratios, and the highest and lowest expressed microRNAs.
    - Use a mean of the remaining log-ratios to compute the scaling factors
  - The underlying assumption is that most microRNAs have similar expression levels in the different samples, and should have similar expression levels after normalization.



# Normalizing small RNA expression levels III

- Quantile normalization (Garmire et al. RNA, 2012)
  - The underlying assumption is that the overall expression distribution is the same in all samples.



- Many other methods exist, developed for RNA-seq or microarrays.
- No consensus about which method to use → always good to try a few different methods.



# Differential expression

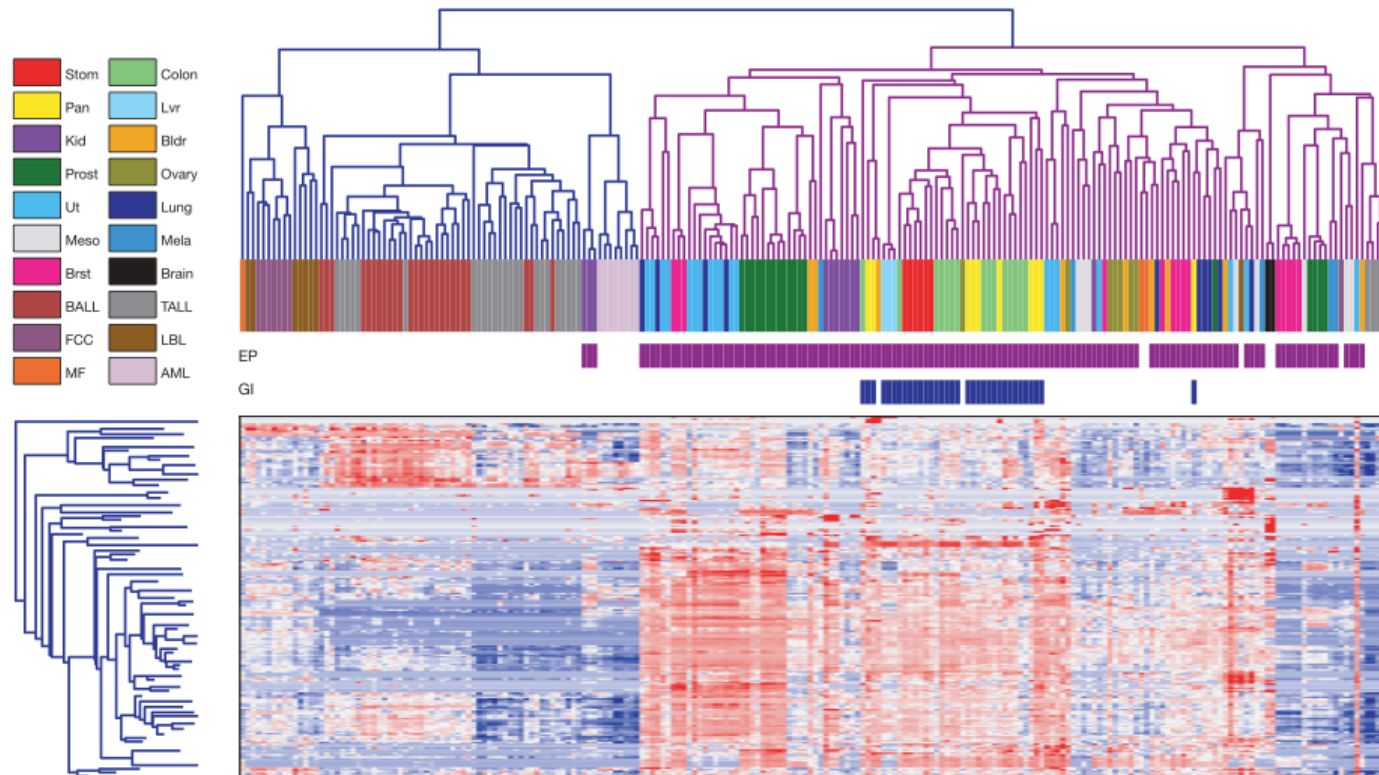
- After the expression levels have been properly normalized, methods for RNA-seq differential expression can be used.
  - ANOVA, t-tests
  - DeSeq, edgeR, voom, limma, etc..
  - No consensus on which method is best.
- Keep in mind: Since microRNA quantification is less reliable than normal RNA-seq:
  - More replicates are needed.
  - More validation experiments are required (Northern blots, in-situ hybridization, etc.).
  - Use caution when interpreting results!



# 3. What can we learn from microRNA expression analysis?



# MicroRNA expression profiles classify human cancers



microRNA expression profiles cluster according to cancer type.

(Lu et al. Nature 2005)



# microRNA profiles can be used to distinguish cancer subtypes

**Table 1. Cancer subtypes that can be distinguished by microRNA or miRNA profiles**

Cancer type	miRNAs <sup>a</sup>	Ref.
<b>Breast</b>		
ER status	miR-26a/b, miR-30 family, miR-29b, miR-155, miR-342, miR-206, miR-191	[38–40,42]
PR status	let-7c, miR-29b, miR-26a, miR-30 family, miR-520g	[41,42]
HER2/ <i>neu</i> status	miR-520d, miR-181c, miR-302c, miR-376b, miR-30e	[38,41]
<b>Lung</b>		
Squamous vs non-squamous cell	miR-205	[33]
Small cell vs non-small cell	miR-17-5p, miR-22, miR-24, miR-31	[32]
<b>Gastric</b>		
Diffuse vs intestinal	miR-29b/c, miR-30 family, miR-135a/b	[35]
<b>Endometrial</b>		
Endometrioid vs uterine papillary	miR-19a/b, miR-30e-5p, miR-101, miR-452, miR-382, miR-15a, miR-29c	[37]
<b>Renal</b>		
Clear cell vs papillary	miR-424, miR-203, miR-31, miR-126	[34,36]
Oncocytoma vs chromophobe	miR-200c, miR-139-5p	[36]
<b>Myeloma</b>		
with t(14;16)	miR-1, miR-133a	[60]
with t(4;14)	miR-203, miR-155, miR-375	[60]
with t(11;14)	miR-125a, miR-650, miR-184	[60]
<b>Acute myeloid leukemia</b>		
with t(15;17)	miR-382, miR-134, miR-376a, miR-127, miR-299-5p, miR-323	[52]
with t(8;21) or inv(16)	let-7b/c, miR-127	[52]
with <i>NPM1</i> <sup>b</sup> mutations	miR-10a/b, let-7, miR-29, miR-204, miR-128a, miR-196a/b	[51,52]
with <i>FLT3</i> ITD	miR-155	[51,52,54]
<b>Chronic lymphocytic leukemia</b>		
ZAP-70 levels and IgVH status	miR-15a, miR-195, miR-221, miR-155, miR-23b	[50]
<b>Melanoma</b>		
with BRAF V600E	miR-193a, miR-338, miR-565	[56]

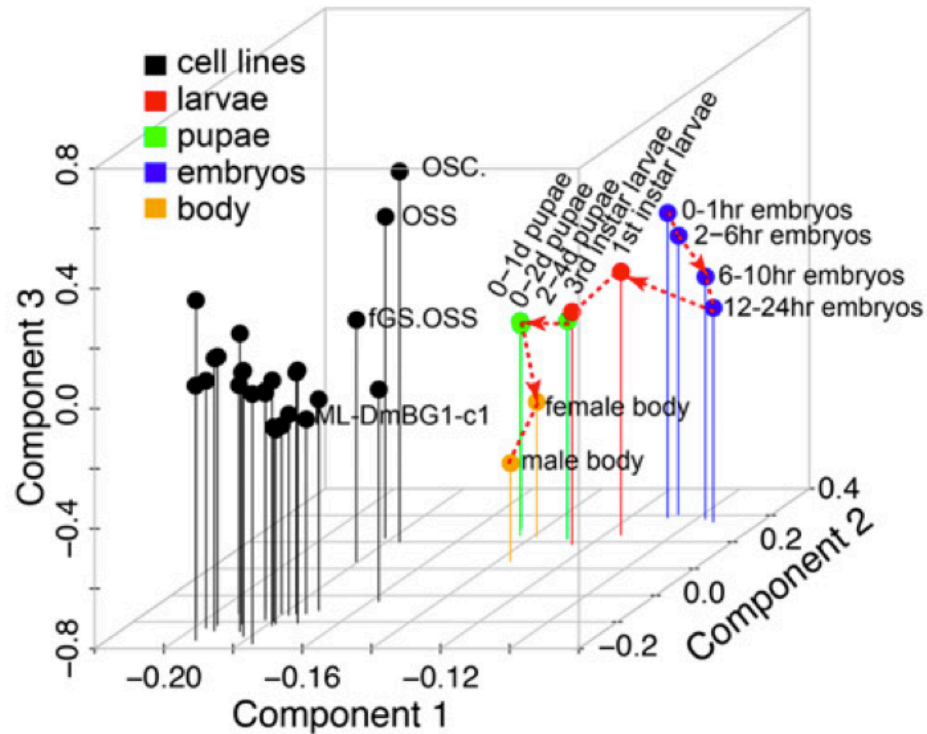
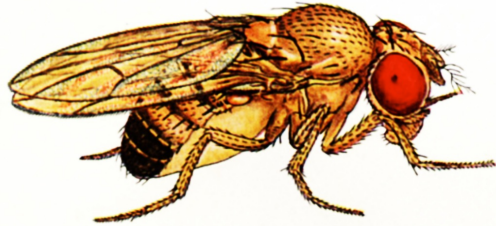
<sup>a</sup>Not all distinguishing miRNAs are represented in this table.

<sup>b</sup>nucleophosmin 1.

(Chan et al. Trends in Molecular Medicine, 2010)



# microRNA profiles in cell lines vs tissues



PCA plot showing that microRNA profiles in most cell lines are more similar to each other than to normal tissues.

(Wen et al. Genome Research 2014)



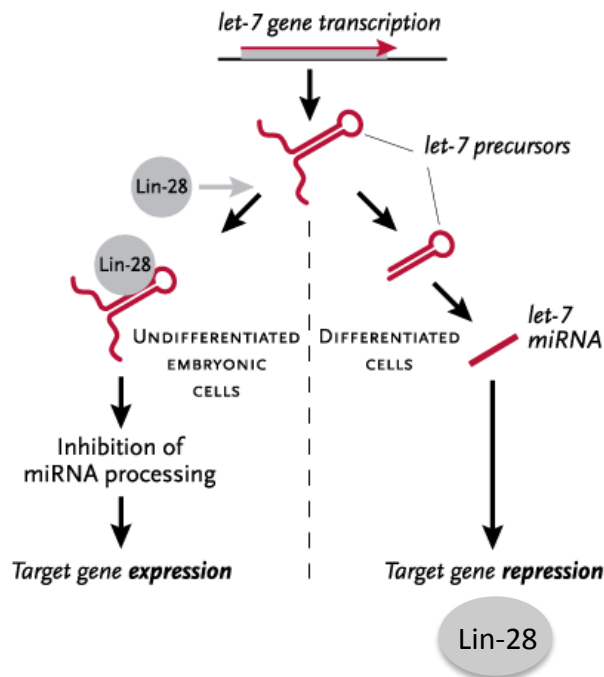
# microRNA regulation in cancer and development

The microRNA let-7 is involved in regulating developmental timing, and is a tumor suppressor.

let-7 is repressed by the protein lin-28.

This repression is post-transcriptional, and is associated with uridylation at the 3' end of let-7.

Using sequencing, such uridylation can be observed for many microRNAs.



(It's also possible to see microRNA editing.)

		<u><i>n</i> clones</u>
<i>let-7*</i>	5'CUAUGCAAUUUUCACCUUACC 3'	23
	5'CUAUGCAAUUUUCACCUUACC <u>U</u> 3'	11
	5'CUAUGCAAUUUUCACCUUACC <u>UU</u> 3'	2

*let-7* genomic 5'ACCGGUGAACUAUGCAAUUUUCACCUUACCGG 3'

(Lehrbach et al. NSMB, 2009)



# Relation between microRNAs and their predicted targets

It is possible to find statistical correlations between expression of microRNAs and of their predicted target genes.

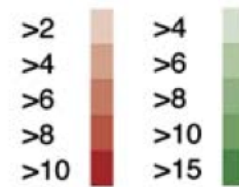
Example: mir-124 is expressed in the nervous system.

Neural genes are depleted for mir-124 target sites in the 3' UTRs.

Genes expressed in epidermis, muscle, gut etc. are enriched for mir-124 sites.

But we cannot be sure that this is true, since we are only looking at predicted targets!

significance (-log combined p values)  
of category avoidance (red)  
and enrichment (green)



	stage 11-12	stage 13-16
miR-124	ventral nerve cord prim. plasmotocytes anlage	CNS gonad ventral nerve cord plasmotocytes brain
	all epidermis prim. dorsal epidermis prim. ventral epidermis prim. tracheal prim. foregut prim. clypeo-labral prim. muscle system prim.	epipharynx hindgut dorsal epidermis tracheal system head epidermis ventral epidermis

(Stark et al. Cell, 2005)



# Discovering new small RNA loci

- microRNAs have very specific patterns, when it comes to
  - Read size and mapping
  - RNA structure
  - Conservation
- This makes it possible to find microRNAs using small RNA sequencing data.

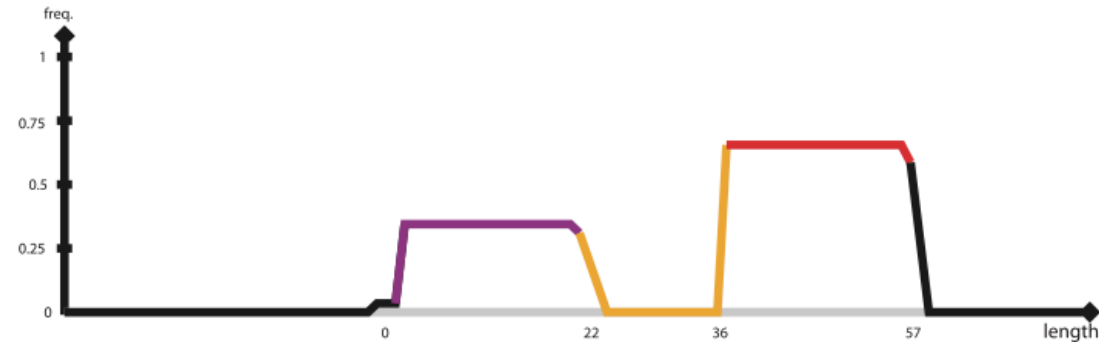


# mirDeep(2)

- The most used program for finding new microRNAs.  
Takes as input:
  - A genome sequence
  - Small RNA sequencing data
  - A set of loci to exclude (optional)
- The basic idea is:
  - Look at sequence data to find a (large) set of possible loci.
  - Look at RNA folding and read mapping patterns to give a score to each candidate
    - Nr reads from both arms
    - Fixed read ends
    - Free energy, base pairing in the hairpin structure, ..



- mirDeep2 is installed on UPPMAX.



Mature

[illegible]

- There are also other programs, e.g. shortStack (Axtell, RNA, 2013) which also finds other small RNAs.

(Friedländer et. al. Nucleic Acids Research, 2011)



# Other strange small RNAs that show up in sequencing data

mirtrons

piRNAs

tRNA fragments

hp-RNAs

TSS-microRNAs

cis-natRNAs

- Some of these are functional
- Some are by products of RNA processing, and can be informative (e.g. microRNA loop sequences).
- Some are probably just “noise”.



# THE END



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