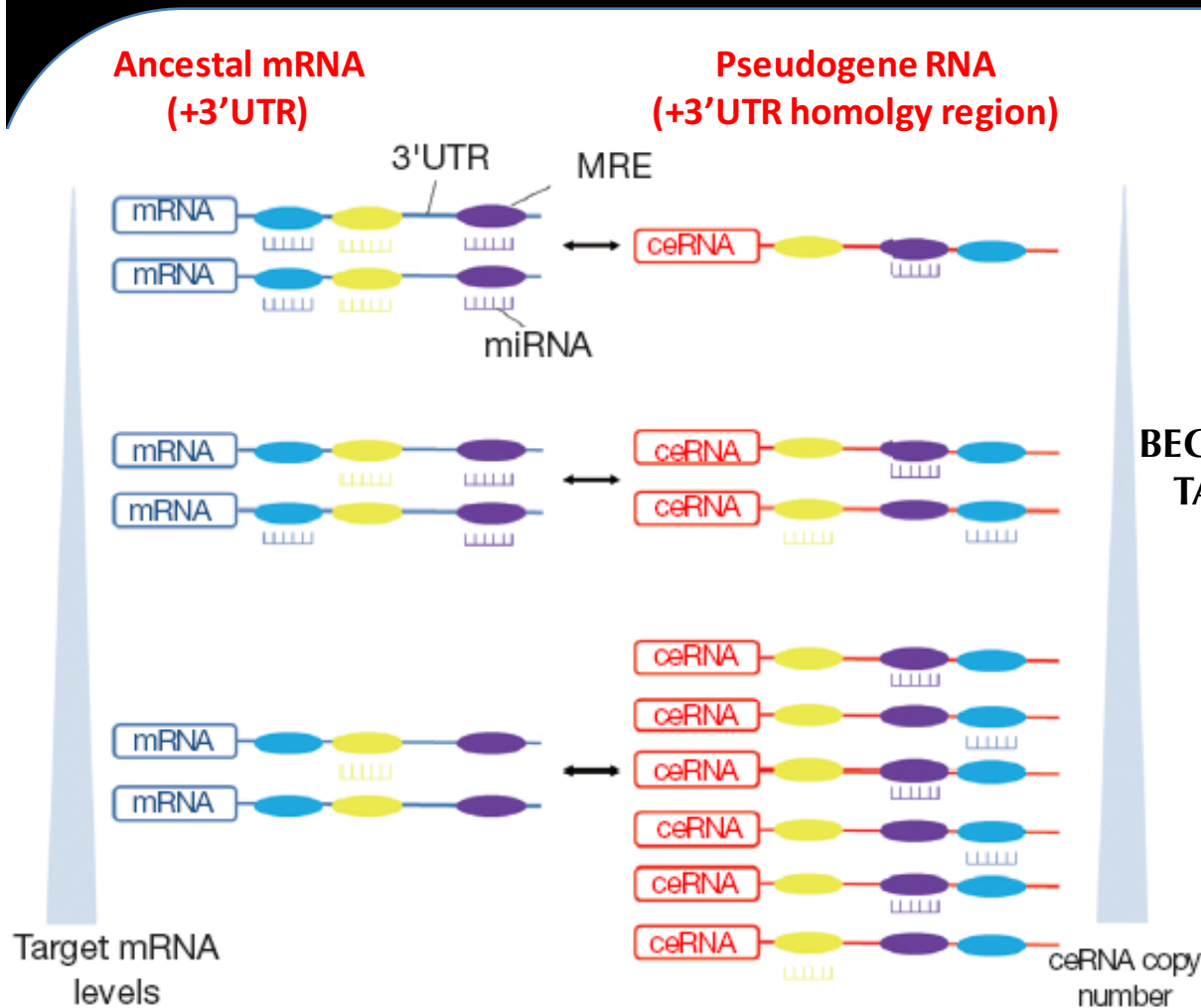


**Circular RNAs (circRNAs) act a stable
miRNA sponges**

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 is in sense with CDR1 miRNA**



The EMBO Journal (2011) 30, 4414–4422 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA

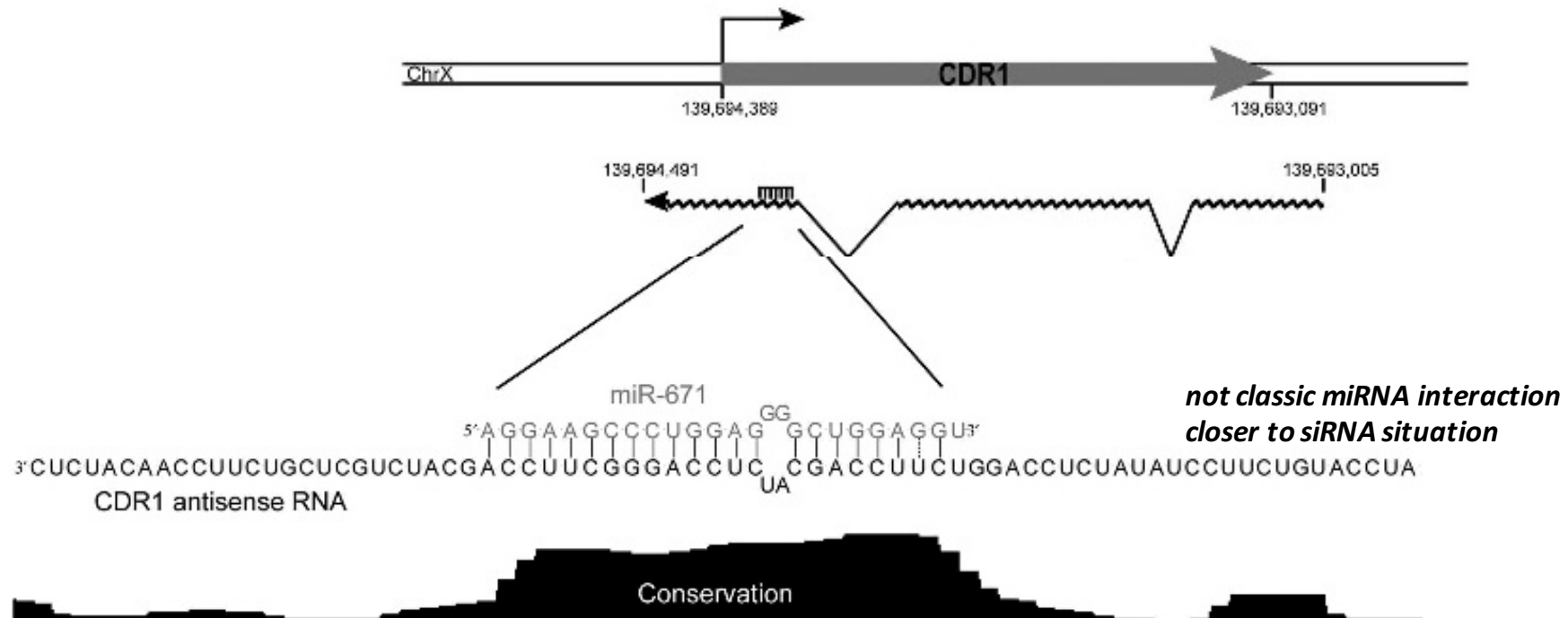
Thomas B Hansen¹, Erik D Wiklund^{1,2},
Jesper B Bramsen¹, Sune B Villadsen¹,
Aaron L Statham², Susan J Clark² and
Jørgen Kjems^{1,*}

¹Department of Molecular Biology, Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Aarhus, Denmark and ²Epigenetics 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*, 200
RISC activity has been detected in
(Langlois *et al*, 2005; Robb *et al*
miRNAs are predominantly nucle
et al, 2010), suggesting that miR
biological functions distinct from
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)

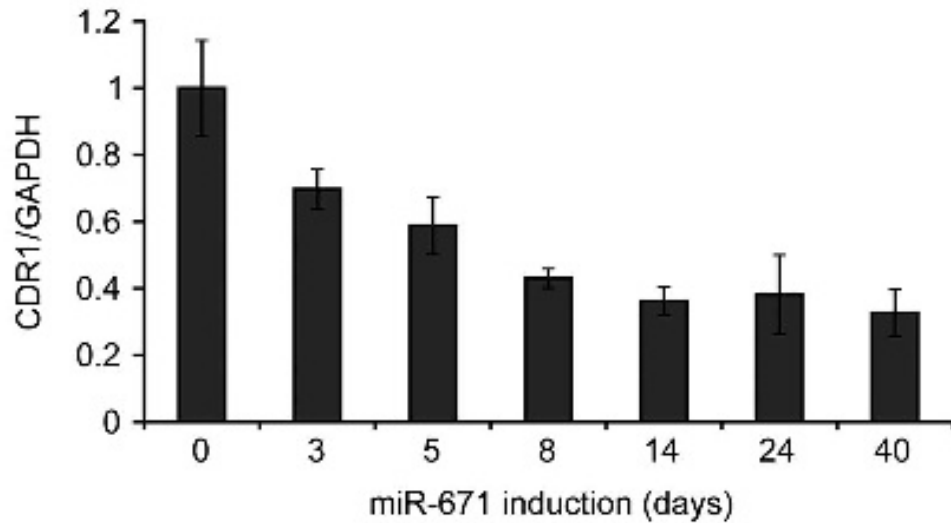


- CDK1 antisense RNA shows highest conservation at miR-671 target site in CDK1 as RNA

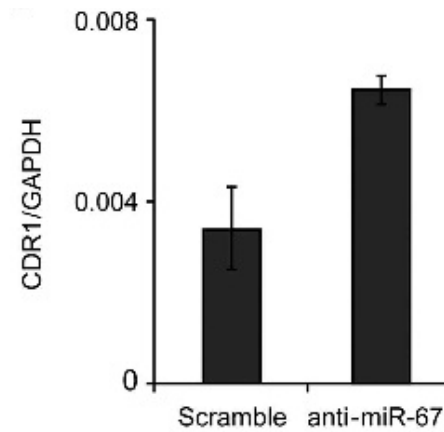
DISCOVERY - OBSERVATION

Circ-AS-CDR1 RNA stabilizes sense CDR1 RNA

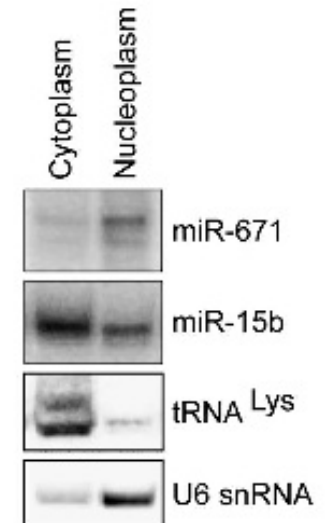
miR-671 overexpression during 40 day
Reduces CDR1 expression



miR-671 knock-down
Increases CDR1 expression



miR-671 is
enriched in the
nucleus

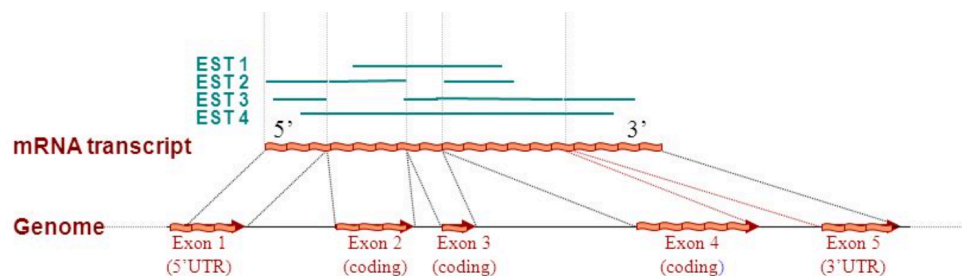


DISCOVERY - OBSERVATION

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

1. Sequence alignment: 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



RNA → RT → (cloning) → sequencing

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

UCSC Genome Browser

Our tools

- **Genome Browser**
interactively visualize genomic data
- **BLAT**
rapidly align sequences to the genome
- **Table Browser**
download data from the Genome Browser database
- **Variant Annotation Integrator**
get functional effect predictions for variant calls
- **Data Integrator**
combine data sources from the Genome Browser database
- **Gene Sorter**
find genes that are similar by expression and other metrics
- **Genome Browser in a Box (GBIB)**
run the Genome Browser on your laptop or server
- **In-Silico PCR**
rapidly align PCR primer pairs to the genome
- **LiftOver**
convert genome coordinates between assemblies
- **VisiGene**
interactively view in situ images of mouse and frog

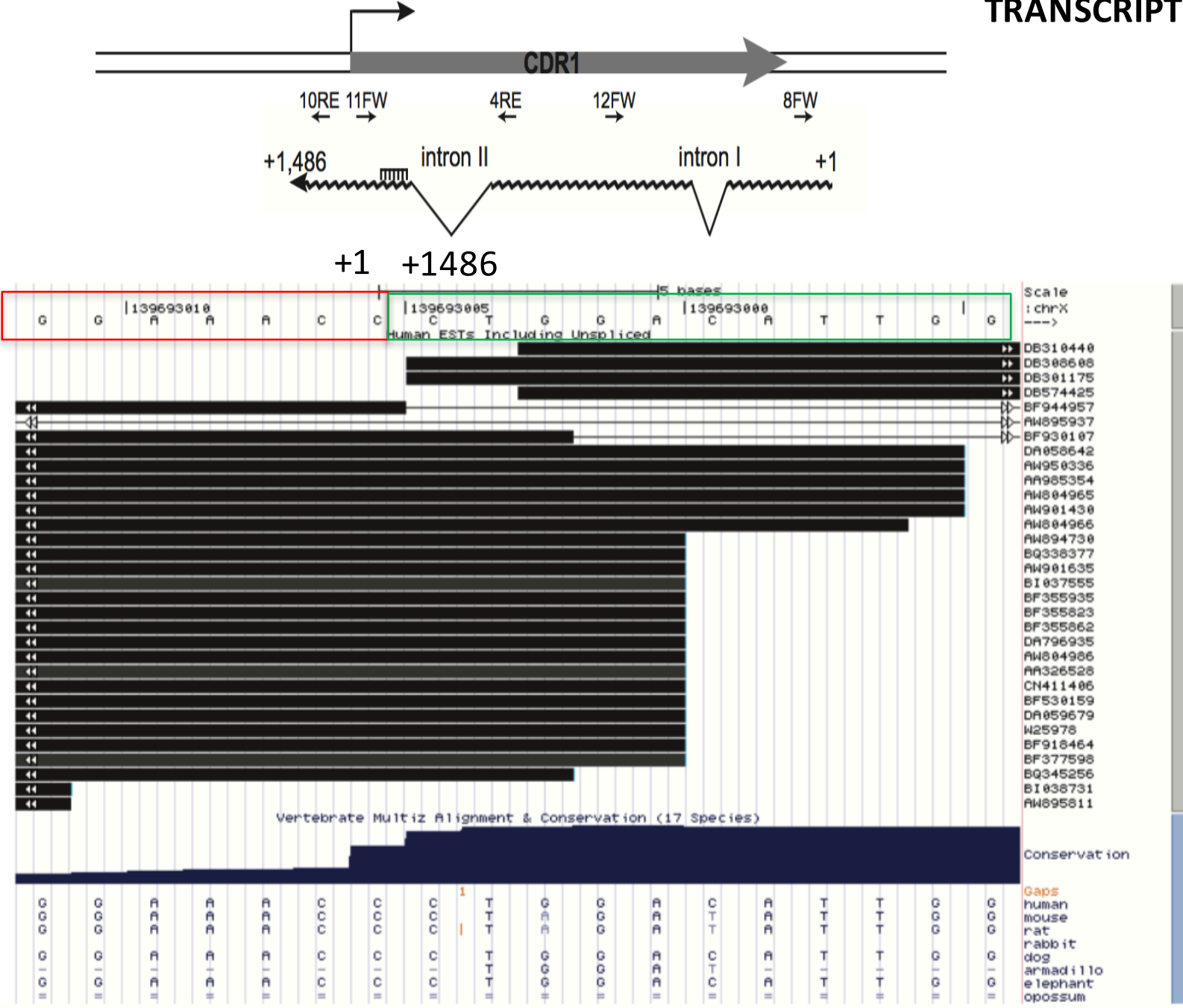
More tools...

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

TRANSCRIPT IDENTITY

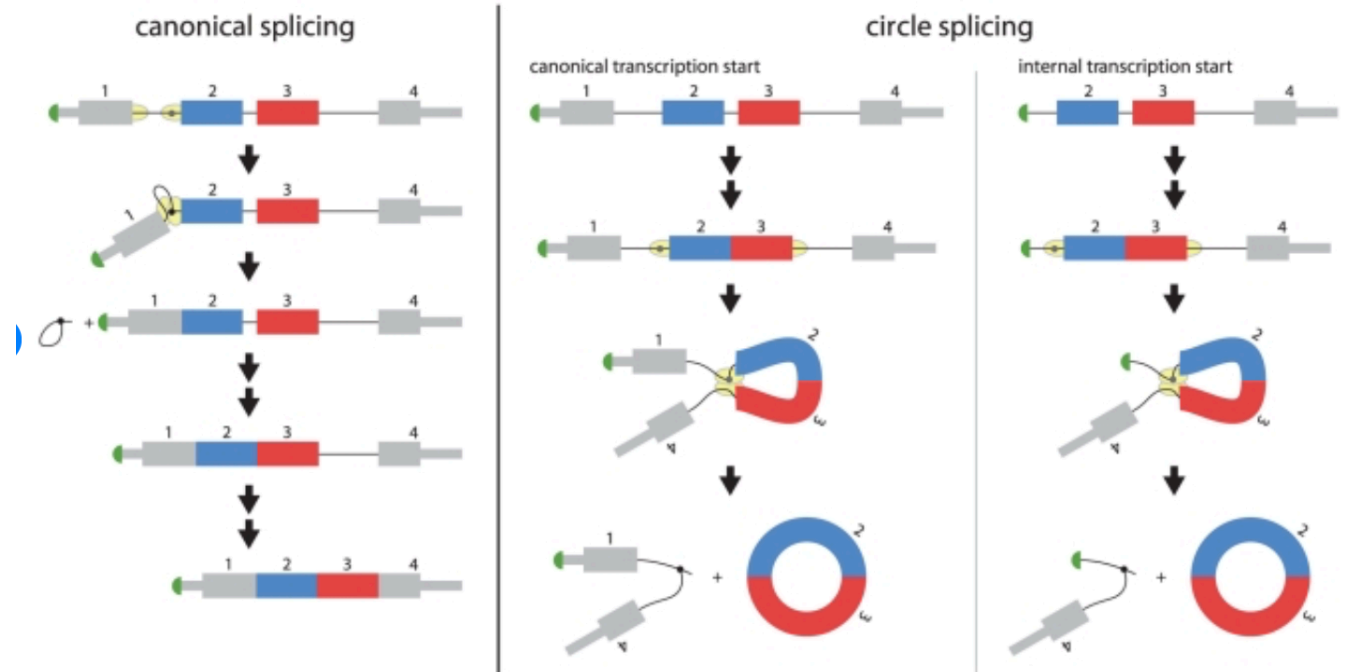
ESTs exist that overlap the 5' and 3' end of AS-CDR1

TRANSCRIPT IDENTITY



“Backsplicing” can produce circular RNAs

LITERATURE, HYPOTHESIS



At left: a schematic diagram of the canonical splicing process splicing out the first intron of the a pre-mRNA of a 4 exon gene, and subsequent removal of introns 2 and 3. Canonical splicing of exon 1 to exon 2 occurs when the splicing machinery catalyzes the formation of the intron lariat and the attack of the free 3' OH of exon 1 on the 3' splice site upstream of exon 2. This produces a lariat containing intron 1 and a pre-mRNA with exons 1 and 2 spliced together. At right: a model for the production of circular transcripts. If there is a canonical transcriptional start, and if intron excision does not proceed sequentially in time from the 5' to 3' direction of the pre-mRNA, non-canonical pairing of 3' and 5' splice sites could be generated. Since the sequences of each 5' splice site of the pre-mRNA contain the same splicing signals, it is possible that the 3' splice site upstream of exon 2 is paired with the 5' splice site downstream of exon 3 and splicing proceeds as if this 5' splice site were paired with the 3' splice site upstream of exon 4. In this case, exon 3 would be spliced upstream of exon 2, creating a pre-mRNA intermediate comprised of these two exons and intron 2. Canonical splicing would be predicted to excise this intron, leaving a circular RNA composed of exons 2 and 3. Non-canonical transcription start, as suggested in [25], could produce an orphan 3' splice site corresponding to the first transcribed exon. This splice site could be paired with a downstream 5' splice site, generating a circular RNA. In both models, the excised intron would be linear and branched, and expected to be quickly degraded.

“Backsplicing” can produce circular RNAs

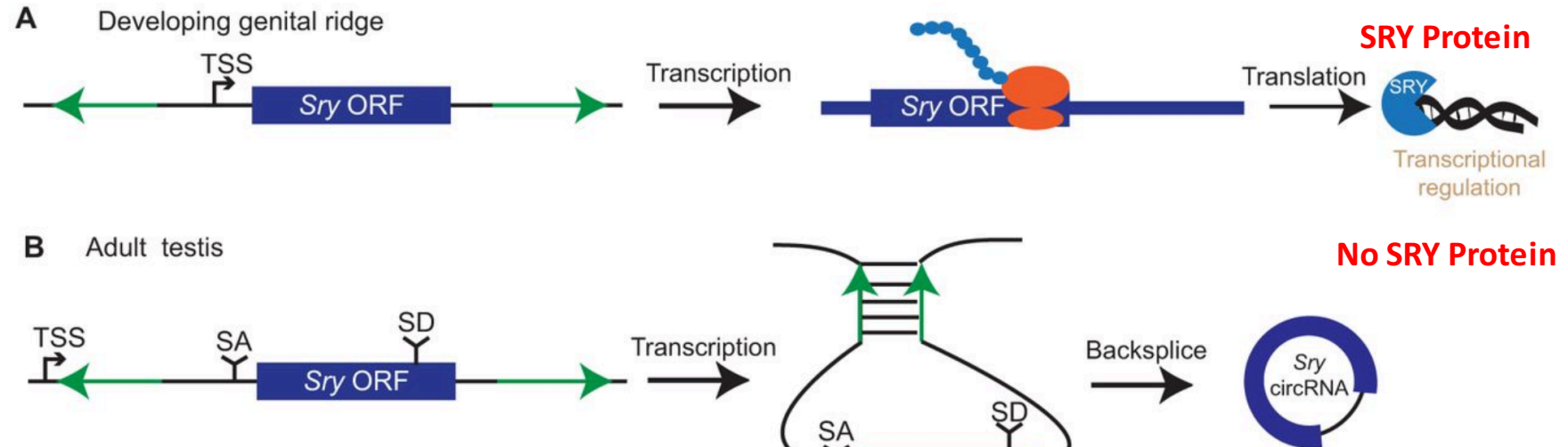
HYPOTHESIS

Most famous example:

SRY: Y-linked transcription factor for male sex development:

ON: during development

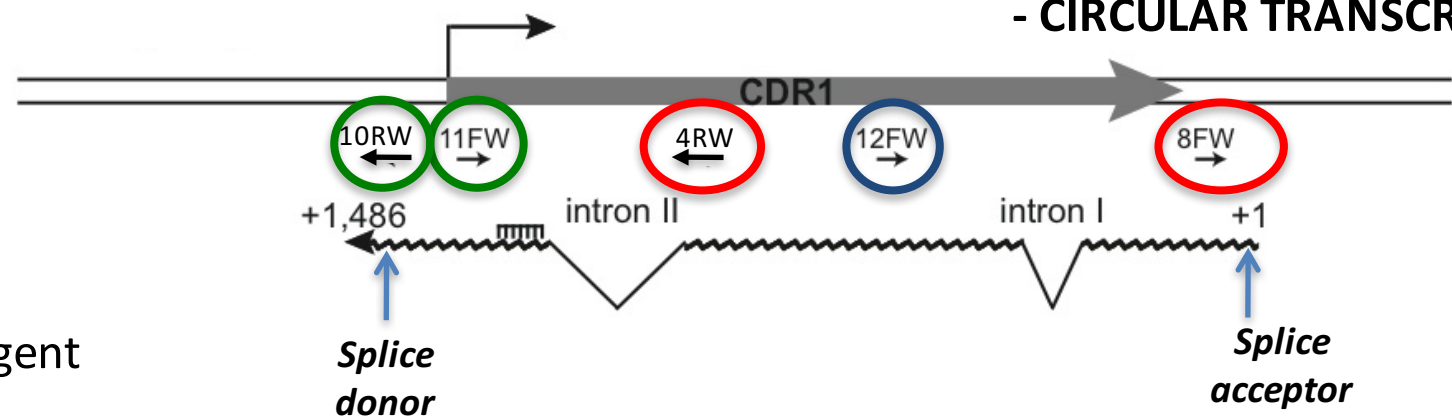
OFF: in adult



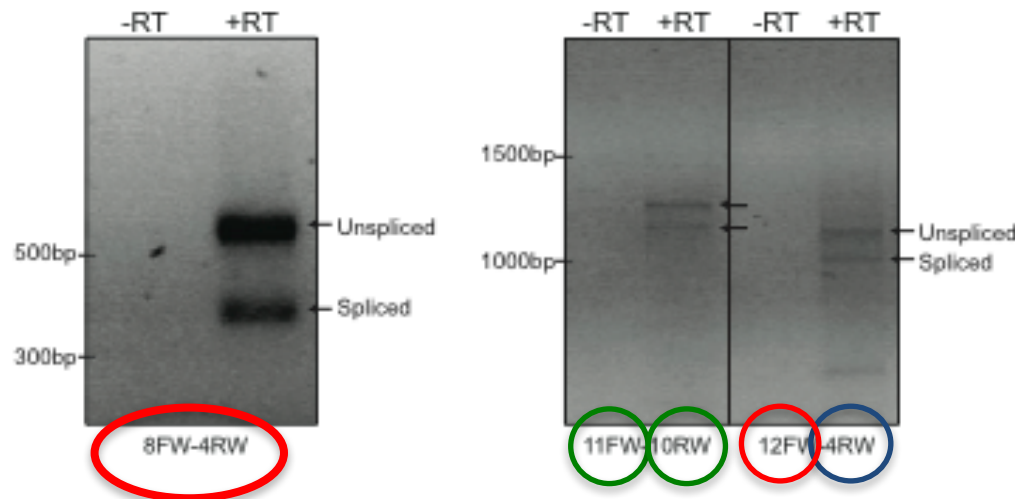
Developmentally regulated expression of SRY. (A) In the genital ridge of the developing mouse embryo, the *Sry* transcription start site (TSS) occurs proximal to the open reading frame (ORF), yielding a translatable mRNA that gives rise to SRY protein, a transcription factor involved in sex determination. (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 DETECT circRNAs?

VALIDATE HYPOTHESIS - CIRCULAR TRANSCRIPT



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

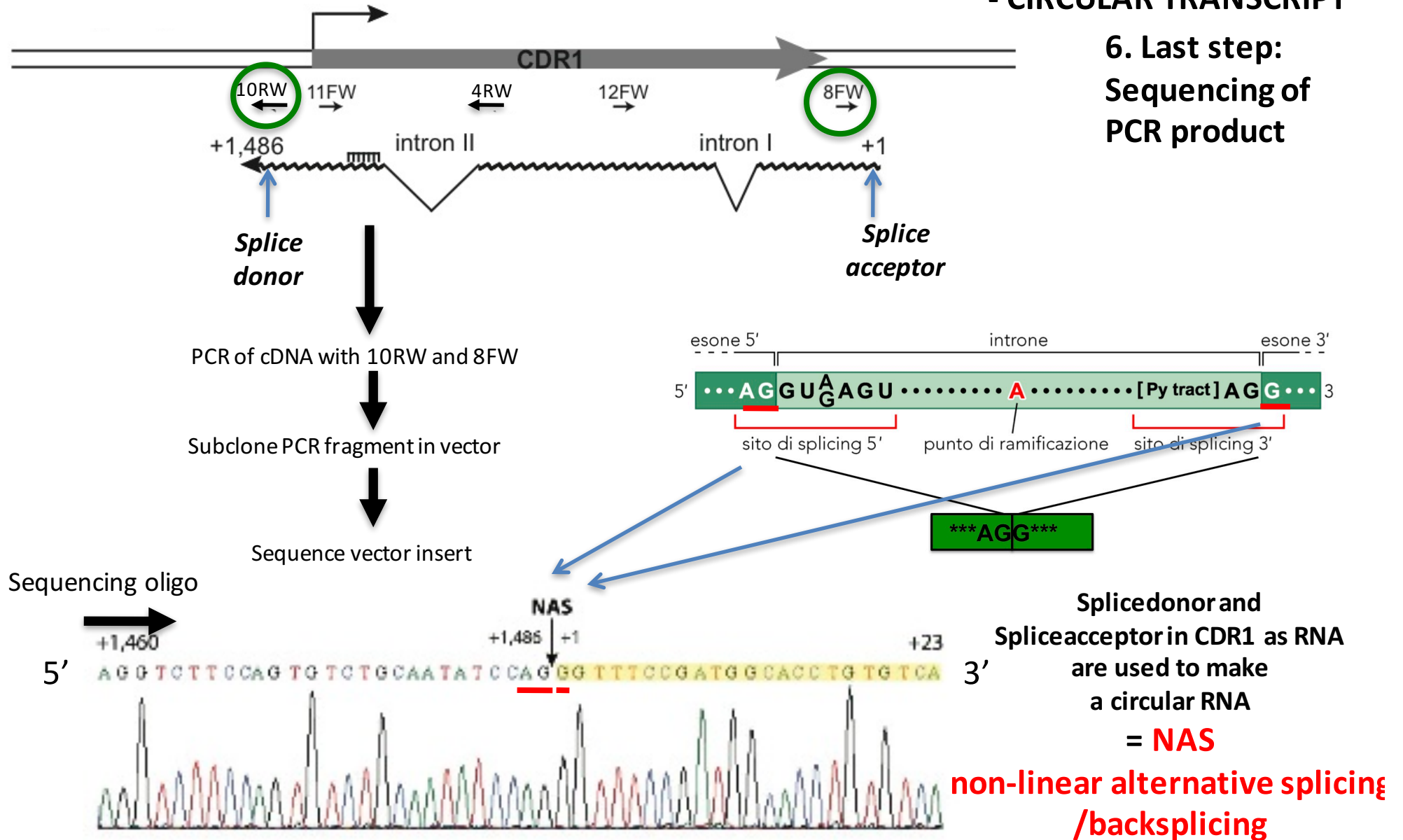


anti-sense CDR1 is a circular RNA

HOW TO DETECT circRNAs?

VALIDATE HYPOTHESIS - CIRCULAR TRANSCRIPT

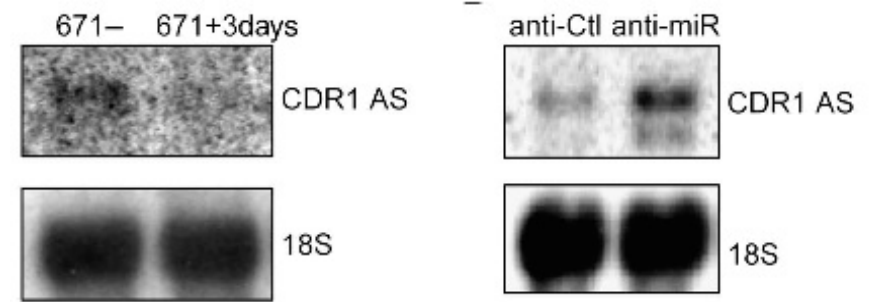
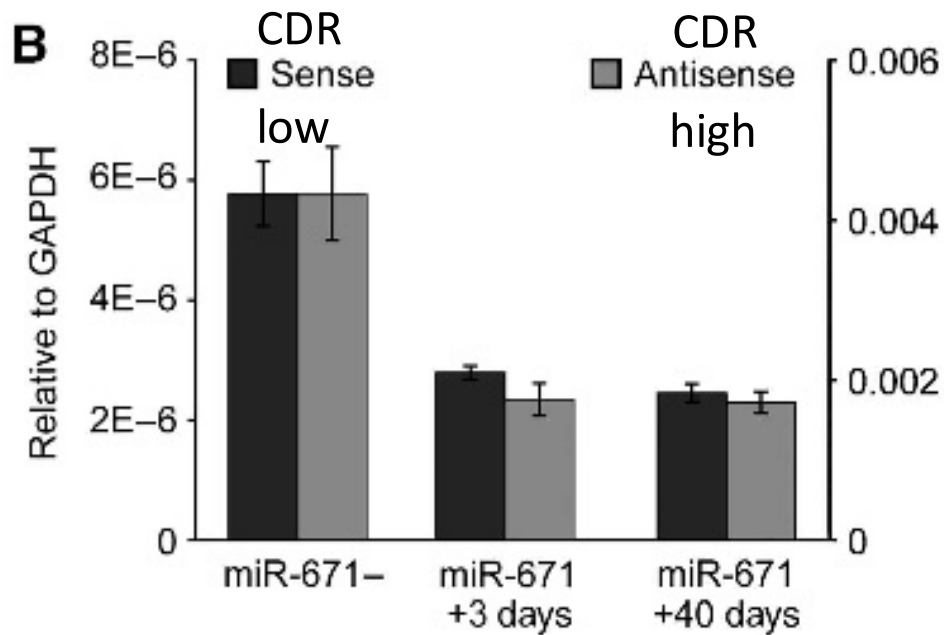
6. Last step:
Sequencing of
PCR product



WHAT IS THE FUNCTION OF miR-671/AS-CDR1: EPIGENETIC REGULATION BY AS CDR1??

FUNCTION OF miRNA ON CDR1 s/as

miR-671 overexpression reduces CDR1 but also CDR1 AS expression
(AS transcript is the predominant transcript)



Ectopic miR-671 decreases CDR1 as
Reducing endogenous miR-671 increases CDR1 as

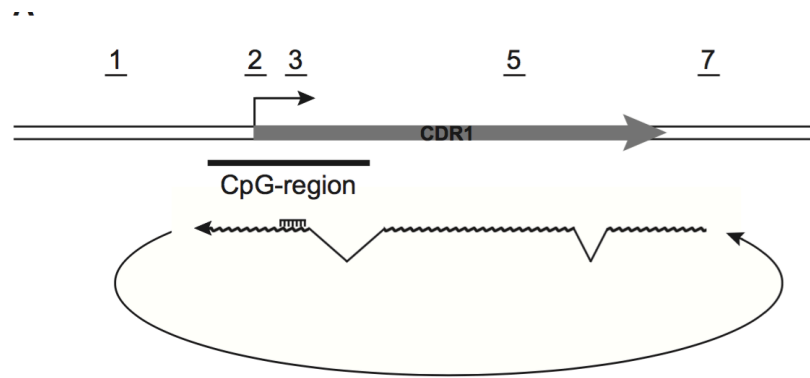


Epigenetics on CDKR1 promoter?

EPIGENETIC REGULATION BY AS CDR1??

HYPOTHESIS:

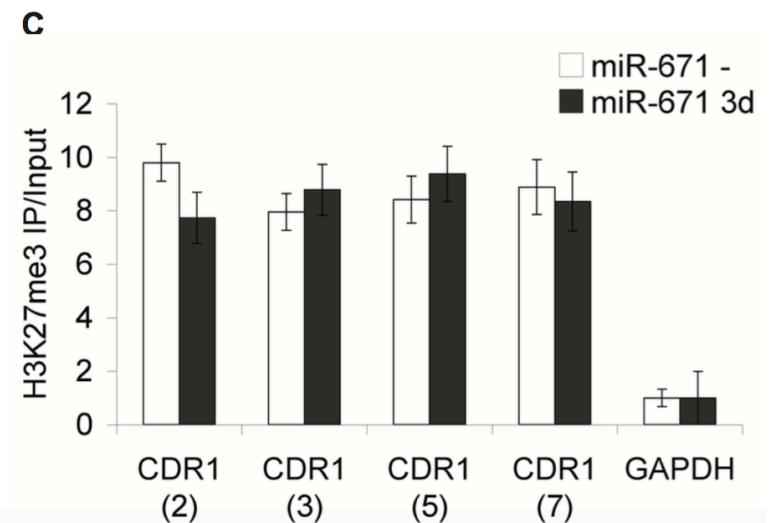
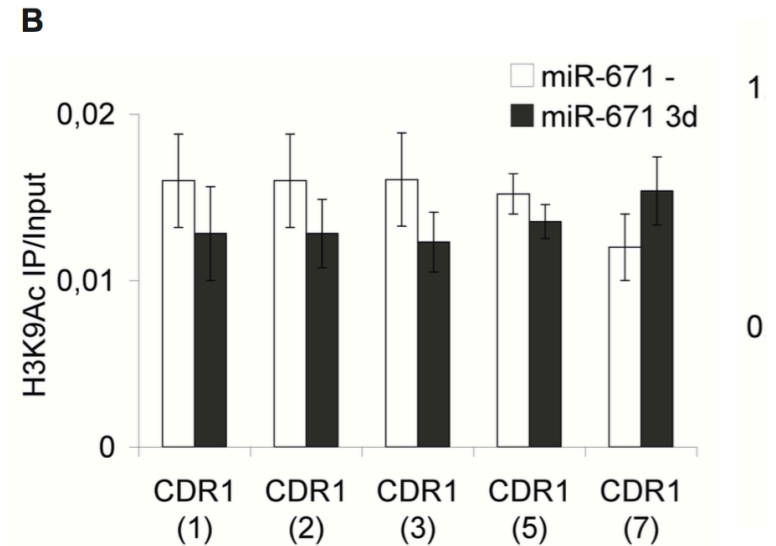
miR-671 and AS CDR1 are involved in epigenetic regulation??



ChIP using PCR oligos 1-7

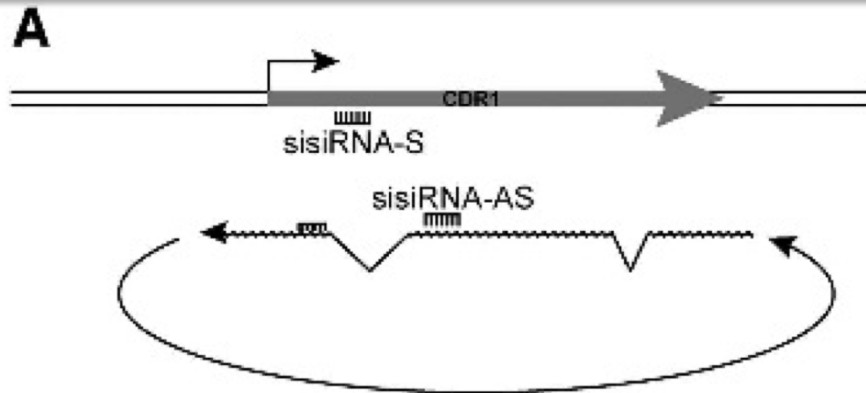
→ Answer: NO
→ NO CHROMATIN CHANGE

**FUNCTION OF miRNA
ON CDR1 s/as**



miRNAs can target nuclear antisense transcripts

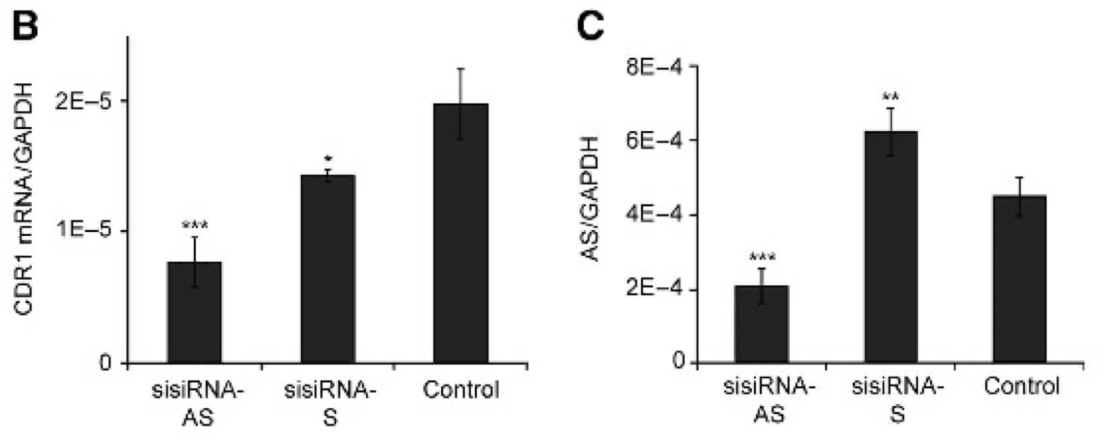
Circ-AS-CDR1 RNA stabilizes sense CDR1 RNA



RNAi specific for sense and anti-sense RNA

siRNA-S: targets sense CDR-1

siRNA-AS: targets AS-CDR-1



→ AS CDR-1 supports S CDR1 expression

→ miR-671 suppresses AS CDR1 expression

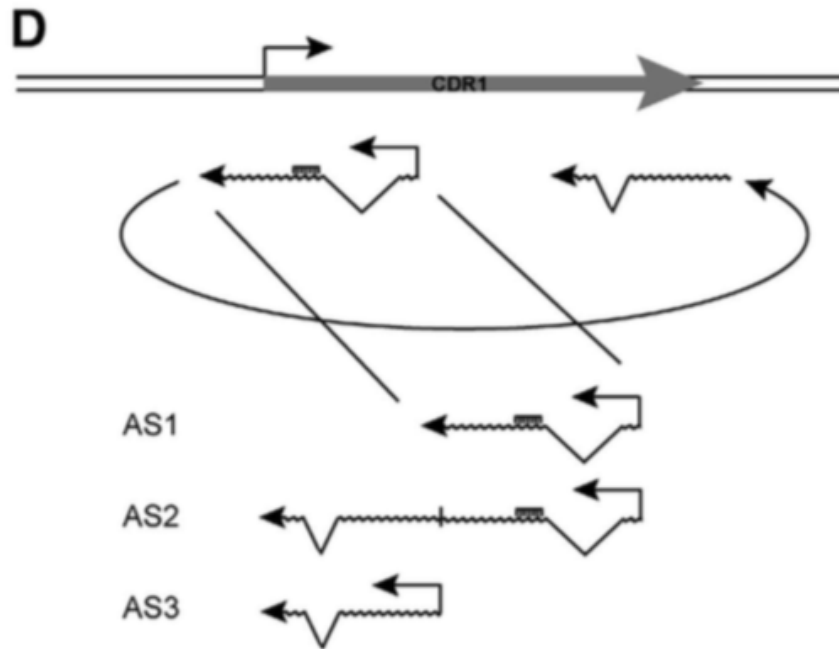
CDR1 sense: reduced by siCDR1 and si-AS-CDR1

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



FUNCTION OF CDR AS

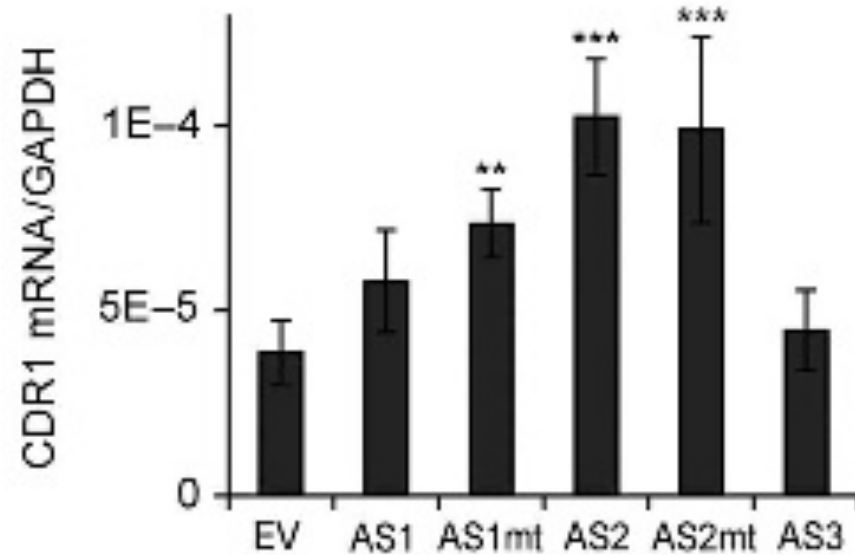
miRNAs can target nuclear antisense transcripts
Circ-AS-CDR1 RNA stabilizes sense CDR1 RNA



Expression of engineered linear transcripts

E

FUNCTION OF CDR AS



Overexpression of AS-CDR1 that “mimics” the structure of the circRNA: Reduced sense-CDR-1 expression

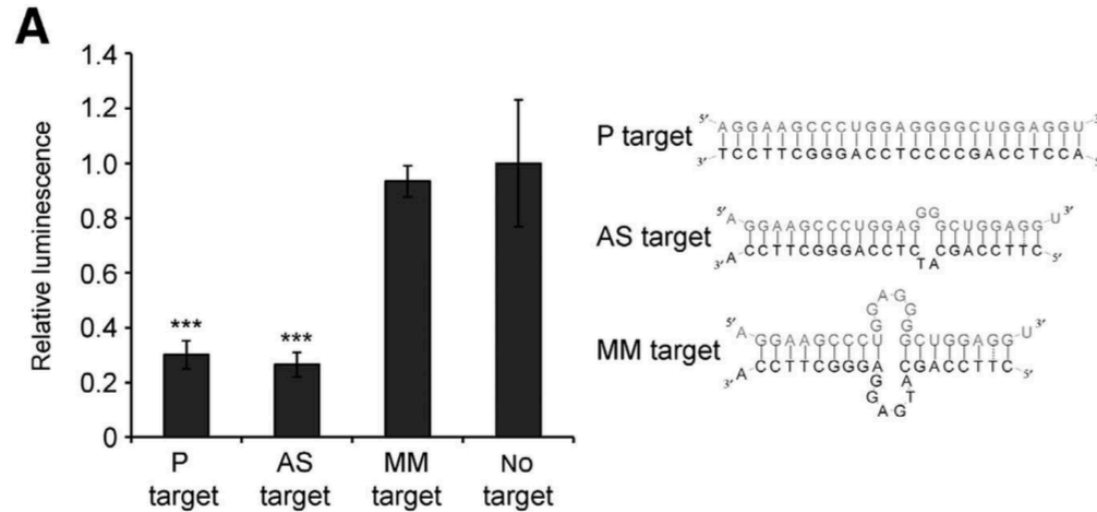
Mutation of the miR-671 target site does not impact on the AS2 effect (miR-671 is not required for AS-CDR1 NAT function)



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?

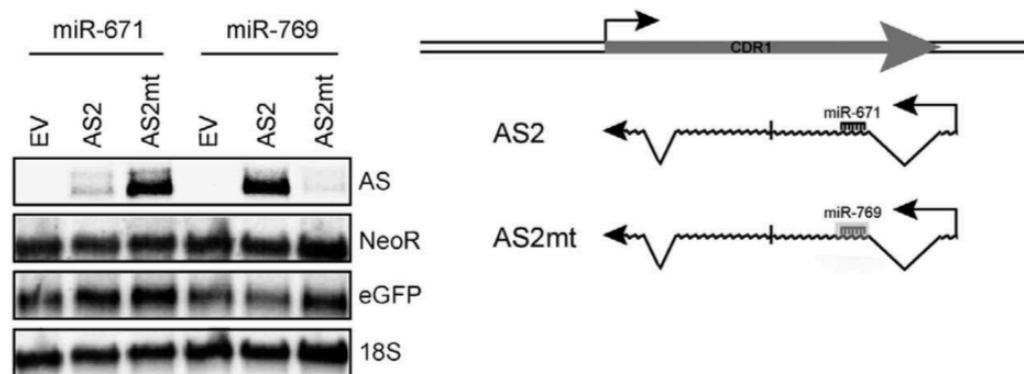
Luciferase assay: AS-CDR1 sequence variants (below) fused to luciferase + mi-671



Perfect match

Endogenous situation

Missmatch situation



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

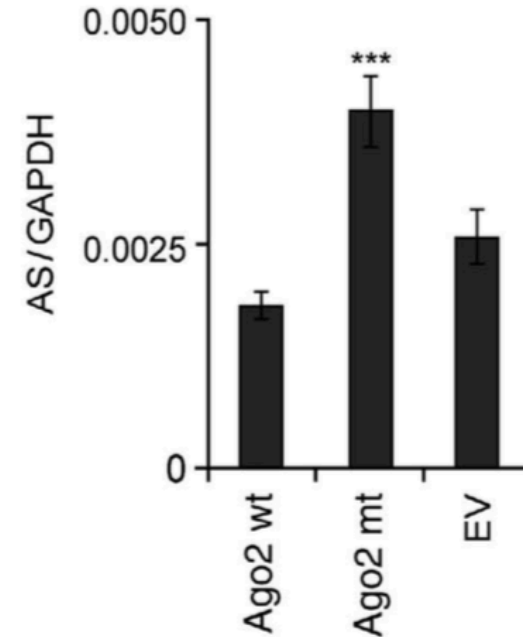
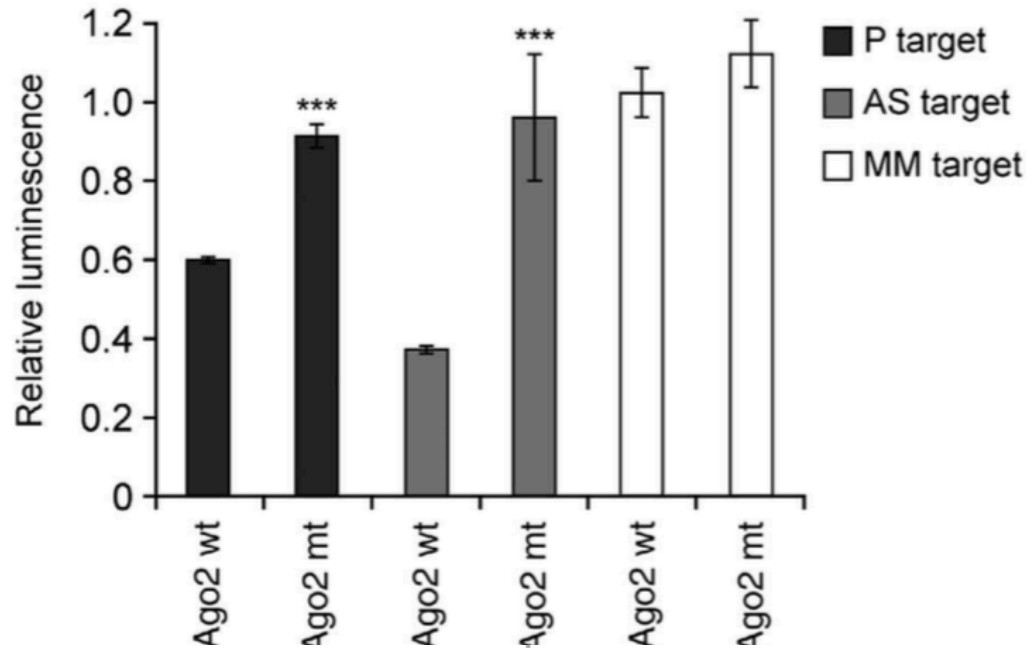
Northern blot

FUNCTION OF CDR1 AS – miRNA INTERACTION

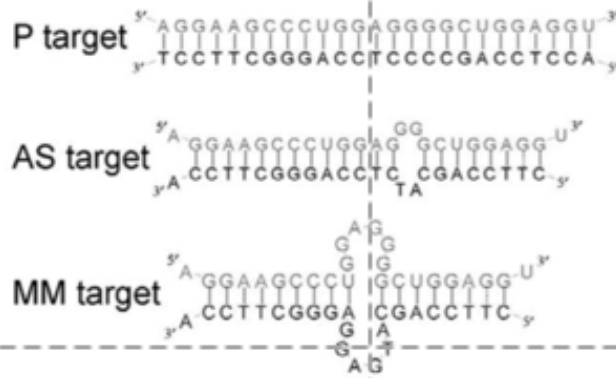
Slicer deficiency protects AS-CDR1 from targeting by miR-671

Luciferase assay: miRNA wt/mutant target sites fused to luciferase + mi-671

RT-PCR



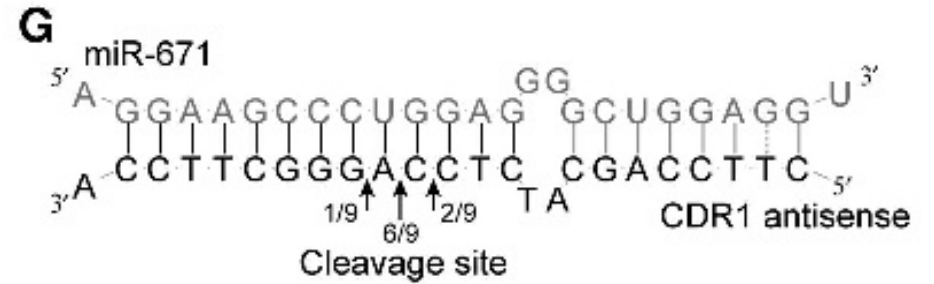
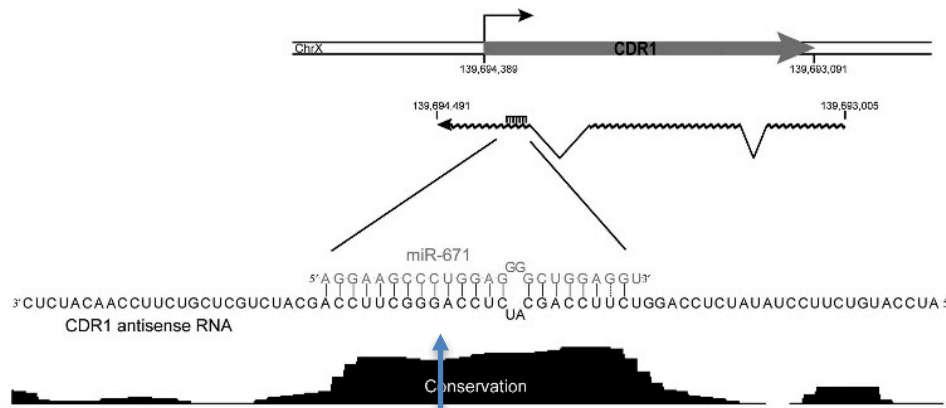
Cells,
Ago wt and
Ago mut



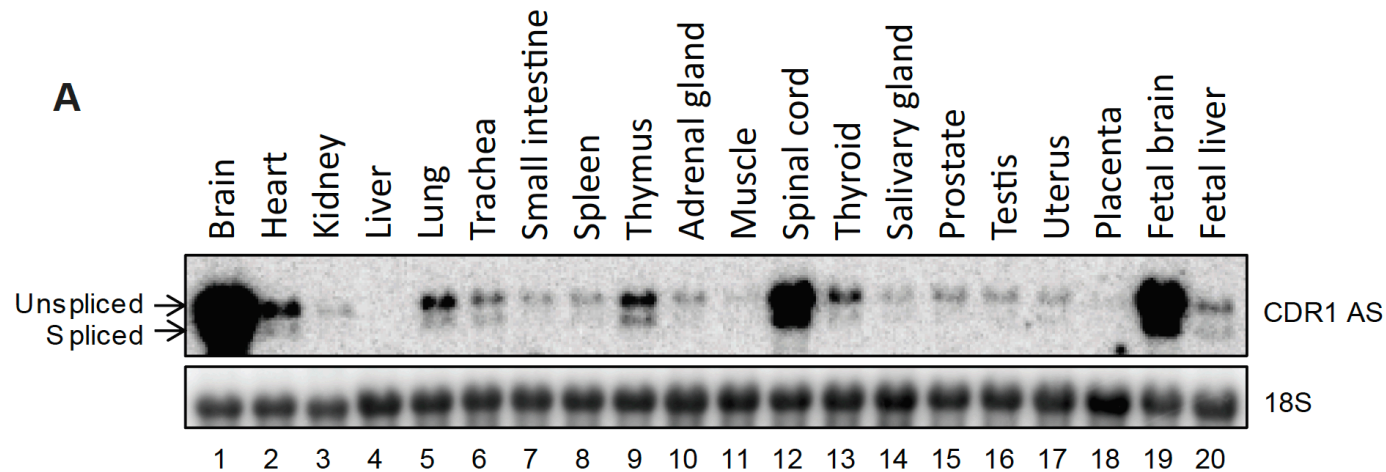
Antisense-specific qRT-PCR on RNA from HEK293 cells transiently transfected with miR-671 along with EV,

FUNCTION OF CDR1 AS – miRNA INTERACTION

The discovery of a circular RNAs



Ago2 cuts AS-CDR1 RNA



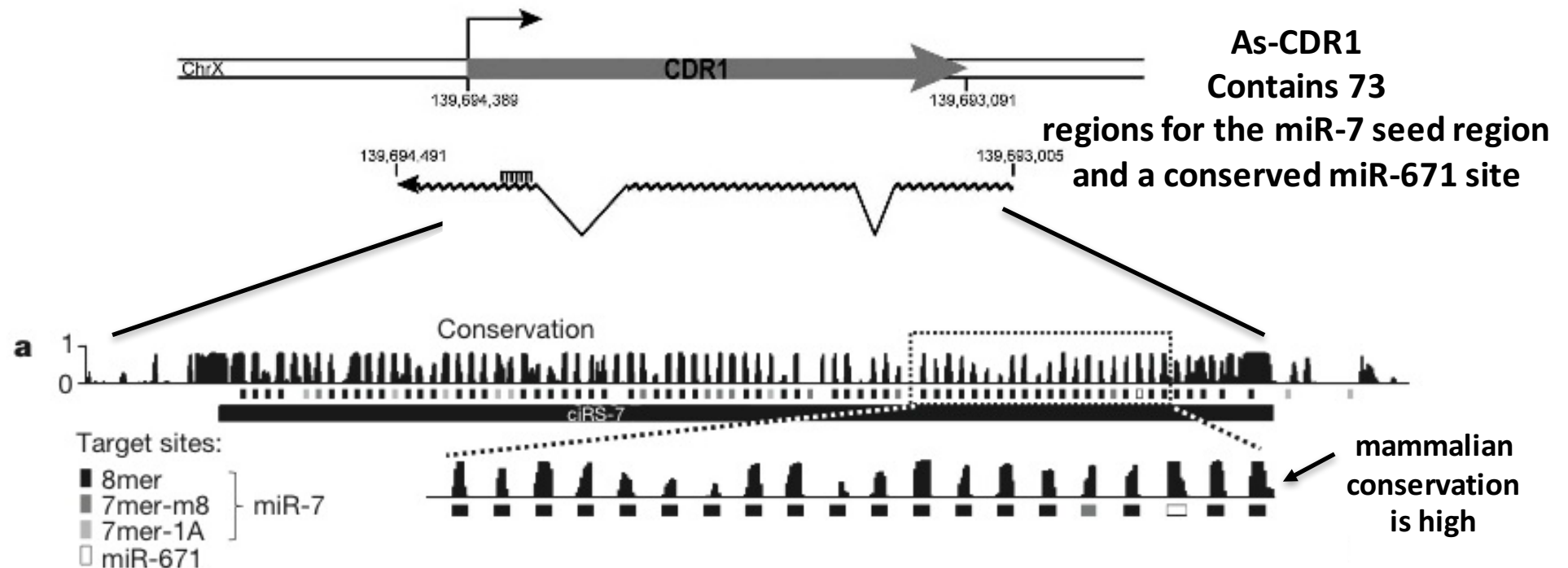
The discovery of a circular RNAs



sisiRNA-mediated strand-specific knockdown. However, the mechanistic link between miR-671 cleavage of the circular NAT and repression of the sense mRNA is unclear. One hypothesis could be that the abundance of *CDR1* NAS antisense may titrate an miRNA from acting on the *CDR1* mRNA, that is a sponge model (Poliseno *et al*, 2010). However, a search for single 7mer putative target seeds sequences, shared between *CDR1* 3'UTR and the antisense, was negative (based on miRBase ver. 17), suggesting that *CDR1* NAS antisense is not acting as a decoy. As the mechanism is epigenetically independent and the *CDR1* mRNA can be increased by ectopic AS expression, we argue that the effect is more likely to be post-transcriptional and that the NAT transcript confers *CDR1* mRNA stability possibly via direct base pairing as observed previously (Faghihi *et al*, 2008).

Natural RNA circles function as efficient microRNA sponges

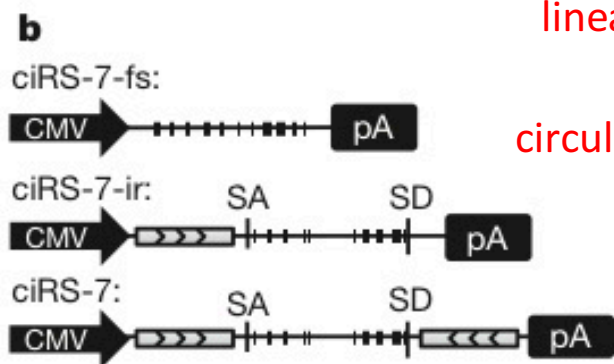
Thomas B. Hansen¹, Trine I. Jensen¹, Bettina H. Clausen², Jesper B. Bramsen^{1,3}, Bente Finsen², Christian K. Damgaard¹ & Jørgen Kjems^{1,3}



cIRS-7: circular RNA sponge for miR-7

ciRS-7 is resistant to miR-7

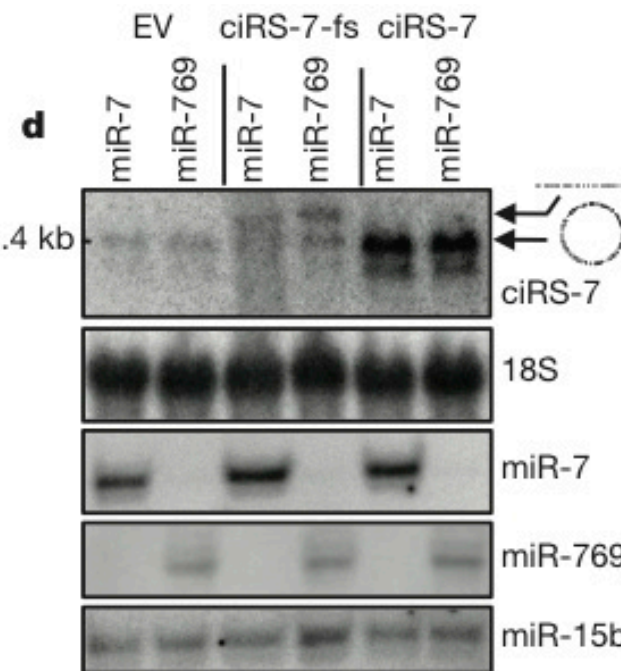
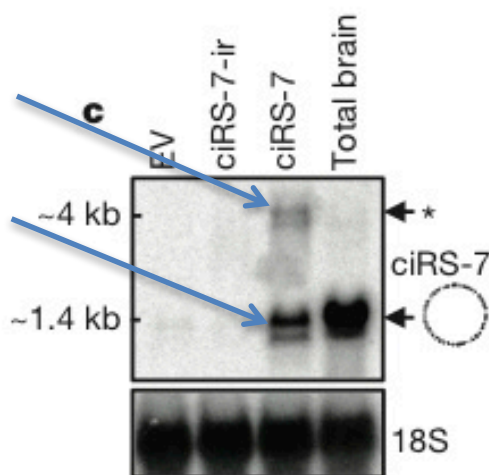
ciRS-7: circular RNA sponge for miR-7



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

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

linear
 circular



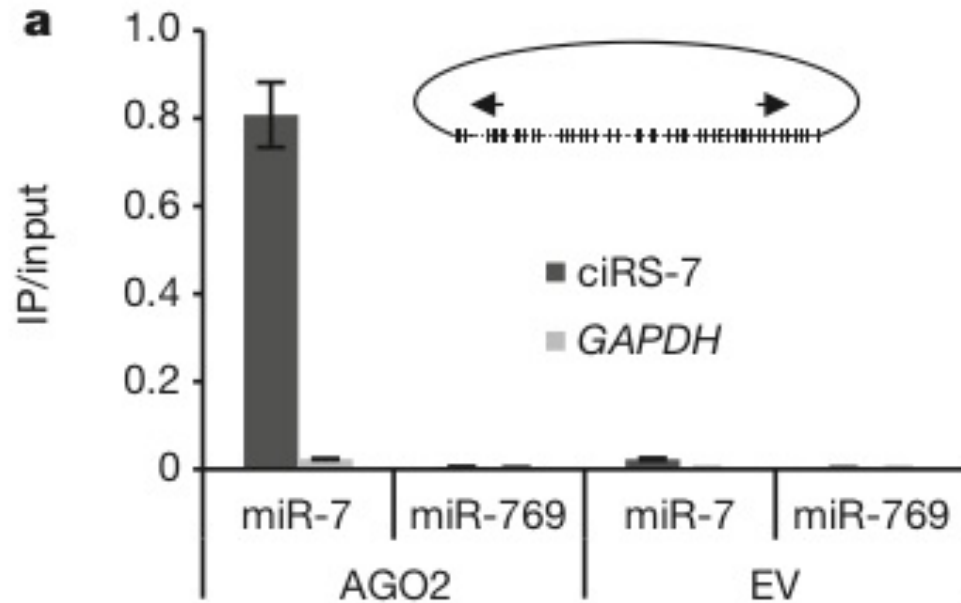
cells transfected with vectors expressing miRNAs and ciRS-7 vectors

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



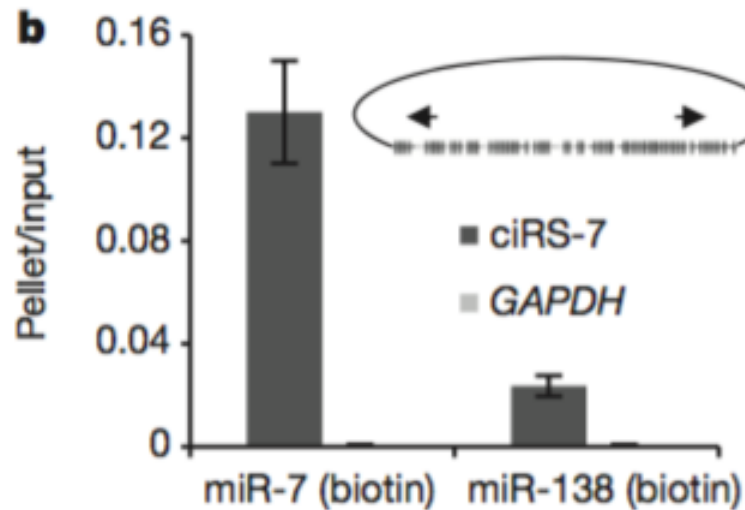
RNA immunoprecipitation

Cells: transfected with Ago2 + miR

IP: anti-AGO2

RT-PCR: ciRS-7 (endog.); gapdh

AGO2 is associated with ciRS-7 and miR-7

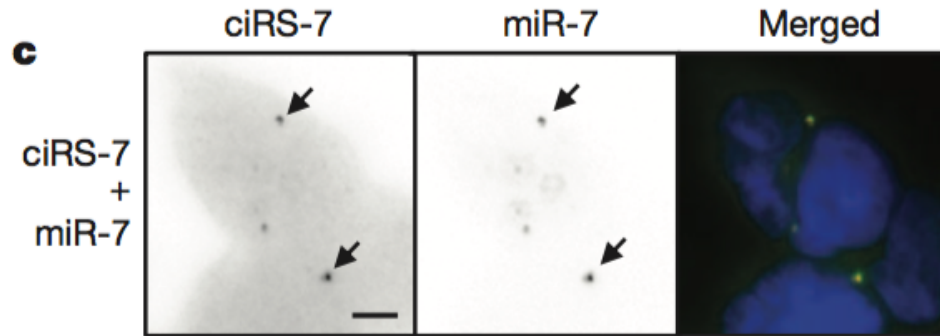


Transient transfection of biotinylated miR-7 or miR-138 and RT-PCR for ciRS-7
Circular RNA

miR-7 is associated with ciRS-7

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

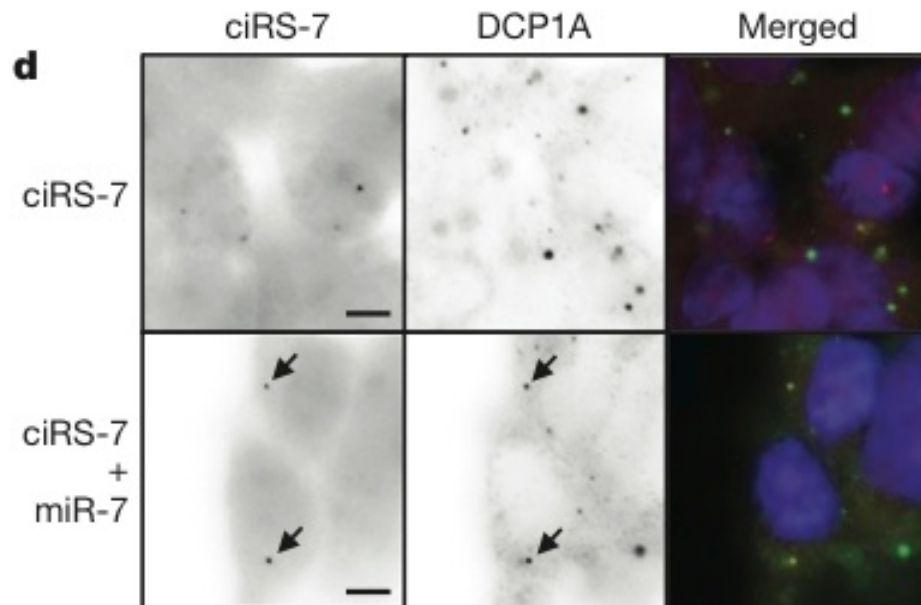
RNA-FISH for miR-7 + ciRS-7



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

Cells:
 - cells + ciRS7 +miR-7

RNA-FISH for miR-7 + Immunostaining for DCP1A



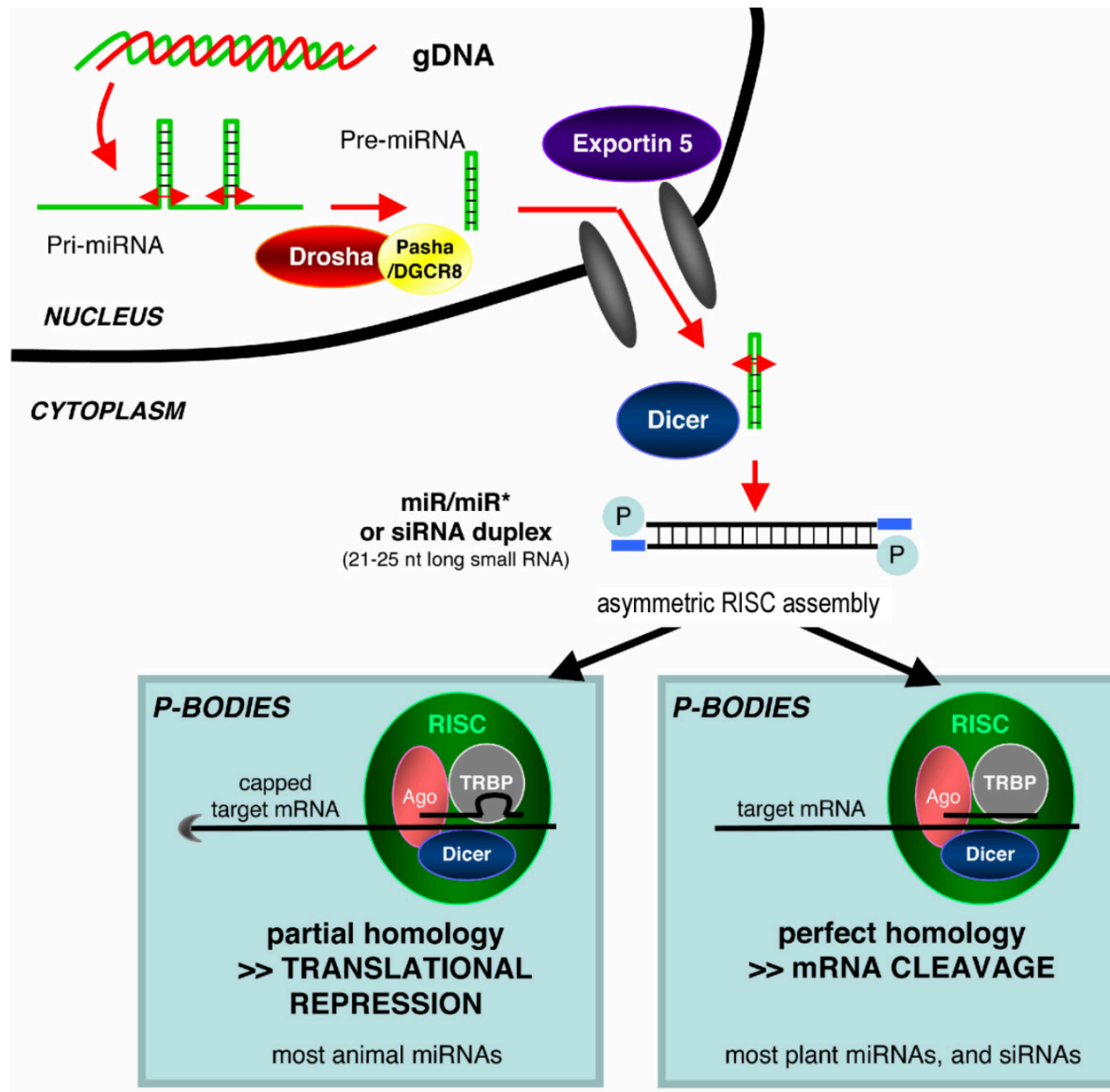
ciRS-7 localize to P-bodies
 (anti-DCP1A/ciRS-7 Immuno-RNA-FISH)

co-localisation only when miR-7 at high levels

Cells:
 - cells + ciRS7 +miR-7

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

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

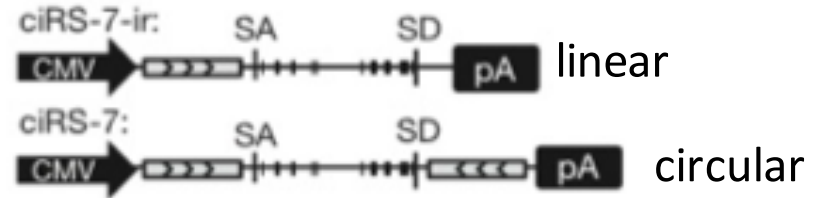
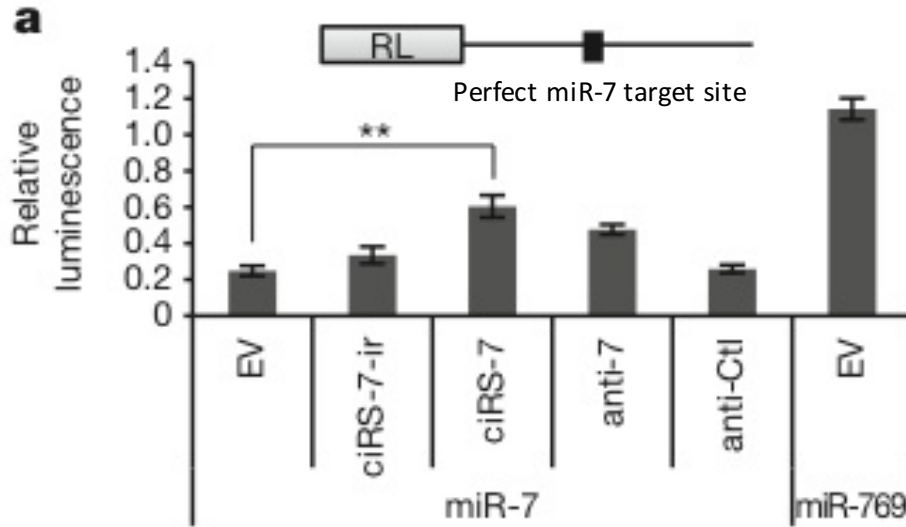


P-bodies: RNA_Protein bodies

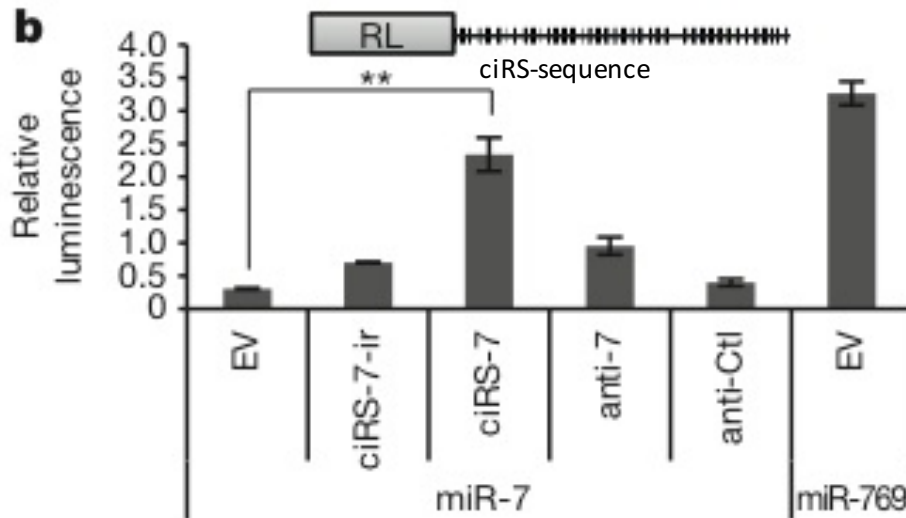
Processing bodies (P-bodies) are distinct foci within the cytoplasm of the eukaryotic cell consisting of many enzymes involved in mRNA turnover. P-bodies have been observed in somatic cells originating from vertebrates and invertebrates, plants and yeast. To date, P-bodies have been demonstrated to play 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

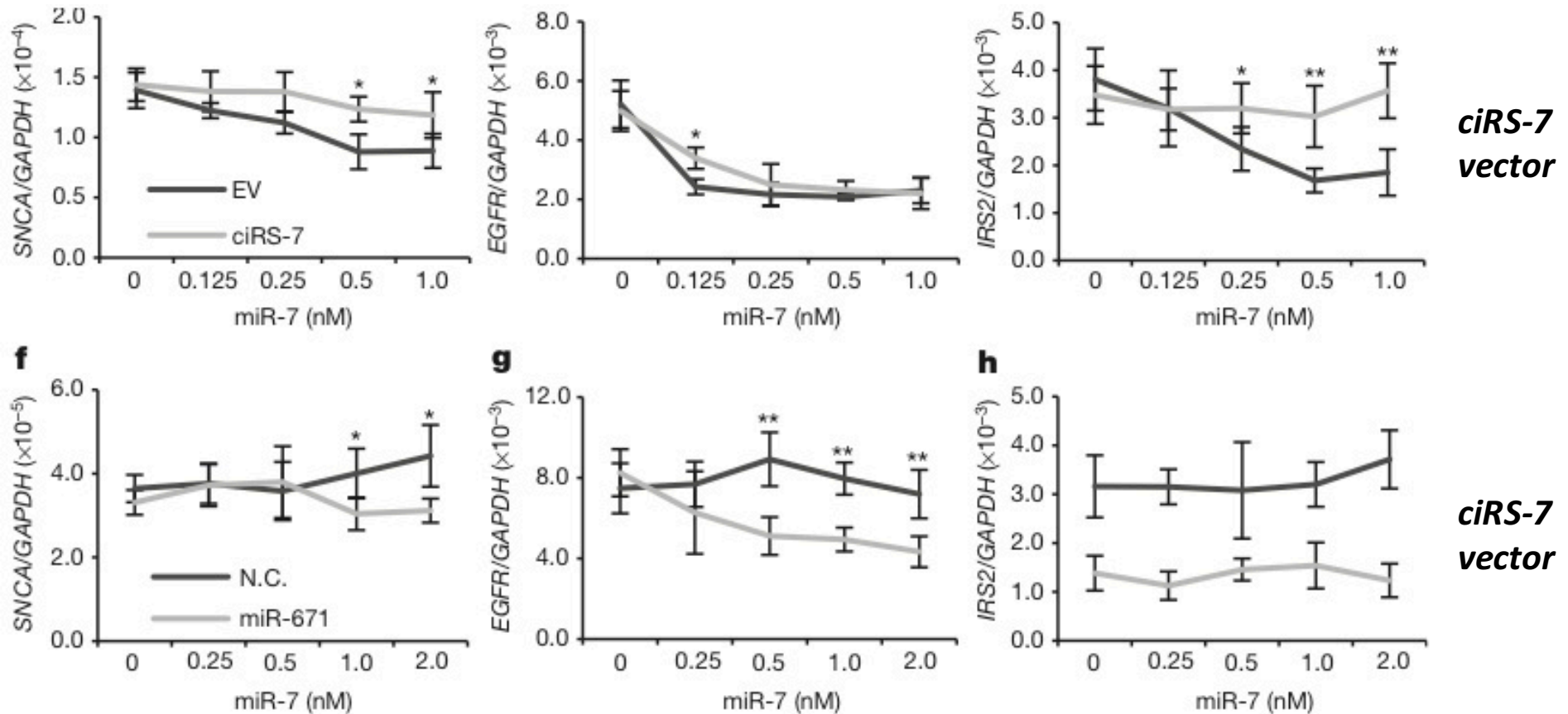


ciRS-7 increases luc activity when
 Luc was fused to a the linearized ciRS-7 RNA sequence

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

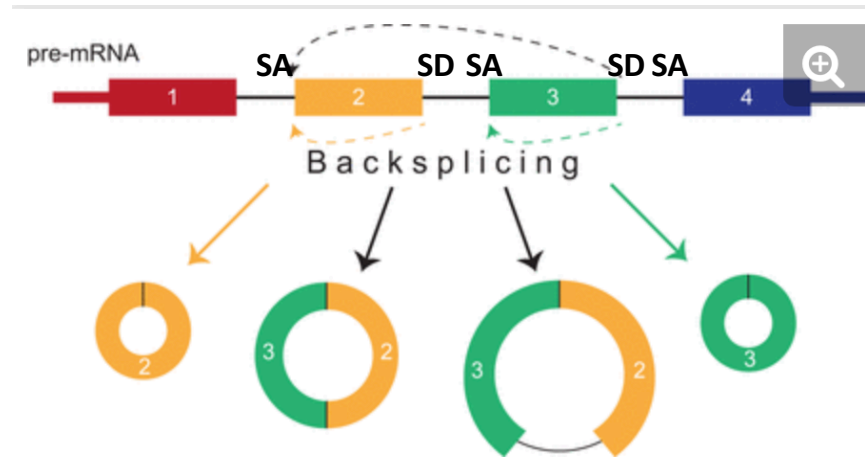
SNCA, EGFR, IRS2 are miR-7 target genes: does ciRS-7 sponge miR-7 ??

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

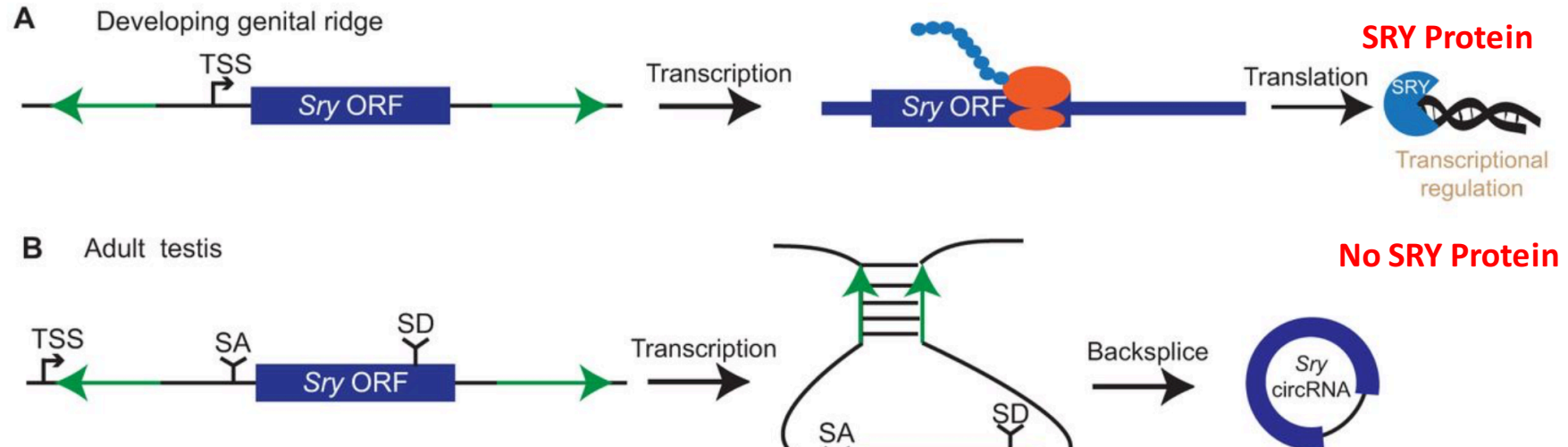


miR-671 degrades ciRS-7

“Backsplicing” can produce circular RNAs



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

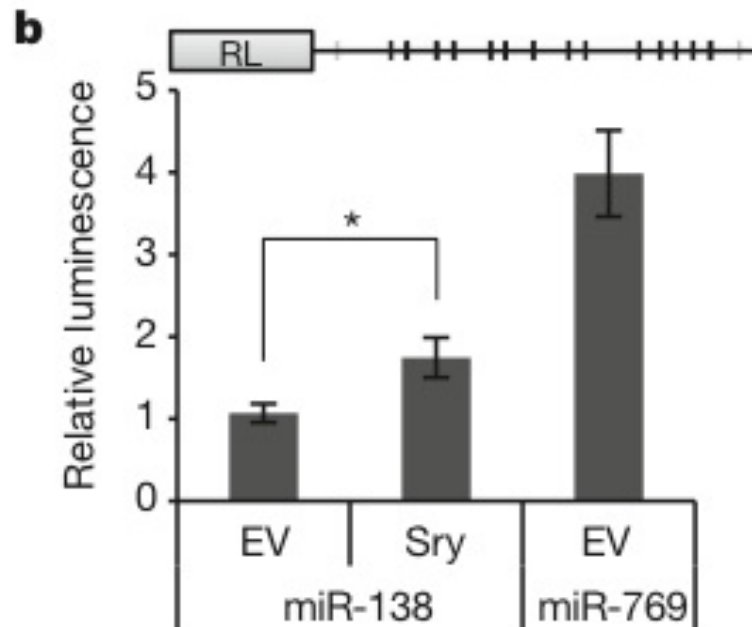
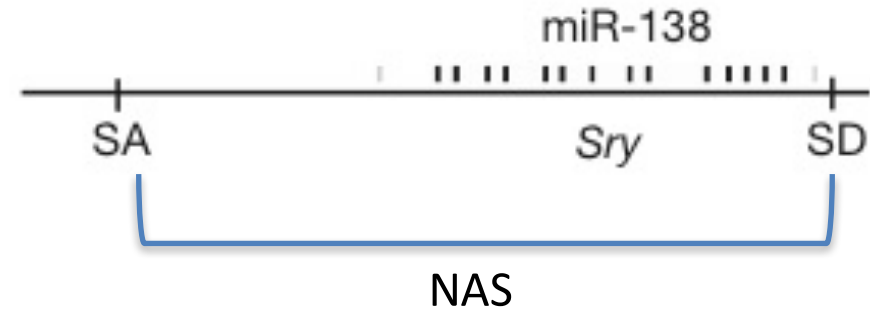


Developmentally regulated expression of SRY. (A) In the genital ridge of the developing mouse embryo, the *Sry* transcription start site (TSS) occurs proximal to the open reading frame (ORF), yielding a translatable mRNA that gives rise to SRY protein, a transcription factor involved in sex determination. (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.

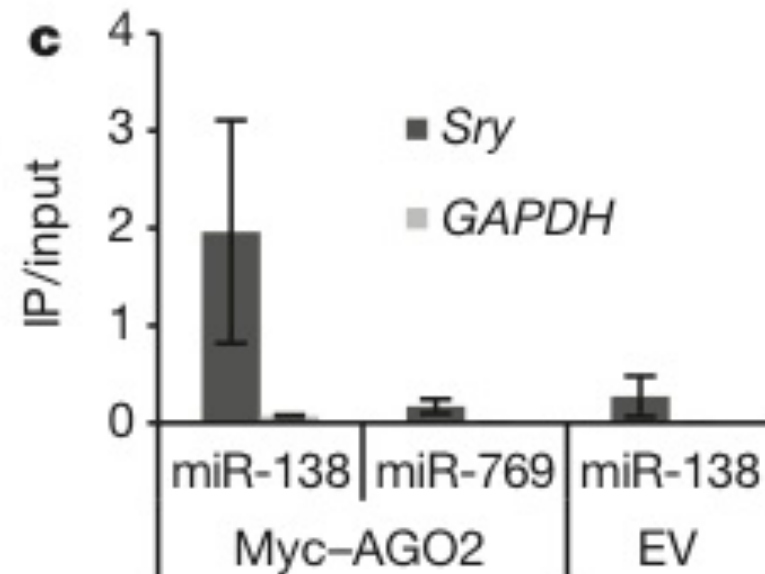
ciRNAs act as sponges for miRNAs – another example

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

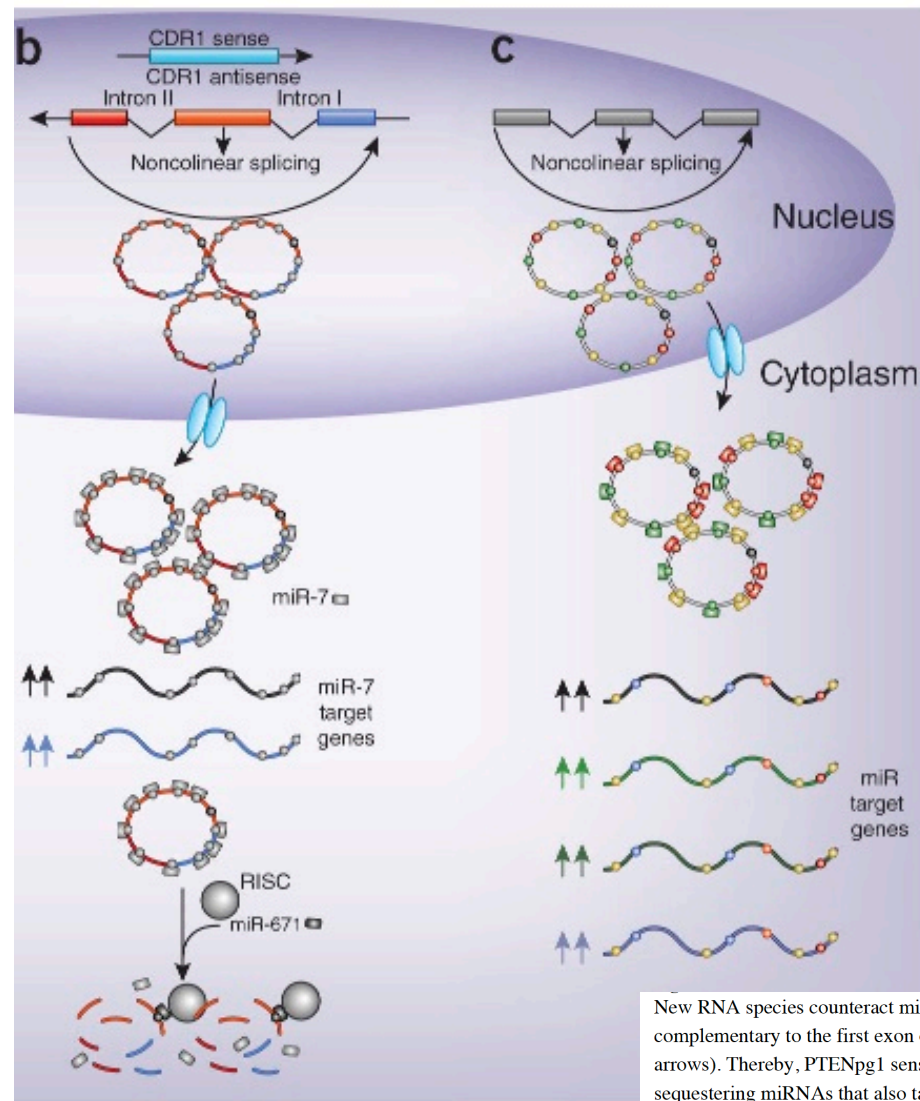


ciR-138 increases
luc-activity in miR-138
Transfected cells



AGO2 associates with ciR-138 in miR-138
transfected cells

Circular RNAs are efficient miRNA sponges

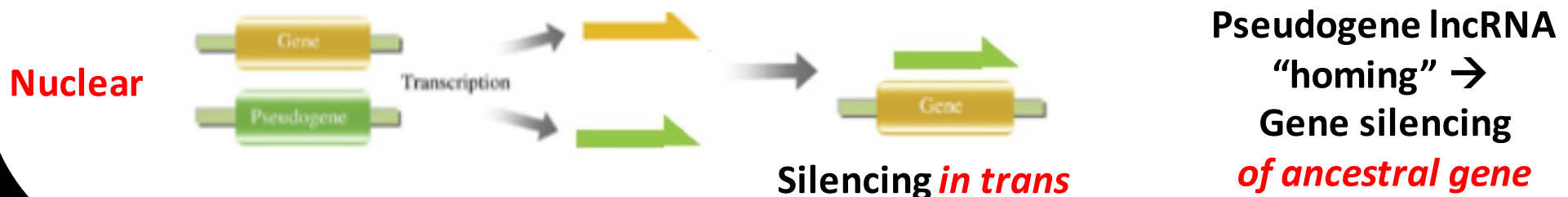
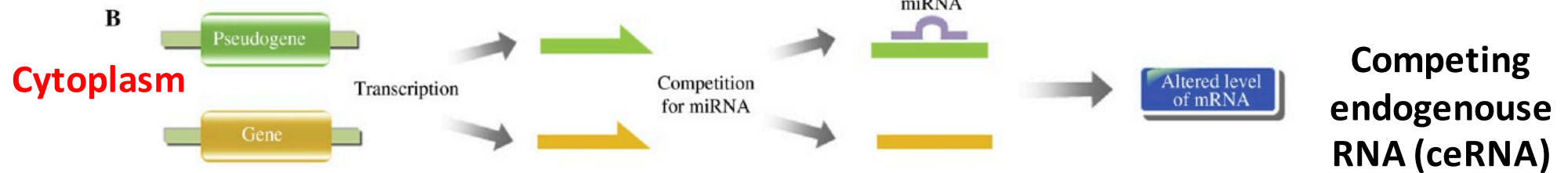


Because circular RNA sponges are characterized by high expression levels, stability, and a large number of miRNA binding sites, they are likely to be more effective sponges than those that are linear.

New RNA species counteract miRNA function. (a) PTENpg1 asRNA β is partially complementary to the first exon of PTENpg1 sense and promotes its stabilization (purple arrows). Thereby, PTENpg1 sense increases *PTEN*-mRNA abundance (red arrows) by sequestering miRNAs that also target *PTEN*. (b) circRNA CDR1 as (ciRS-7) arises from head-to-tail splicing of its precursor. CircRNA CDR1 as (ciRS-7) contains ~70 MREs for miR-7 and increases the expression of miR-7 target genes (black and blue arrows) by sequestering miR-7. CircRNA CDR1 as (ciRS-7) is under miR-671 regulation. miR-671 is almost perfectly complementary to CDR1 as (ciRS-7), and, although circularization protects from canonical nucleases, this is not sufficient to counteract AGO2 slicer activity in RISC. (c) circRNAs containing several distinct MREs can sequester different miRNA families and increase the expression of all genes (colored arrows) under miRNA regulation. MREs and miRNAs are indicated as color-coded circles and rectangles, respectively, on the RNA molecules.

**Small RNAs from endogenous loci
endo-siRNAs**

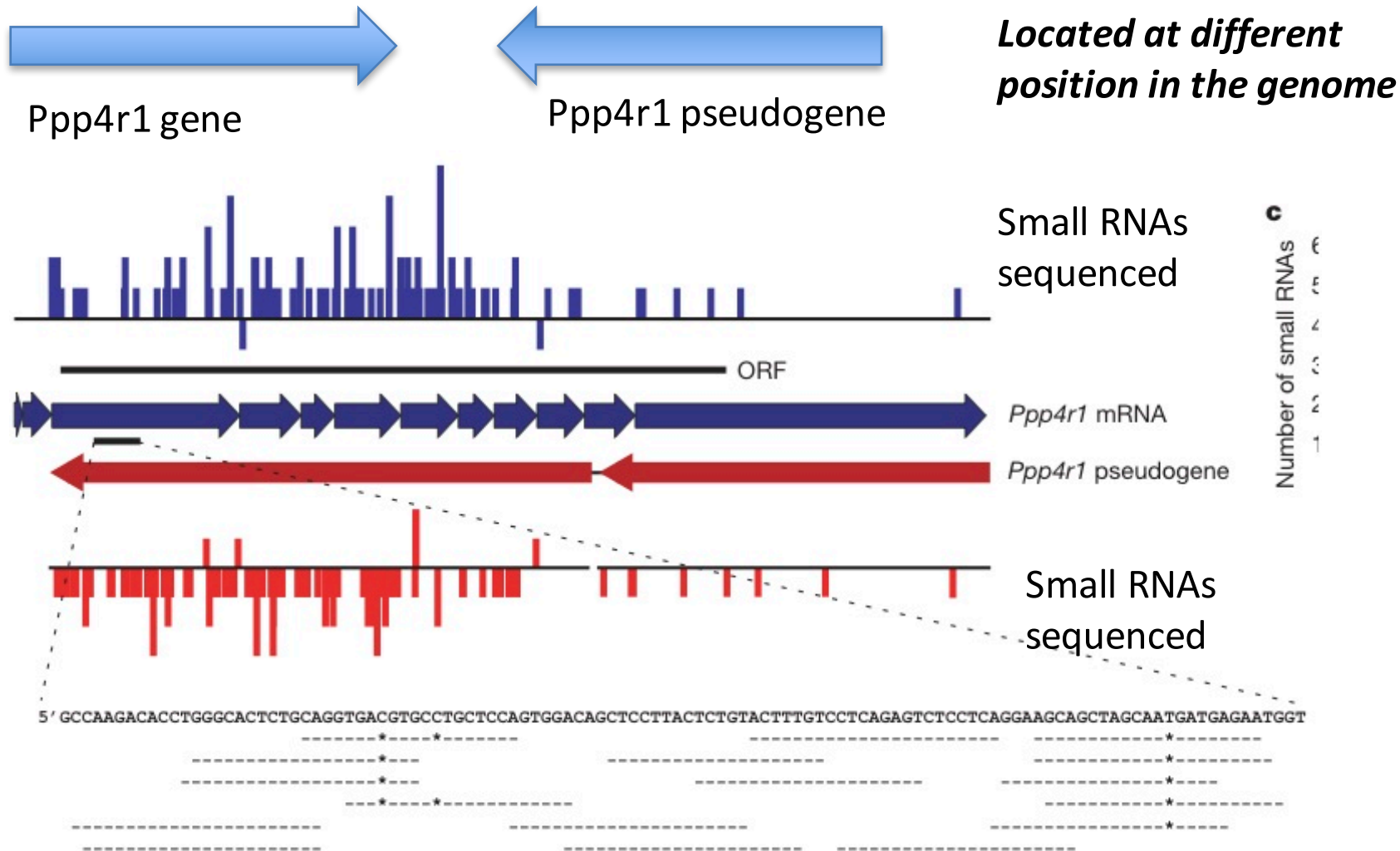
pseudogenes are powerful regulators of gene expression



Discovery of pseudogene derived endo-siRNAs

Massive parallel sequencing of small RNAs

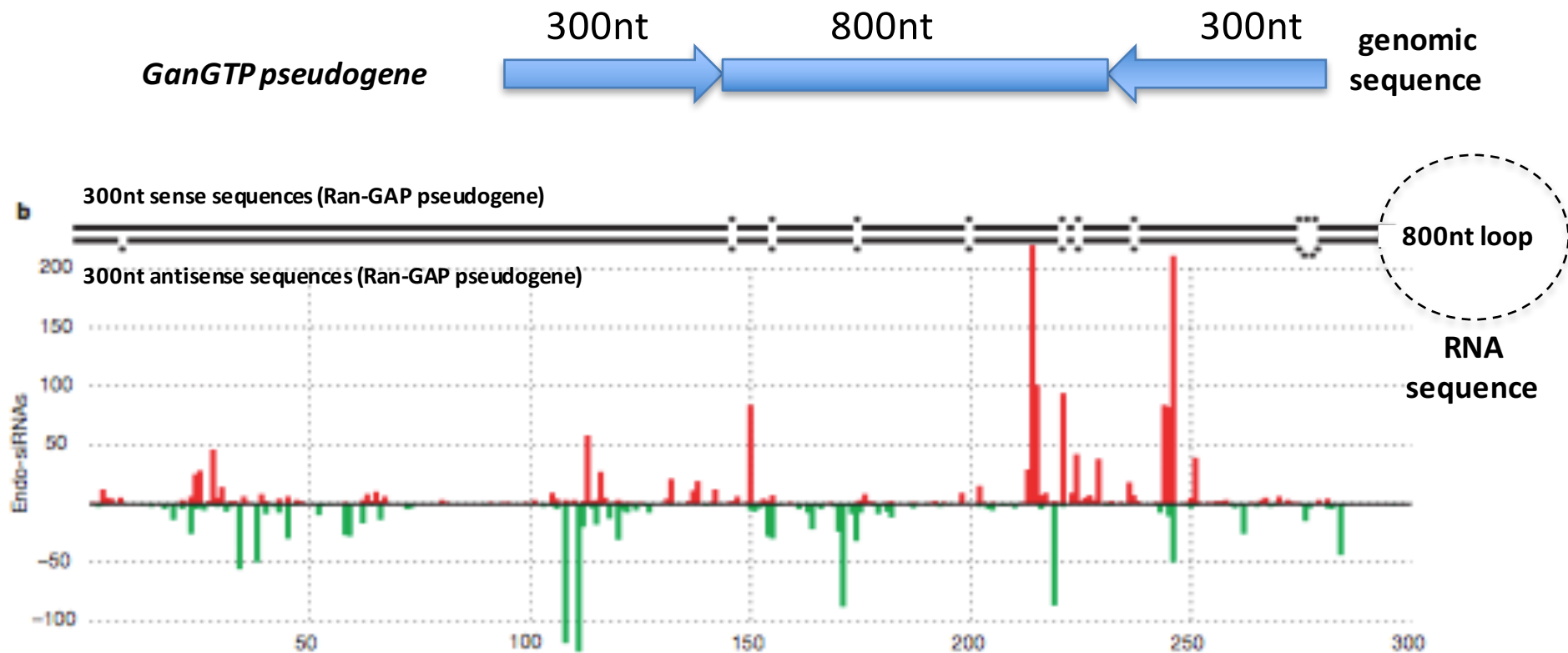
A small fraction of RNAs (21-22nt) was found to map to pseudogenes
siRNA map only to regions of complementarity (not to miRNA genes)



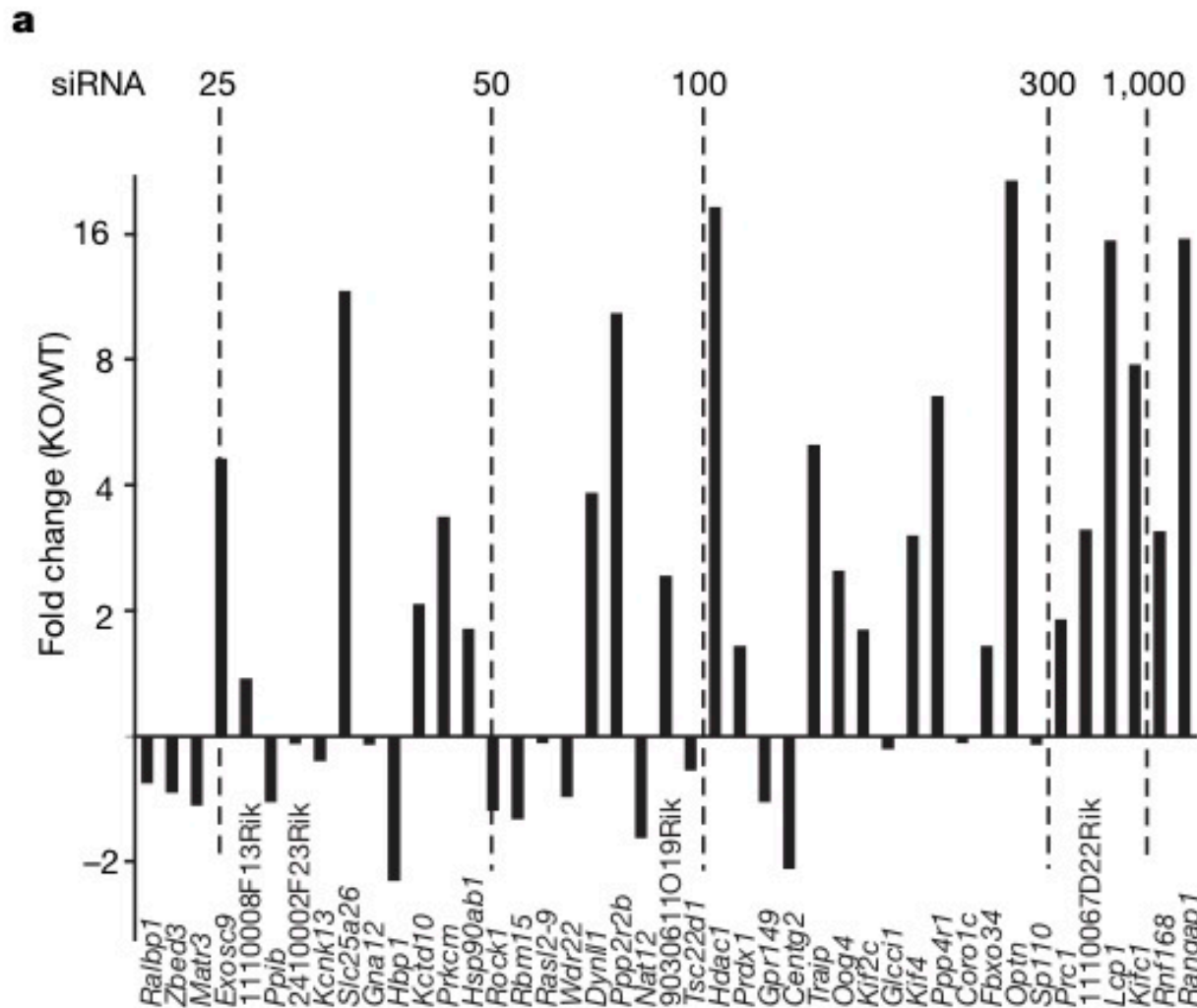
Discovery of pseudogene derives endo-siRNAs

PseudogeneGTPase-activating protein for Ran (Ran-GAP)

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



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



How to elegantly demonstrate the action of siRNAs

Take small RNA sequencing data

- list of siRNA sequences
- Match to annotated mRNAs
- Select candidate genes expressed in ES cells

Use Dicer wt and Dicer^{-/-} Embryonic stem cells

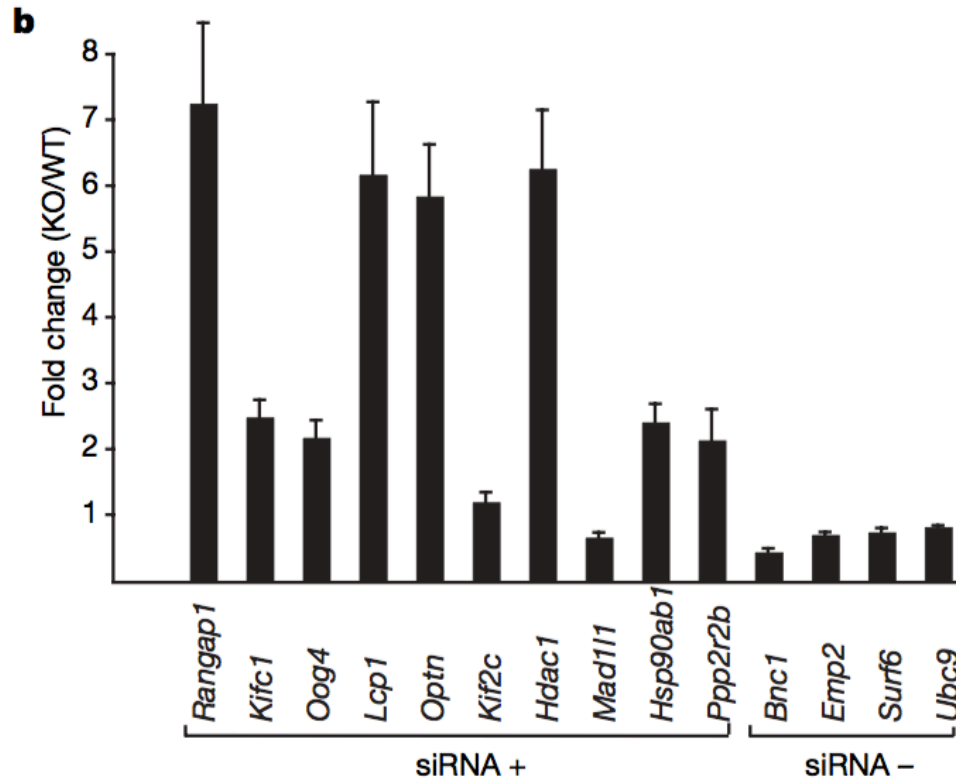
- Prepare total RNA

- 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



Genes with corresponding
siRNA reads found in
sequencing data

*Higher expression
in Dicer-/- versus
Dicer wt ES cells*

Genes with no corresponding
siRNA reads found in
sequencing data

*Same expression
in Dicer-/- versus
Dicer wt ES cells*

Embryonic stem cells
(murine)

Dicer wild type
Dicer^{-/-}

- Prepare total RNA

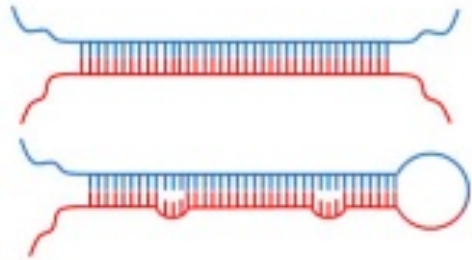
- Make reverse transcription

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

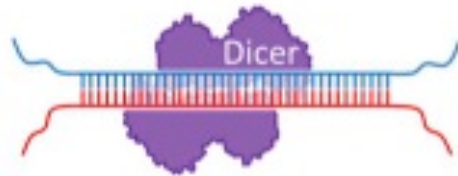
Generation of endo-siRNAs

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 occurring 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