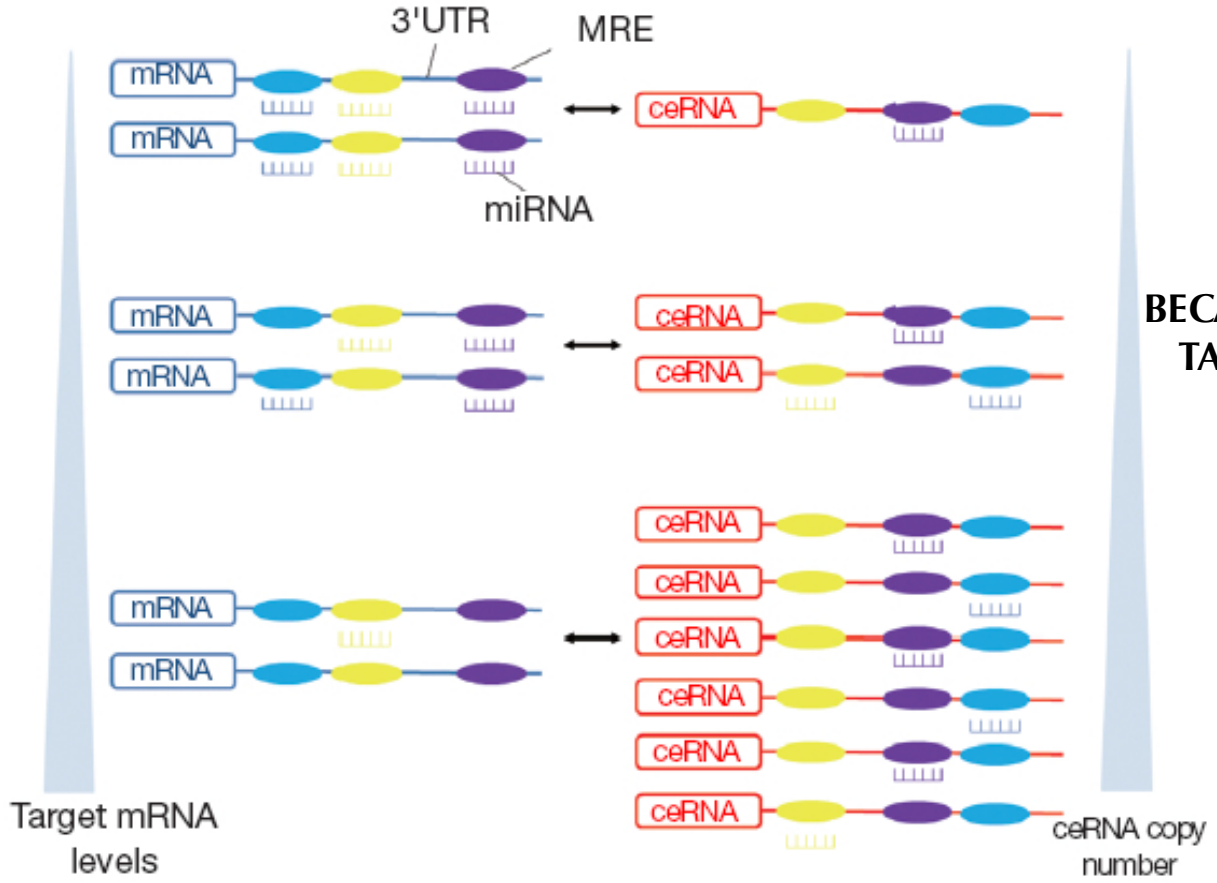
# Circular RNAs (circRNAs) act a stable miRNA sponges

# ceRNAs compete for miRNAs




**The model holds true for all RNAs that share a miRNA binding site =ceRNAs**

**PSEUDOGENES ARE POTENT BECAUSE THEY SHARE MORE THEN 1 miRNA TARGET SITE WITH A CORRESPONDING mRNA FROM AN ANCESTRAL GENE**

**Evolution of ncRNAs to fine-tune the expression of ancestral genes**

# The discovery of a circular RNAs

1. Question: Can miRNAs control the activities of gene promoters
2. Approach: Identify miRNAs that are highly complementary with DNA sequences that are located on vicinity of promoters (ca. +/- 5000nt)
3. CANDIDATE GENE: **CDR1** (intronless) and **miR-617**
4. **HOWEVER: miR-617 targets the template strand of CDR1**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## miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA

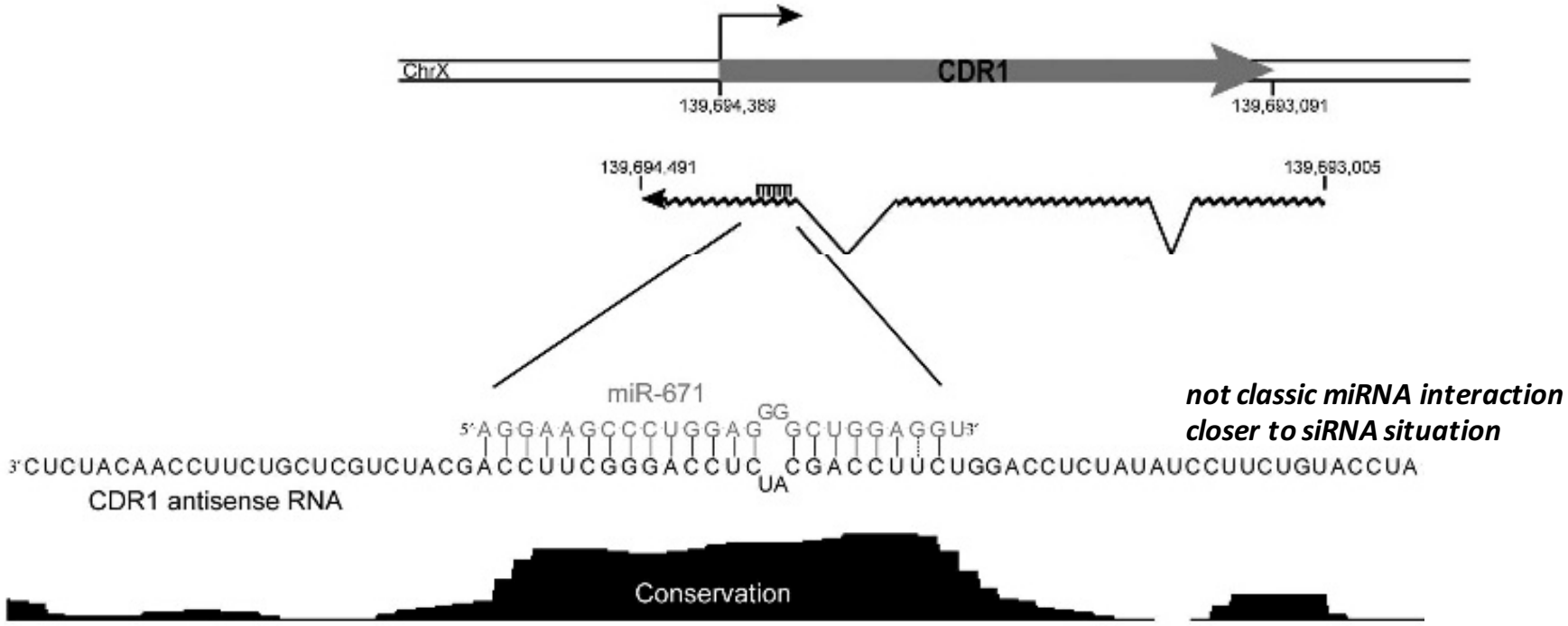
**Thomas B Hansen[1], Erik D Wiklund[1,2], Jesper B Bramsen[1], Sune B Villadsen[1], Aaron L Statham[2], Susan J Clark[2] and Jørgen Kjems[1,*]**

[1]Department of Molecular Biology, Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Aarhus, Denmark and [2]Epigenetics Laboratory, Cancer Program, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia

sites in the 5′ UTR and ORF of m
Orom *et al*, 2008; Tay *et al*, 20(
RISC activity has been detected in
(Langlois *et al*, 2005; Robb *et a*
miRNAs are predominantly nucle
*et al*, 2010), suggesting that miR
biological functions distinct fror
mRNA repression.

# The discovery of a circular RNAs

-CDK1 has an anti-sense transcript
-AS CDK1 contains 2 introns and is spliced
- ca 40% of human genes have evidence for a NATURALLY OCURRING ANTISENSE TRANSCRIPTS (NATs)

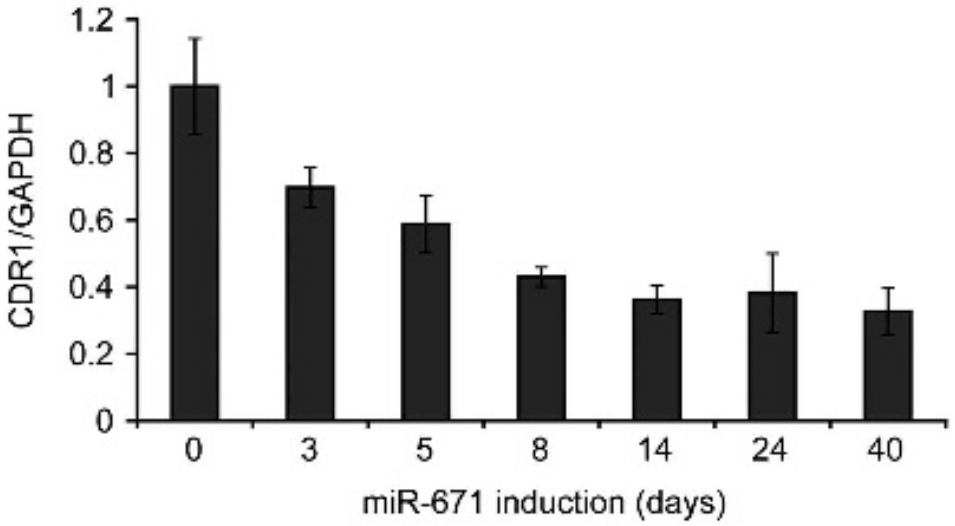



*not classic miRNA interaction*
*closer to siRNA situation*

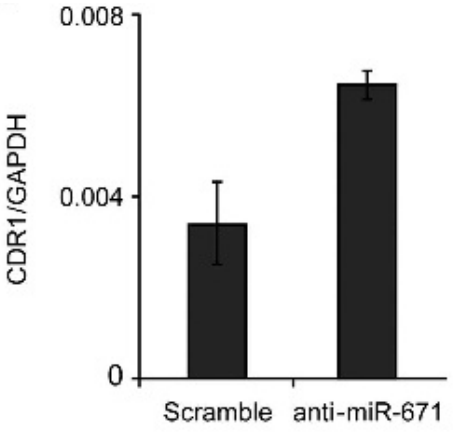
- CDK1 antisense RNA shows highest conservation at miR-671 target site in CDK1 as RNA

# Circ-AS-CDR1 RNA stabilizes sense CDR1 RNA

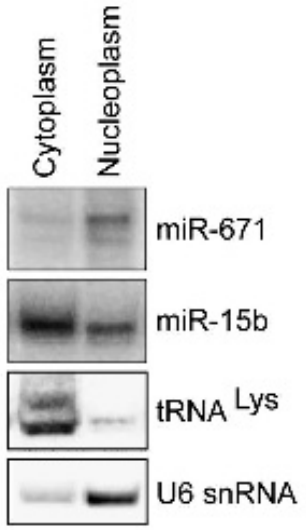
**miR-671 overexpression during 40 day**
**Reduces CDR1 expression**




**miR-671 knock-down**
**Increases CDR1 expression**




**miR-671 is enriched in the nucleus**

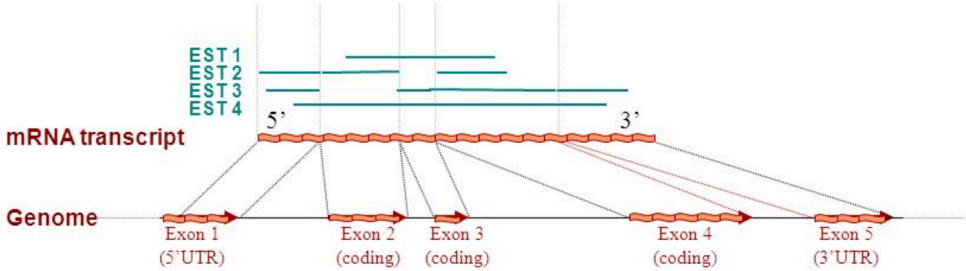



miR-671 ⊣ AS CDR-1 → CDR-1

# AS-CDR1: what transcripts are known….?

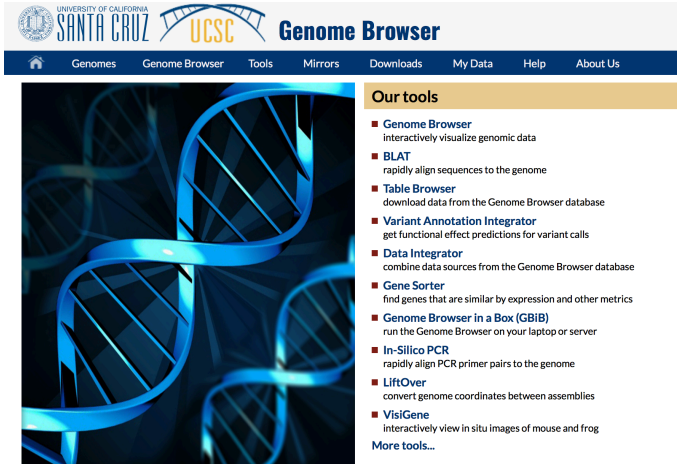
## 1. Sequence alignement: Insert AS-CDR1 in UCSC genome browser: look for ESTs that overlap the AS-CDR1 region

- Mapping of ESTs to the genome via the (predicted) mRNA transcripts
  - map each of the ESTs on the set of (predicted) mRNA transcripts, or genes with known genomic locations
  - align the EST against the genomic fragment containing the gene for the EST with an exact alignment method



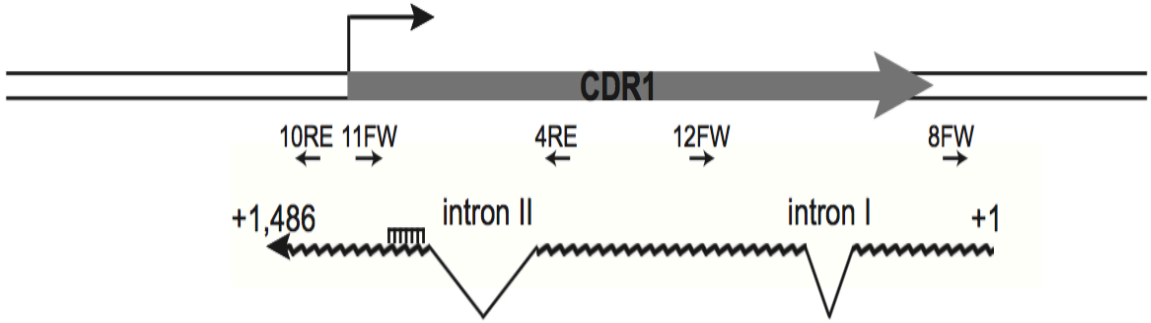

- Faster than exact mapping
- Can be used to improve *existing* gene models, but not to discover new ones

***RNA → RT → (cloning) → seqeuncing***

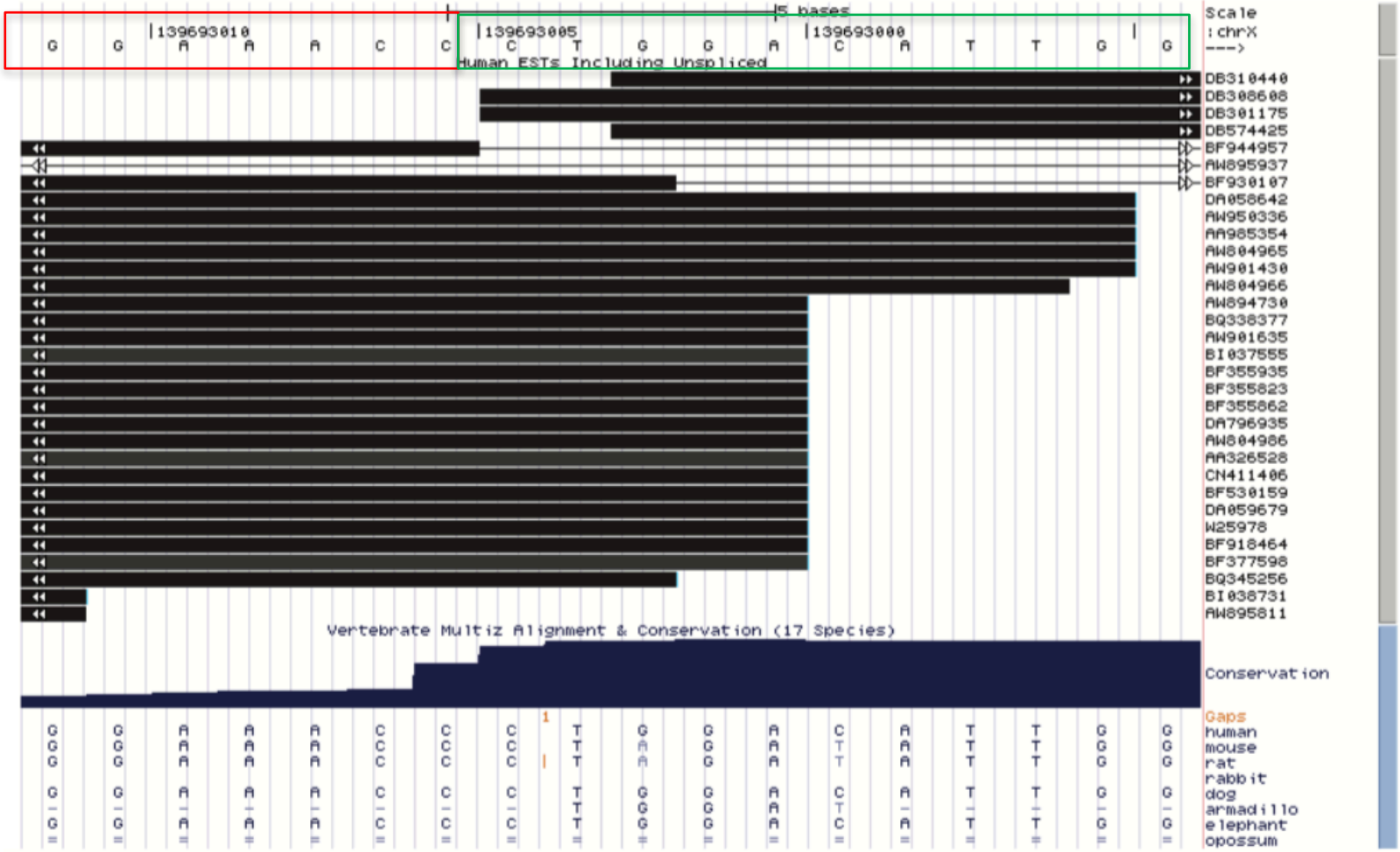



**Easy to use tool for genomics analysis (RNA, DNA, Chromatin, etc…)**

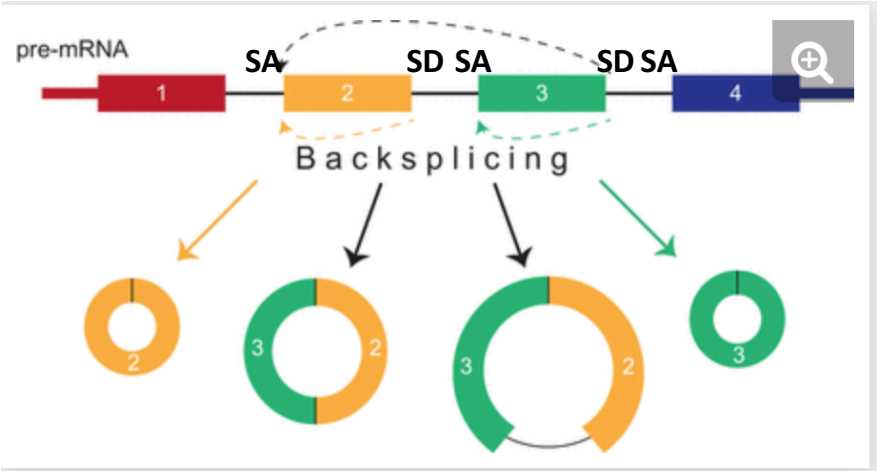
# ESTs exist that overlap the 5’ and 3’ end of AS-CDR1



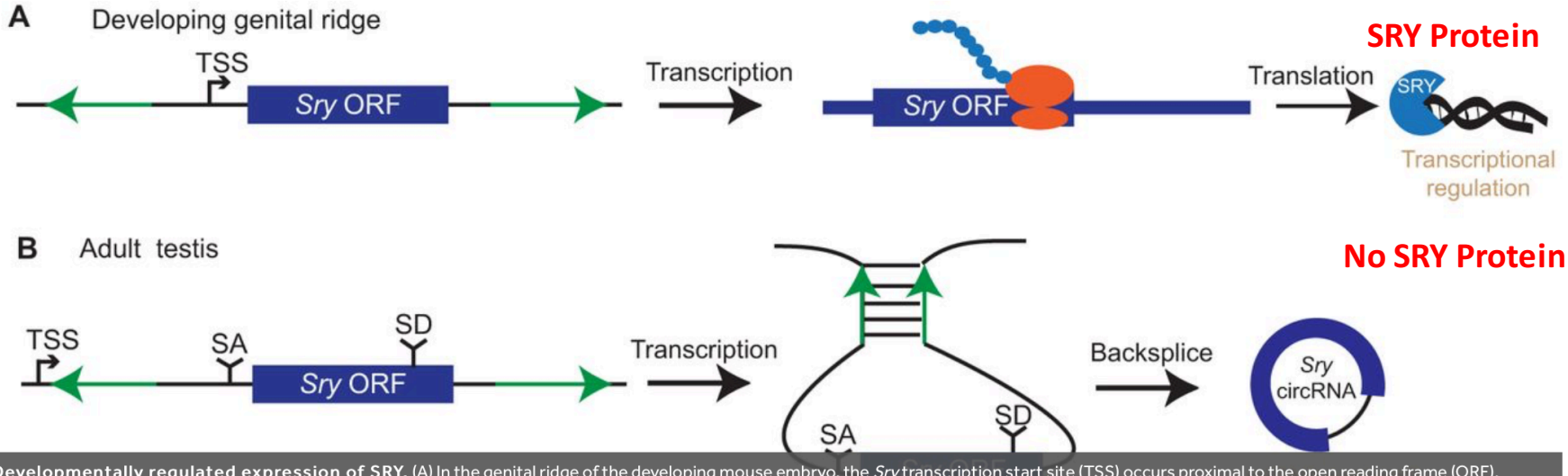

+1 +1486



# “Backsplicing” can produce circular RNAs



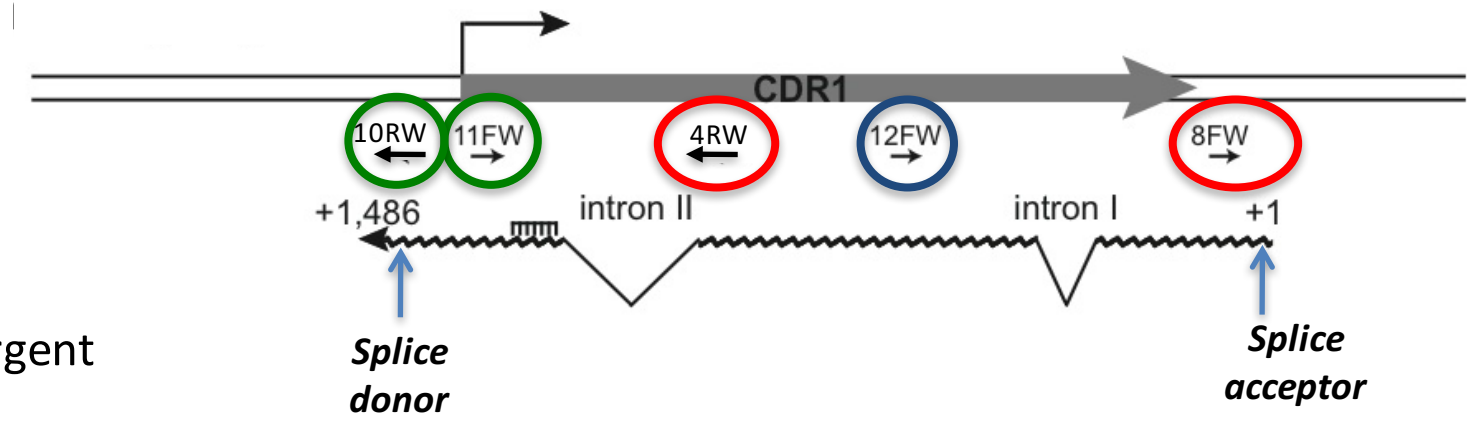

***Most famous example: SRY: Y-linked transcrition factor for male sex development***

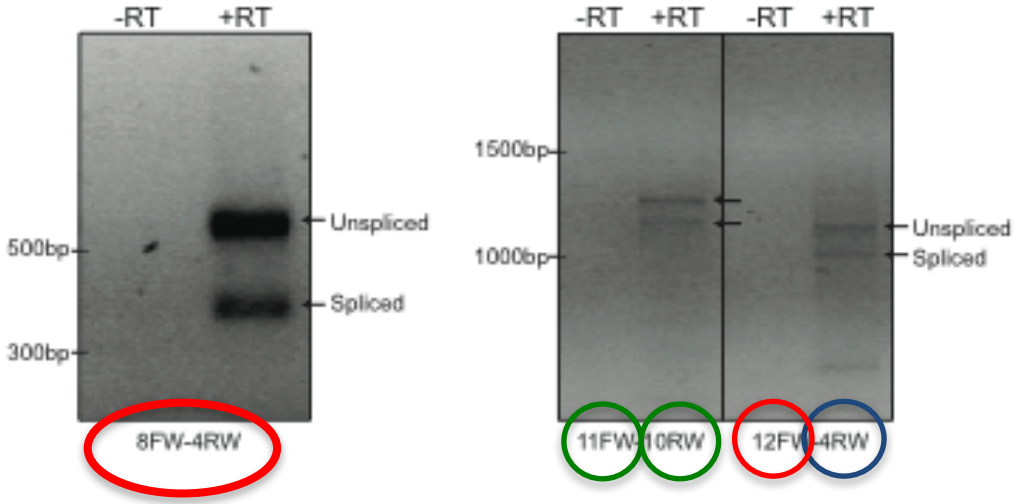



**Developmentally regulated expression of SRY.** (A) In the genital ridge of the developing mouse embryo, the *Sry* transcription start site (TSS) occurs proximal to the open reading frame (ORF), yielding a translatable mRNA that gives rise to SRY protein, a transcription factor involved in sex determination. (B) In the adult testis, the TSS occurs far upstream, yielding a long transcript containing large inverted repeats (green arrows). This transcript is backspliced to form a circRNA, which might function as a miR-138 sponge. SA, splice acceptor; SD, splice donor.

# HOW TO DETCT circRNAS?T

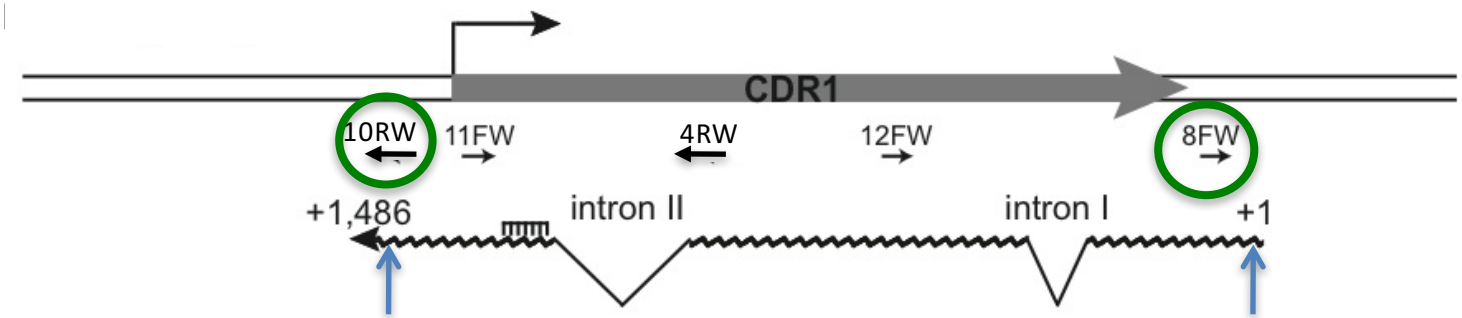
1. RNA + RT
2. PCR using divergent primers
3. Agarose gel
4. To validates sequence identity: Sothern blot using a radioactive CDR1 probe




**anti-sense CDR1 is a circular RNA**

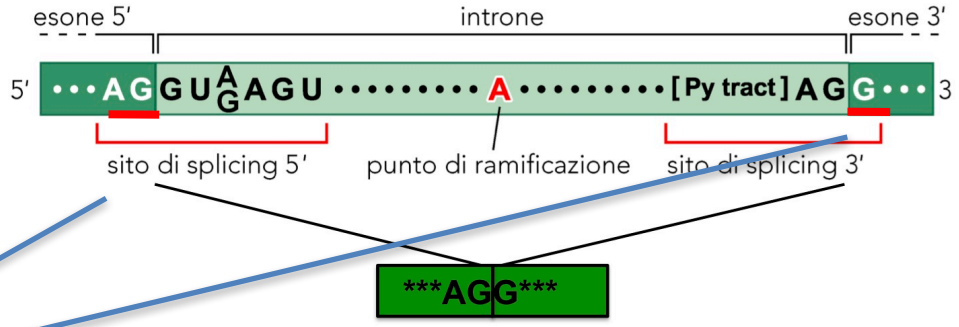
# HOW TO DETCT circRNAS?T



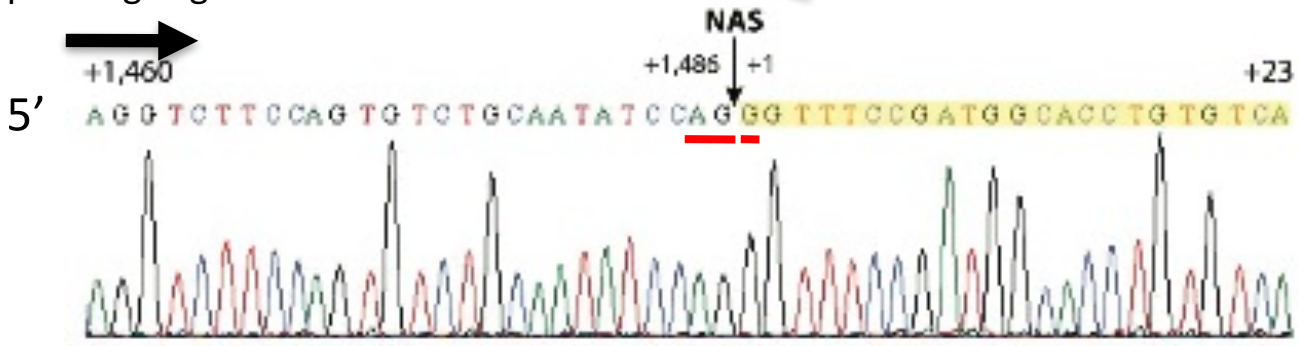

PCR of cDNA with 10RW and 8FW

Subclone PCR fragment in vector

Sequence vector insert




Sequencing oligo




**6. Last step: Sequencing of PCR product**

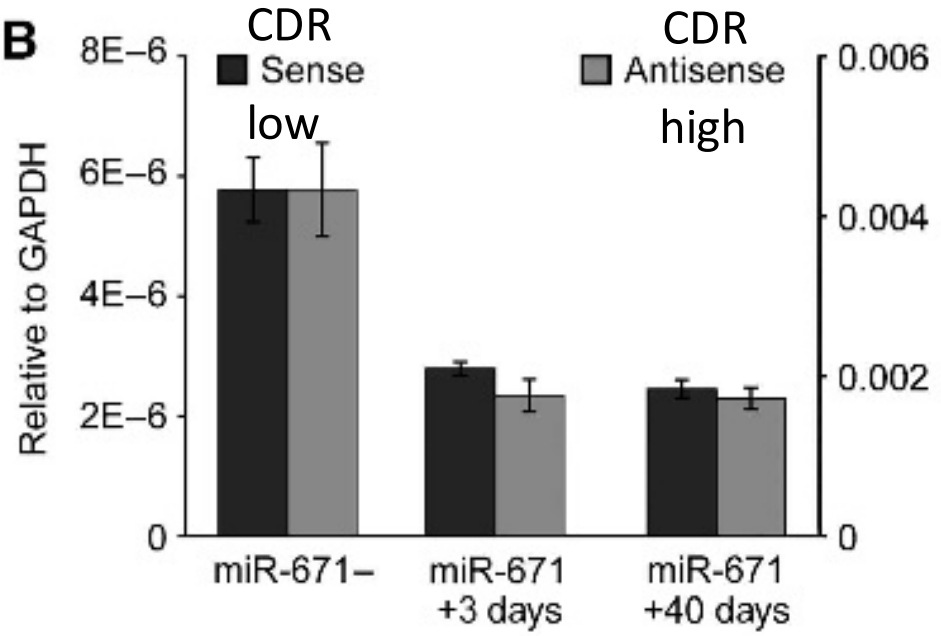
**Splicedonor and Spliceacceptor in CDR1 as RNA are used to make a circular RNA**

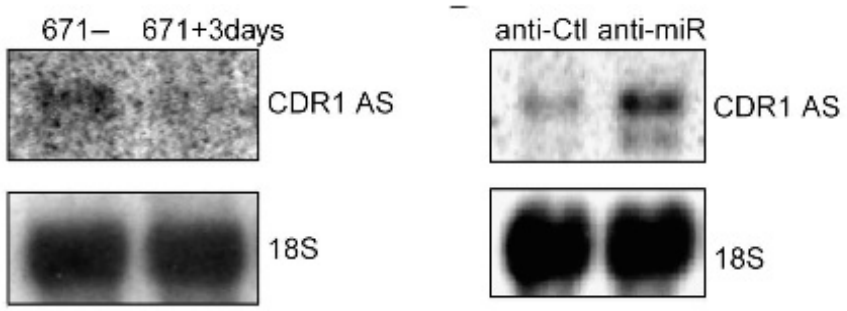
**= NAS**

**non-linear alternative splicing /backsplicing**

# WHAT IS THE FUNCTION OF miR-671/AS-CDR1: EPIGENTIC REGUALTION BY AS CDR1??

**miR-671 overexpression reduces CDR1 but also CDR1 AS expression (AS transcript is the pre-dominant transcript**




Ectopic miR-671 decreases CDR1 as

Reducing endogenous miR-671 increases CDR1 as

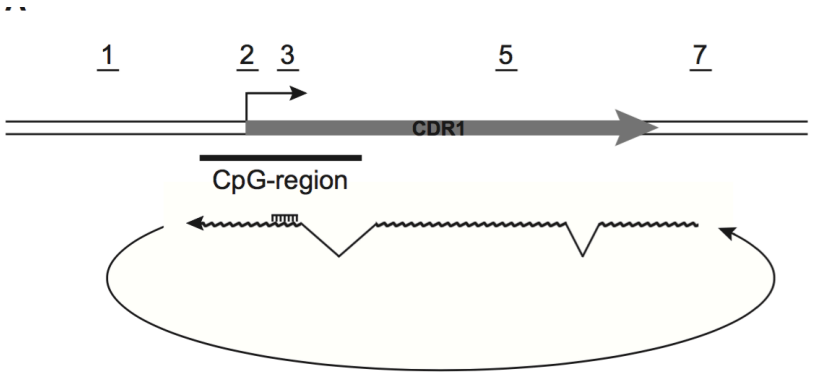
miR-671 ⊣ AS CDR-1 → CDR-1

**Epigenetics on CDKR1 promoter?**

# EPIGENTIC REGUALTION BY AS CDR1??

**HYPOTHESIS:**

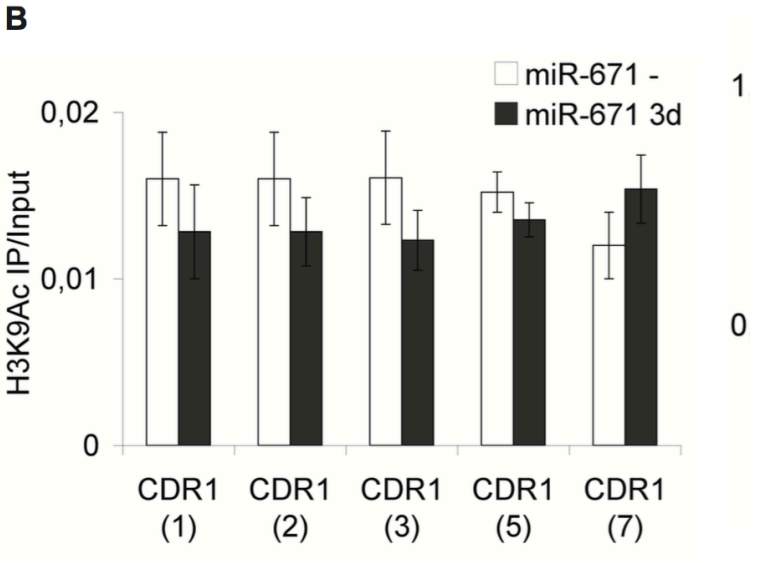
miR-671 and AS CDR1 are involved in epigenetic regulation??

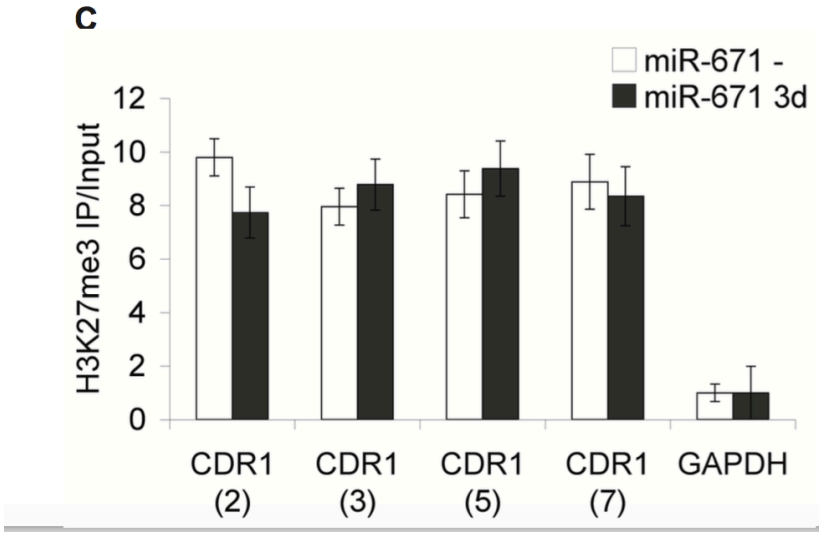



***ChiP using PCR oligos 1-7***

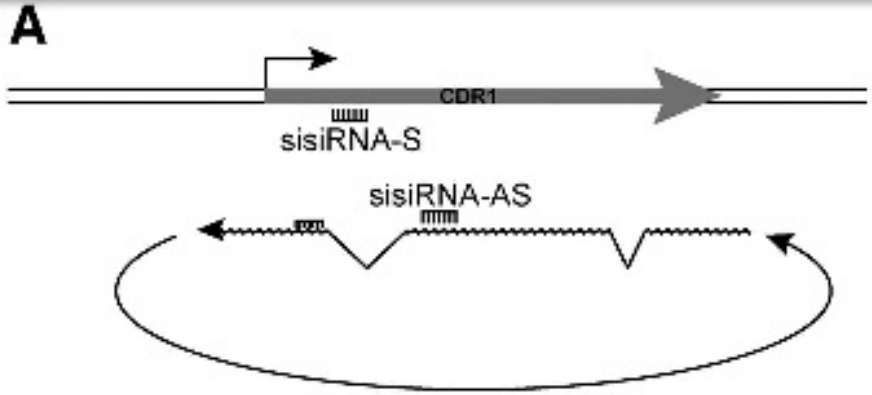
→Answer: NO

→NO CHROMATIN CHANGE



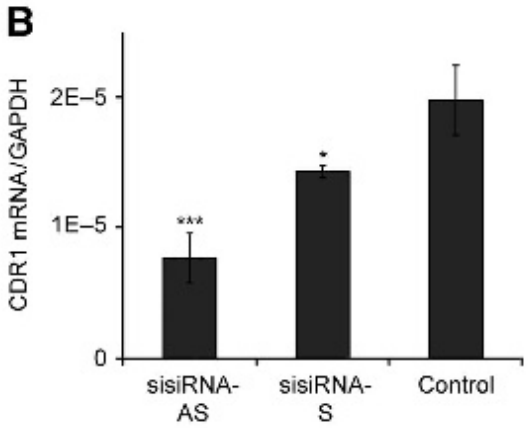



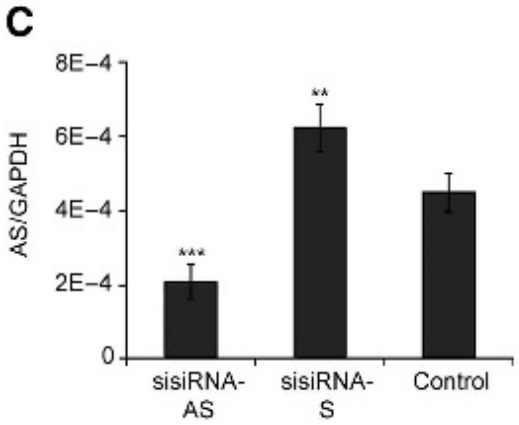
# miRNAs can target nuclear antisense transcripts
# Circ-AS-CDR1 RNA stabilizes sense CDR1 RNA




**RNAi specific for sense and anti-sense RNA**

sisiRNA-S: targets sense CDR-1
sisiRNA-AS: targets AS-CDR-1



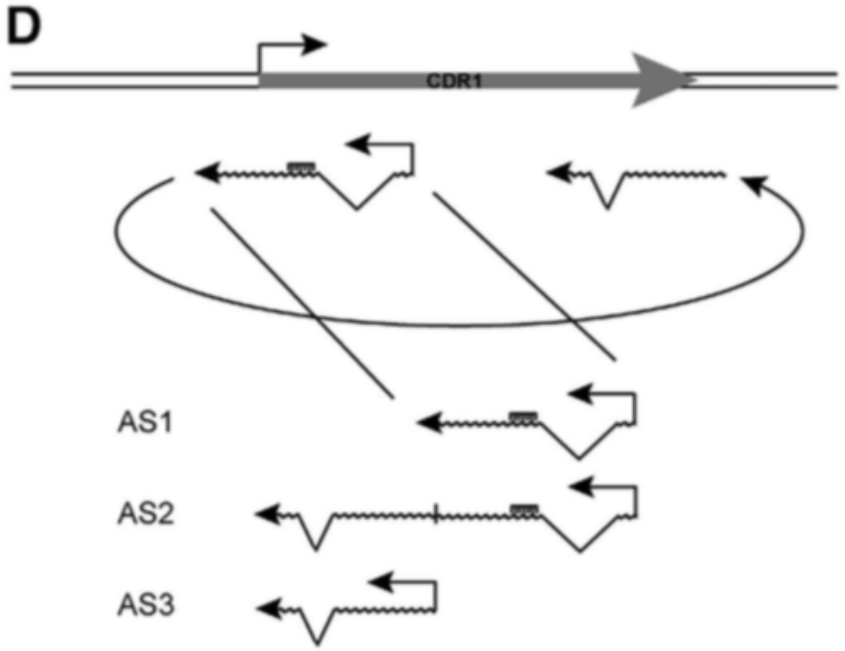

→ AS CDR-1 supports S CDR1 expression
→ miR-671 suppresses AS CDR1 expression

CDR1 sense: reduced by siCDR1 and si-AS-CDR1

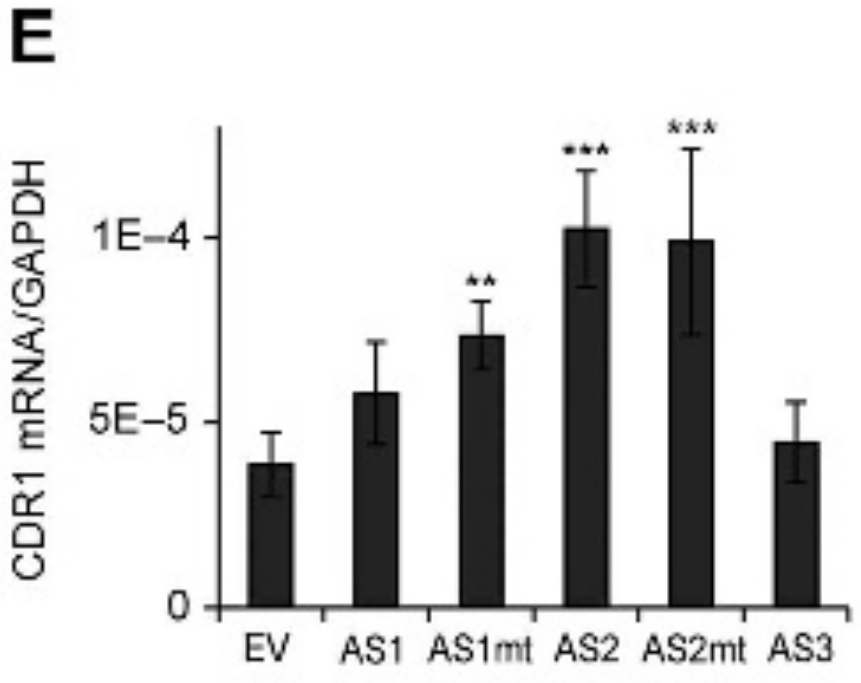
CDR1 anti-sense: reduced only by si-AS-CDR1

miR-671 ⊣ AS CDR-1 → CDR-1

# miRNAs can target nuclear antisense transcripts
# Circ-AS-CDR1 RNA stabilizes sense CDR1 RNA




Expression of engineered: linear transcripts




Overexpression of AS-CDR1 that "mimics" the struture of the circRNA: Reduced sense-CDR-1 expression

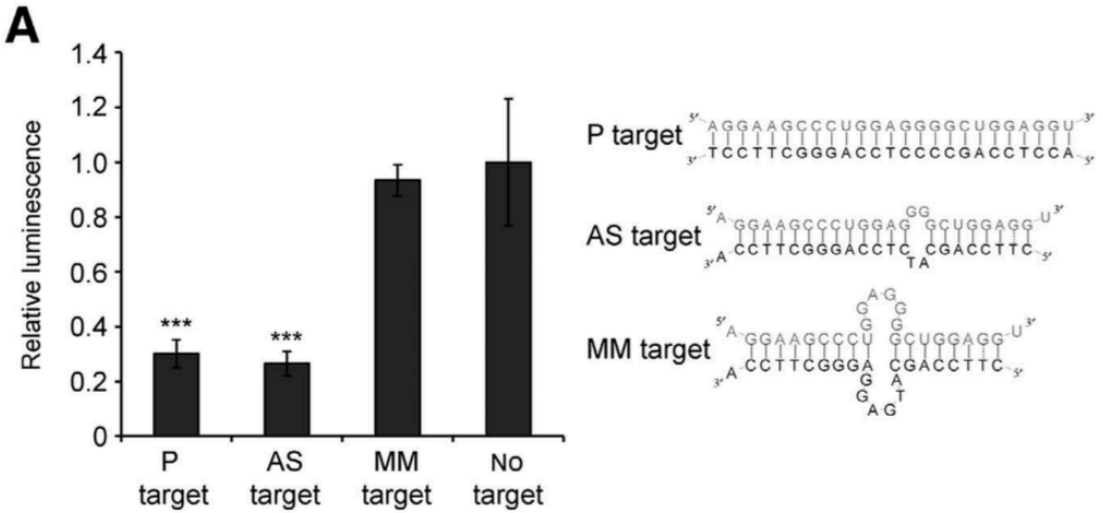
Mutation of the miR-671 target site does not impact on the AS2 effect (miR-671 is not required for AS-CDR1 NAT function

**miR-671** ⊣ **circ-asCDR1 RNA** → **Sense CDR RNA expression**

***The regulatory effect on CDR1 expression comes directly from the sense antisense orientation of transcripts: miR-671 only controls AS-CDR1 expression***

# miR-671 acts as a “siRNA” to control CDR1 AS RNA expression?

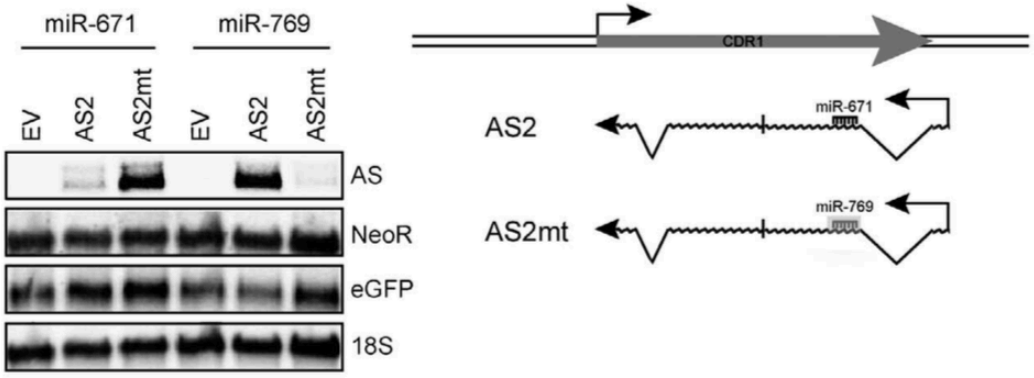
**Luciferase assay: AS-CDR1 seqeucne variants (below) fused to luciferase + mi-671**




Perfect match

Endogenous situation

Missmatch situation

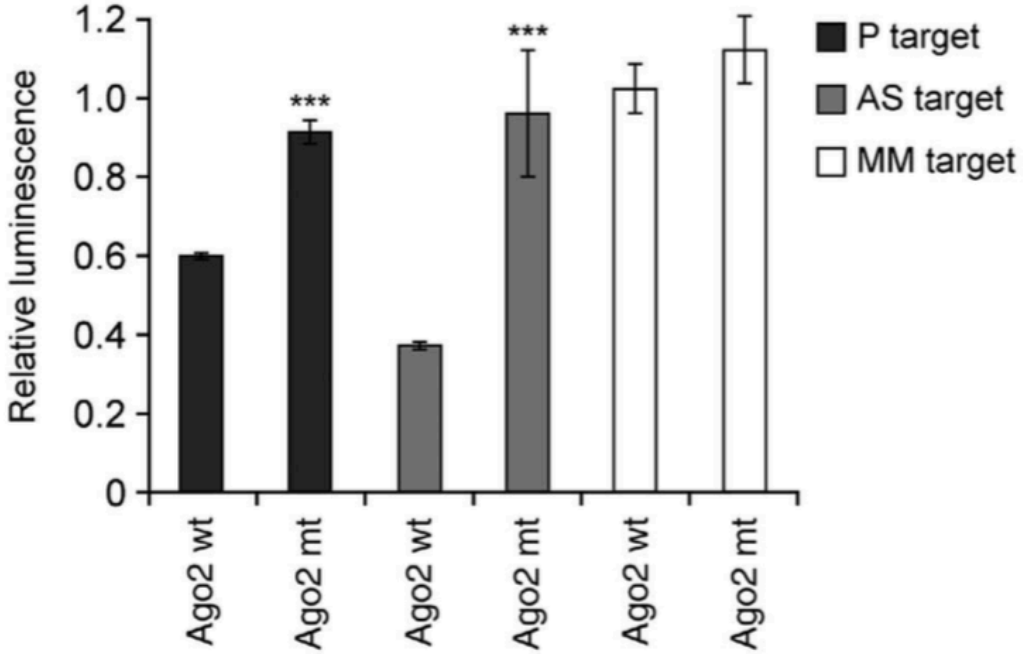



Northern blot

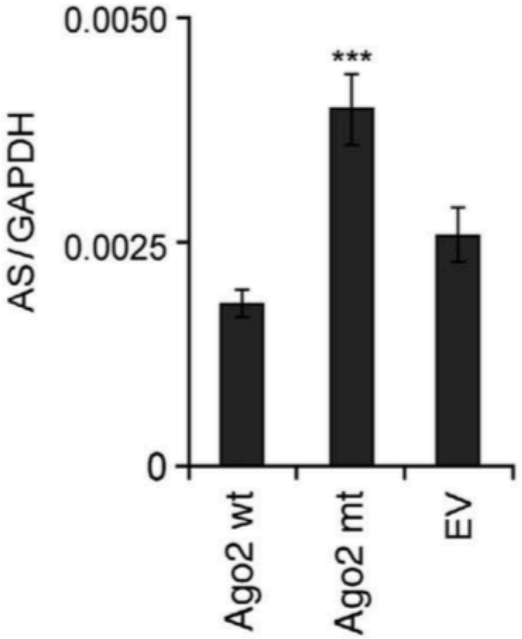
miR-671 slices AS-CDR1
Another miRNA (miR-769) does not

# Slicer deficiency protects AS-CDR1 from targeting by miR-671

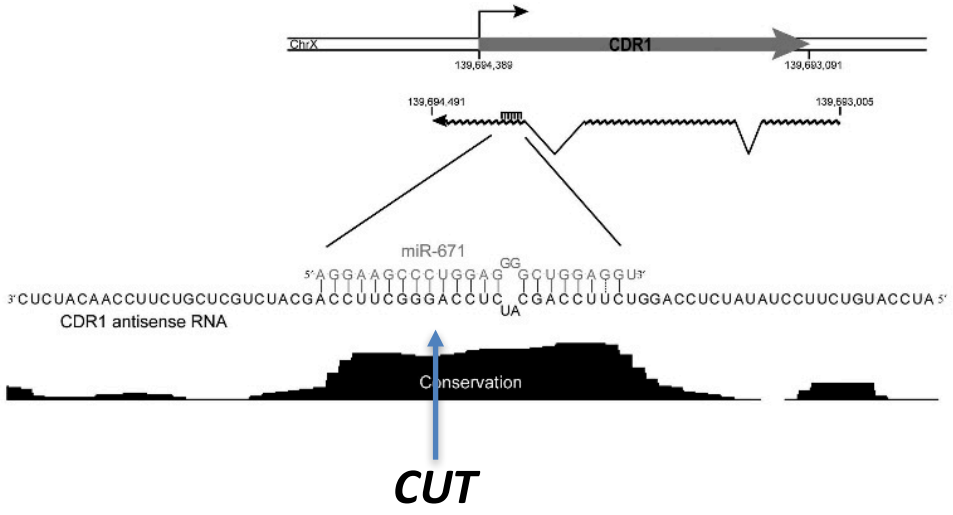
**Luciferase assay: AS-CDR1 seqeucne variants (below) fused to luciferase + mi-671**

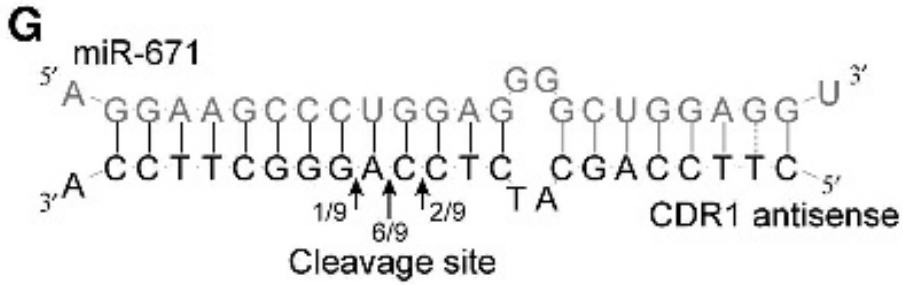



**Northern blot**



# The discovery of a circular RNAs




***Ago2 cuts AS-CDR1 RNA***

**miR-671** —Ago2⊣ **circ-asCDR1 RNA** → **Sense CDR RNA expression**

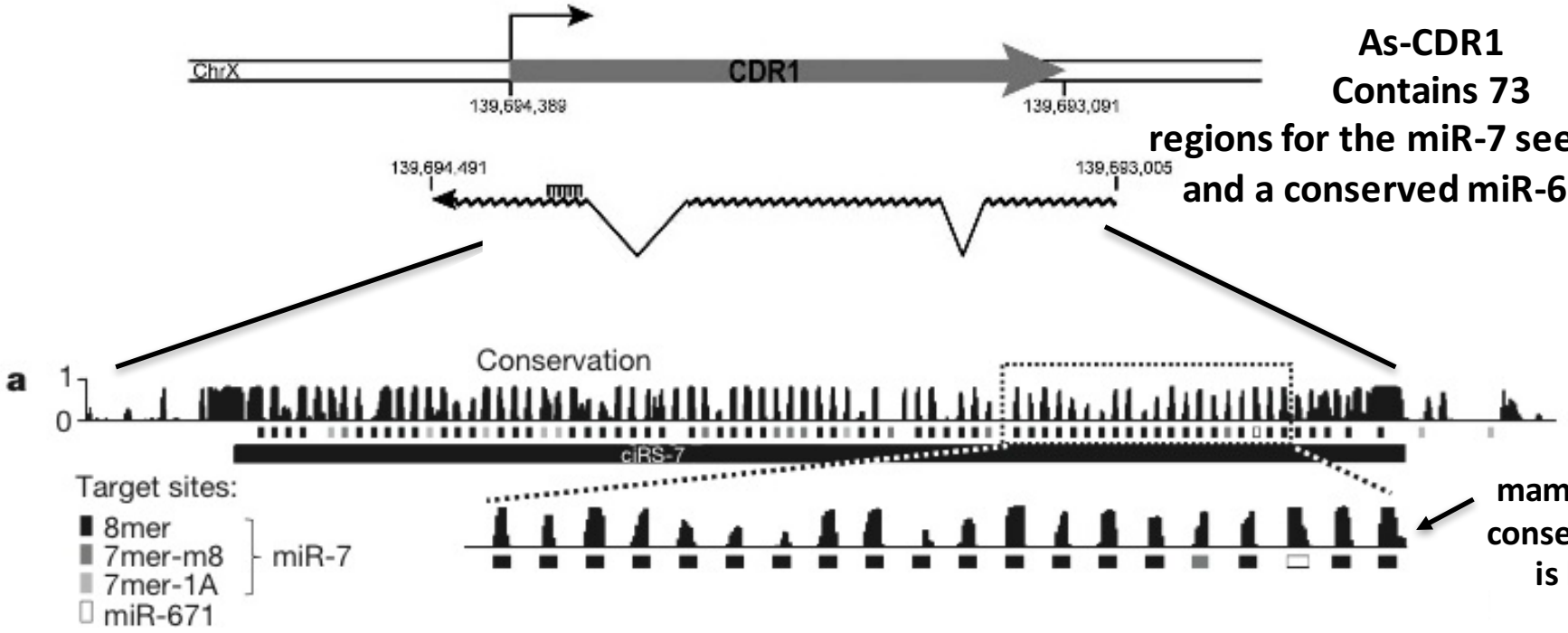
# Circ-RNAs act as miRNA sponges

LETTER

doi:10.1038/nature11993

## Natural RNA circles function as efficient microRNA sponges

Thomas B. Hansen[1], Trine I. Jensen[1], Bettina H. Clausen[2], Jesper B. Bramsen[1,3], Bente Finsen[2], Christian K. Damgaard[1] & Jørgen Kjems[1,3]



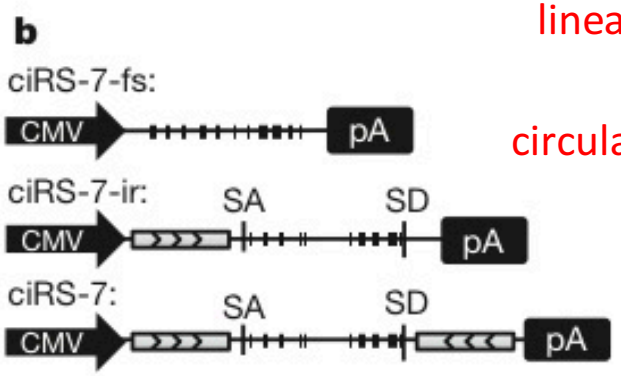

**As-CDR1 Contains 73 regions for the miR-7 seed region and a conserved miR-671 site**

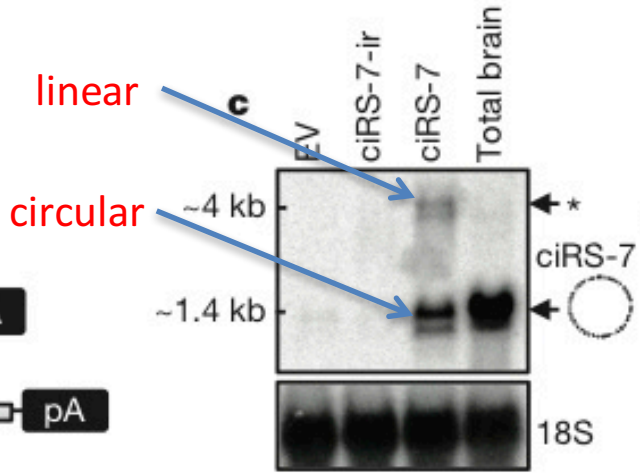
**mammalian conservation is high**

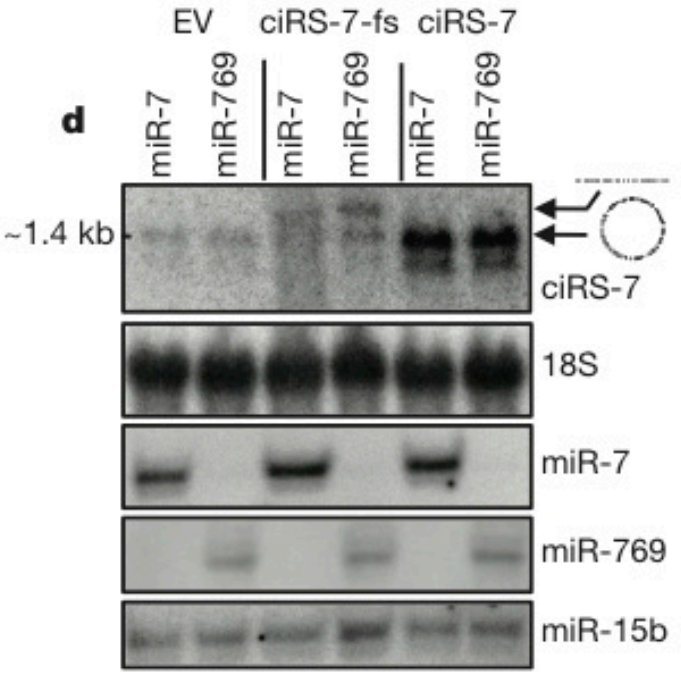
**ciRS-7: circular RNA sponge for miR-7**

# ciRS-7 is resistant to miR-7

***ciRS-7: circular RNA sponge for miR-7***




cells transfected with vectors expressing miRNAs and ciRS-7 vectors

ciRS-7:Linear (no splice, No inverted repeats)
ciRS-7-ir: splice, 5' endogenous repeate; 3' end no inverted repeat
→ inefficient circularization
ciRS-7: splice , endogenouse inverted repeats cloned up- and downstream of splice sites

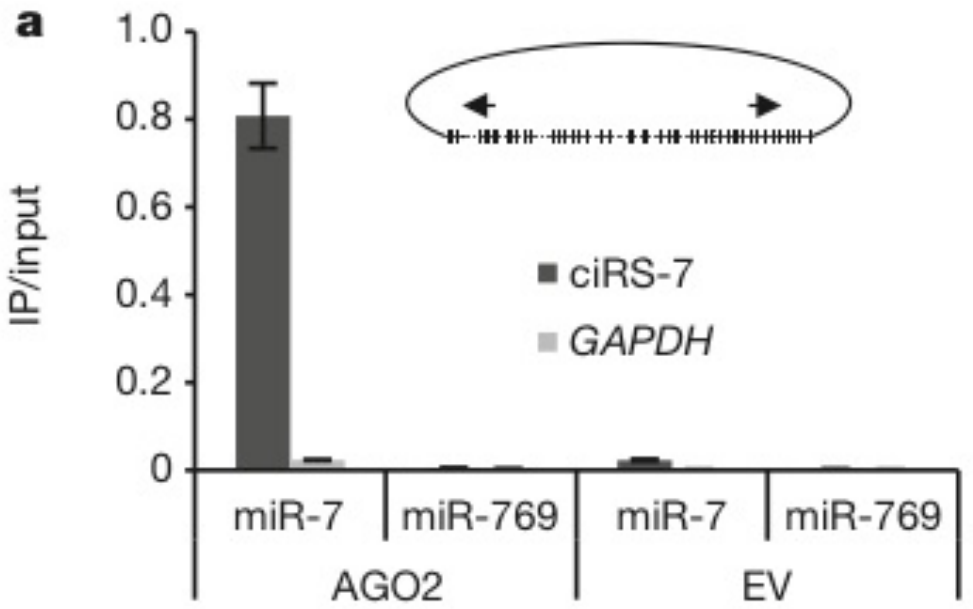
Note: the addition of IR (inverted repeats), promotes splicing related circRNA formation

**miR-7 does not cause reduction of ciRS-7 (miR-769 is a negative control RNA)**

**→ miR-7 is not able to degrade the ciRS-7 RNA**

**→ miR-7 targets only ciRS-fs (linear): -40%**

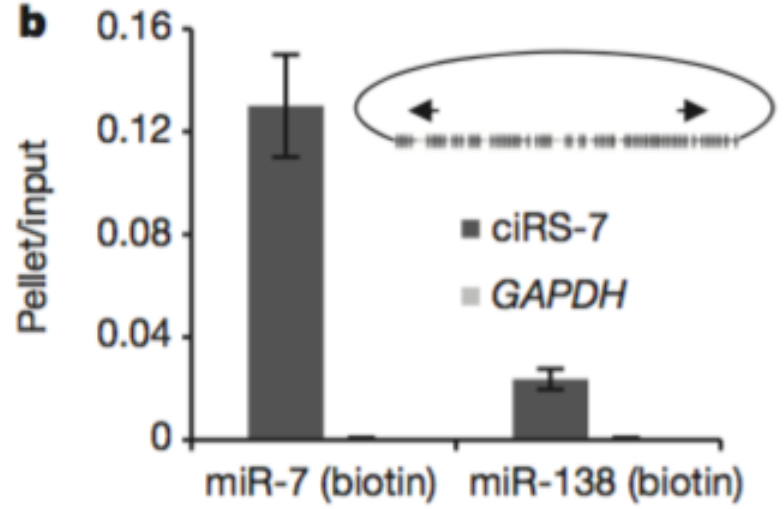
# ciRS-7 – miR-7 is associated with the miRNA machinery




***RNAimmunoprecipitation***

***Cells: transfected with Ago2 + miR***
***IP: anti-AGO2***
***RT-PCR: ciRS-7 (endog.) ; gapdh***

**AGO2 is associated with ciRS-7 and miR-7**

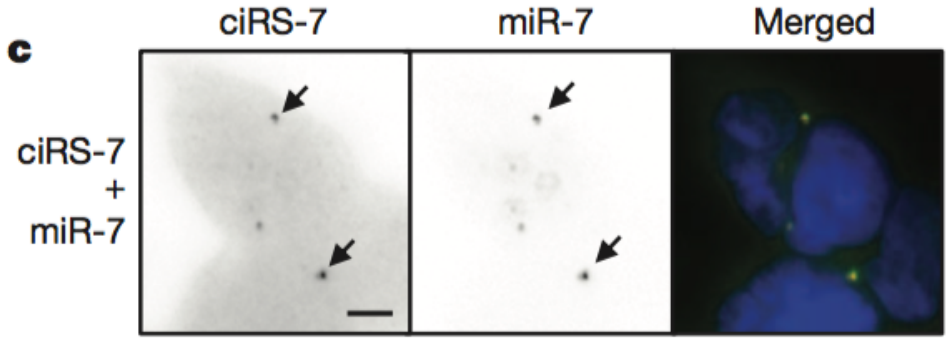



***Transient transfection of biotinylated miR-7 or miR-138 and RT-PCR for ciRS-7 Cicular RNA***

**miR-7 is associates with ciRS-7**

# ciRS-7 – miR-7 is associated with the miRNA machinery

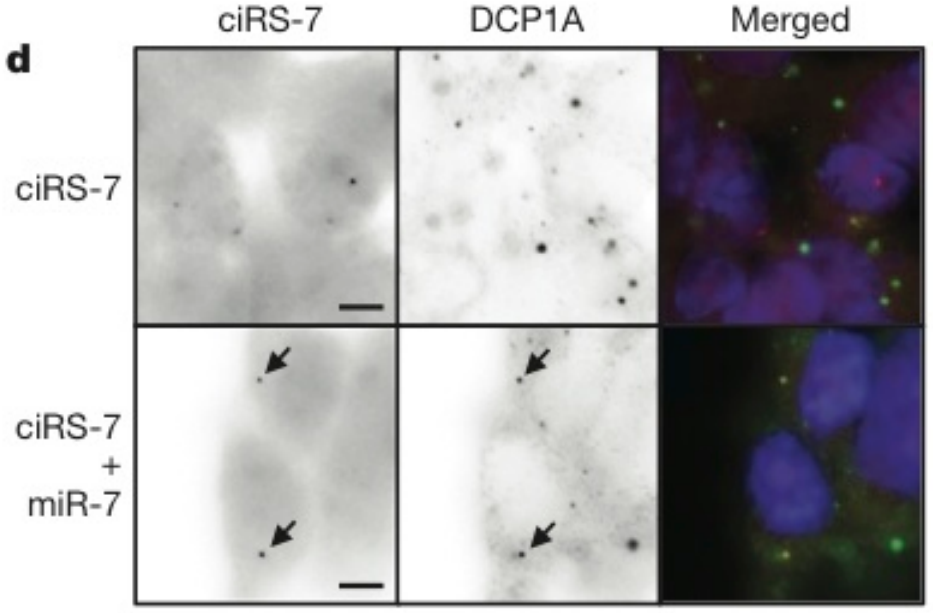
## RNA-FISH for miR-7 + ciRS-7




**Probes:**
- ciRS-7 Cy5
- miR-7 Cy3

**Cells:**
- cells + ciRS7 +miR-7

## RNA-FISH for miR-7 + Immunostaining for DCP1A



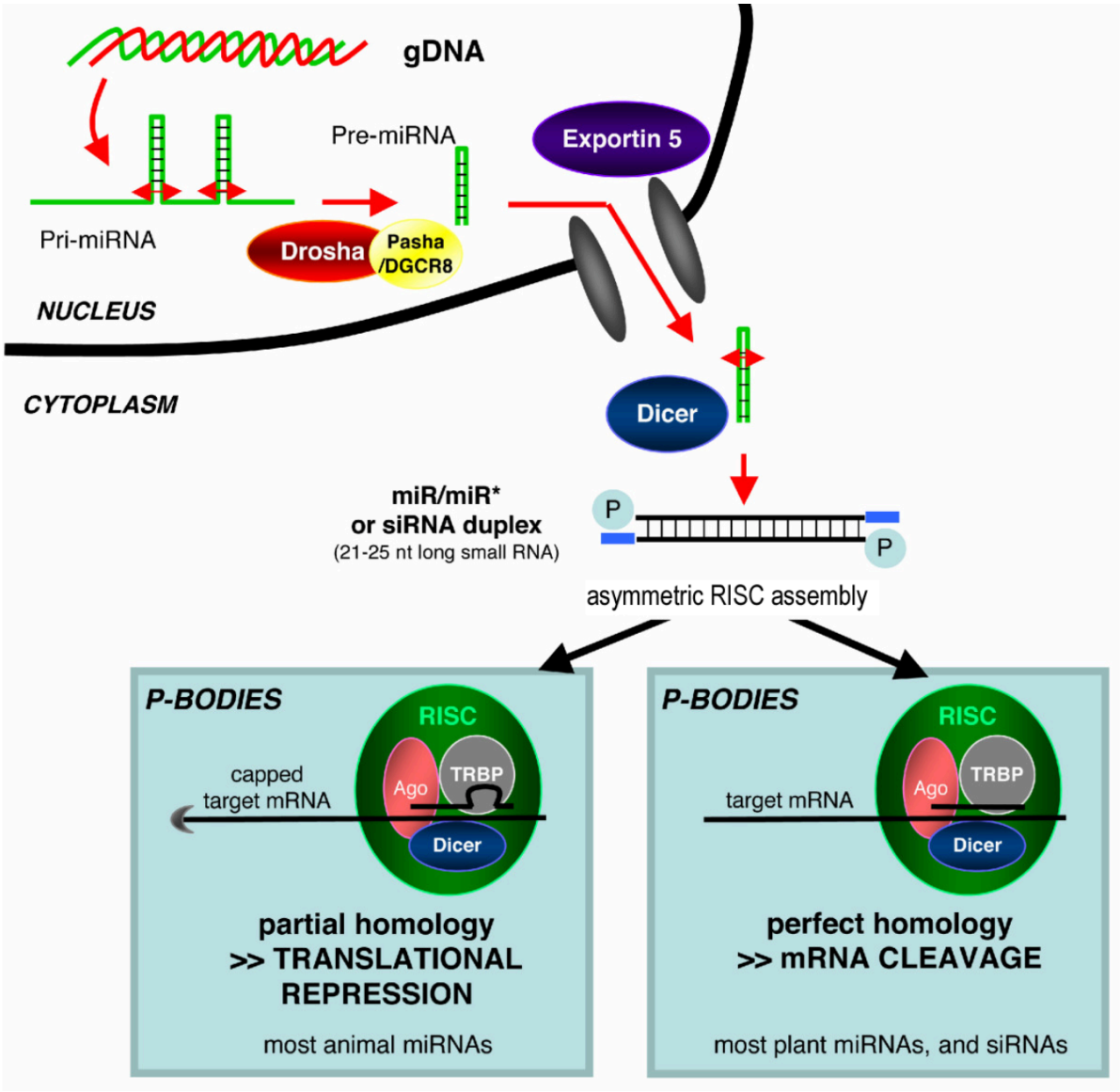

**ciRS-7 localize to P-bodies (anti-DCP1A/ciRS-7 Immuno-RNA-FISH)**

**co-localisation only when miR-7 at high levels**

**Cells:**
- cells + ciRS7 +miR-7

miRNA effector machiary concentrates in P-bodies:
--> DCP1A is a P-body protein

# ciRS-7 – miR-7 is associated with the miRNA machinery

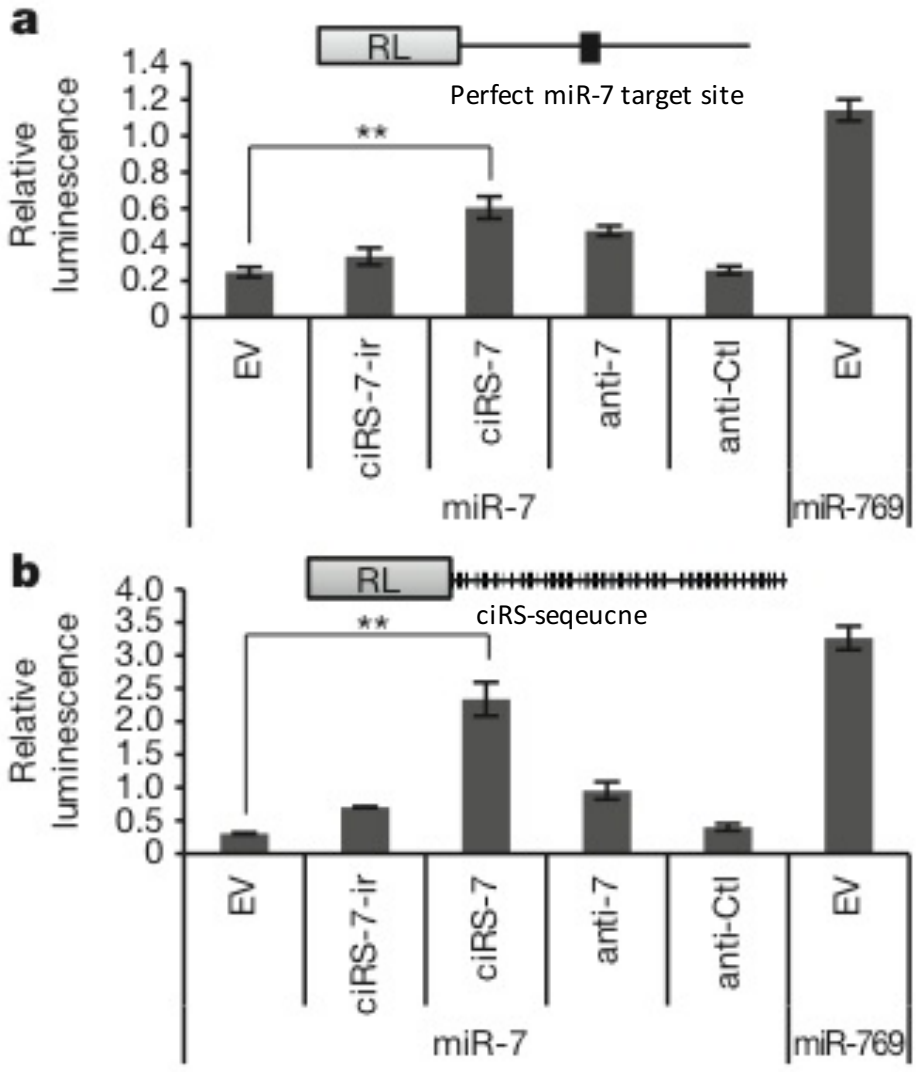



## P-bodies: RNA_Protein bodies

Processing bodies (P-bodies) are distinct foci within the cytoplasm of the eukaryotic cell consisting of many enzymes involved in mRNA turnover. P-bodies have been observed in somatic cells originating from vertebrates and invertebrates, plants and yeast. To date, P-bodies have been demonstrated to play fundamental roles in general mRNA decay, nonsense-mediated mRNA decay, adenylate-uridylate-rich element mediated mRNA decay, and microRNA induced mRNA silencing. Not all mRNAs which enter P-bodies are degraded, as it has been demonstrated that some mRNAs can exit P-bodies and re-initiate translation. The link to P-bodies comes by the fact that many, if not most, of the proteins necessary for miRNA gene silencing are localized to P-bodies, as reviewed by Kulkarni et al. (2010)

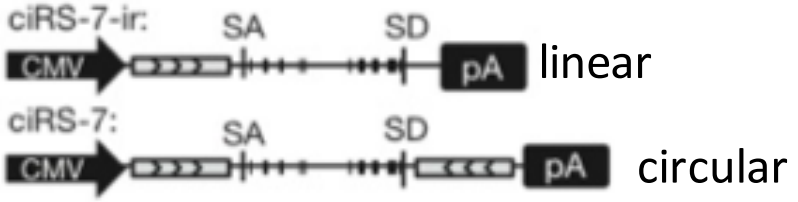
# ciRS-7 expression increases expression of a miR-7 target RNA

***Luciferase reporter assays***





**ciRS-7 increases luc activity when Luc was fused to a perfect miR-7 target site**

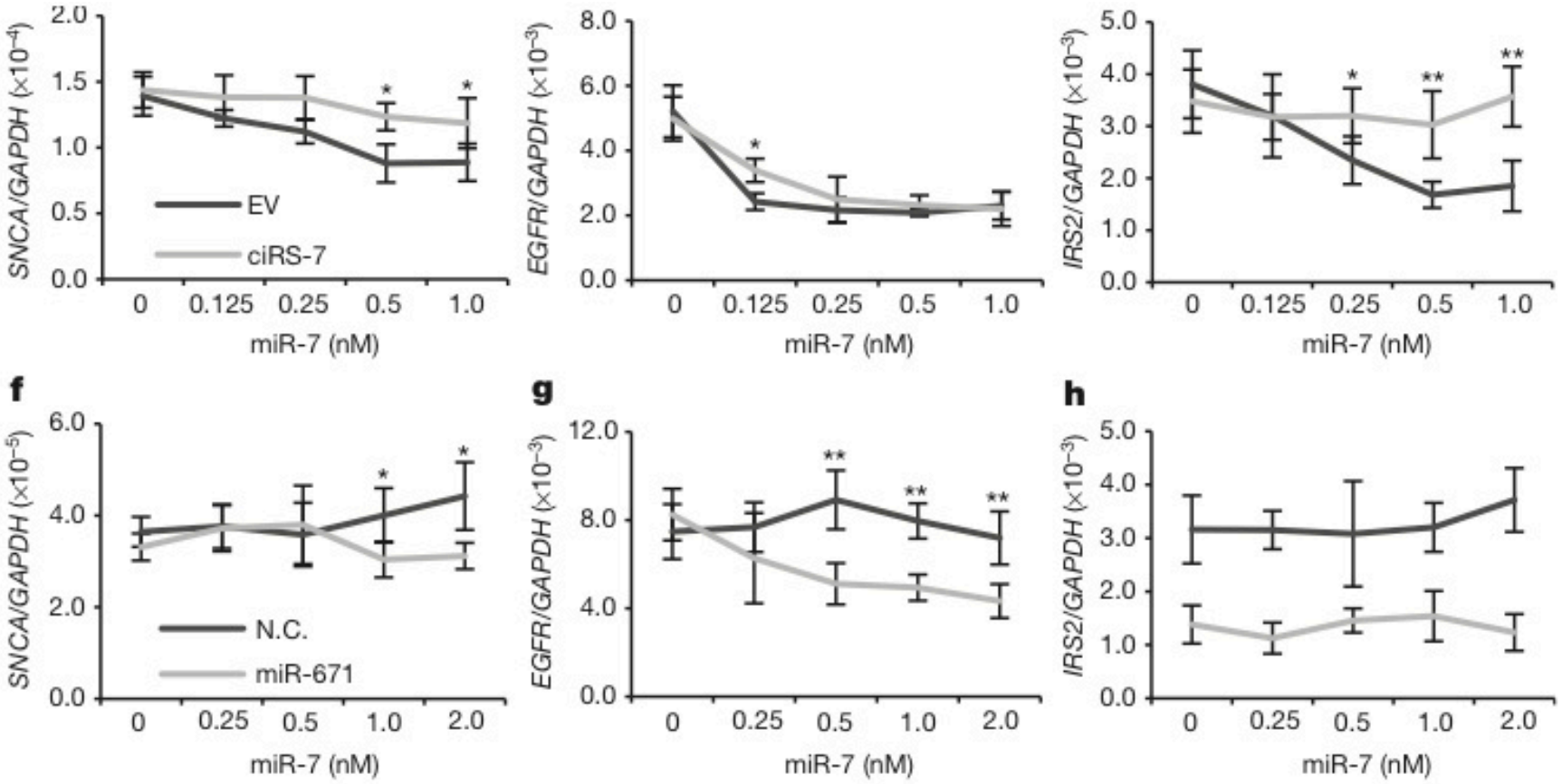



**ciRS-7 increases luc activity when Luc was fused to a the linearized ciRS-7 RNA sequence**

# ciRS-7 expression increases the expression of miR-7 target genes

**SNCA, EGFR, IRS2 are miR-7 target genes: does ciRS-7 sponge miR-7 ??**

**cells stably transfected with ciRS-7 vector are transfected with miR-7 at different concentrations**




***ciRS-7 vector***

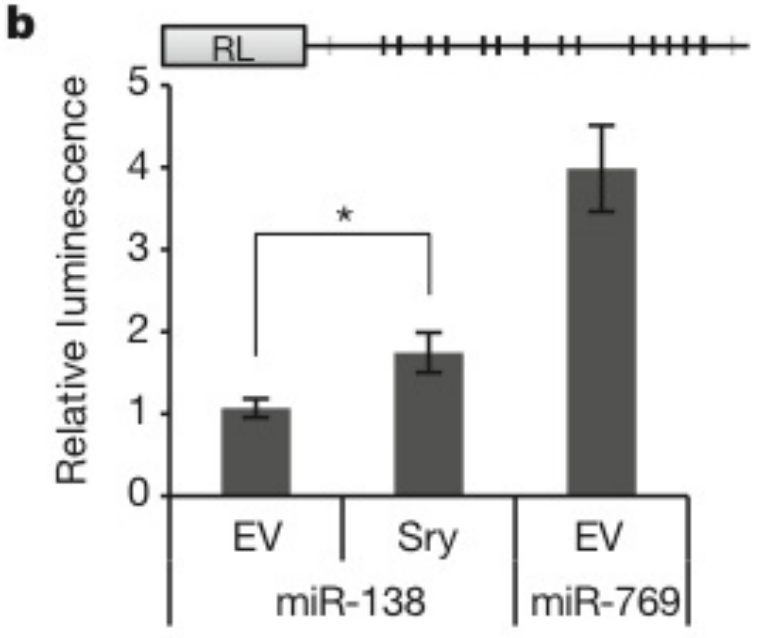
***Control vector***

# ciRNAs act as sponges for miRNAs – another example

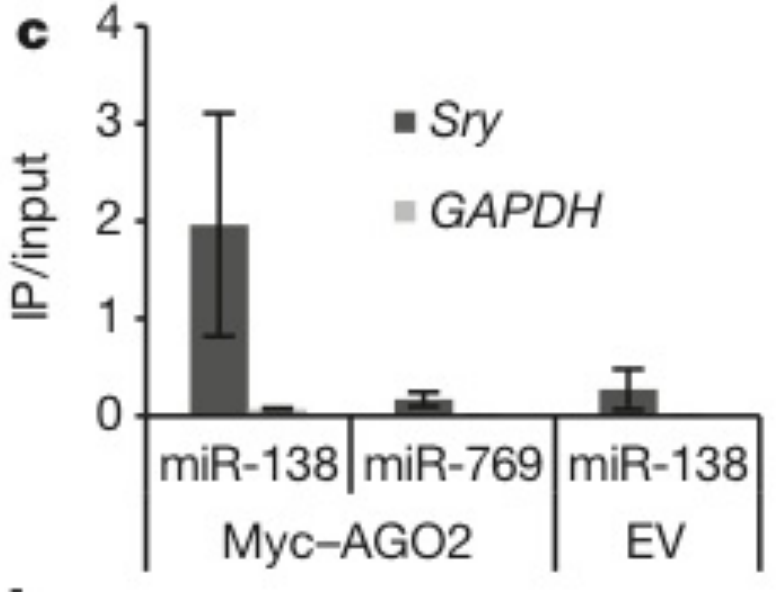
**Another notable circular miRNA sponge is SRY**. SRY, which is highly expressed in murine testes, functions as a miR-138 sponge. In the genome, SRY is flanked by long inverted repeats (IRs) over 15.5 kilobases (kb) in length. When one or both of the IRs are deleted, circularization does not occur. It was this finding that introduced the idea of inverted repeats enabling circularization.

Sry has 16 target sites for miR-138


NAS



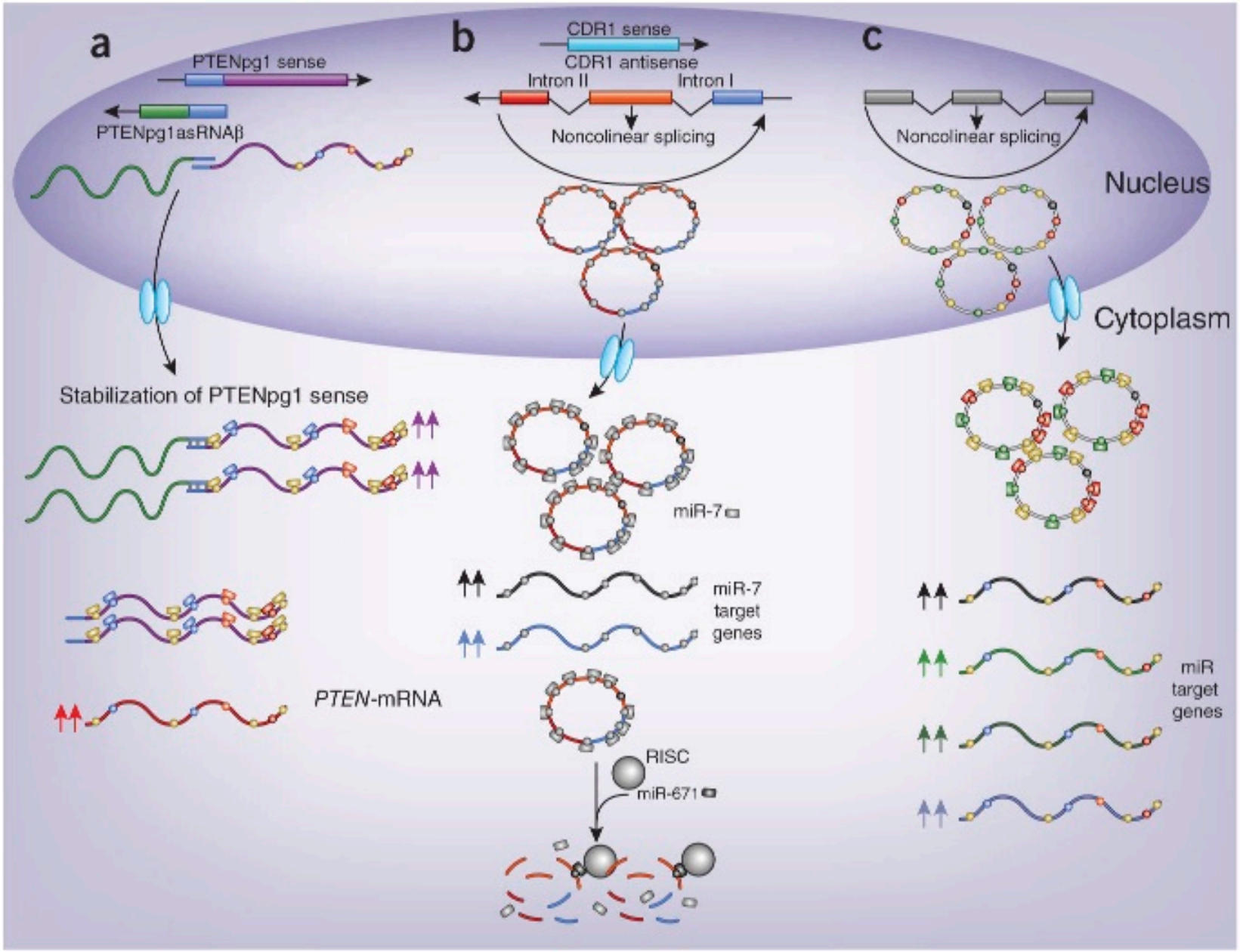

**ciR-138 increases luc-activity in miR-138 Transfected cells**




**AGO2 associates with ciR-138 and miR-138 transfected cells**

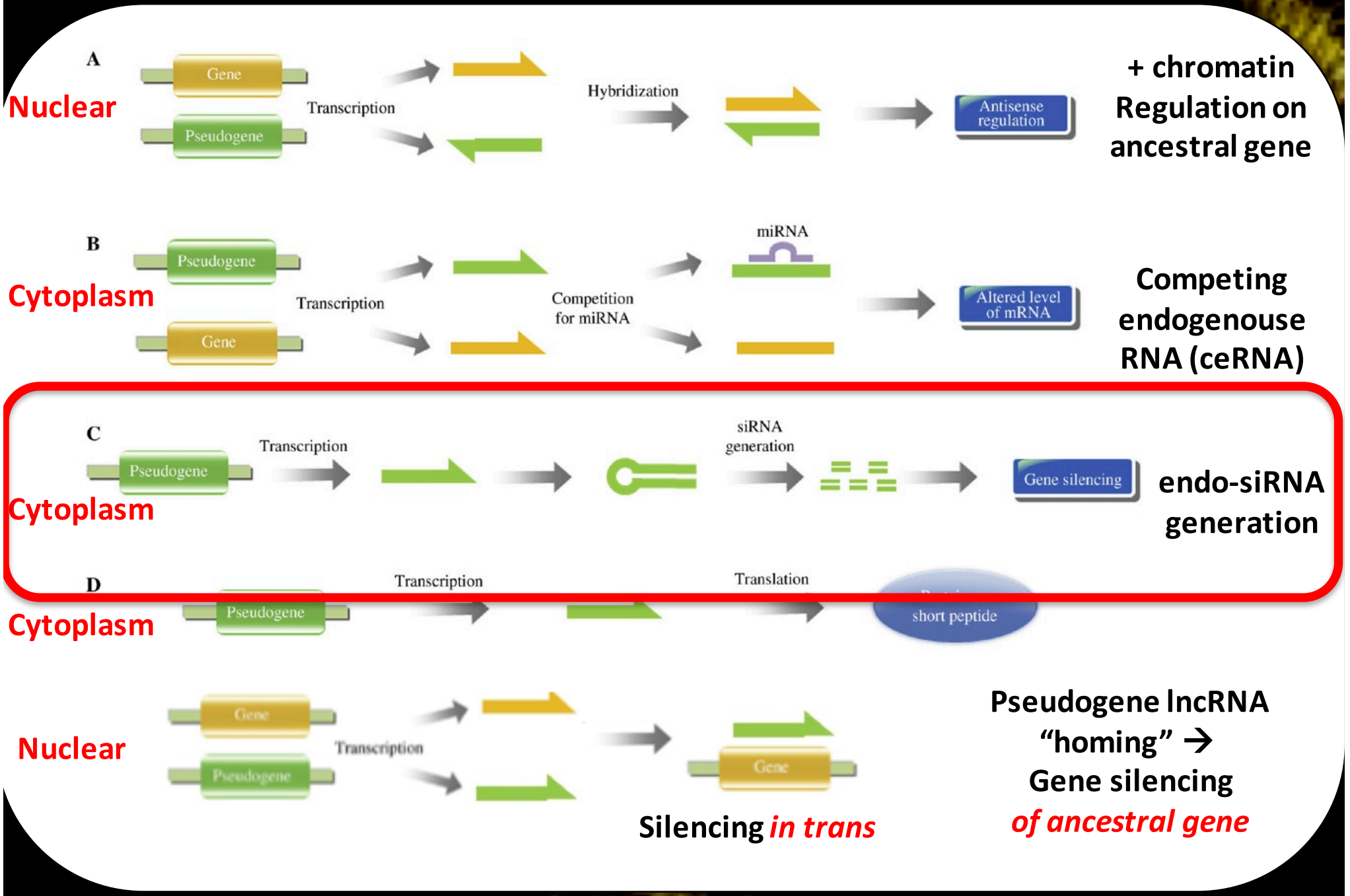
# Circular RNAs are efficient miRNA sponges

Because circular RNA sponges are characterized by high expression levels, stability, and a large number of miRNA binding sites, they are likely to be more effective sponges than those that are linear.[6]



# Small RNAs from endogenous loci
# endo-siRNAs

# seudogenes are powerful regulators of gene expressio



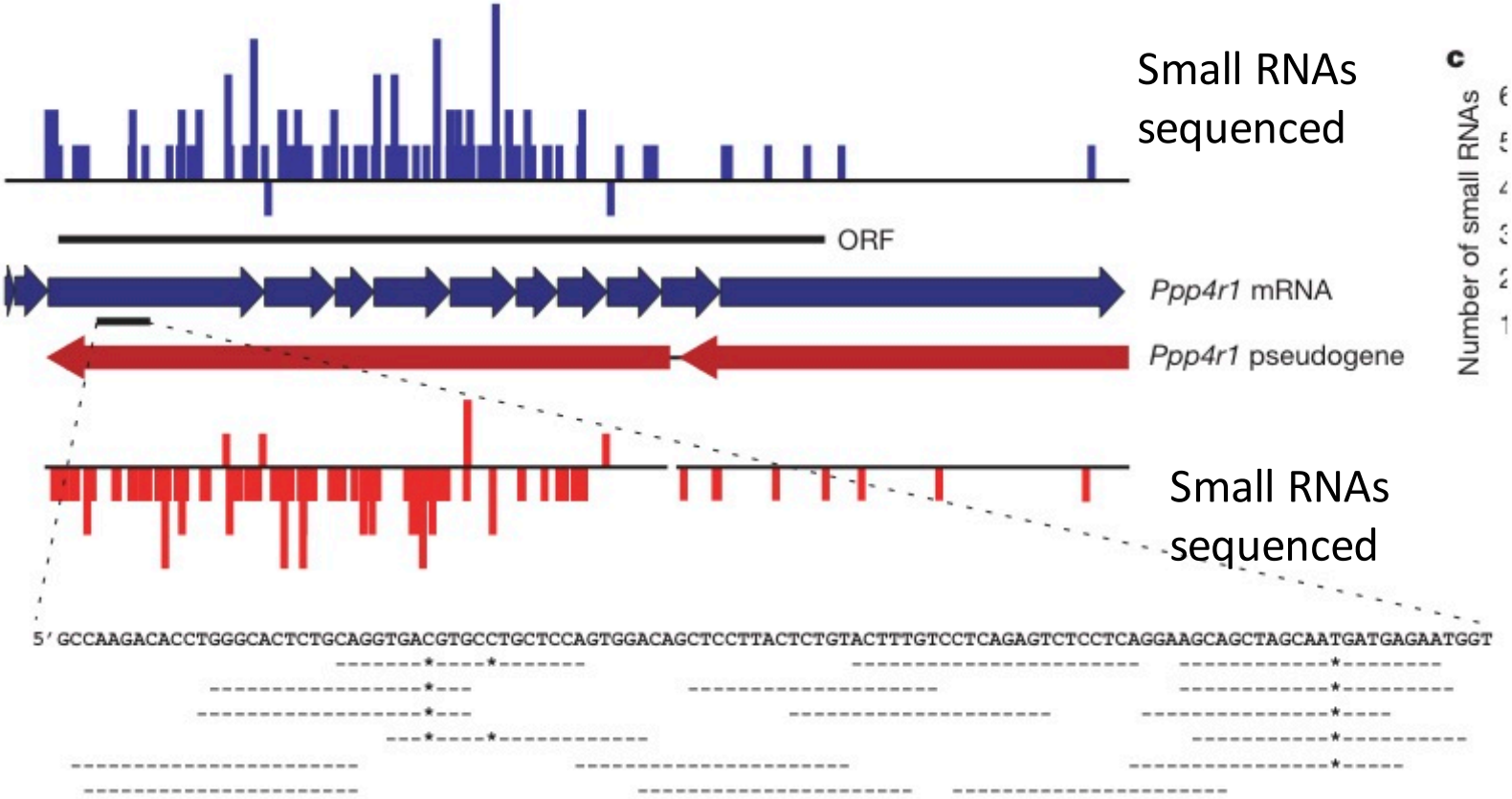
# Discovery of pseudogene derives endo-siRNAs

Massive parallel sequencing of small RNAs
A small fraction of RNAs (21-22nt) was found to map to pseudogenes
siRNA map only to regions of complementarity

Ppp4r1 gene

Ppp4r1 pseudogene

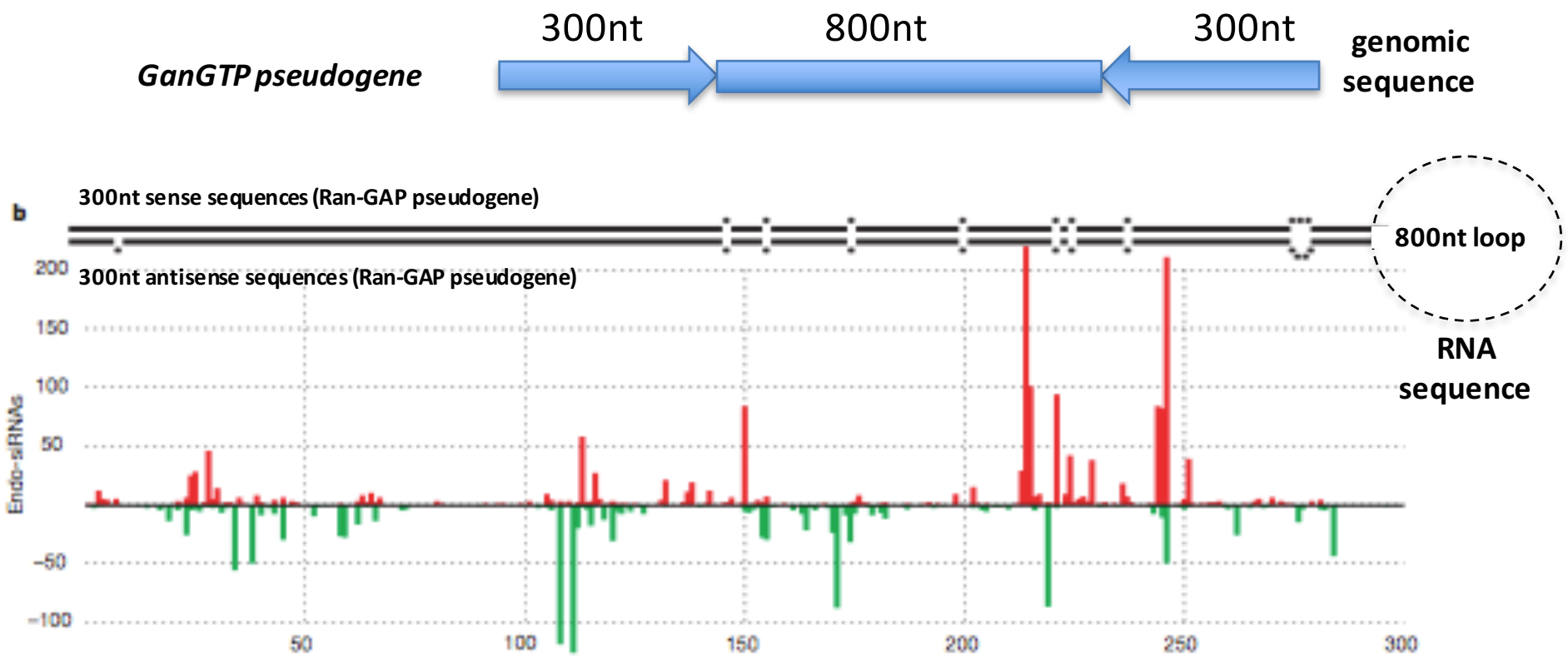
***Located at different position in the genome***



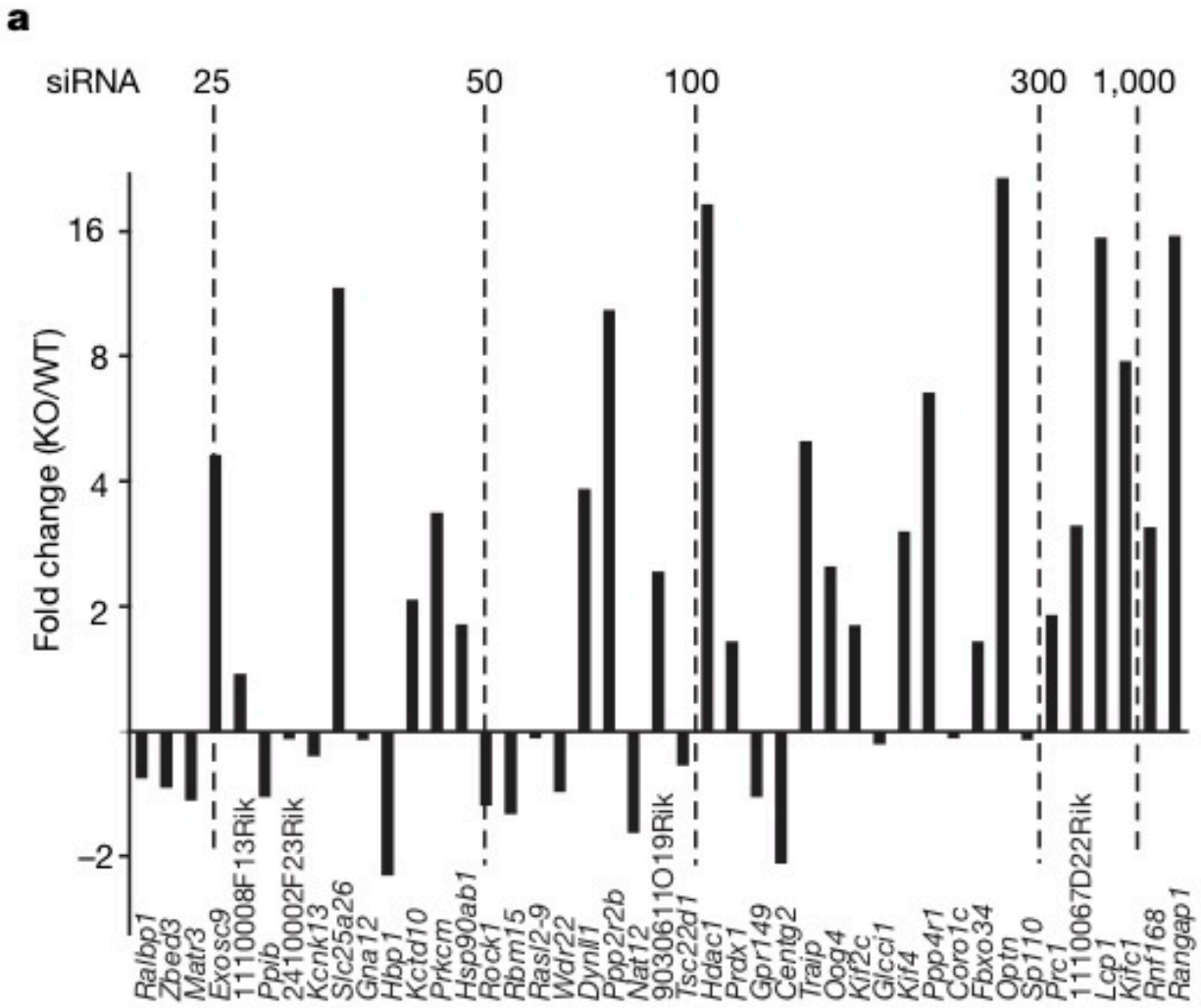
# Discovery of pseudogene derives endo-siRNAs

**PseudogeneGTPase-activating protein for Ran (Ran-GAP)**

Pseudogene contains a 300 bp inverted repeat and and intervening 800 bp loop
siRNAs can be detected on regions were RNAs from inverted repeats overlap



# A large set of endo-siRNAs are involved in gene regulation




**Embryonic stem cells (murine)**

**Dicer wild type**
**Dicer $^{-/-}$**

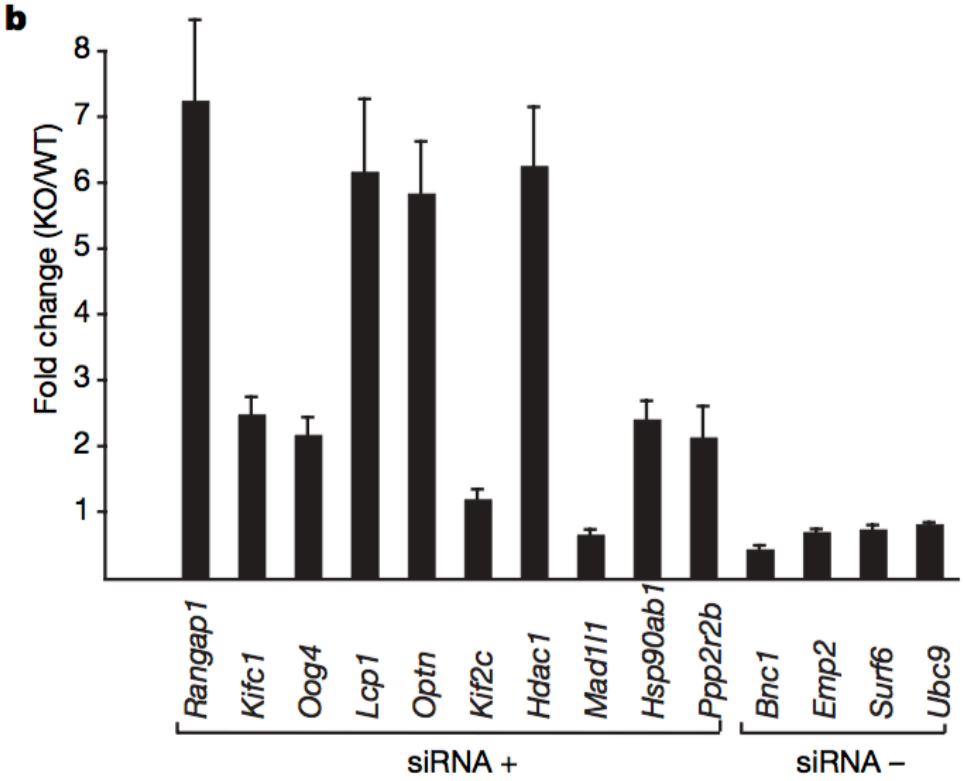
**- Prepare total RNA**

**- Make reverse transcription**

**-Measure the expression of genes with overlapping siRNAs**

**A large proportion of genes associated with endo-siRNAs is upregulated**

# A large set of endo-siRNAs are involved in gene regulation




**Embryonic stem cells (murine)**

**Dicer wild type**
**Dicer $^{-/-}$**

**- Prepare total RNA**

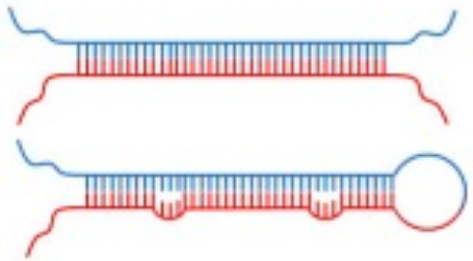
**- Make reverse transcription**

**-Measure the expression of genes with overlapping siRNAs (siRNA +) or without Overlapping siRNAs (siRNA-)**

# Generation of endo-siRNAs

## Human/mouse

Endogenous dsRNA precursors



dsRNA, long hairpins
sense/antisense hybrids



Ago-1
Ago-2
Ago-4?

Post –transcriptional repression
Transposon control
Chromatin modification

**A source for anti-sense transcripts:**
**-Antisense pseudogenes**
**-Transcribed inverted repeats**
**-NATs: naturally ocurring antisense transcripts**
**-Frequently also antisense transcripts of transposable elements**

**sense and antisense transcripts can base-pair and form dsRNA**

↓

**Processing by Dicer**

↓

**siRNA formation**

↓

**Target RNA slicing**

Endo-siRNA levels are low in vertebrate species:
**no siRNA amplification loop because no RNA dependent Polymerase present!!!**
Higher relevance in biological Situations where endo-siRNAs reach higher levels:
→Control of transposable elements
→DNA damage associated expression of small RNAs
→Biological situations
→Associated with the upregulation of sense – antisense forming transcripts