INVESTIGATING FUNCTIONS OF circRNAs

Francesca Rossi

[franc.rossi@uniroma1.it]

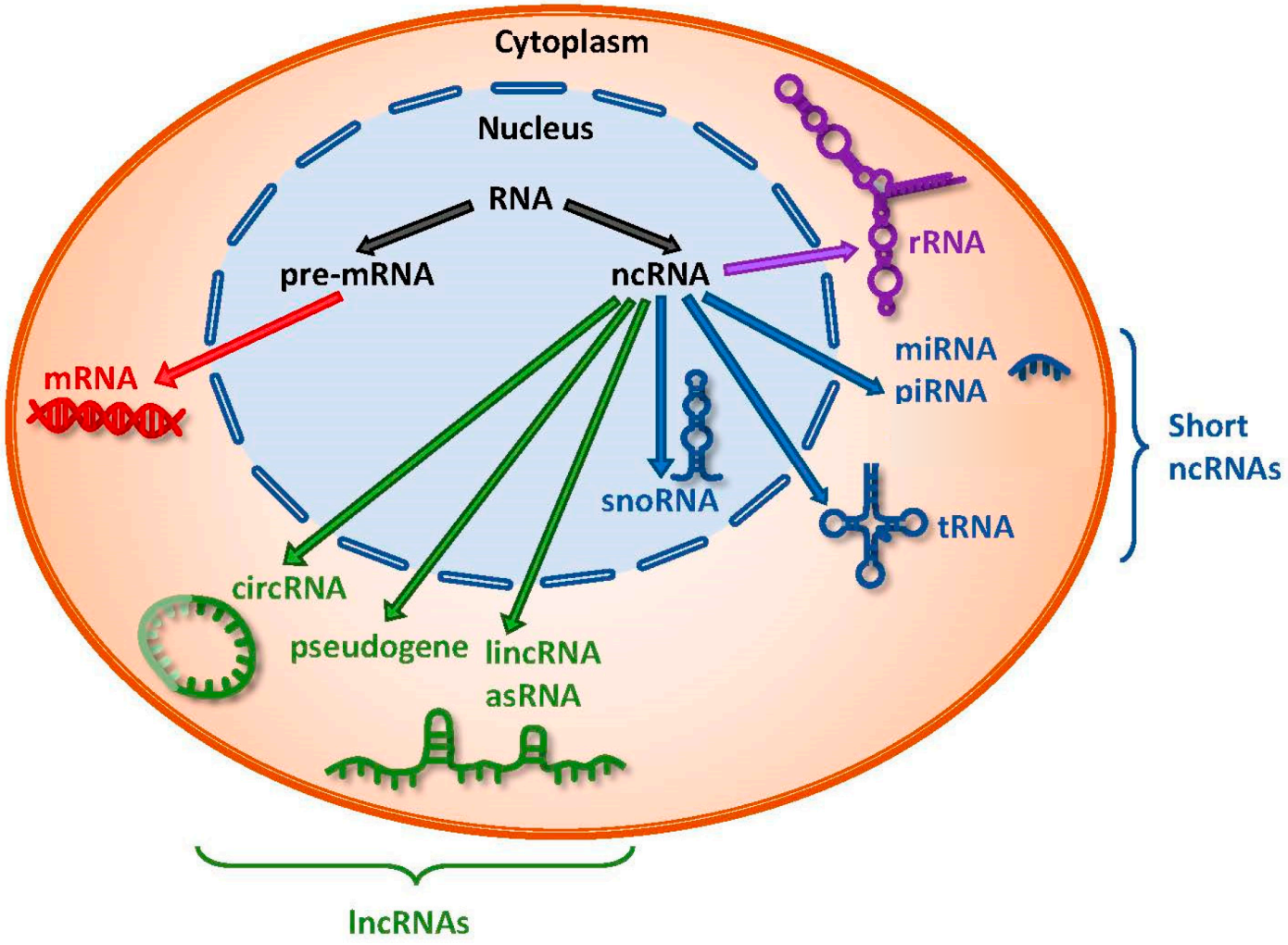
Summary

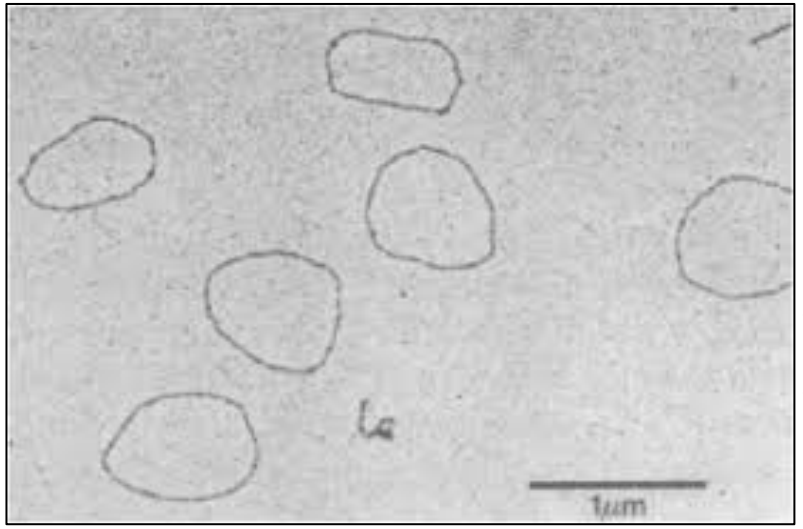
1. (Very brief) review about circRNA biogenesis
2. How to study circRNA molecular functions
3. Molecular functions of circRNAs
4. CircRNAs and their role in cancer
5. CircRNAs as biomarkers in body fluids

Summary

1. (Very brief) review about circRNA biogenesis
2. How to study circRNA molecular functions
3. Molecular functions of circRNAs
4. CircRNAs and their role in cancer
5. CircRNAs as biomarkers in body fluids

CircRNAs belong to long (non-)coding RNAs

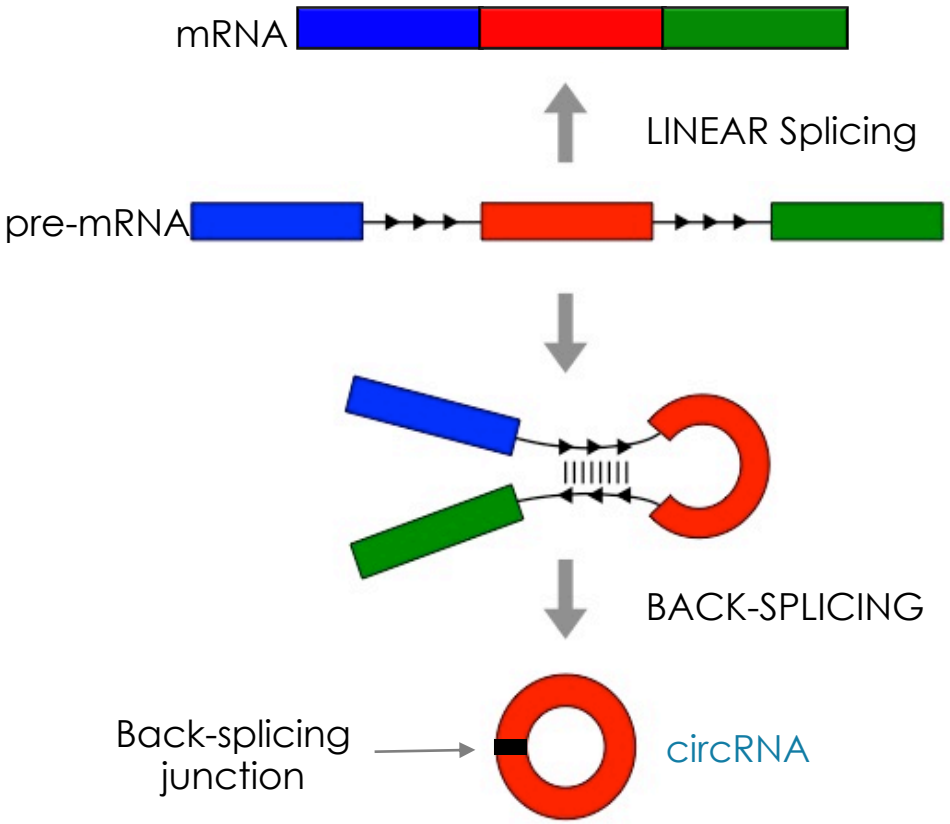




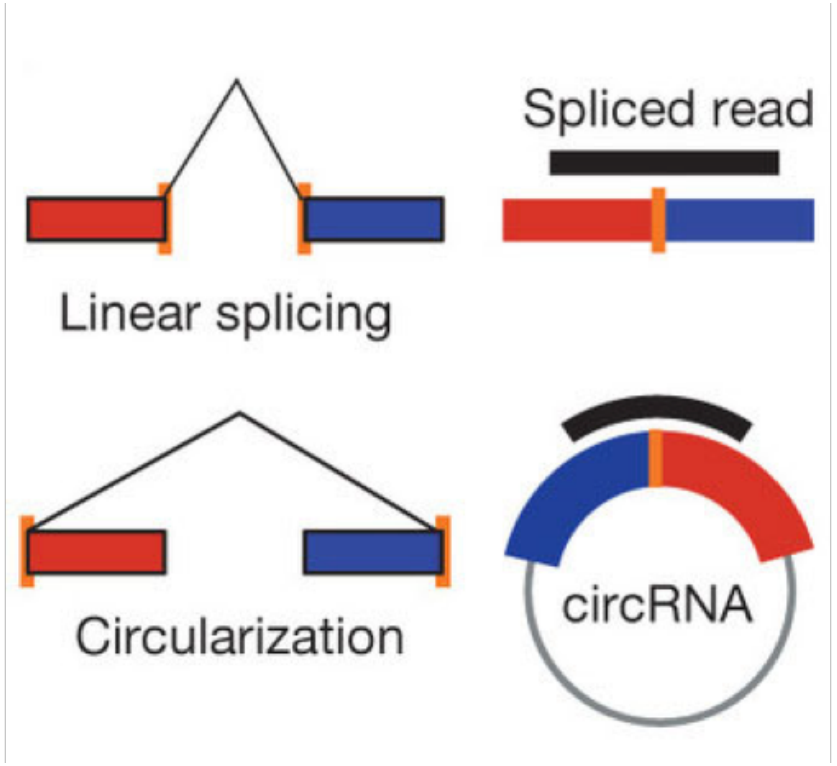
Matsumoto et al. (1990) PNAS

CircRNAs were first reported in **1979** by Hsu & Coca-Prados in an article entitled *Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells*

CircRNA biogenesis



Adapted from Liang *et al.*, 2014

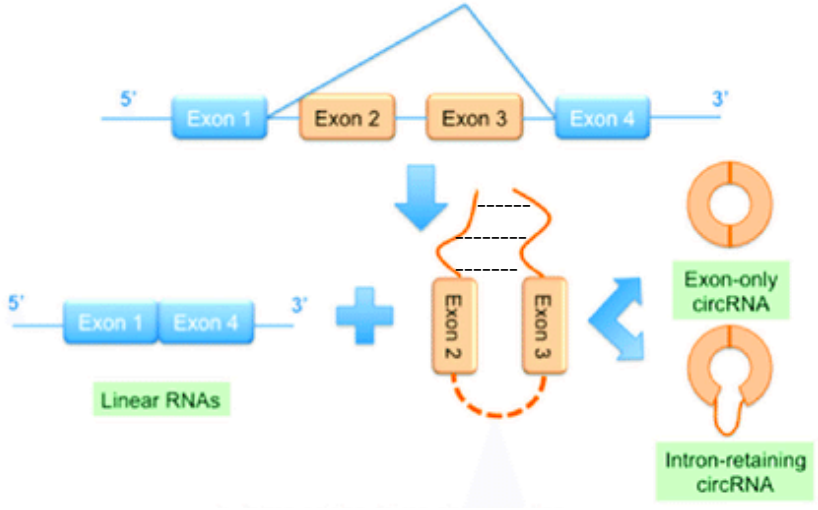


Memczak *et al.*, 2013

In early 1990s Nigro *et al.* (1991) and Coquerelle *et al.* (1993) identified these new RNA species having an **inverted exon order** with respect to the reference genome, which they called "scrambles exons"

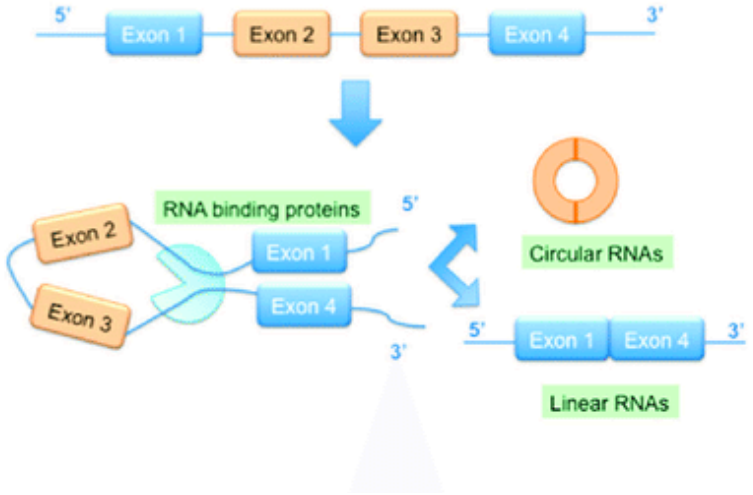
Back-splicing as a new alternative splicing event

EXON SKIPPING



Intron-pairing driven circularization

EXON SKIPPING

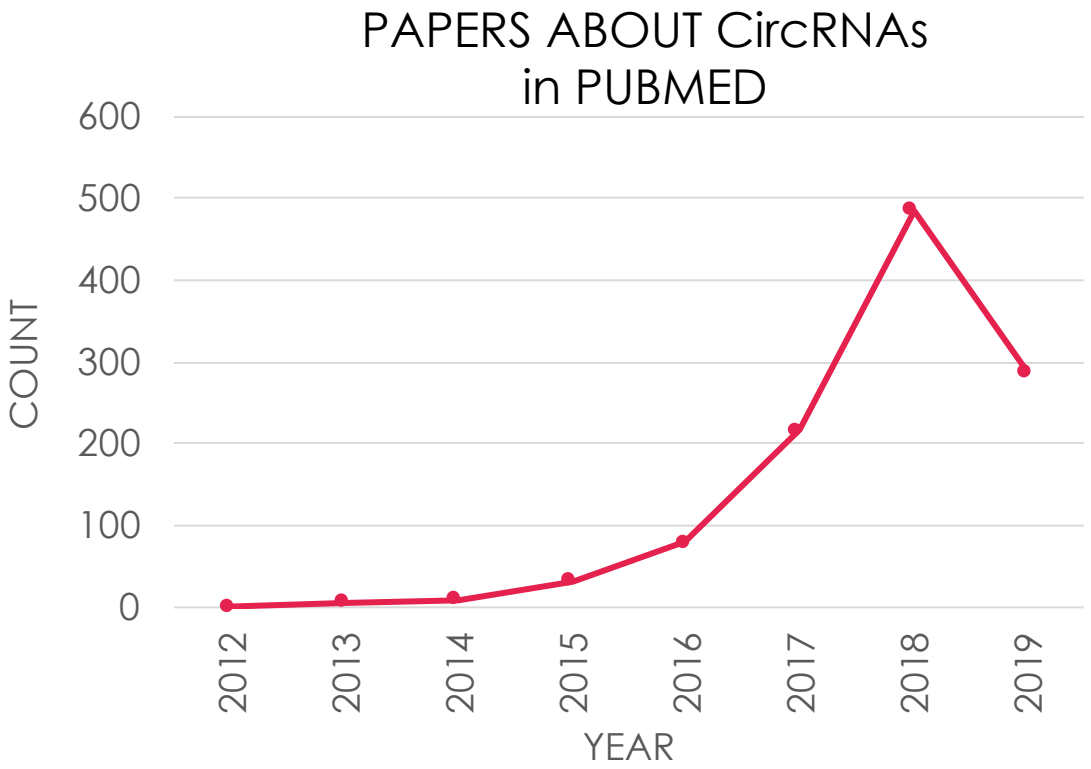


RNA-binding protein driven circularization

Summary

1. (Very brief) review about circRNA biogenesis
2. How to study circRNA molecular functions
3. Molecular functions of circRNAs
4. CircRNAs and their role in cancer
5. CircRNAs as biomarkers in body fluids

CircRNA functions



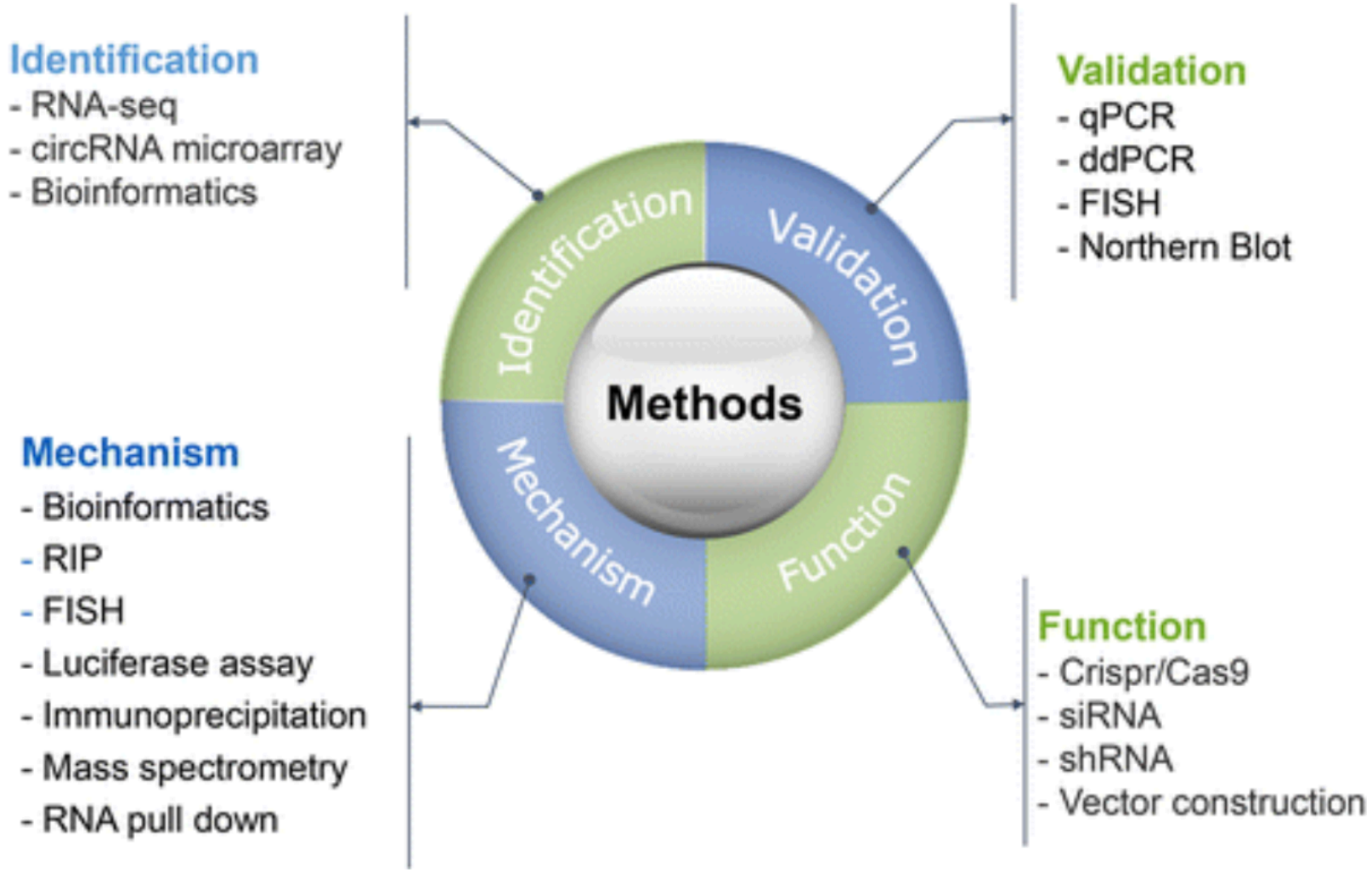
CircRNAs are broadly expressed, conserved, modulated in response to cellular stimuli, and a large fraction is also tissue-/cell type-specific (i.e. Jeck *et al*., 2013; Memczak *et al*., 2013; Salzman *et al*., 2012).

They can be very abundant, as for example in nervous system.

These characteristics strongly suggest possible biological functions for circRNAs.

Although a general mechanism of action has not been found, so far we have several examples of functional circRNAs.

How to study circRNA functions



Zhang et al, 2017

How to study circRNA functions

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

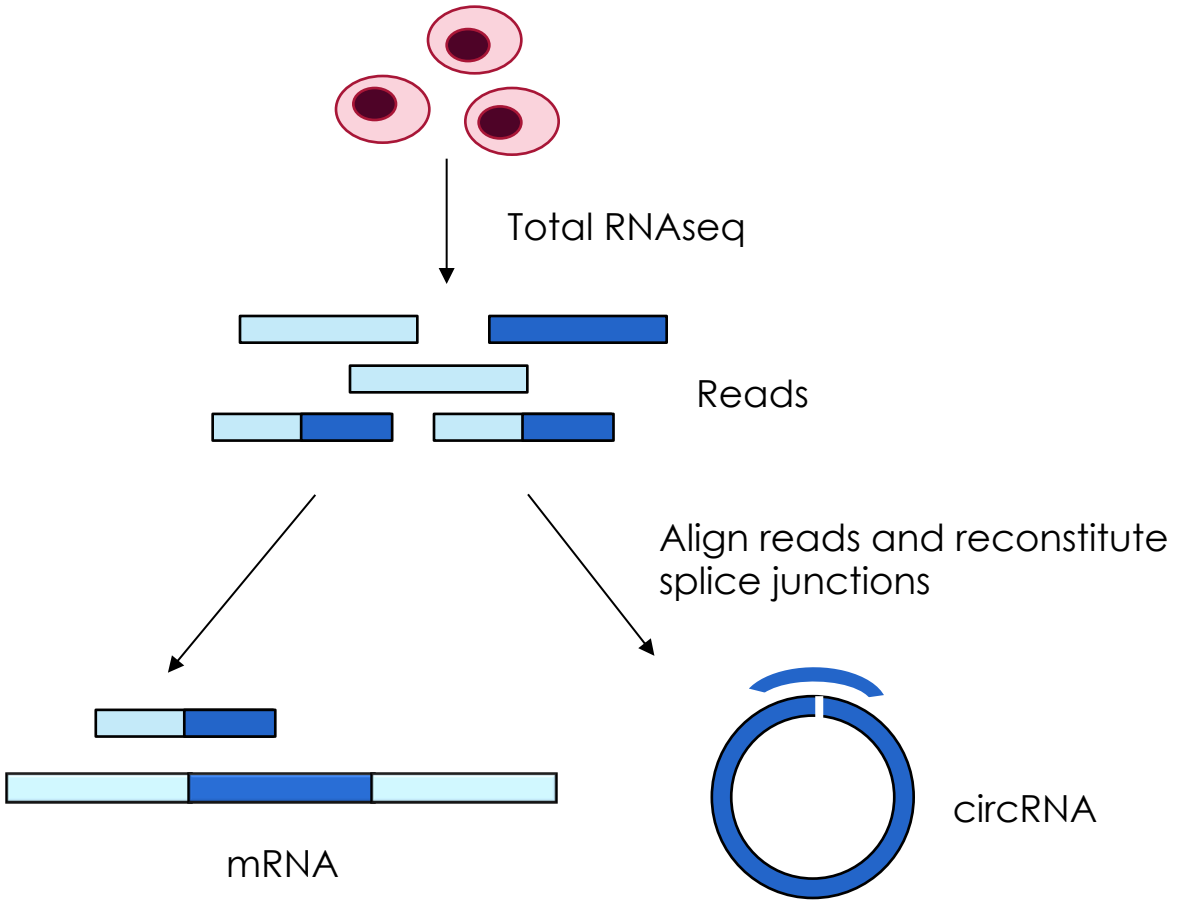
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

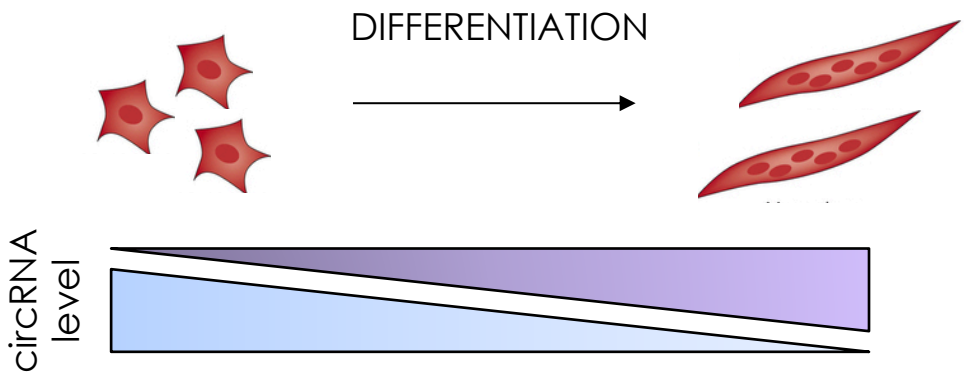
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

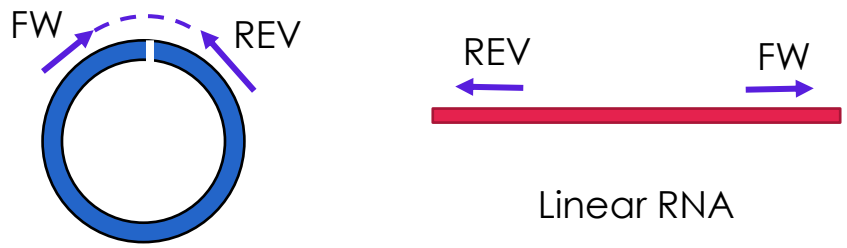
E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions

PCR with **divergent primers**



circRNA

PCR amplification

Linear RNA

No PCR amplification

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

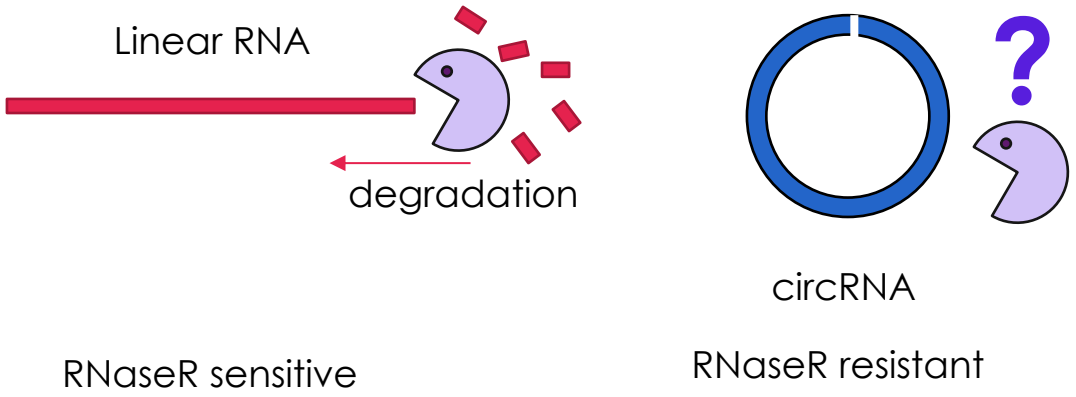
E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions

RNaseR resistance assay



A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

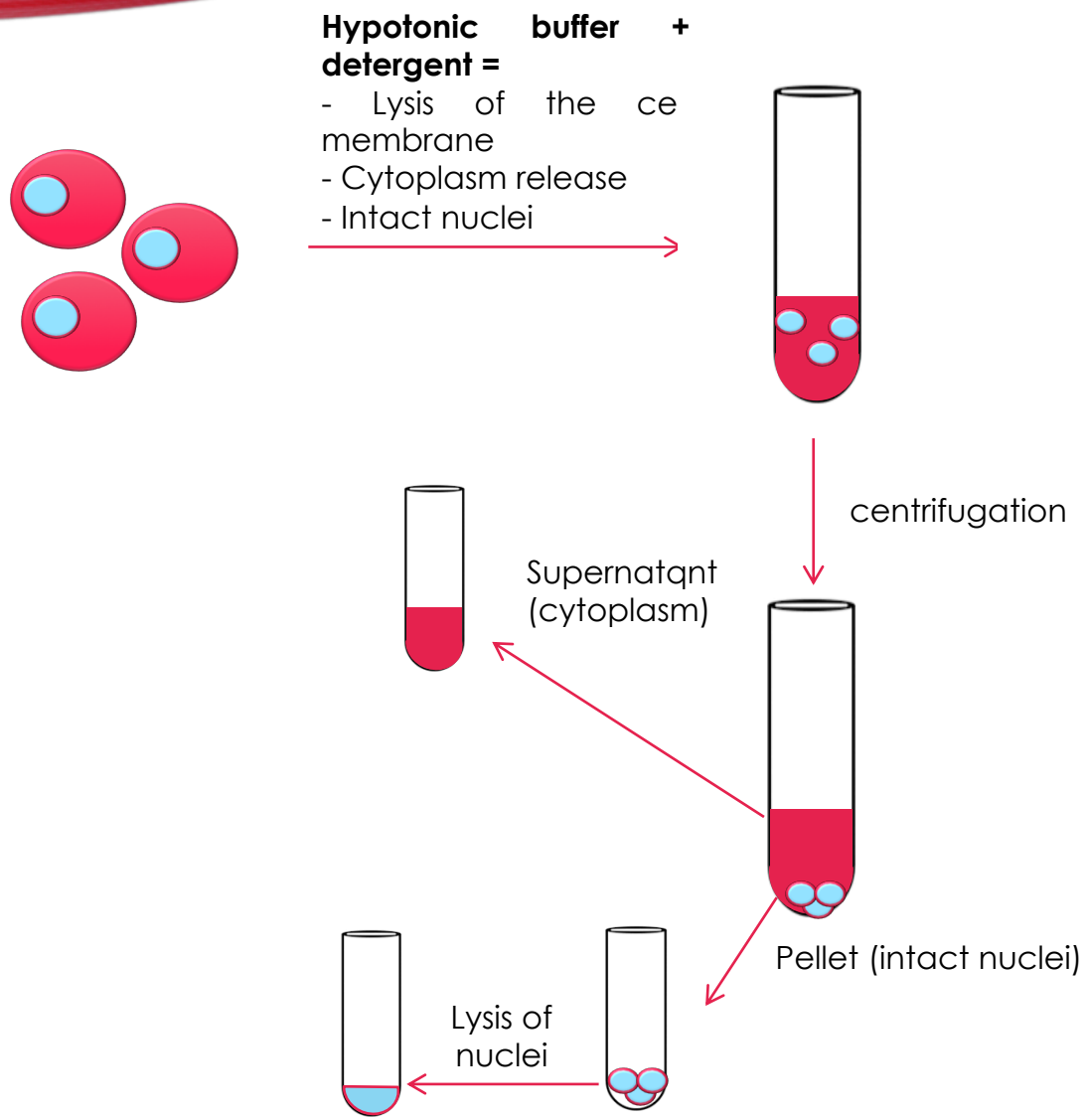
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

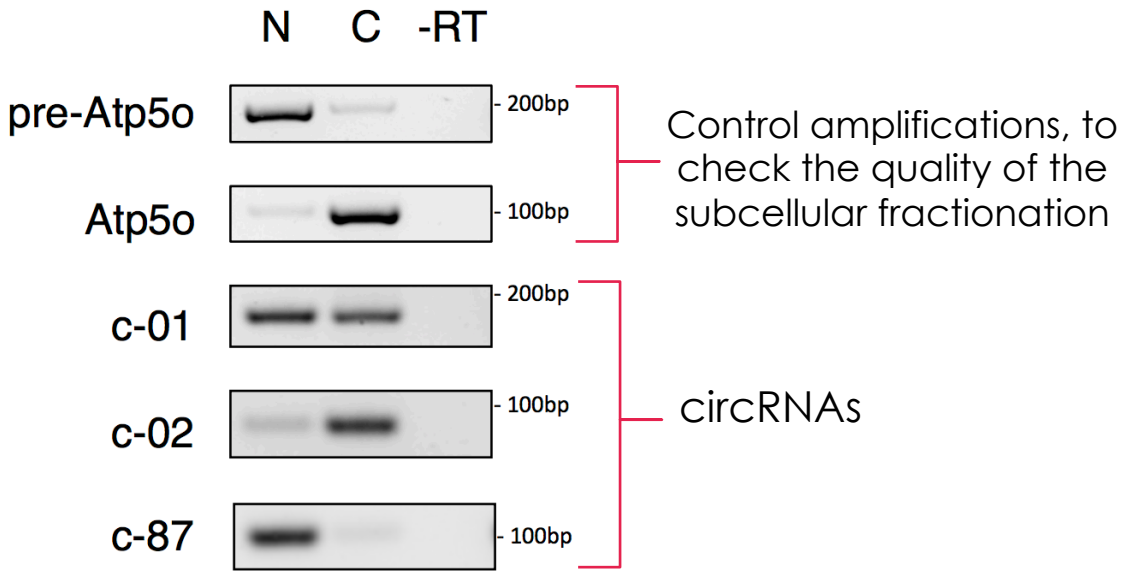
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



Adapted from
Errichelli *et al.*, 2017

N = nucleus
C = cytoplasm
-RT = negative control (amplification on RNA -> no reverse transcription before PCR amplification)

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

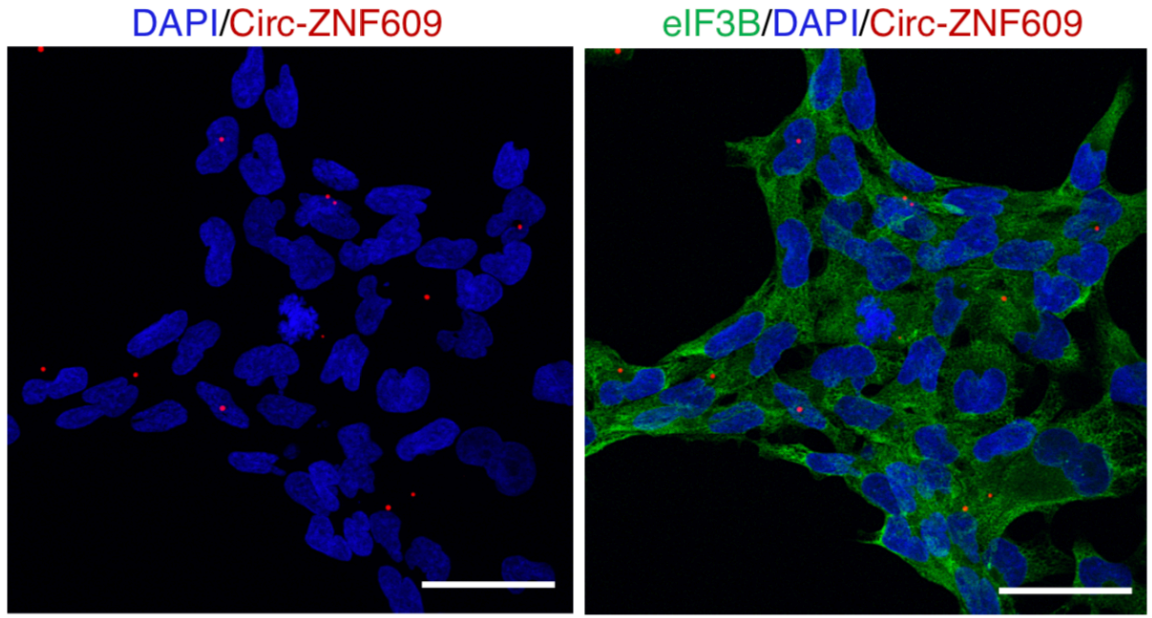
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

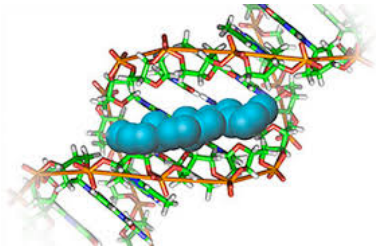
F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



Adapted from Rossi *et al.*, 2019



DAPI is a fluorescent stain that binds strongly A/T-rich regions in DNA. When bound to double-stranded DNA, DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue)

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

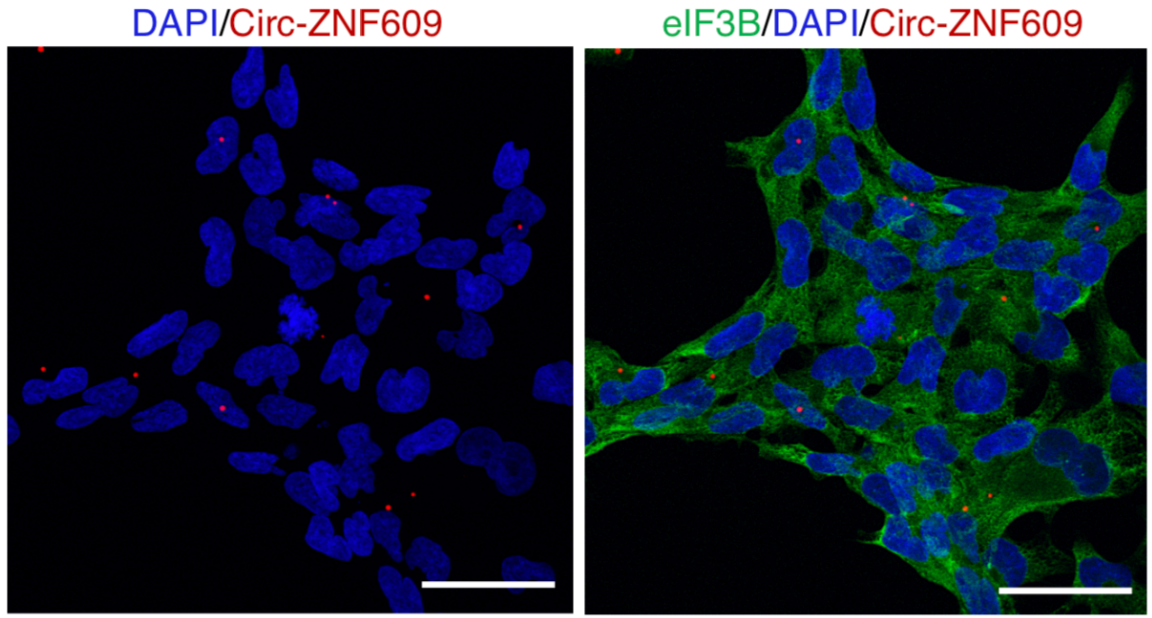
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



Adapted from Rossi *et al.*, 2019

eIF3B: RNA-binding component of the eukaryotic translation initiation factor 3 (eIF3) complex, which is required for several steps in the initiation of protein synthesis

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions

Exonic circular RNAs are enriched in cytoplasm (Lasda *et al.*, 2014).

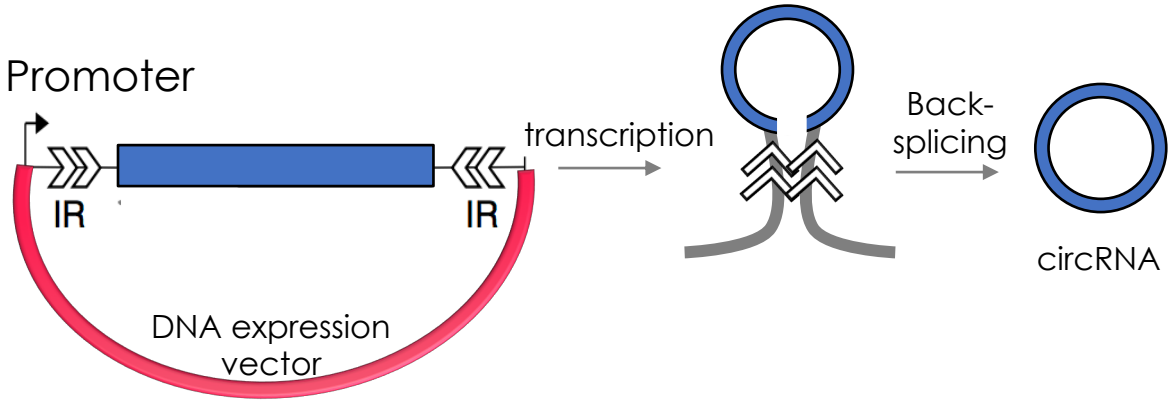
circRNAs can be enriched in cell-type specific sub-compartments -> synapses, as for instance circ-STAU2 and circ-RMST.
The localization of their linear counterpart is different: STAU2 mRNA localizes to cytoplasm, whereas lnc-RMST is nuclear (Rybak-Wolf *et al.*, 2015).

circRNAs have been found also in exocytosis vesicles -> maybe for eliminating the excess of circular RNAs from cellular cytoplasm (Lasda and Parker, 2016).

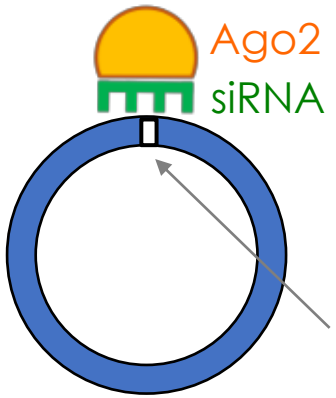
A. CHOOSE A MODEL SYSTEM
B. IDENTIFY circRNAs EXPRESSED -> RNAseq
C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…
E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?
F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec
G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions

OVEREXPRESSION



KNOCK-DOWN



siRNA has a perfect match on the back-splicing junction region -> slicing and degradation of the circRNA

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

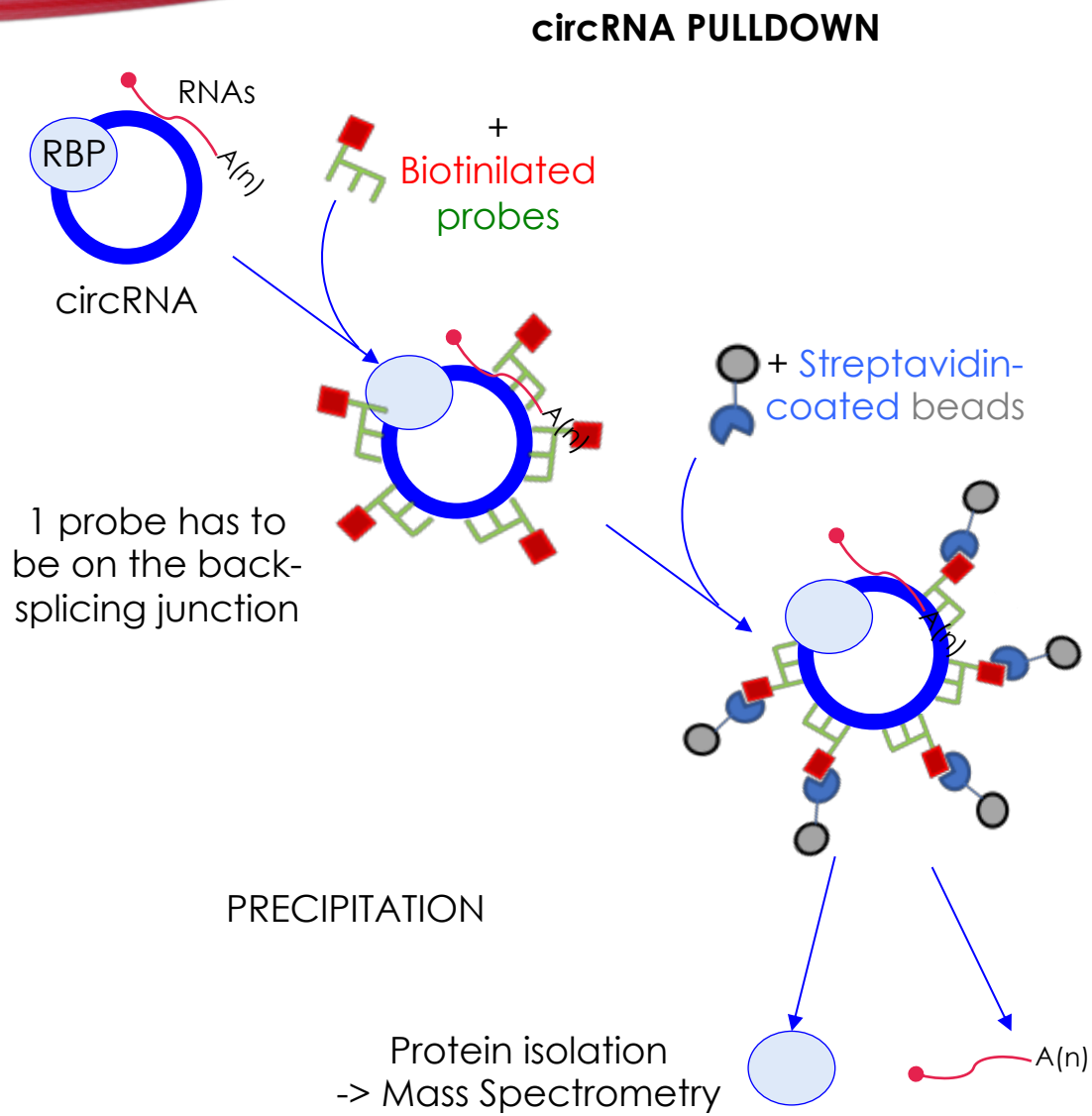
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions



A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

How to study circRNA functions

A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

E. SET UP A FUNCTIONAL SCREENING -> overexpression, knock-down, knock-out… -> phenotype ?

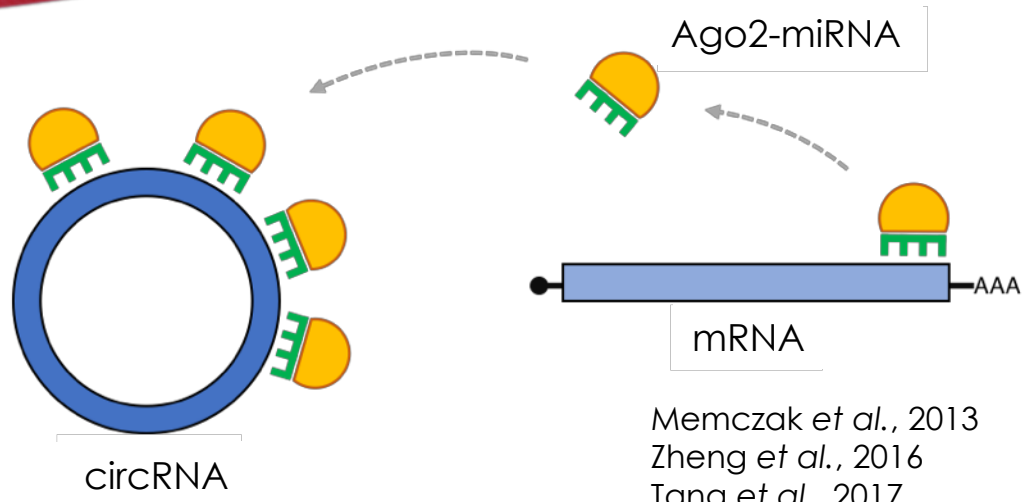
F. IDENTIFY MOLECULAR INTERACTORS -> RNA-pulldown + RNAseq / MassSpec

G. HYPOTHESIZE A MECHANISM OF ACTION -> validate it!

Summary

1. (Very brief) review about circRNA biogenesis
2. How to study circRNA molecular functions
3. Molecular functions of circRNAs
4. CircRNAs and their role in cancer
5. CircRNAs as biomarkers in body fluids

Molecular functions of circRNAs

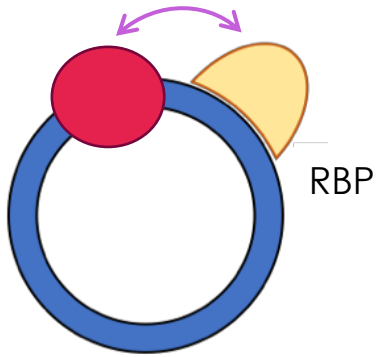


Memczak *et al.*, 2013
Zheng *et al.*, 2016
Tang *et al.*, 2017

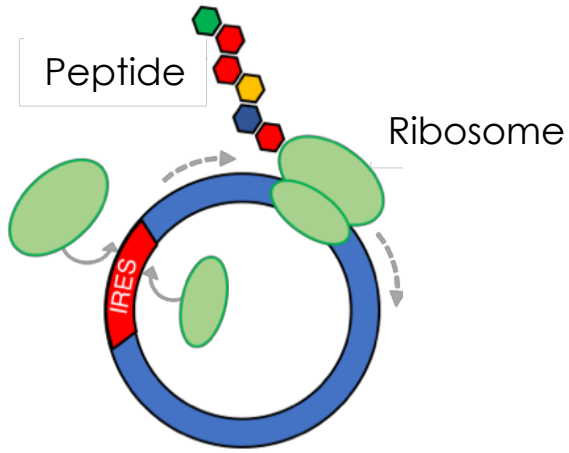
A. miRNA "sponges" = competing endogenous RNA, de-repressing miRNA targets

B. RNA-Binding Protein (RBP) interactors and modulators

C. Templates for cap-independent translation

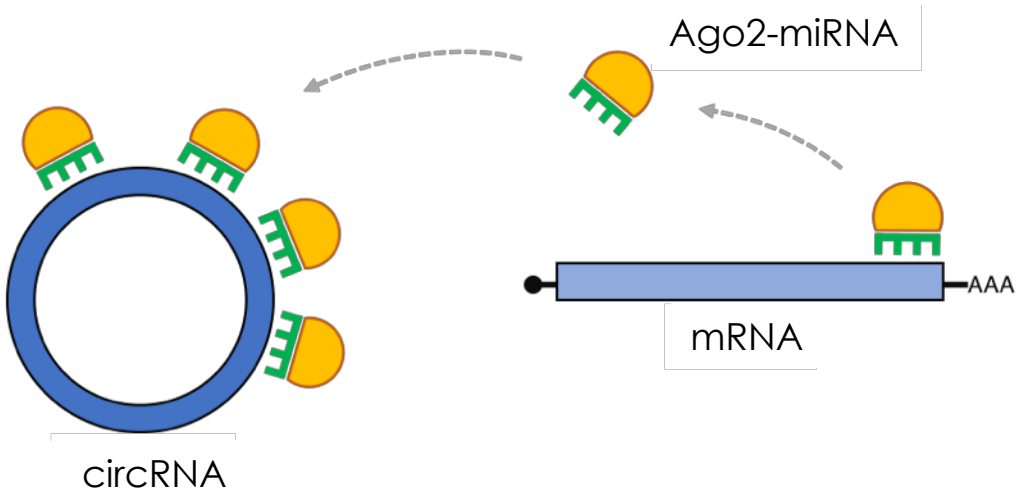


Du *et al.*, 2016
Schneider *et al.*, 2016



Legnini *et al.*, 2017
Pamudurti *et al.*, 2017

miRNA sponges

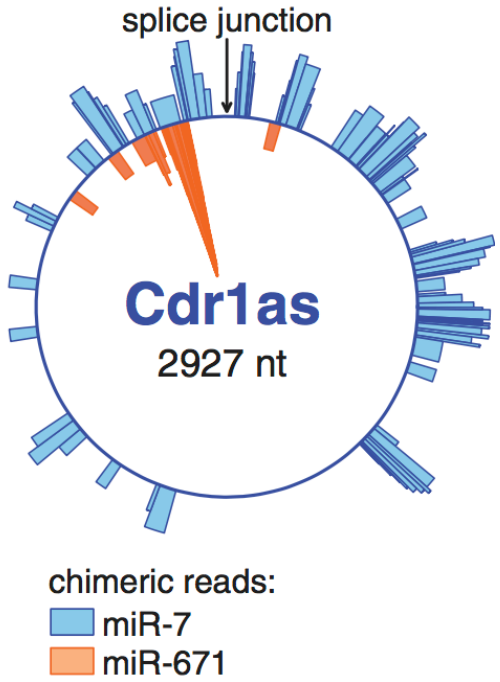


The first active circRNA to be identified is CDR1as. It has 74 miR-7 seed matches and most them are conserved in at least one more species (Memczak *et al.*, 2013).

Another circRNA which acts as a microRNA inhibitor is the one coming from SRY gene, that has 38 binding sites for miR-138 (Hansen *et al.*, 2013).

Apart from these two circRNAs, others seem to be microRNA sponges, but those identified don't have as many binding sites for the same miRNA as CDR1as or circSRY.

CDR1as



Cdr1as is densely bound by AGO: miRNA complexes containing miR-7 and miR-671. Bars on the circle represent circRNA:miRNA chimeric reads from AGO2 HITS-CLIP data from mouse brains.

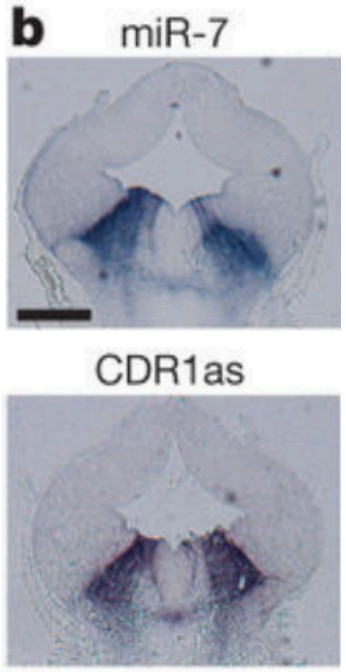
Adapted from Piwecka *et al.*, 2017

CDR1as has 74 miR-7 seed matches, of which 63 are conserved in at least one more species.

The binding sites are not perfect, meaning CDR1as-miR-7 is likely **not** sliced by Ago2.

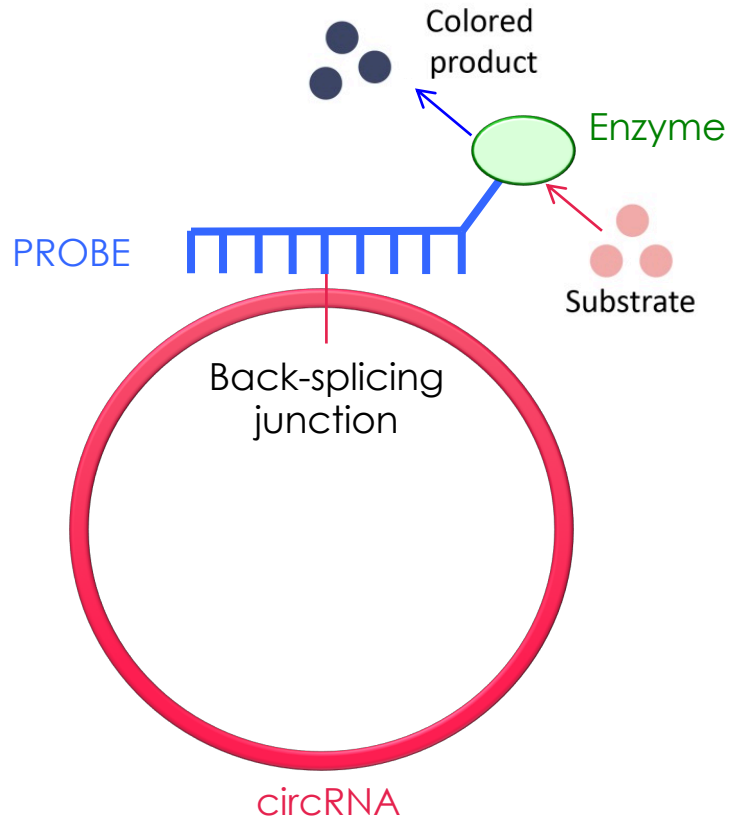
CDR1as also has an almost perfect binding site for miR-671, suggesting it may function to **slice** CDR1as for releasing its miR-7 cargo.

CDR1as



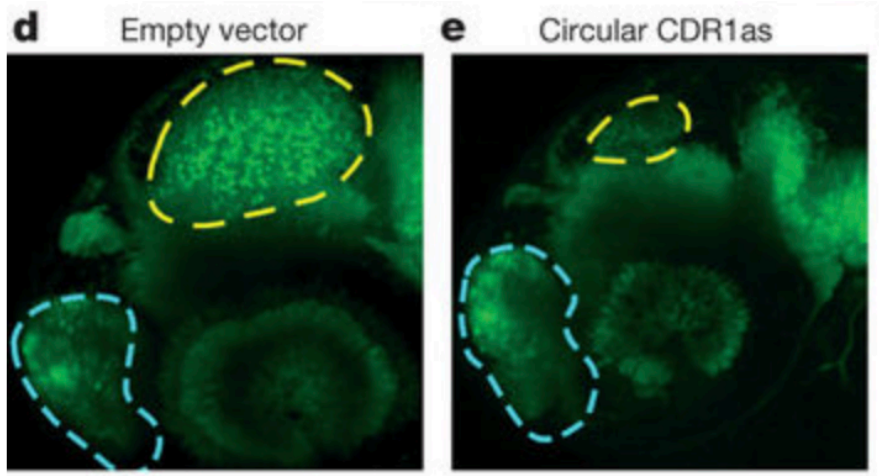
In situ staining of CDR1as and miR-7 in mouse embryo brain E13.5. Scale bar, 1 mm.

CDR1as and miR-7 are co-expressed in the brain.



Adapted from Memczak *et al.*, 2013

CDR1as



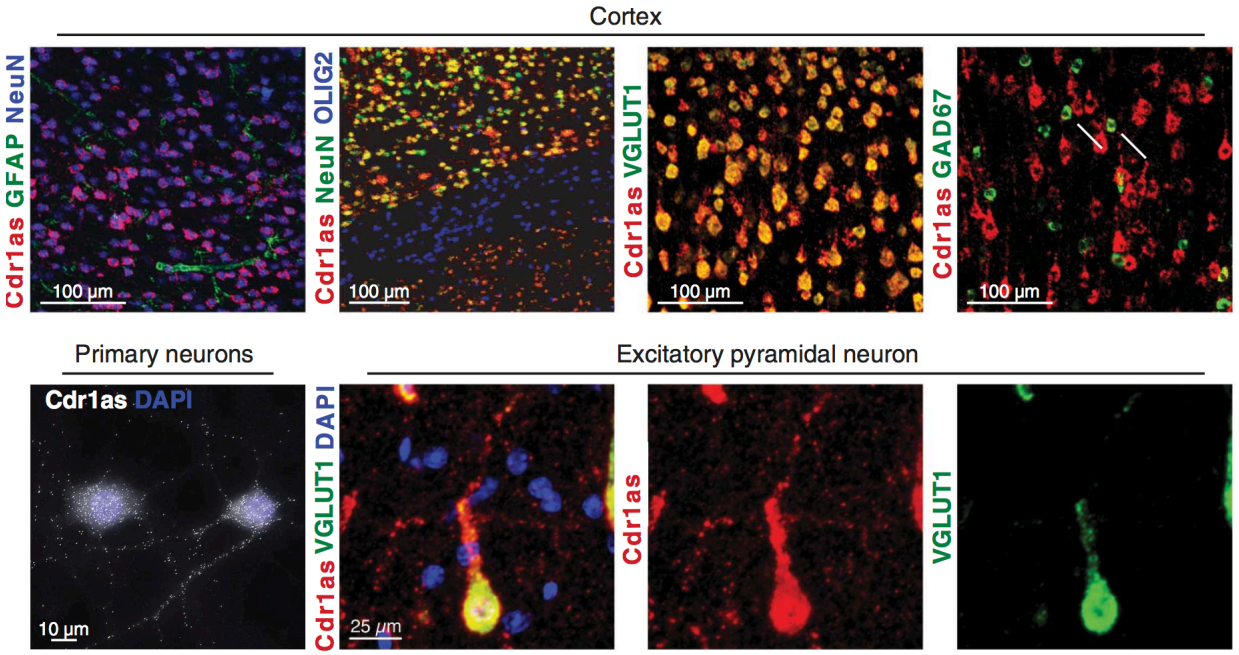
What are the *in vivo* effects of CDR1as ?

Zebrafish (*Danio rerio*) has lost the *cdr1* locus, whereas miR-7 is conserved and highly expressed in the embryonic brain.

- O.E. of CDR1as -> **MIDBRAIN DEFECTS**

Memczak *et al.*, 2013

CDR1as: SPONGE or BOAT?



Markers: GFAP, astrocytes; NeuN, neurons, OLIG2, oligodendrocytes; VGLUT1, excitatory neurons; GAD67, inhibitory neurons. Arrows mark Cdr1as expression overlap with inhibitory neurons.

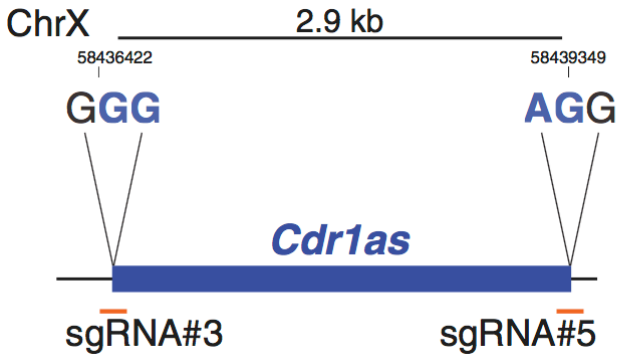
Piwecka *et al.*, 2017

CDR1as was highly expressed in neurons but not expressed in glial cells such as oligodendrocytes and astrocytes.

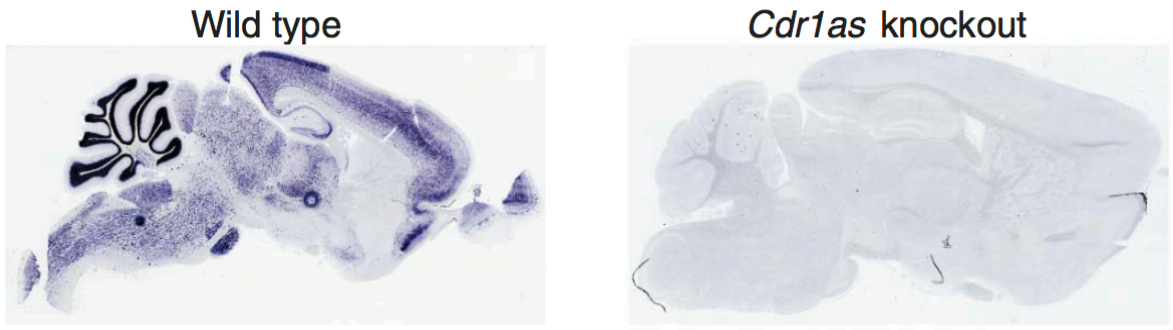
CDR1as was predominantly expressed in excitatory neurons.

Single-molecule RNA FISH in primary cortical neurons revealed CDR1as expression in both soma and neurites, indicating a possible functional role of CDR1as in different subcellular localizations.

CDR1as: SPONGE or BOAT?

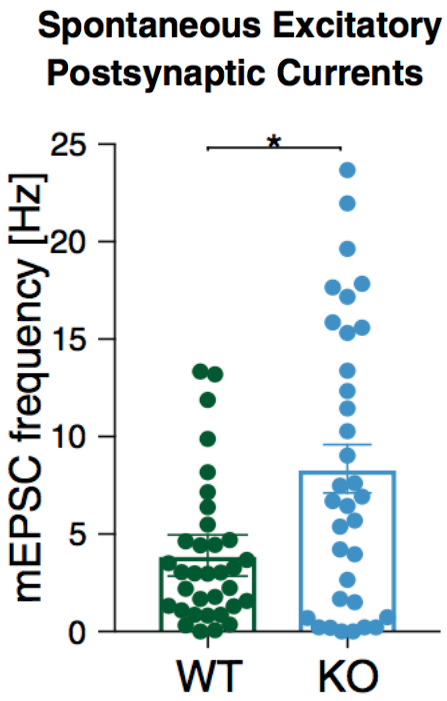


In vivo phenotype: CDR1as KNOCKOUT mice obtained with CRISPR-CAS9 technology.



Piwecka *et al.*, 2017

CDR1as: SPONGE or BOAT?



Cdr1as KO neurons showed increased spontaneous vesicle release

Piwecka *et al.*, 2017

Physiological effects of CDR1as removal?

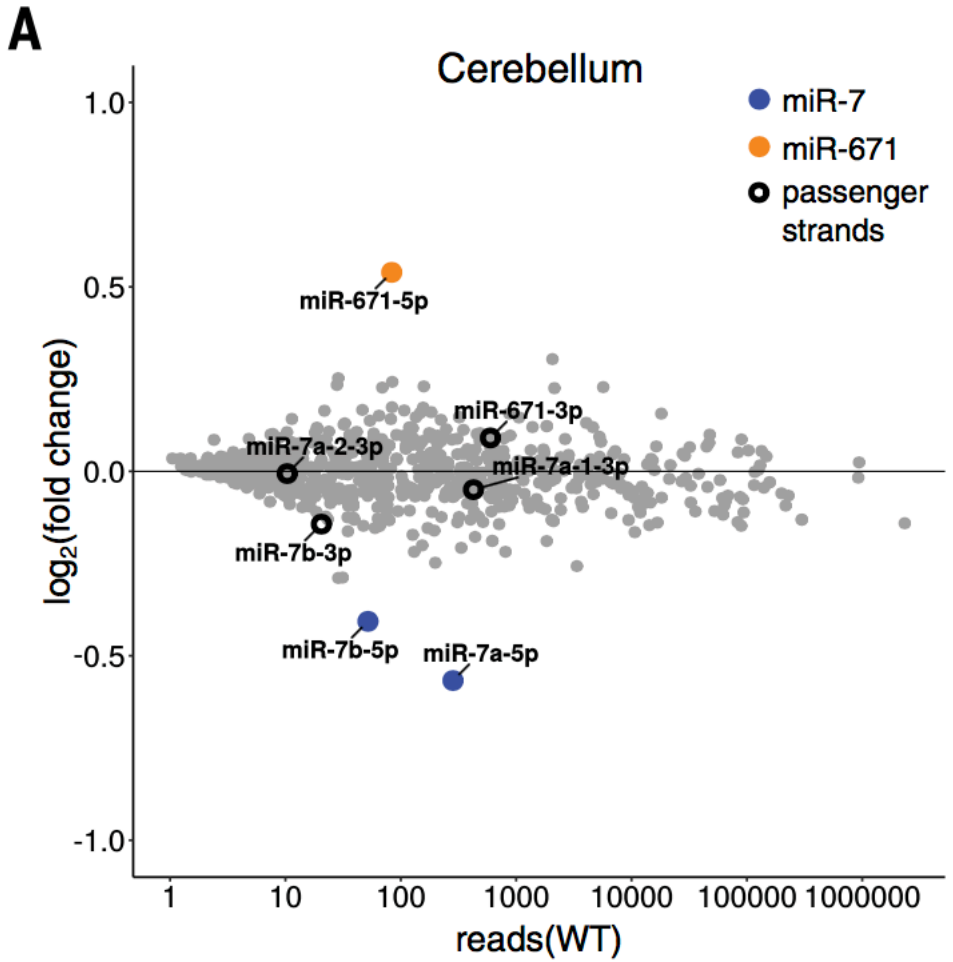
↓

Study excitatory postsynaptic currents (EPSCs) in single hippocampal neurons

↓

Cdr1as deficiency leads to a dysfunction of excitatory synaptic transmission

CDR1as: SPONGE or BOAT?



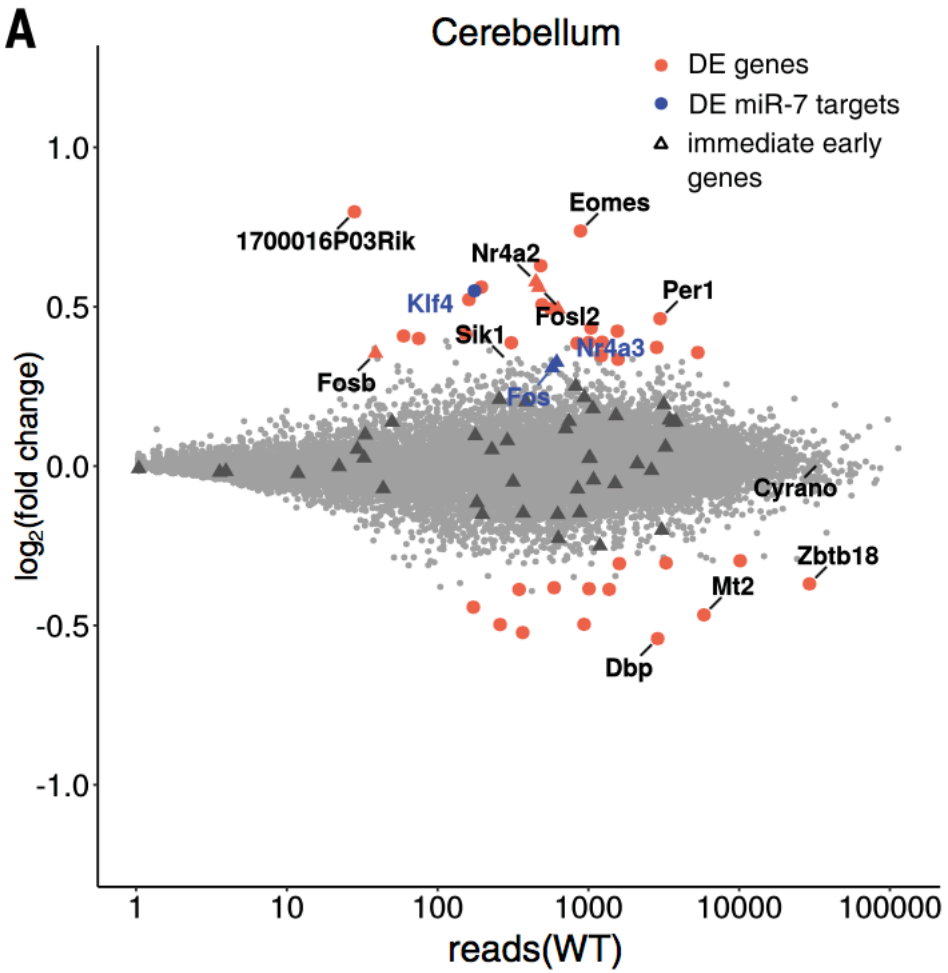
Piwecka *et al.*, 2017

miR-7 was consistently and markedly down-regulated in KO mice.

miR-7 down-regulation was post-transcriptional -> none of the three miR-7 passenger strands were significantly deregulated.

Unlike miR-7 expression, miR-671-5p expression in KO animals was up-regulated.

CDR1as: SPONGE or BOAT?



Piwecka *et al.*, 2017

What about miR-7 targets in CDR1as KO mice?

Conserved miR-7 targets were up-regulated in the cortex, cerebellum, and olfactory bulb!!!!

Different from what expected according to the "sponge" model…

CDR1as: SPONGE or BOAT?

miR-7 -> **non-perfect** binding to Cdr1as -> **no slicing** of the circRNA -> maybe miR-7 is stabilized by Cdr1as -> when we remove Cdr1as, miR-7 is **degraded**.

miR-671 -> 1 binding site with almost **perfect** complementarity -> **slicing** of Cdr1as and may cause removal of miR-671 -> when we remove Cdr1as, miR-671 is **stabilized**.

«miR-671 therefore may provide an "unlocking" mechanism that serves to slice Cdr1as under specific conditions within the cell to release the cargo (sponged miR-7:AGO complexes)».

Piwecka *et al.*, 2017

CDR1as: SPONGE or BOAT?

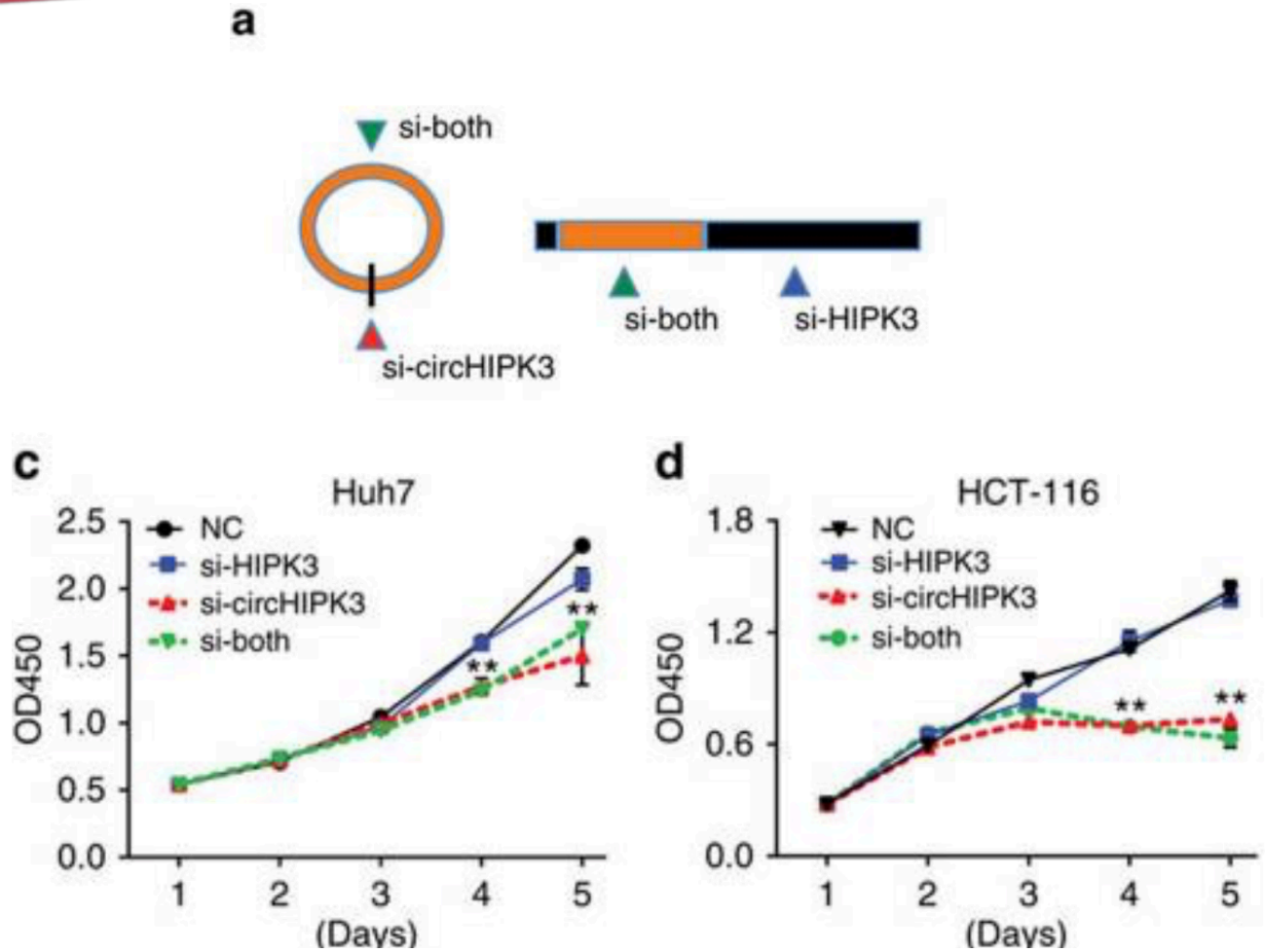
CDR1as can be important for the regulation of neurological processes via sequestering miR-7.

It transports miR-7 to the synapses to regulate mRNA encoding proteins that control synaptic functioning.

Unbound miR-7 would otherwise be targeted for degradation in the cytoplasm.

CDR1as could not act as a canonical ‘sponge’ but as a ‘boat’: it prevents its passengers from degradation and also moves them on to new subcellular localization.

CircHIPK3 affects cell proliferation

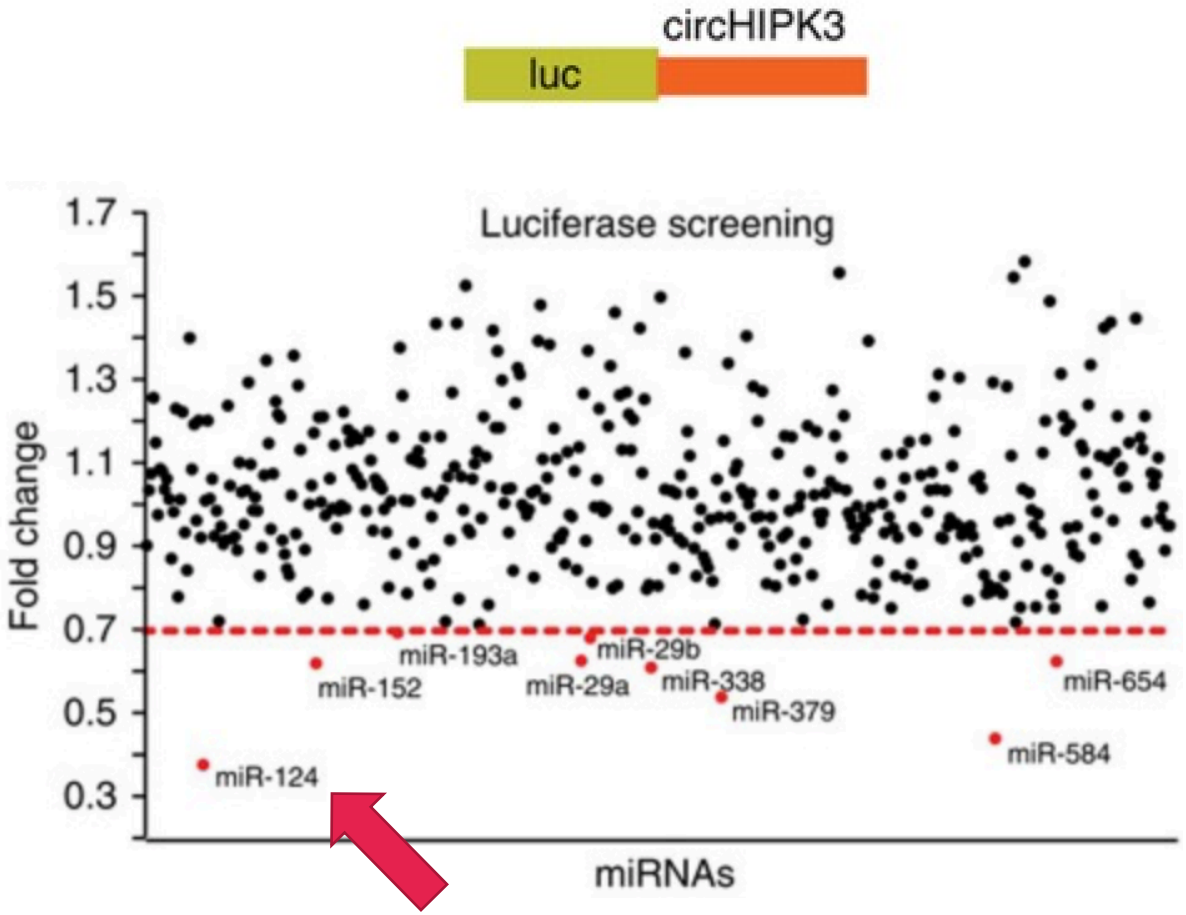


Zheng *et al.*, 2016

circHIPK3 was significantly upregulated in liver cancer compared with matched normal tissues

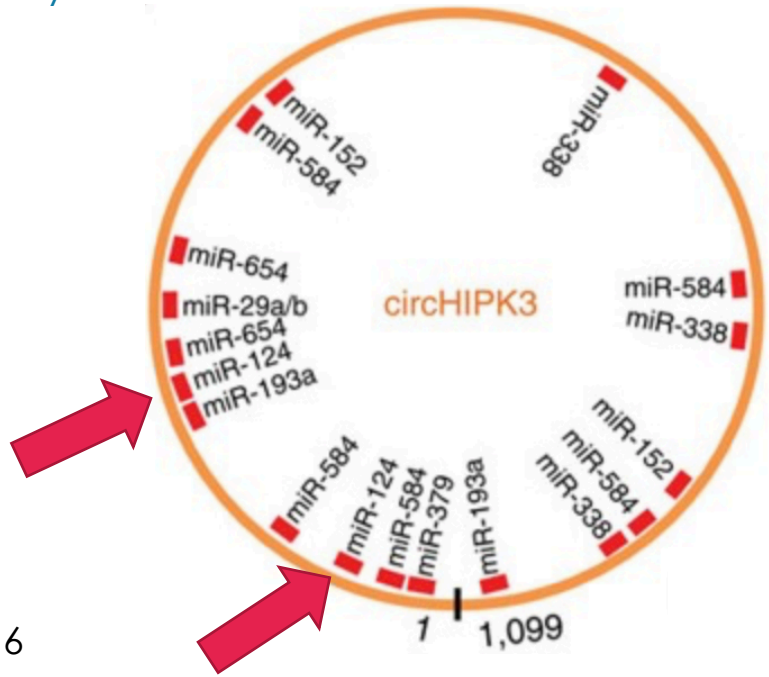
What is the mechanism through which it affects cell proliferation?

CircHIPK3: miR-124 sponge



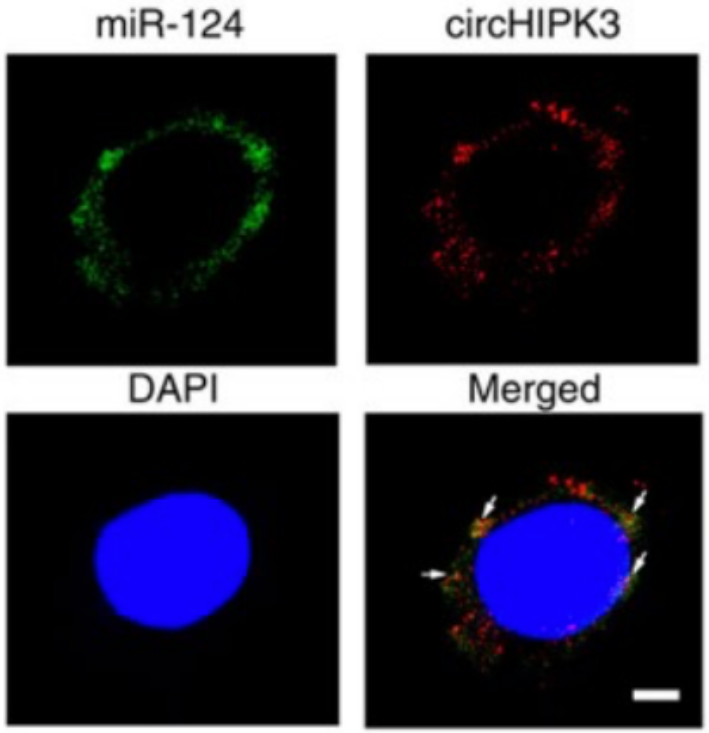
An AGO2-RIP was conducted and endogenous circHIPK3 was pulled-down and specifically enriched in the IP.

circHIPK3-associated miRNAs can inhibit luciferase activity.



Zheng *et al.*, 2016

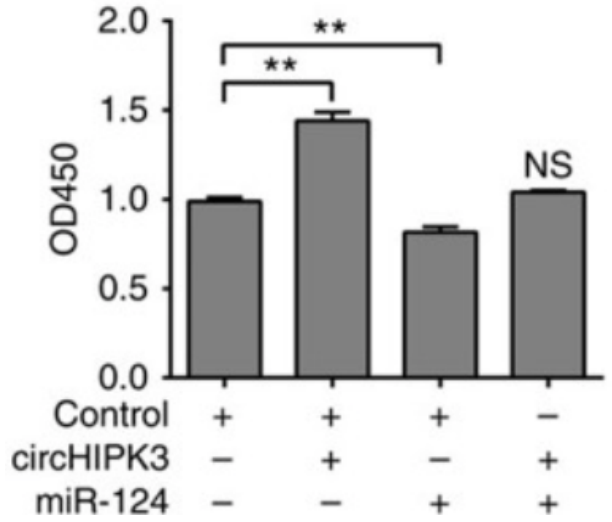
CircHIPK3 can be a sponge for miR-124 and has a role in cell proliferation



miR-124 and circHIPK3 expression and localization are similar.

miR-124 is an anti-proliferative miRNA: its overexpression inhibits cell growth.

circHIPK3 has a pro-proliferative effect: its overexpression promotes cell growth.



Zheng *et al.*, 2016

RNA-Binding Protein (RBP) interactors and modulators

Molecular Cell
Article

circRNA Biogenesis Competes with Pre-mRNA Splicing

Reut Ashwal-Fluss,[1,3] Markus Meyer,[2,3] Nagarjuna Reddy Pamudurti,[1,3] Andranik Ivanov,[2] Osnat Bartok,[1] Mor Hanan,[1] Naveh Evantal,[1] Sebastian Memczak,[2] Nikolaus Rajewsky,[2,*] and Sebastian Kadener[1,*]

Journal
RNA Biology
Volume 14, 2017 - Issue 3

Research Paper

Identification of HuR target circular RNAs uncovers suppression of PABPN1 translation by *CircPABPN1*

Kotb Abdelmohsen, Amaresh C. Panda, Rachel Munk, Ioannis Grammatikakis, Dawood B. Dudekula, Supriyo De, ...show all

Pages 361-369 | Received 18 Nov 2016, Accepted 30 Dec 2016, Accepted author version posted online: 12 Jan 2017, Published online: 12 Jan 2017

Download citation http://dx.doi.org/10.1080/15476286.2017.1279788 Check for updates

Nucleic Acids Research

Nucleic Acids Res. 2016 Apr 7; 44(6): 2846–2858. PMCID: PMC4824104
Published online 2016 Feb 9. doi: 10.1093/nar/gkw027

Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2

William W. Du,[1,2,†] Weining Yang,[1,†] Elizabeth Liu,[1,2] Zhenguo Yang,[1,2] Preet Dhaliwal,[1,2] and Burton B. Yang[1,2,*]

nature COMMUNICATIONS

ARTICLE

Received 31 Oct 2015 | Accepted 1 Jul 2016 | Published 19 Aug 2016 DOI: 10.1038/ncomms12429 OPEN

Circular non-coding RNA *ANRIL* modulates ribosomal RNA maturation and atherosclerosis in humans

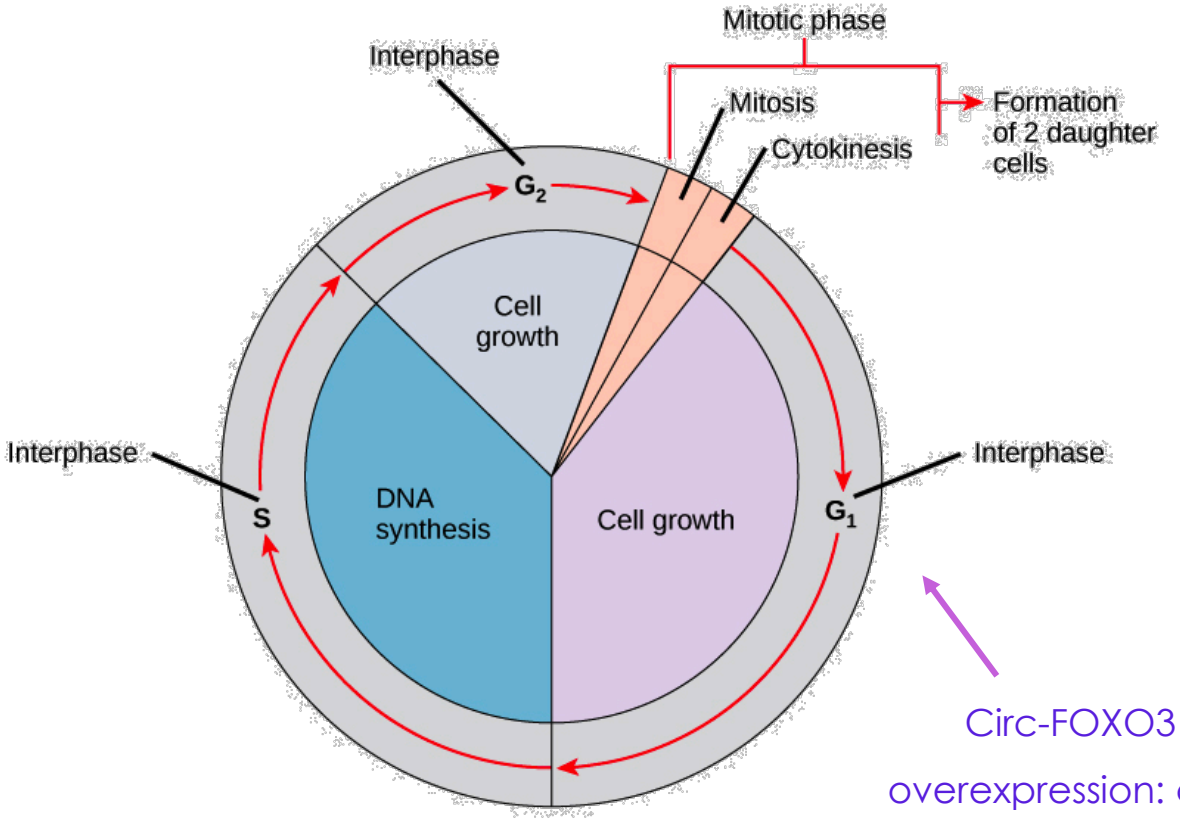
Lesca M. Holdt[1,2], Anika Stahringer[1], Kristina Sass[1], Garwin Pichler[3], Nils A. Kulak[3], Wolfgang Wilfert[1], Alexander Kohlmaier[1], Andreas Herbst[1], Bernd H. Northoff[1], Alexandros Nicolaou[1], Gabor Gäbel[4], Frank Beutner[2,5], Markus Scholz[2,6], Joachim Thiery[2,5], Kiran Musunuru[7,8], Knut Krohn[2,9], Matthias Mann[3] & Daniel Teupser[1,2]

Many circRNAs are predicted to interact with RNA binding proteins (RBPs).

Web tools allow to investigate predicted RBP-circRNA interactions.

I.e. CircInteractome (circRNA interactome) searches public circRNA, miRNA, and RBP databases to provide bioinformatic analyses of binding sites on circRNAs and additionally analyzes miRNA and RBP sites on junction and junction-flanking sequences (Dudekula *et al.*, 2016).

RNA-Binding Protein (RBP) interactors and modulators



Circ-FOXO3 overexpression: cells are **unable** to enter S phase

CircFOXO3 was one of the first circRNAs to be shown as a scaffold for cell cycle-related proteins.

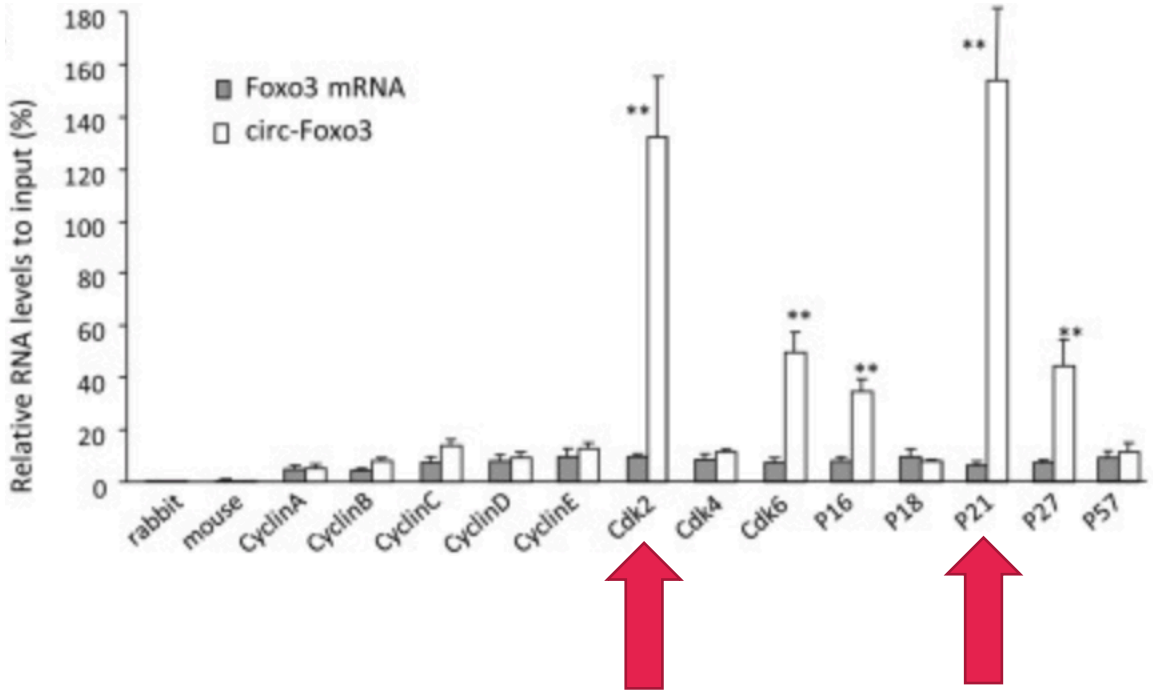
CircFOXO3 has a role in cell cycle: its overexpression increases cells in G1, while reduces cells in S and G2 phases.

What is the mechanism through which it affects cell proliferation?

Du *et al.*, 2016

CircFOXO3 can bind cell cycle modulators

IP of cell cycle related proteins



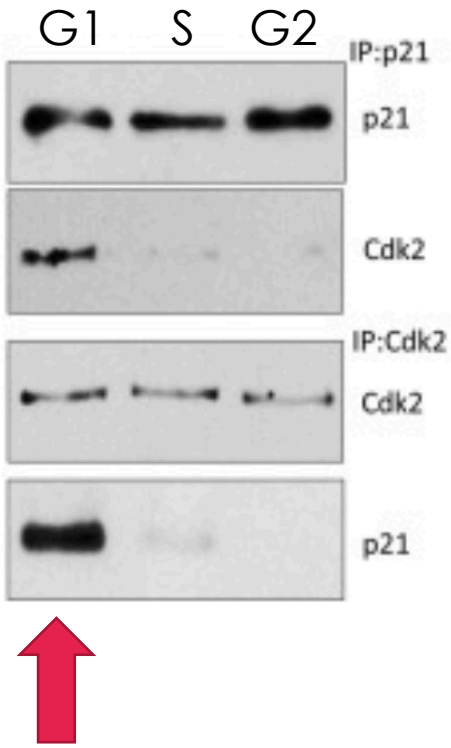
Du *et al.*, 2016

Look for potential interactions of circ-Foxo3 with cell cycle associated proteins.

CDK2 and p21 showed the greatest difference, suggesting their important role in circ-Foxo3-mediated cell cycle progression.

CircFOXO3 can bind cell cycle modulators

Cells overexpressing circ-FOXO3



Immunoprecipitation with antibody against p21 or CDK2.

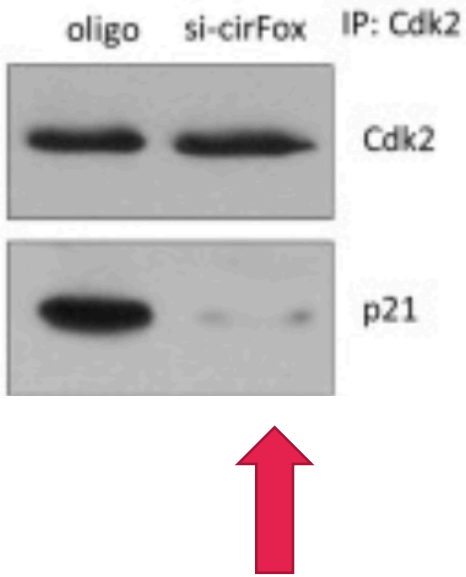
Anti-p21 antibody pulled-down more Cdk2 and anti-CDK2 antibody pulled-down more p21 in the cells transfected with circ-Foxo3.

The interaction of p21 and CDK2 mediated by circ-Foxo3 occurred in G1 phase.

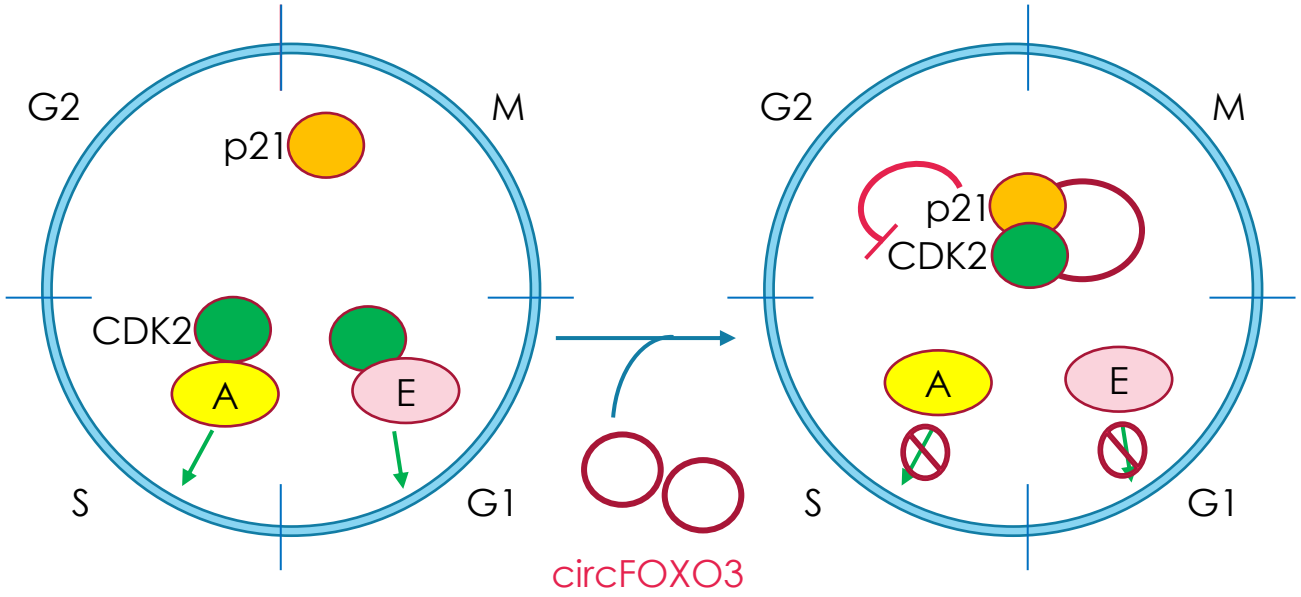
Adapted from Du *et al.*, 2016

CircFOXO3 regulates cell cycle progression

DISRUPT THE COMPLEX -> circ-FOXO3 KD

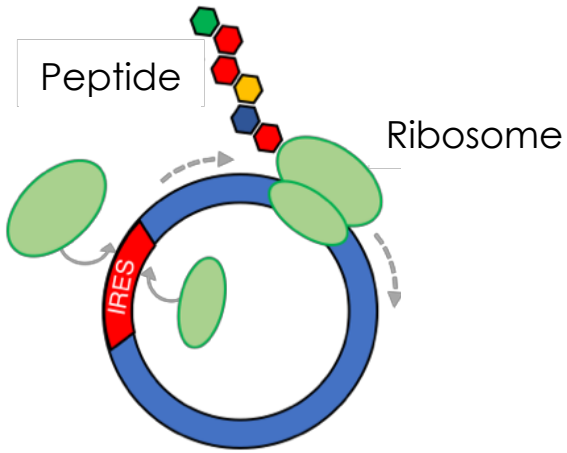


circ-Foxo3 acts as a scaffold to modulate protein-protein interaction. It interacts with p21 and CDK2, facilitating the inhibition of CDK2 by p21, and repressing cell cycle progression at the G1 stage.



Adapted from Du *et al.*, 2016

CircRNAs as templates for cap-independent translation

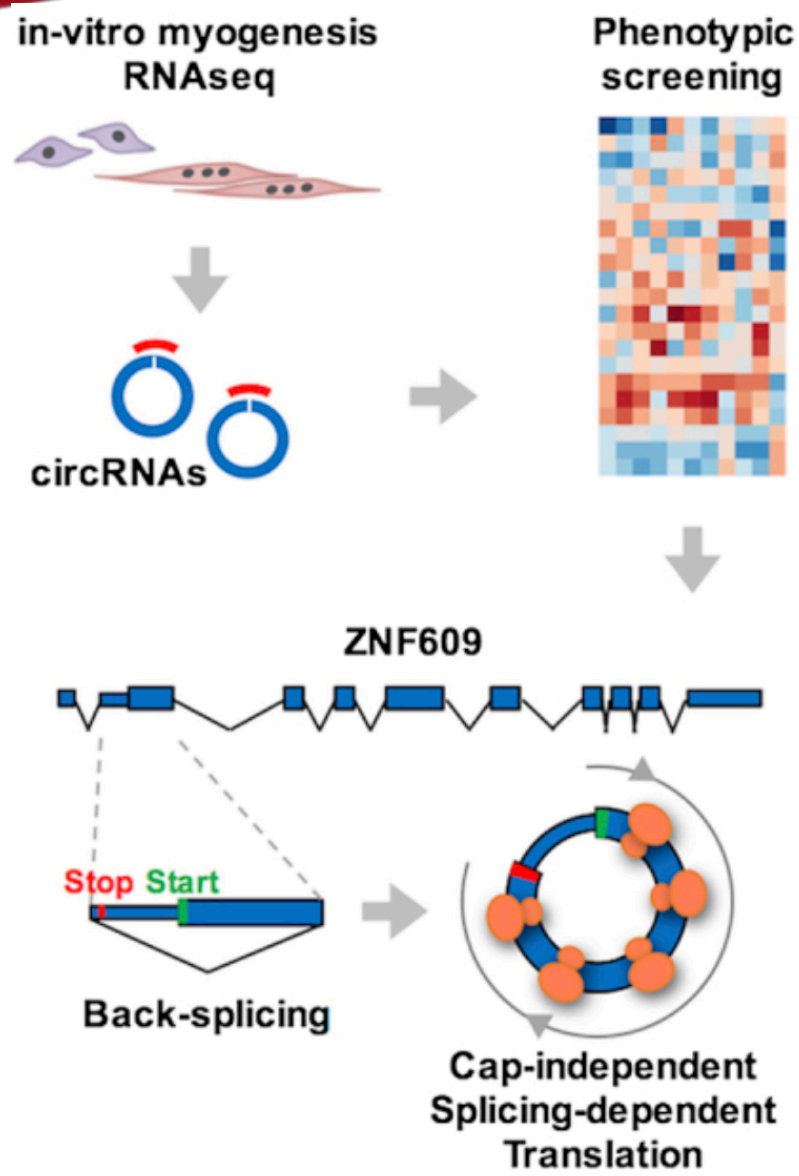


As most of circRNAs originate from exons and are cytoplasmic, we can hypotesize that they could be translated into proteins.

Artificial circRNAs with an internal ribosomal entry site (IRES) can be translated *in vitro* or *in vivo* (Chen and Sarnow, 1995 - Wang and Wang, 2015).

And what about translation of endogenous circRNA with an Open Reading Frame (ORF)?

CircZNF609 can be translated and functions in myogenesis



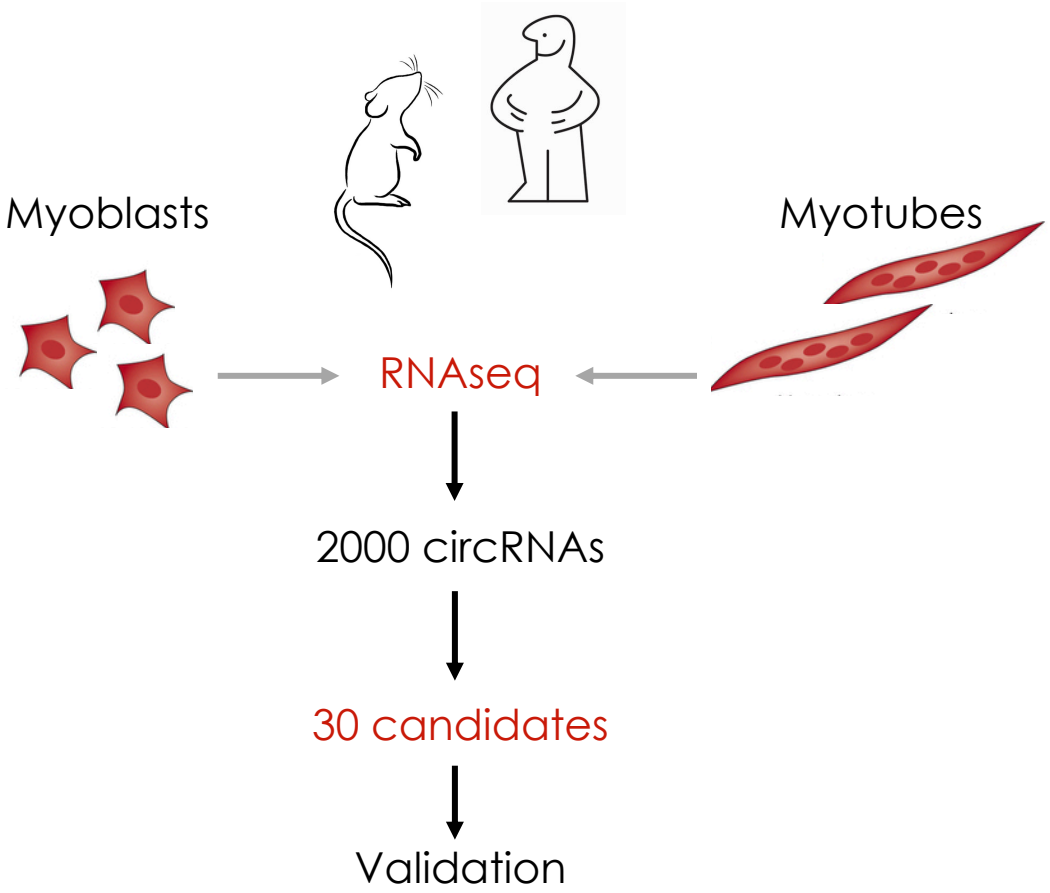
We identified several circRNAs expressed and modulated during human and murine myogenesis.

Some of these circRNAs have a role in myoblast proliferation and differentiation.

We also discovered the first mammalian circRNA that can be translated: circZNF609.

Legnini *et al.*, 2017

CircRNA discovery in human and mouse muscle cells

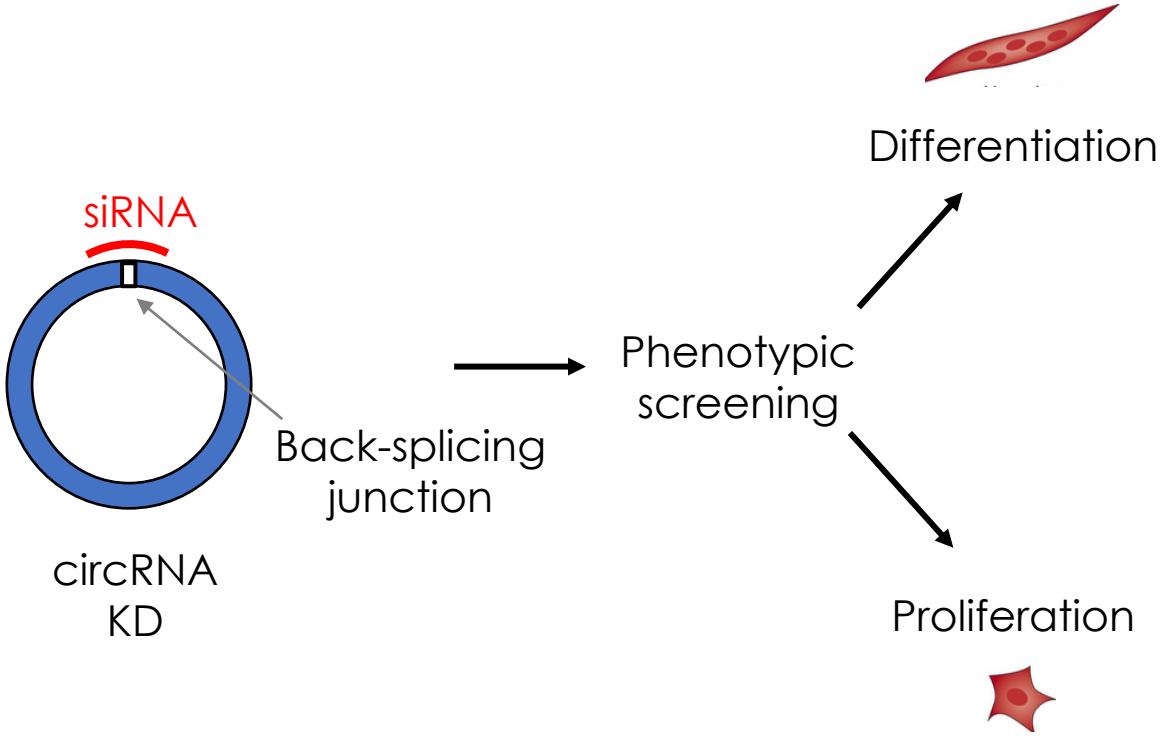


A paired-end total RNA-seq was performed on human and murine proliferating myoblasts and differentiated myotubes.

Thanks to a specific computational pipeline (Memczak *et al.*, 2013) we discovered about 2000 circRNAs, of which almost 600 were conserved in both species.

We selected 30 candidates whose expression level was highest, which were conserved in human and mouse, and either differentially regulated during myogenesis or more expressed than their linear counterpart.

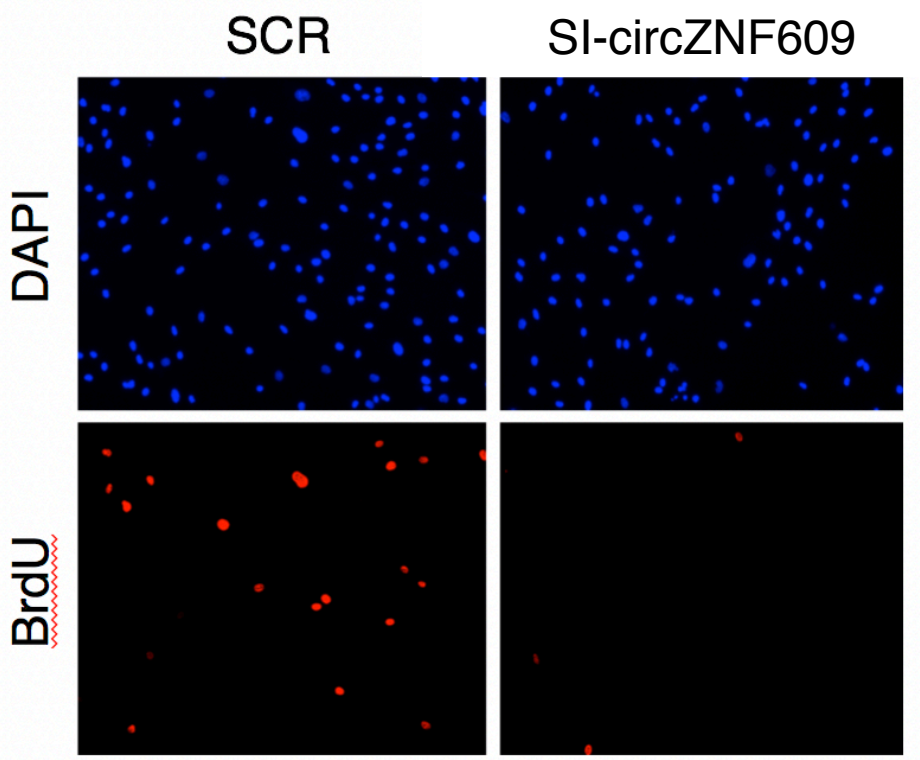
CircRNA role in myogenesis



To investigate their role in myogenesis, we conducted an siRNA-based phenotypic screening.

In order to analyze the effect of circRNA-specific depletion on myoblast proliferation or differentiation, siRNAs targeting the back-splicing junction were used.

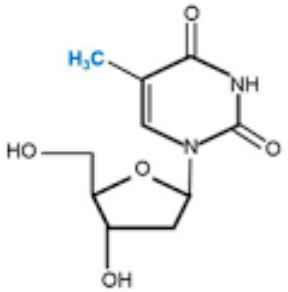
CircZNF609 has role in myoblast proliferation



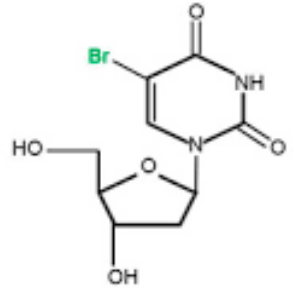
Adapted from Legnini *et al.*, 2017

Among the circular RNAs whose knockdown produces a specific phenotype, there is circZNF609.

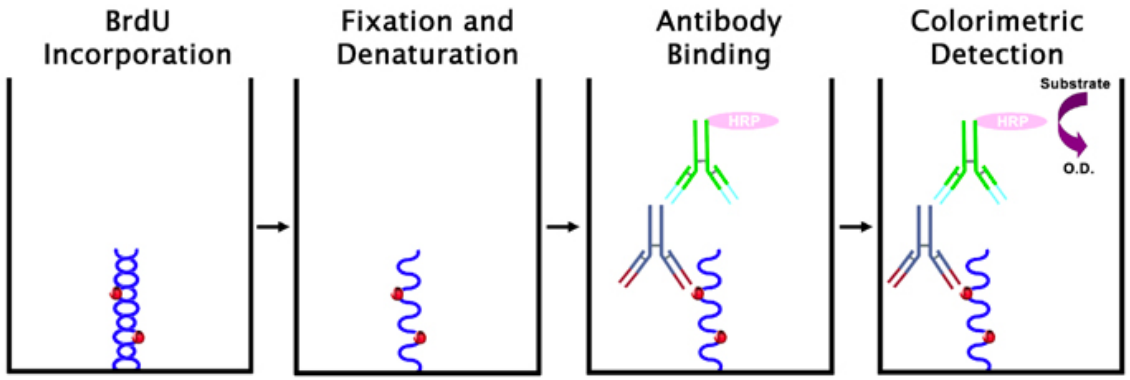
Its specific knock-down strongly reduces the number of BrdU+ cells (= proliferating cells).



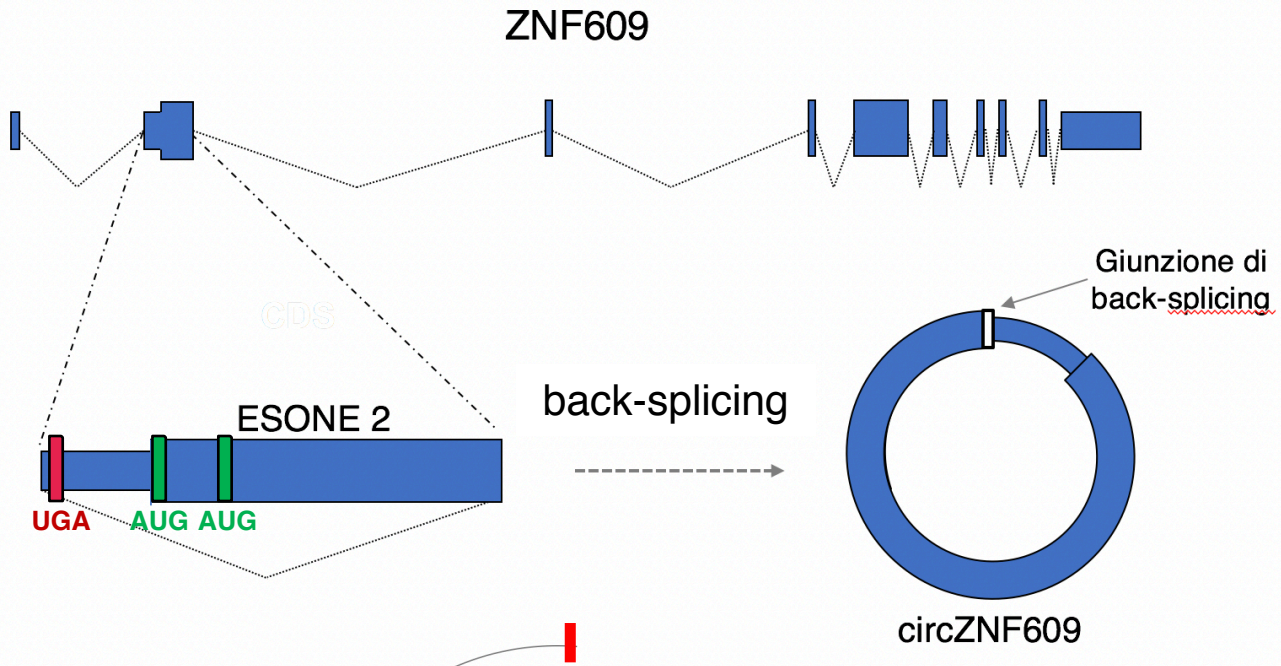
Thymidine

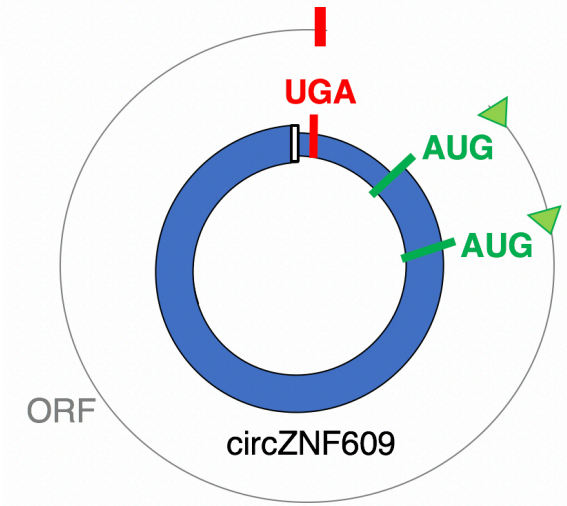


5'-bromo-2'-deoxyuridine (BrdU)



Who is circZNF609?





circZNF609 derives from the circularization of the second exon of ZNF609 gene, which encodes a zinc-finger transcription factor.

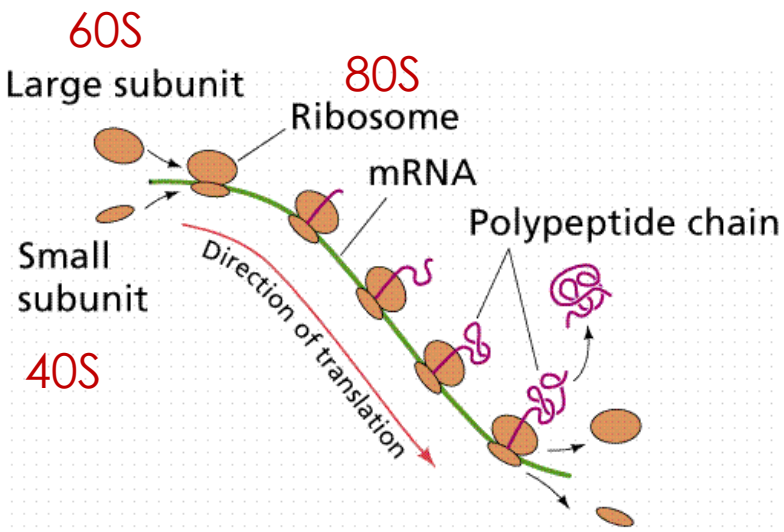
The 2nd exon of its host gene contains the last part of its 5'UTR, and the beginning of its CDS, which opens with the start codon.

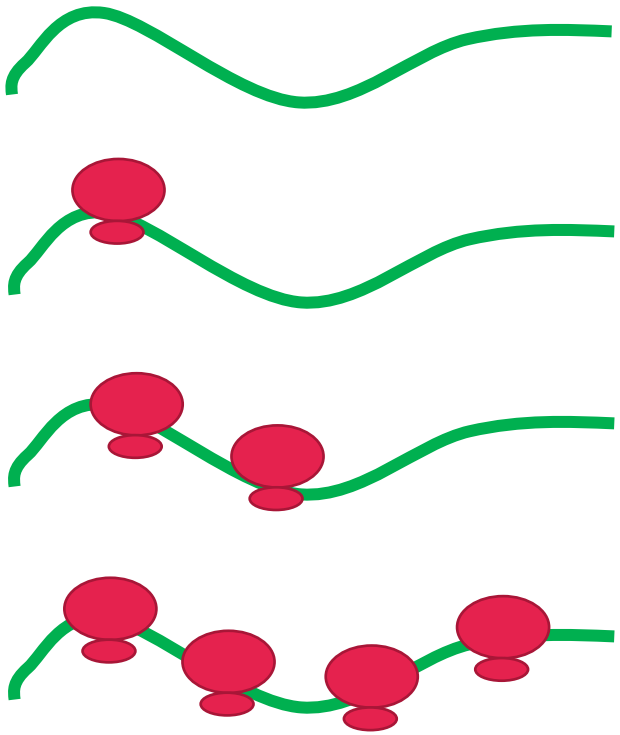
Upon circularization, the start codon is in frame with a stop codon which is one triplet downstream the back-splicing junction, producing an ORF of 753 nucleotides.

Is the ORF functional?

When an RNA is translated into a protein, it is bound to ribosomes.

When many ribosomes are associated to an RNA, we talk about «POLYSOMES»





Ribosome-free RNA

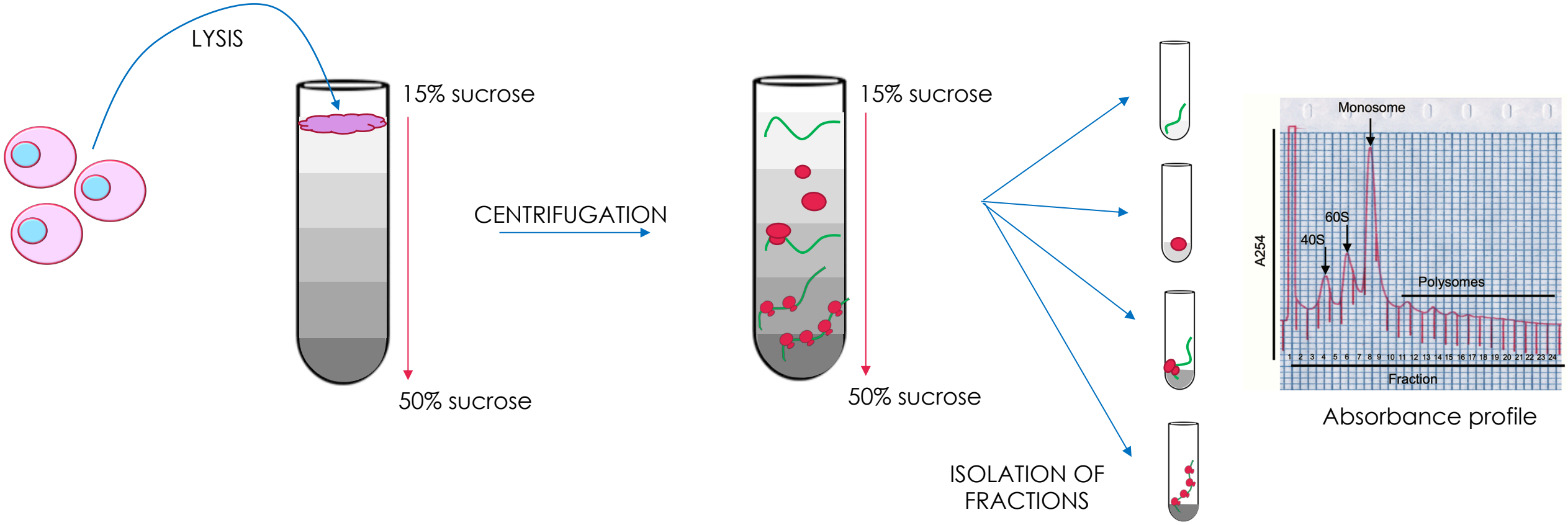
RNA associated to 1 ribosome (80S or MONOSOME)

RNA associated to many ribosomes (POLYSOMES)

Is the ORF functional?

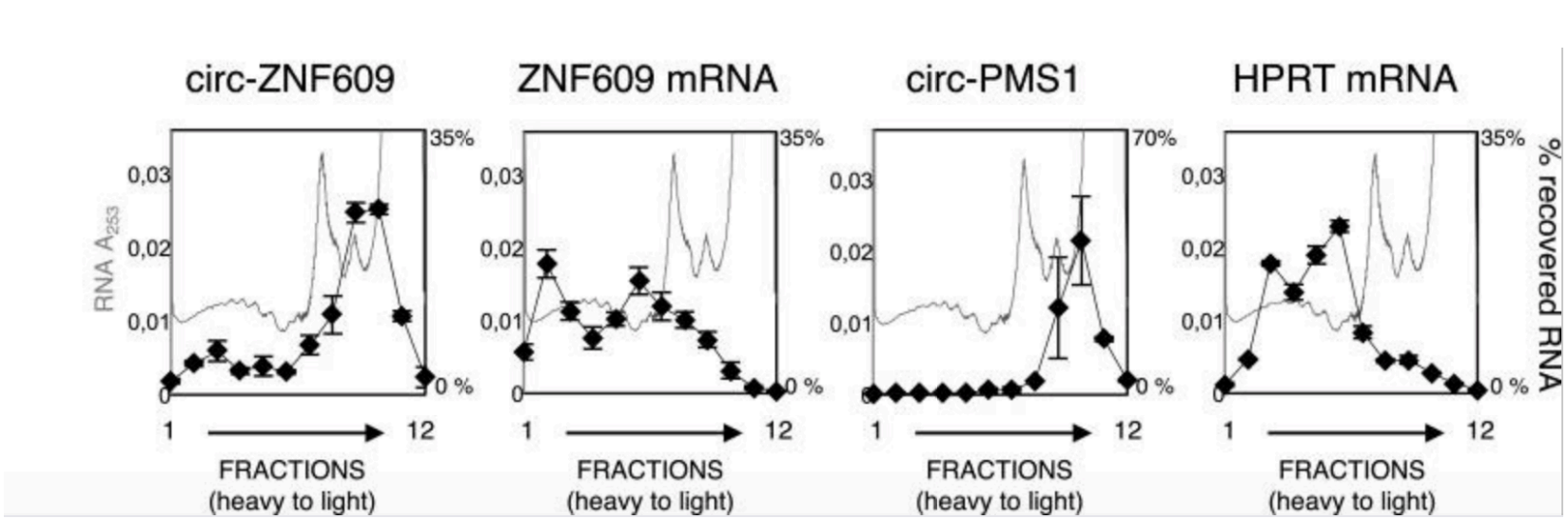
When an RNA is translated into a protein, it is bound to ribosomes.

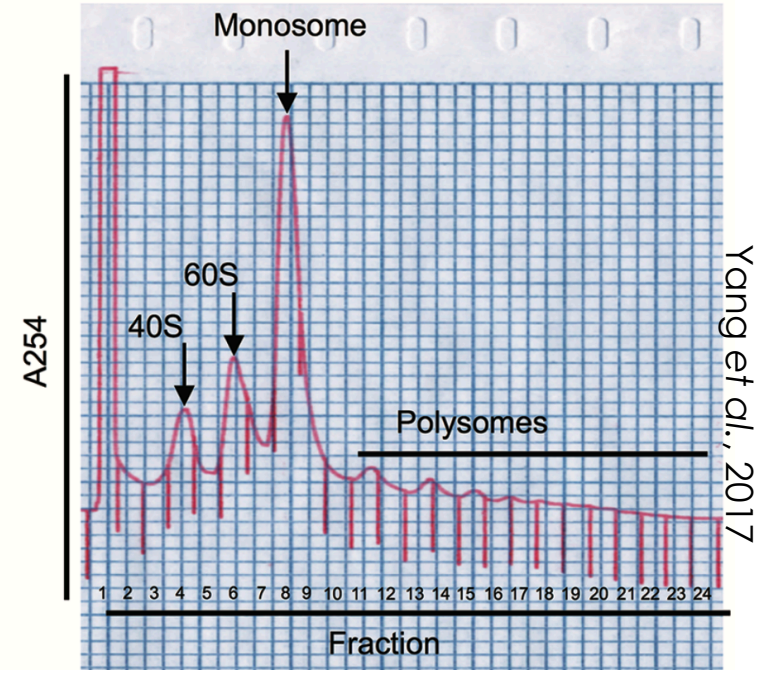
When many ribosomes are associated to an RNA, we talk about «POLYSOMES»



Absorbance profile

Is the ORF functional?



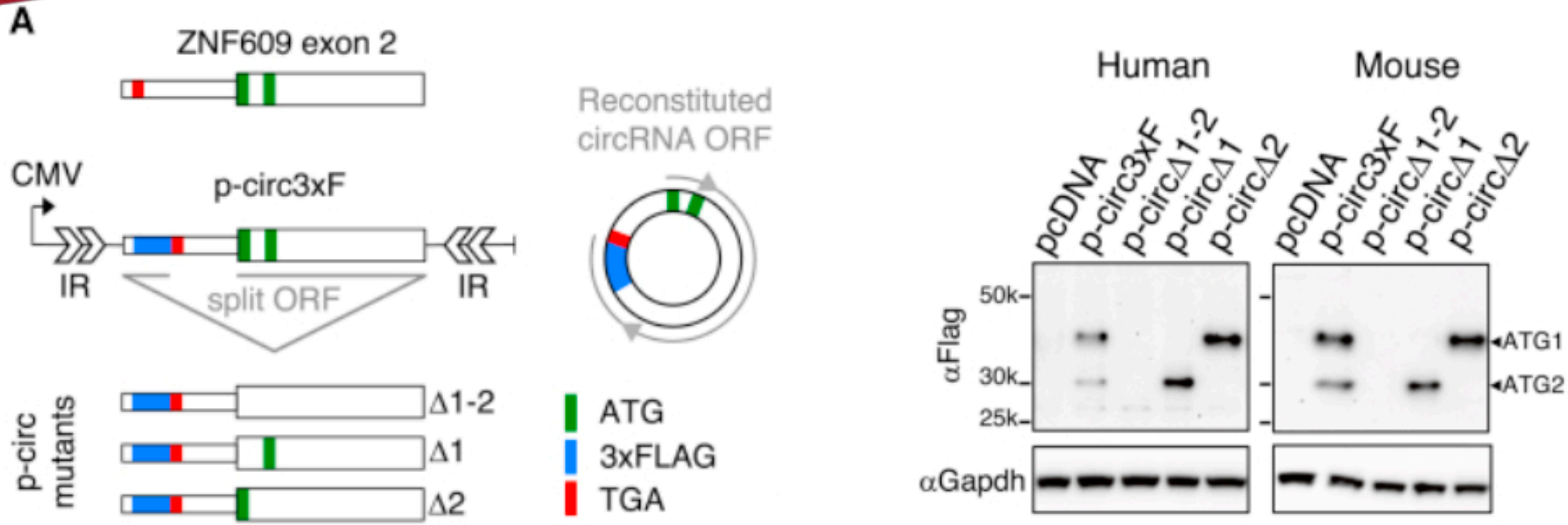


Light <- Heavy

A significant fraction of the circRNA is associated to heavy polysomes and shifts towards lighter fractions upon puromycine treatment, as observed for translated mRNAs.

Legnini *et al.*, 2017

Is the ORF functional?

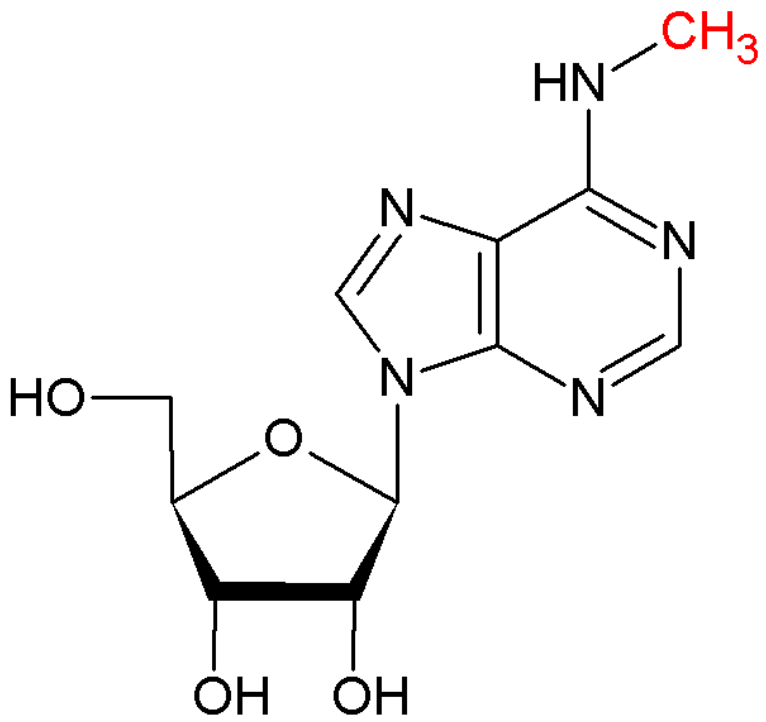


We cloned a 3xFlag-tagged circZNF609 sequence in an expression vector with the 3xFlag-coding sequence is immediately upstream to the stop codon (and upstream to the start codon, in the linear sequence).

When we overexpress the 3XFlag circZNF609, we are able to detect a flag-tagged protein with an anti-Flag antibody, with respect to the control sample.

Legnini *et al.*, 2017

Factors that trigger circRNA cap-independent translation



N6-methyladenosine

RNA methylation -> m⁶A (N6-methyladenosine)

[redacted]

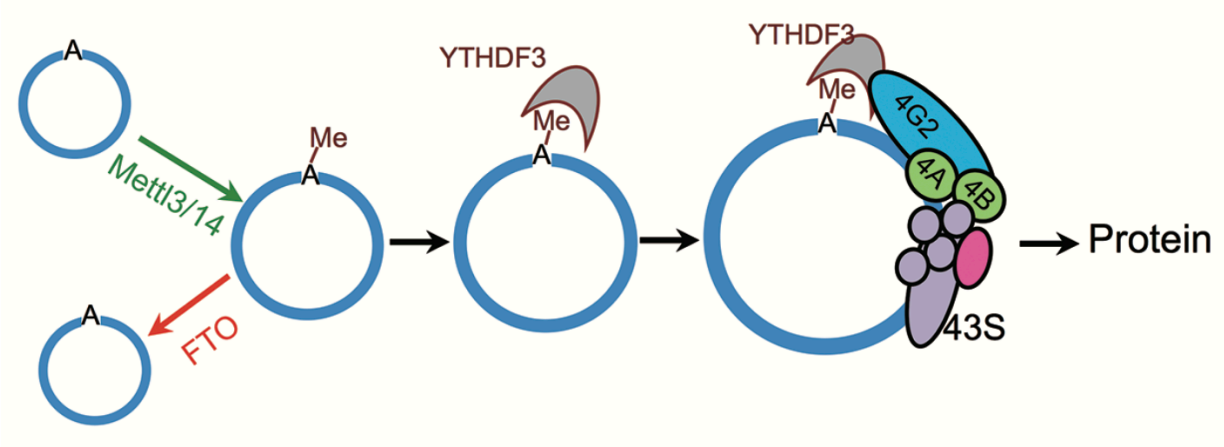
ORIGINAL ARTICLE

Cell Research (2017) 27:626-641.
www.nature.com/cr

Extensive translation of circular RNAs driven by *N*⁶-methyladenosine

Yun Yang[1, 2, 3, 4, *], Xiaojuan Fan[2, *], Miaowei Mao[4, 5, *], Xiaowei Song[2, 4], Ping Wu[6, 7], Yang Zhang[8], Yongfeng Jin[1], Yi Yang[5], Ling-Ling Chen[8], Yang Wang[9], Catherine CL Wong[6, 7], Xinshu Xiao[3], Zefeng Wang[2, 4]

Identification of methylated circRNAs and circular mRNAs



Yang *et al.*, 2017

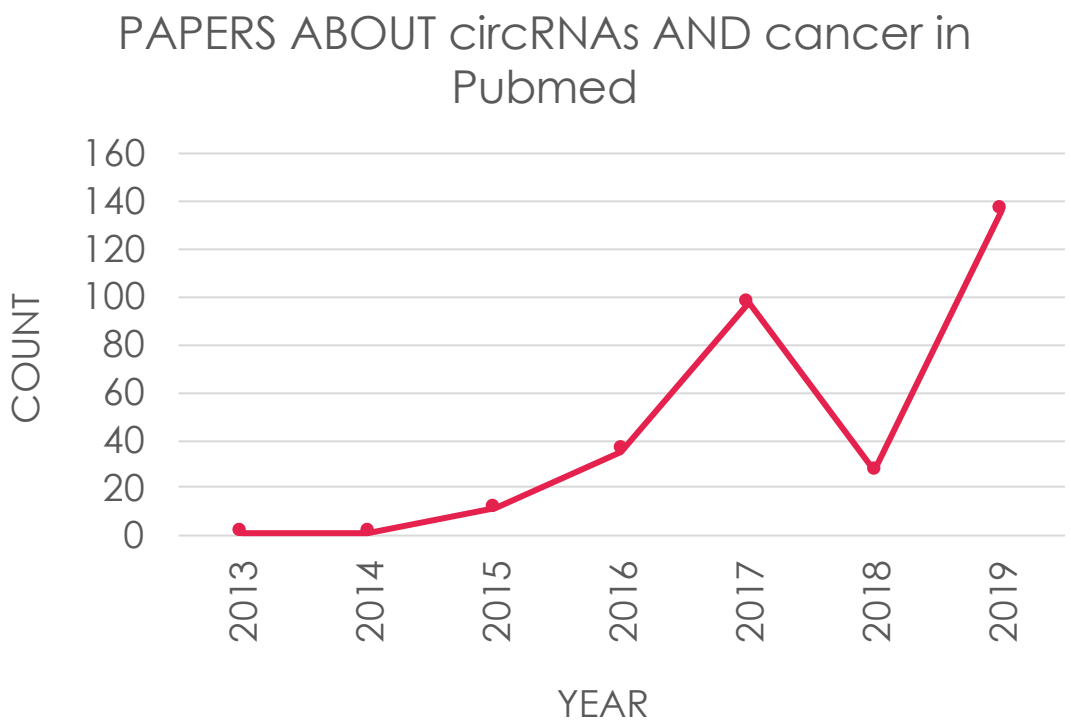
MODEL

circRNAs contain extensive m^{6}A modifications, which are sufficient to drive protein translation in a cap-independent fashion involving the m^{6}A reader YTHDF3 and the translation initiation factors eIF4G2 and eIF3A.

Summary

1. (Very brief) review about circRNA biogenesis
2. How to study circRNA molecular functions
3. Molecular functions of circRNAs
4. CircRNAs and their role in cancer
5. CircRNAs as biomarkers in body fluids

circRNAs as novel disease biomarkers



Given circRNA abundance, stability, together with their importance and specificity in several pathologies, many research groups focused on finding circular RNAs that could be used as novel biomarkers.

Expression and role of circRNAs in cancer

circRNAs and cancer

Gastric Cancer (GC)

Hepatocellular Carcinoma (HCC)

Lung cancer

Fusion-CircRNAs in leukemia

circRNAs and cancer

Gastric Cancer (GC)

Circ-PVT1 is up-regulated in GC tissues because of the amplification of its genomic locus.

It promotes GC cell proliferation by acting as a sponge against members of the miR-125 family (Chen *et al.*, 2016).

Hepatocellular Carcinoma (HCC)

Lung cancer

hsa_circ_0000190 was identify as a biomarker for gastric cancer. It was down-regulated in both GC tissues and plasma from patients with GC.

Compared with Carcinoembryonic antigen and CA19-9, two classic biomarkers for GC, it has more sensitivity and specificity (Chen *et al.*, 2017).

Fusion-CircRNAs in leukemia

circRNAs and cancer

Gastric Cancer (GC)

High expression of ciRS-7 (= CDR1as) in HCC is related to AFP (alpha-fetoprotein, HCC biomarker) levels (Xu *et al.*, 2017).

Hepatocellular Carcinoma (HCC)

Lung cancer

hsa_circ_0001649 has binding sites for miR-182 (Qin *et al.*, 2016).

Fusion-CircRNAs in leukemia

hsa_circ_0005075 has binding sites for miR-93 (Shang *et al.*, 2016).

circRNAs and cancer

Gastric Cancer (GC)

circ-ITCH is overexpressed in lung cancer and inhibits Wnt/β-catenin pathway, acting as a sponge of miR-7 and miR-214 (Wan *et al.*, 2016).

Hepatocellular Carcinoma (HCC)

circ_100876 is up-regulated in non-small cell lung cancer and correlates with lymphnode metastasis and tumour staging (Yao *et al.*, 2017).

Lung cancer

Fusion-CircRNAs in leukemia

circRNAs and cancer

Promyelocytic leukemia/Retinoic Acid Receptor-α (PML/RARα) is a fusion protein that plays an important role in acute promyelocytic leukemia.

In leukemia with PML/RARα translocations, a type of special fusion-circRNA can be generated during the generation of fusion-gene.

Also other solid and liquid tumors (es. leukemia with MLL/AF9 fusion protein) harboring chromosomal translocations also harbor fusion-circRNAs.

Fusion-circRNAs can be tumor promoting, with potential diagnostic and therapeutic implications.

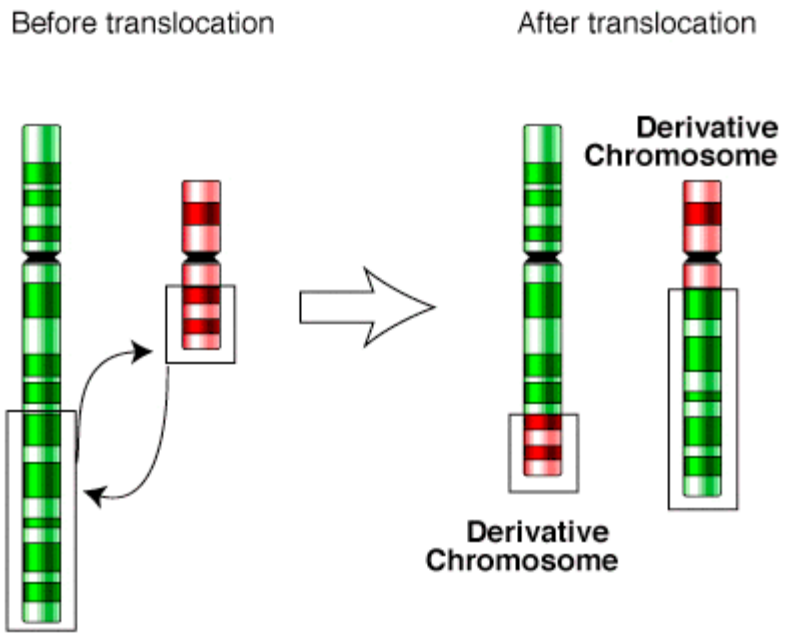
Gastric Cancer (GC)

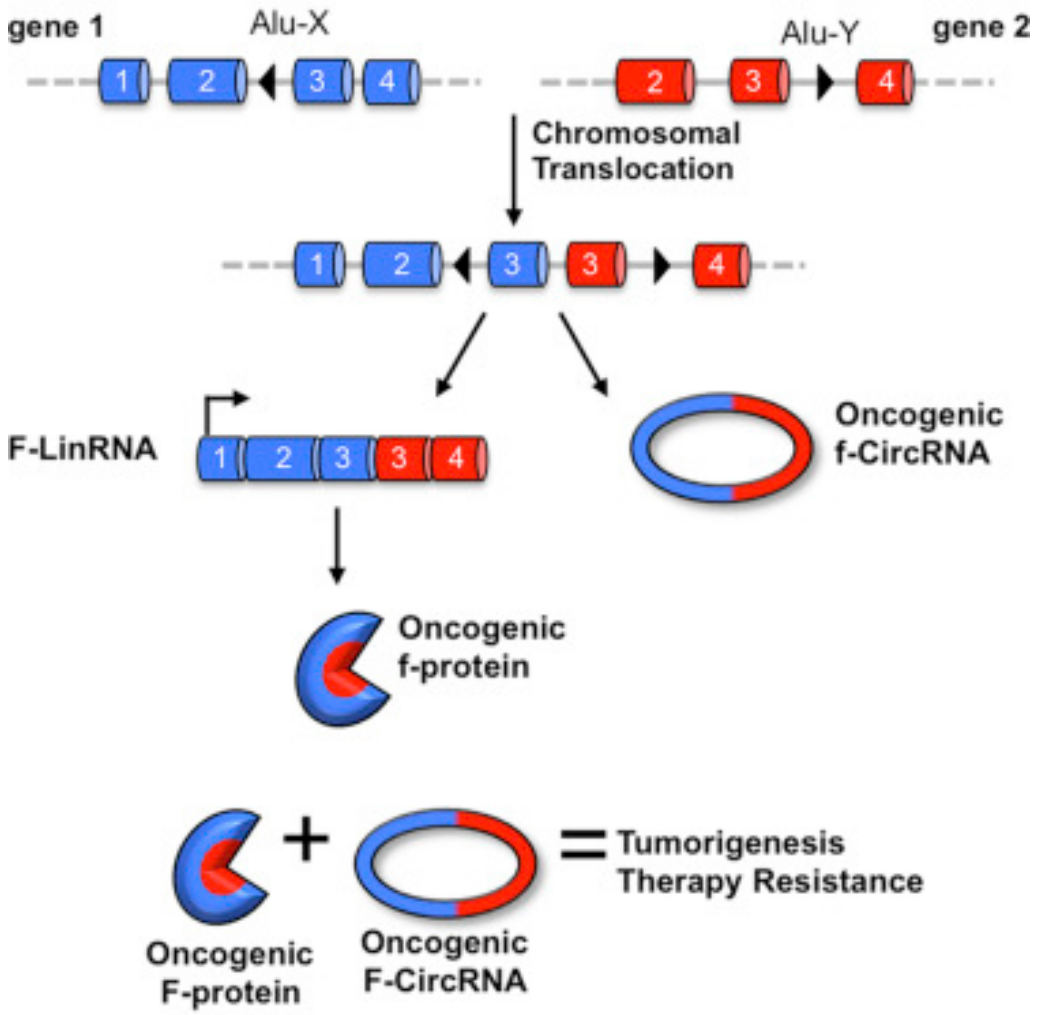
Hepatocellular Carcinoma (HCC)

Lung cancer

Fusion-CircRNAs in leukemia
(Guarnerio *et al.*, 2016)

Fusion-circRNAs in leukemia





Adapted from Guarnerio *et al.*, 2016

Summary

1. (Very brief) review about circRNA biogenesis
2. How to study circRNA molecular functions
3. Molecular functions of circRNAs
4. CircRNAs and their role in cancer
5. CircRNAs as biomarkers in body fluids

CircRNAs can be biomarkers in body fluids

A potent way to evaluate pathological conditions, without invasive approaches, is to analyze a specific marker in blood or in other body fluids.

Bahn *et al*. (2015) provided the first example of the presence of circular RNAs in an extracellular fluid, the saliva.

Memczak *et al*. (2015) identified more than 2000 circRNAs in human whole blood, indicating that circRNAs are very abundant in this tissue, and many of them are here more expressed than their linear counterpart.

CircRNAs can be biomarkers in body fluids

It seems that circRNAs are very enriched and stable in blood because they are contained in exosomes, small membranous vesicles secreted by several cell types.

Li *et al*. (2015) discovered this enrichment and demonstrated that exosome-circRNAs (exo-circRNAs) can distinguish a tumor condition from a healthy one.

Apart from cancer diseases, exo-circRNAs have also been studied in relation to other pathologies, such as coronary artery diseases and type 2 diabetes mellitus (Zhao *et al*., 2016 and 2017).

Zhang *et al*. (2016) identified a subset of circRNAs in blood corpuscles as early predictors of pre-eclampsia, in pregnant women.