Circular RNAs

18 May 2021

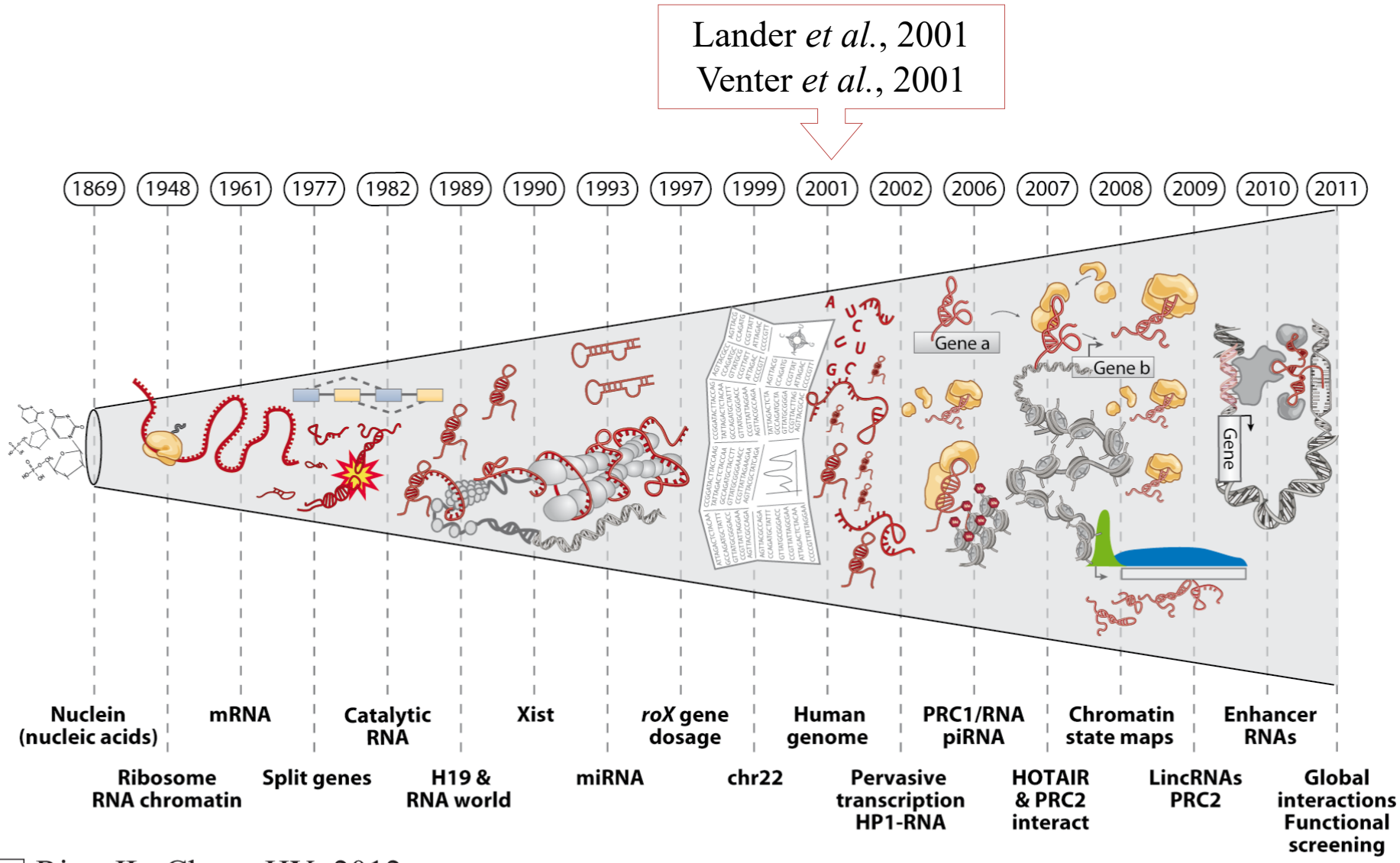
The GENOMIC ERA

– at the beginning of the XXI century, one of the major question was:

how many genes in the human genome?

The huge popular interest in counting the number of genes present in the human genome led even to a public wager named Gene Sweepstake, with an extensive media coverage (nyt Wade 2003)

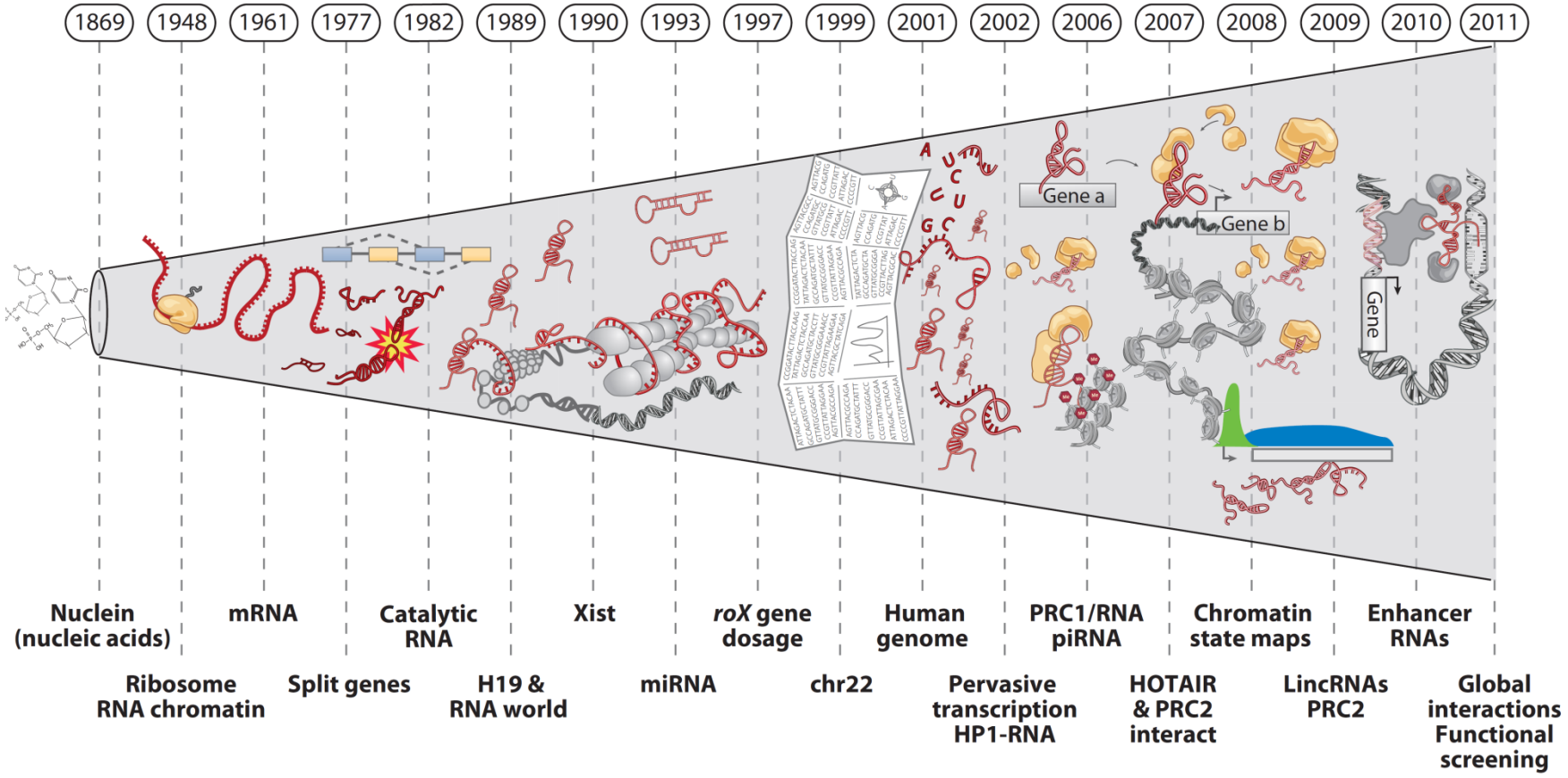
RNA: the most re-discovered molecule



Rinn JL, Chang HY. 2012.
Annu. Rev. Biochem. 81:145–66

RNA: the most re-discovered molecule

The FANTOM3 Consortium, 2005



Rinn & Chang *Annu Rev Biochem* 2012

Transcriptome analysis

ENCODE

ARTICLE

doi:10.1038/nature11247

An integrated encyclopedia of DNA elements in the human genome

The ENCODE Project Consortium*

The Encyclopedia of DNA Elements (ENCODE) Consortium is an international collaboration of research groups funded by the National Human Genome Research Institute (NHGRI). The goal of ENCODE is to build a comprehensive parts list of functional elements in the **human** genome, including elements that act at the protein and RNA levels, and regulatory elements that control cells and circumstances in which a gene is active.

FANTOM5

A promoter level mammalian expression atlas
Alistair R.R. Forrest *et al.*, *submitted*

CAGE analysis of the following libraries:

573 human primary cell samples

128 mouse primary cell samples

250 different cancer cell lines samples

152 human post-mortem tissues samples

271 mouse developmental tissue samples

22000 genes encoding for proteins

Genome Organization

ENCODE

ARTICLE

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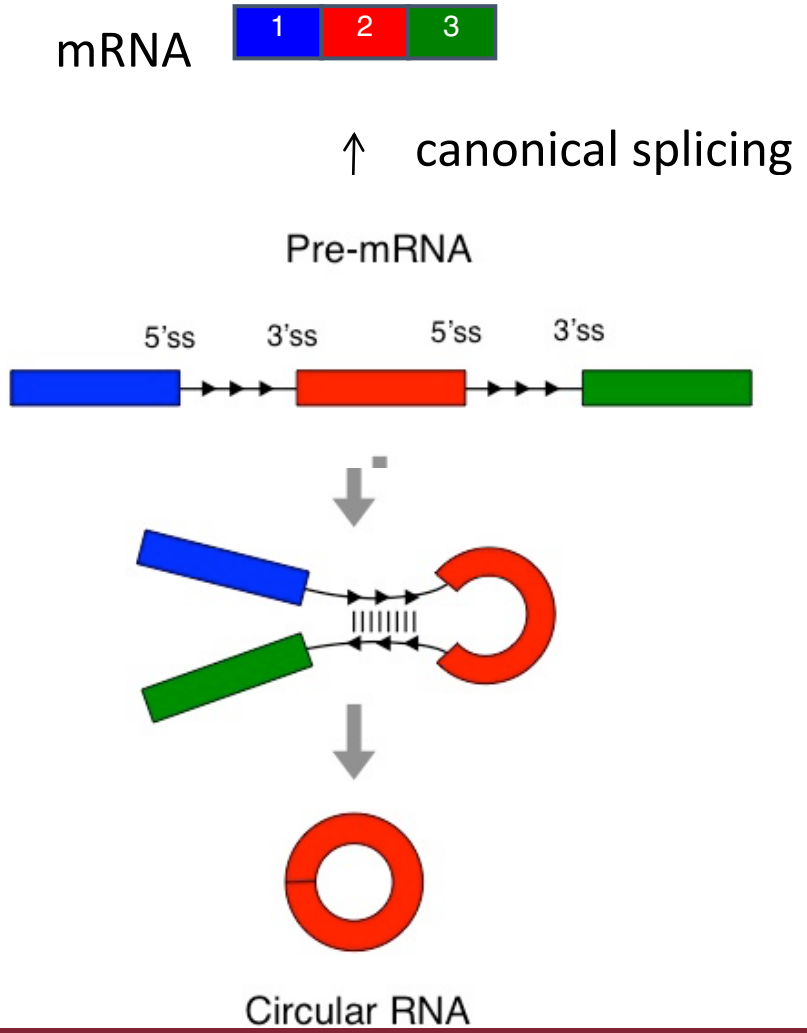
22000 genes that encodes for proteins

>40000 long non-coding RNAs and growing…….
>50% of the genome is functional

…..more non coding (?) RNAs - ***circular RNAs***



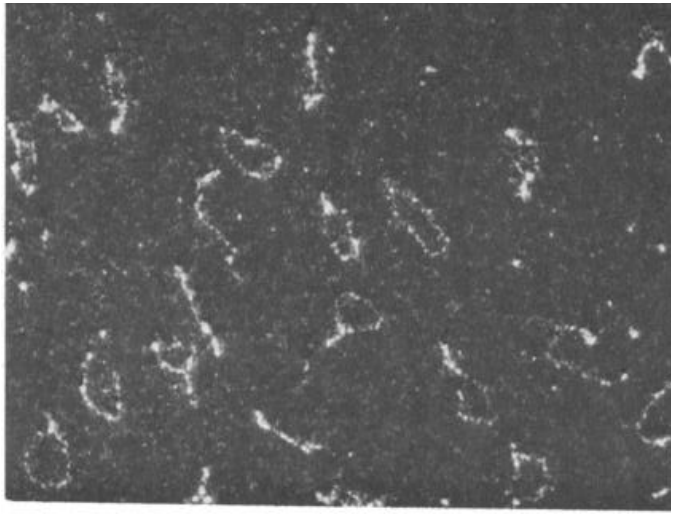
Biogenesis of circular RNAs. A gene can be transcribed and spliced into linear and circular RNAs. Note the unique 'head-to-tail' splice junctions formed by an acceptor splice site at the 5′ end of an exon and a donor site at the 3′ end of a downstream exon.

Adapted from Liang et al., ***Genes and Dev***., 2014

circRNAs

Identified long time ago:

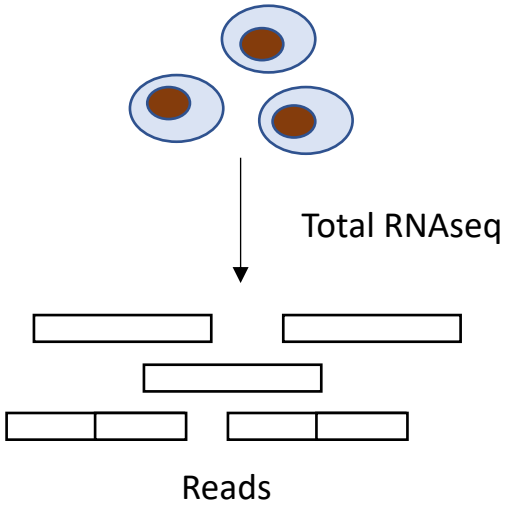
Sanger et al., 1976; PNAS *73*
Kjems and Garrett 1988; Cell *54*
Capel et al., 1993; Cell *73*

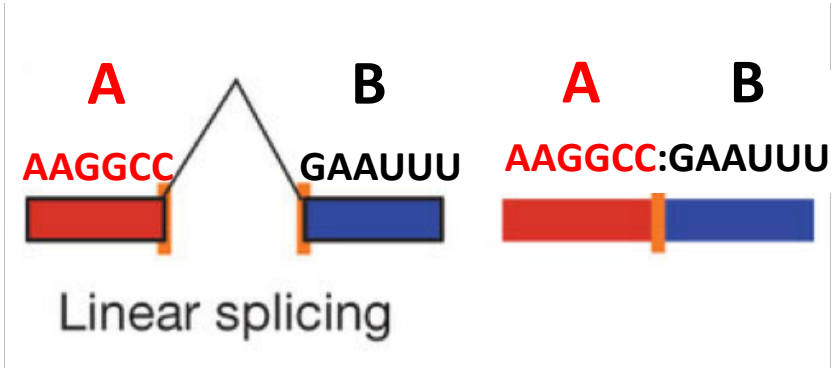


Thousands of circular RNAs from Eukaryotes
(Salzman 2012, Hansen 2013, Memczack 2013)

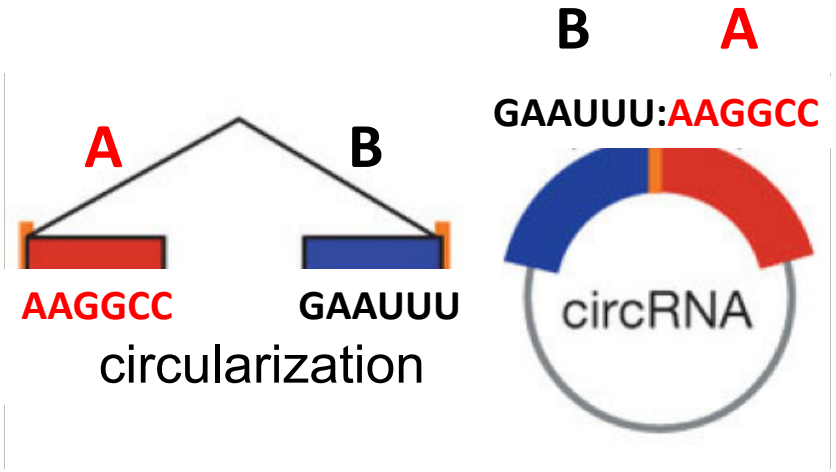
Are they functional molecules?

circRNAs were detected in total RNA seq thanks to the unusual sequence order



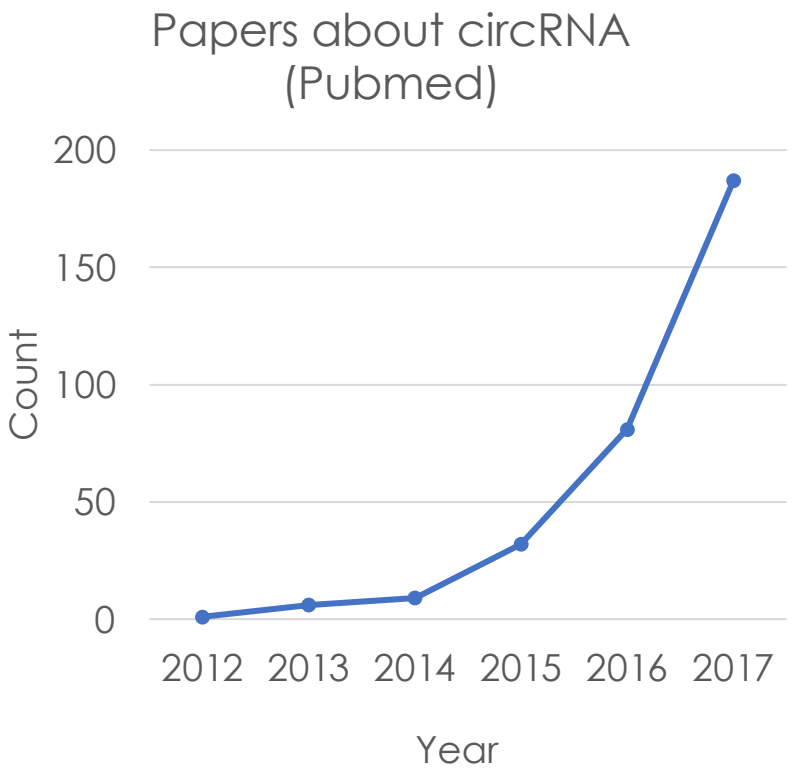


Sequence B should be found downstream to A, instead it was found upstream to it



Adapted from Liang et al., ***Genes and Dev***., 2014

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.

circRNAs regulate gene expression by:

- **Sponging miRNAs**
  - Hansen et al. Nature, 2013
  - Memczak et al. Nature, 2013
  - Piwecka et al. Science, 2017
- **Controlling splicing**
  - Ashwal-Fluss et al. Mol Cell, 2014
  - Zang et al. Cell, 2014
  - Zang et al. Cell Rep, 2016
- **Sponging proteins**
  - Chen et al. Mol Cell, 2017
  - Li et al. Mol Cell , 2017
  - Xia et al. Immunity, 2018
- **Affecting transcription**
  - Zang et al. Mol Cell ,2013
  - Li et al. NSMB, 2015
- **Producing proteins**
  - Legnini et al. Mol Cell, 2017
  - Pamudurti et al. Mol Cell, 2017
  - Yang et al. Cell Res, 2017

circRNA functions

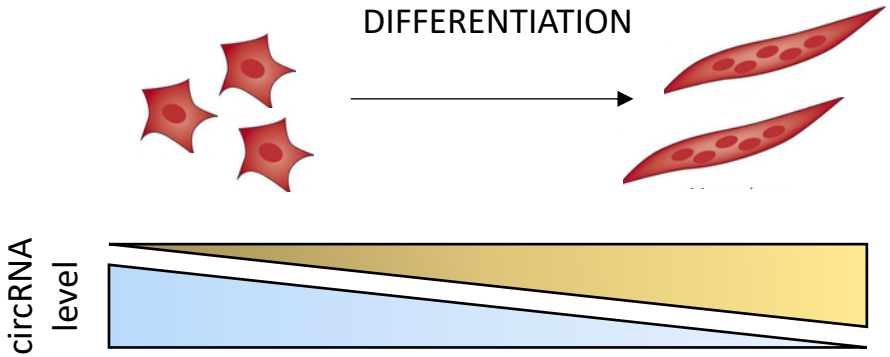
Exonic circular RNAs are enriched in cytoplasm (Lasda *et al.*, 2014).

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).

How to study circRNA functions



A. CHOOSE A MODEL SYSTEM

B. IDENTIFY circRNAs EXPRESSED -> RNAseq

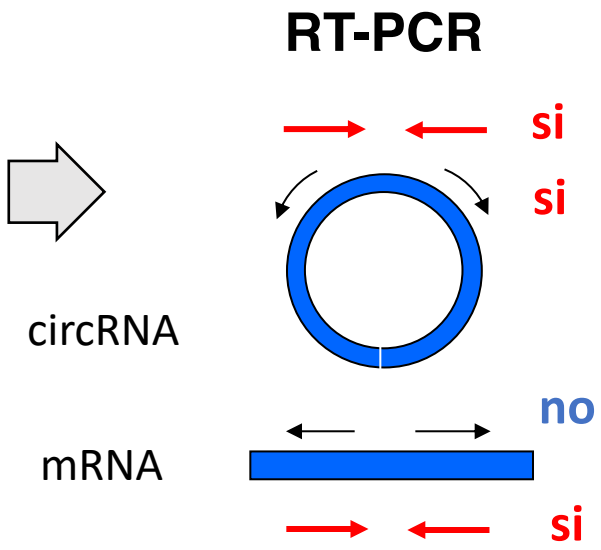
C. SELECT AND VALIDATE INTERESTING CANDIDATES -> expression levels, conservation, modulation…

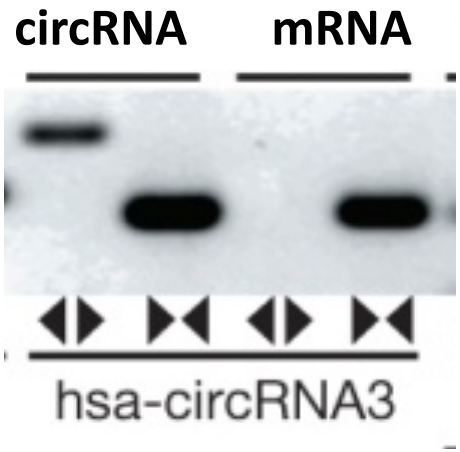
D. SUBCELLULAR LOCALIZATION -> subcellular fractionation, *in situ* hybridization…

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

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

How to study circRNA functions





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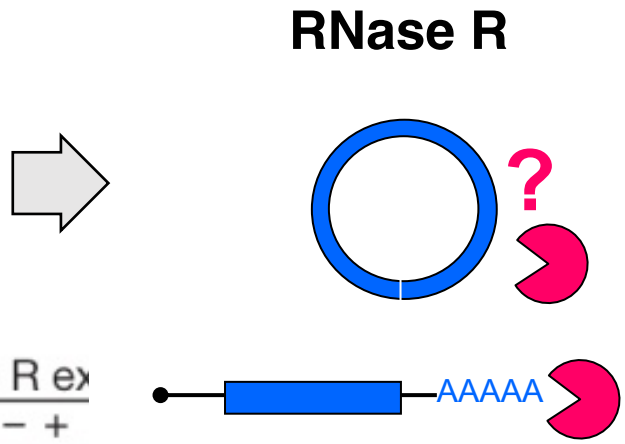

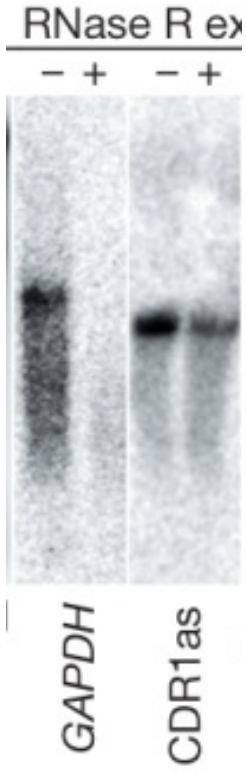

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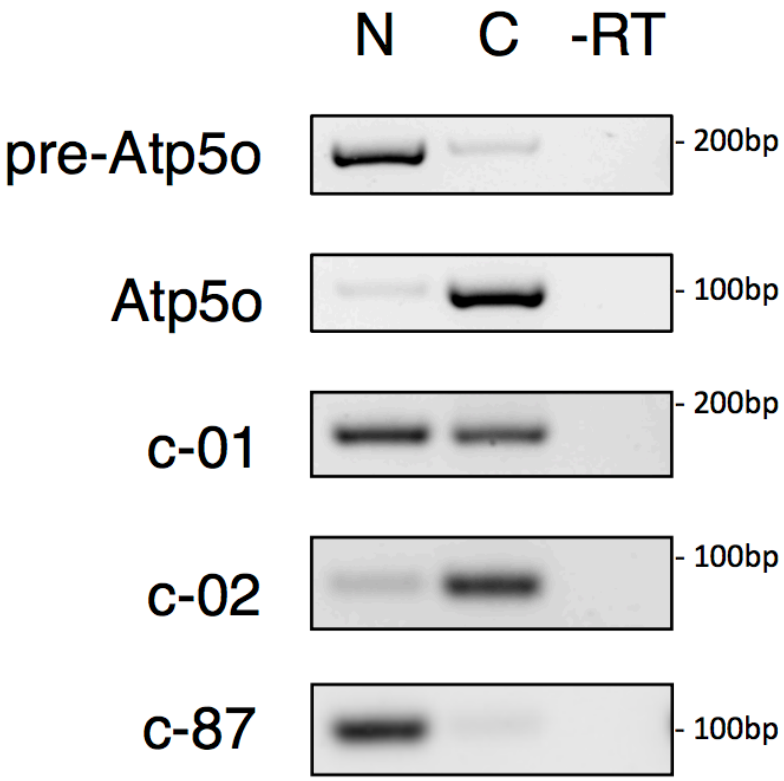

Adapted from Errichelli *et al.*, 2017

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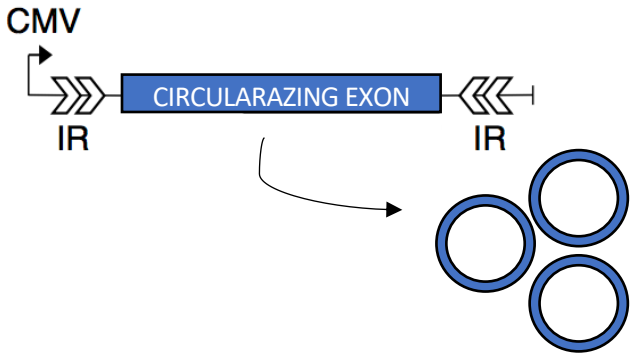
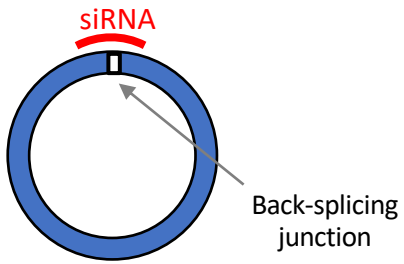
How to study circRNA functions

OVEREXPRESSION



KNOCK-DOWN



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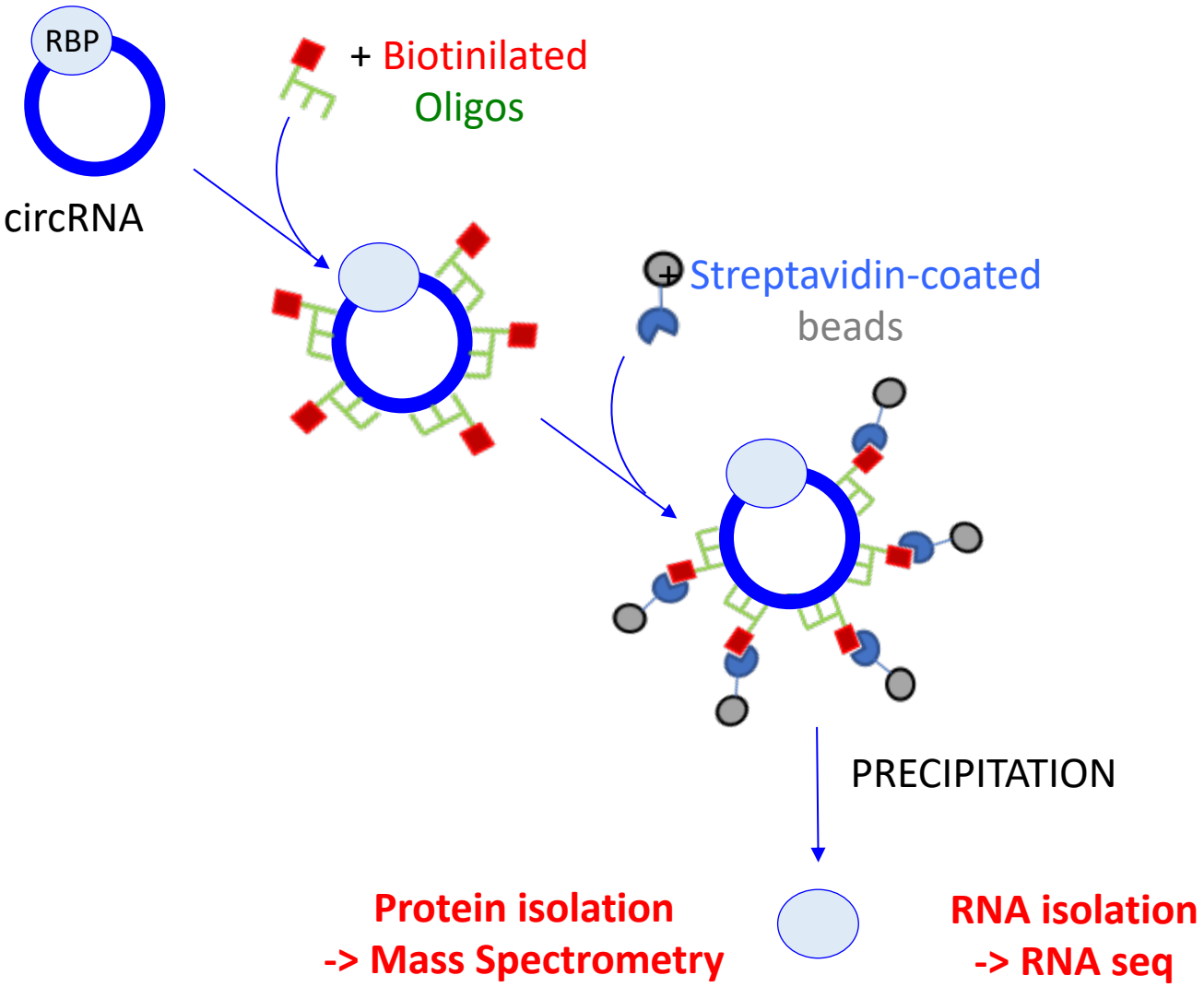
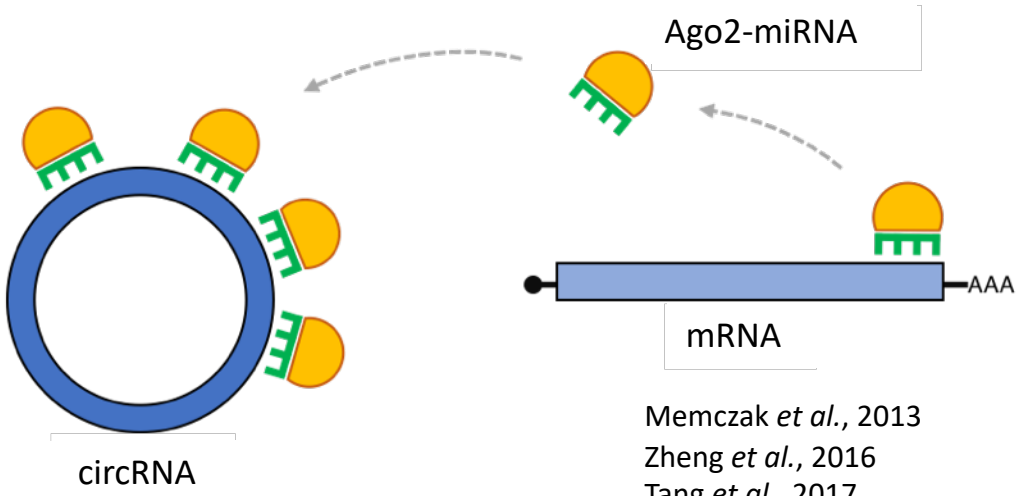
circRNA functions – identify the interactors



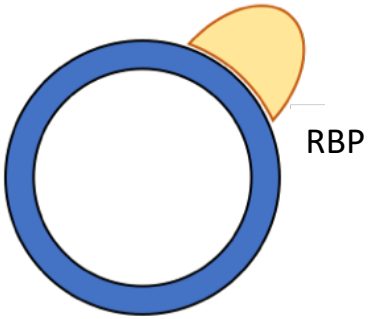
circRNA functions



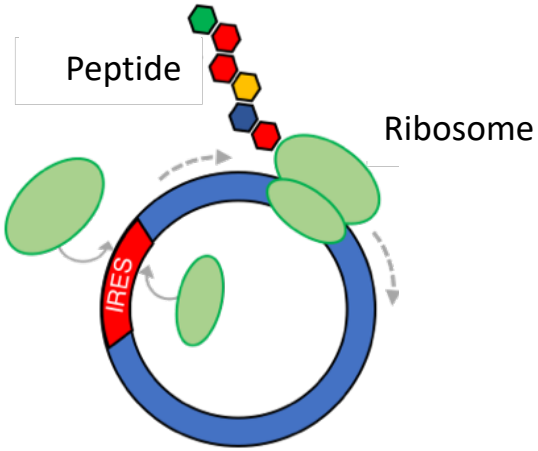
Memczak *et al.*, 2013
Zheng *et al.*, 2016
Tang *et al.*, 2017

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

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



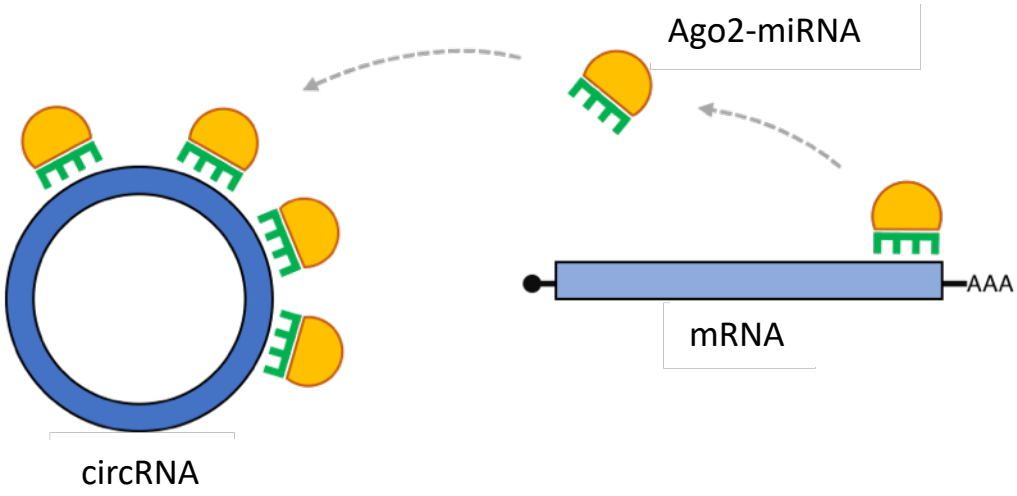
Du *et al.*, 2016
Schneider *et al.*, 2016



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

C. Templates for cap-independent translation

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.

Which factors control backsplicing?..... The FUS protein

SCIENTIFIC REPORTS

OPEN

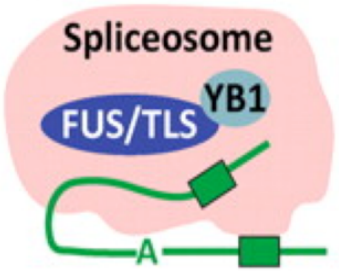
SUBJECT AREAS:
BIOINFORMATICS
BIOCHEMISTRY
COMPARATIVE GENOMICS
NEURODEGENERATION

Widespread binding of FUS along nascent RNA regulates alternative splicing in the brain

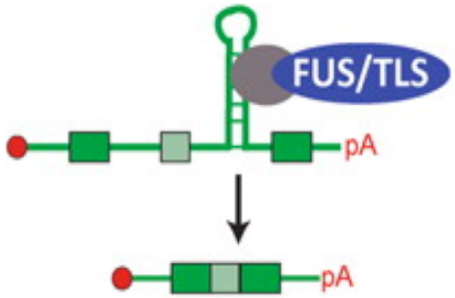
Boris Rogelj[1,6*], Laura E. Easton[2*], Gireesh K. Bogu[3], Lawrence W. Stanton[3], Gregor Rot[4], Tomaž Curk[4], Blaž Zupan[4], Yoichiro Sugimoto[2], Miha Modic[2], Nejc Haberman[2], James Tollervey[2,7], Ritsuko Fujii[5], Toru Takumi[5], Christopher E. Shaw[1*] & Jernej Ule[2*]

- Mutations in FUS are linked to ALS
- FUS regulates alternative splicing

CANONICAL



ALTERNATIVE

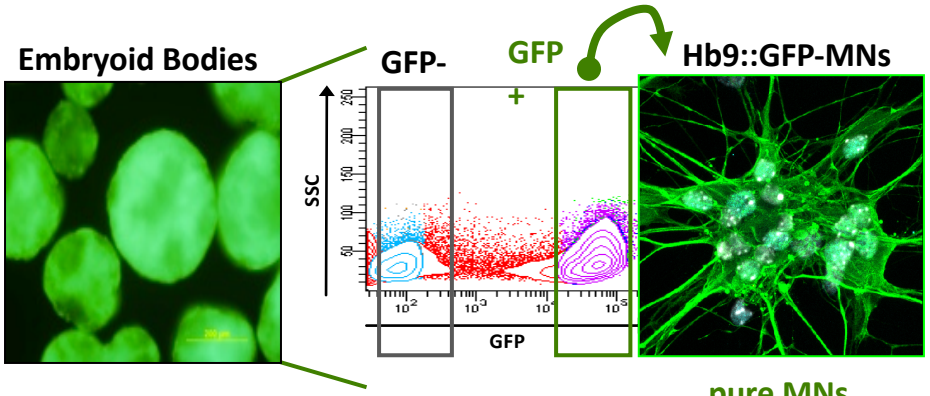


In vitro motor neuron differentiation

P. Laneve

mESC & hiPSC

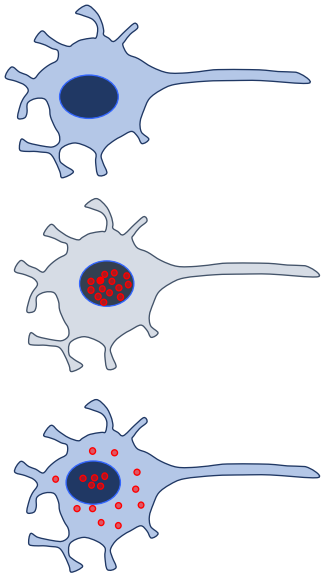
A. Rosa



FUS^{KO}

FUS^{WT}

FUS^{mut}



gain/loss of function

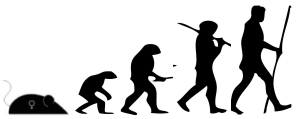
transcriptome

mRNAs

small RNAs (microRNA)

circular RNAs

long non coding RNAs



Conservation

Association with ALS

SAPIENZA UNIVERSITÀ DI ROMA

FUS KO affects circRNA levels in mESC-derived motor neurons

nature COMMUNICATIONS

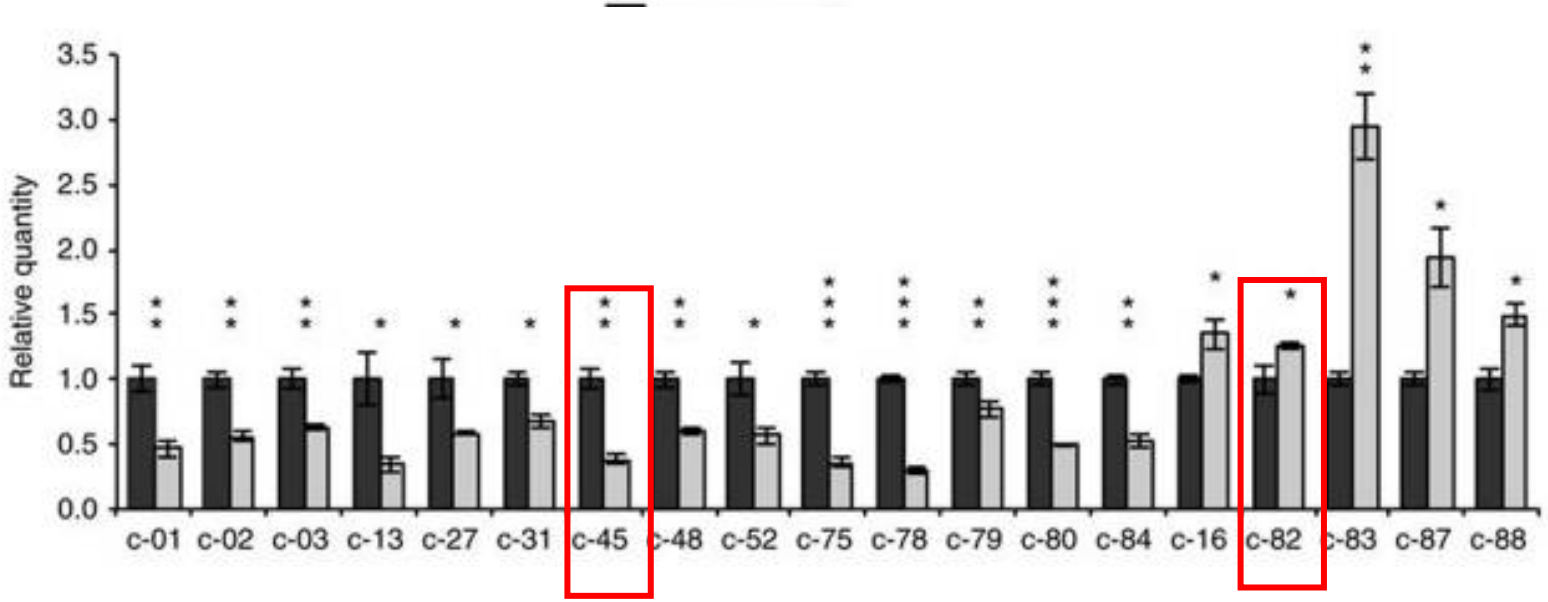
ARTICLE

Received 24 Jun 2016 | Accepted 26 Jan 2017 | Published 30 Mar 2017

DOI: 10.1038/ncomms14741 OPEN

FUS affects circular RNA expression in murine embryonic stem cell-derived motor neurons

Lorenzo Errichelli[1,2,*], Stefano Dini Modigliani[1,*], Pietro Laneve[1], Alessio Colantoni[2], Ivano Legnini[2], Davide Capauto[1,2], Alessandro Rosa[1,2], Riccardo De Santis[1,2], Rebecca Scarfò[2], Giovanna Peruzzi[1], Lei Lu[3], Elisa Caffarelli[1,4], Neil A. Shneider[3], Mariangela Morlando[2] & Irene Bozzoni[1,2,4,5]



M. Morlando & L. Errichelli

FUS binds neighboring intronic regions

nature COMMUNICATIONS

ARTICLE

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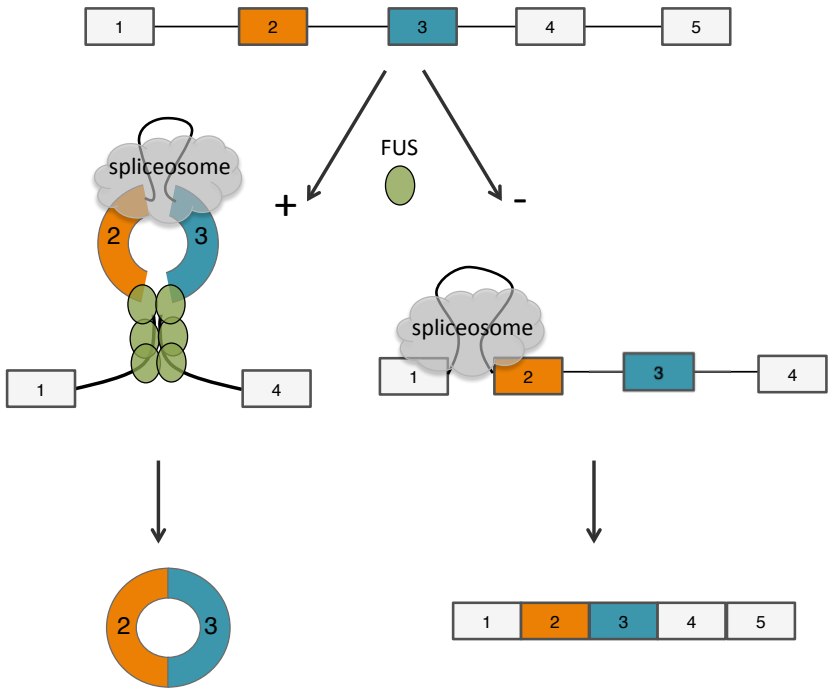
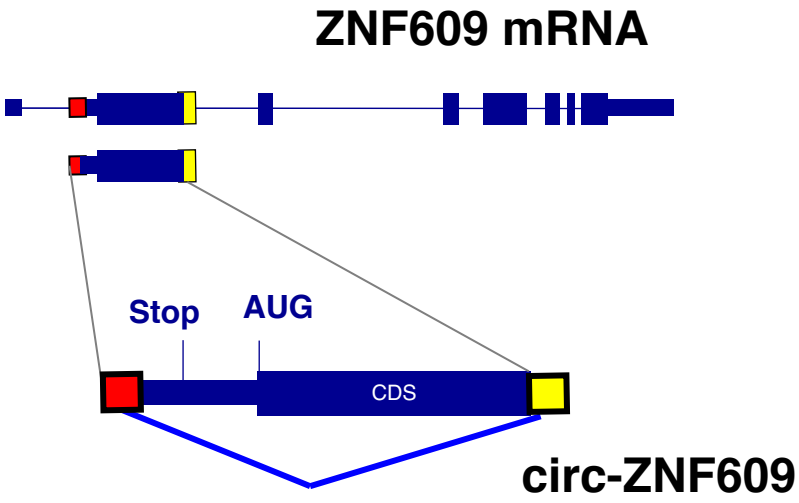
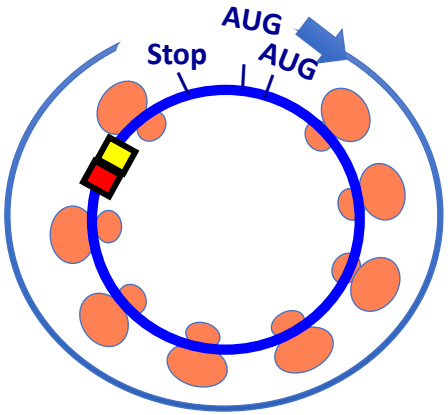
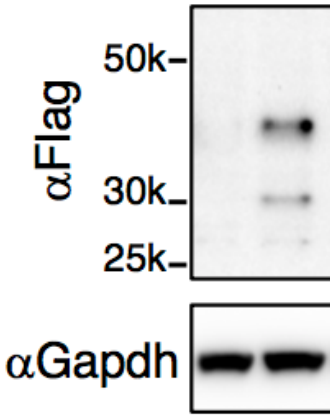


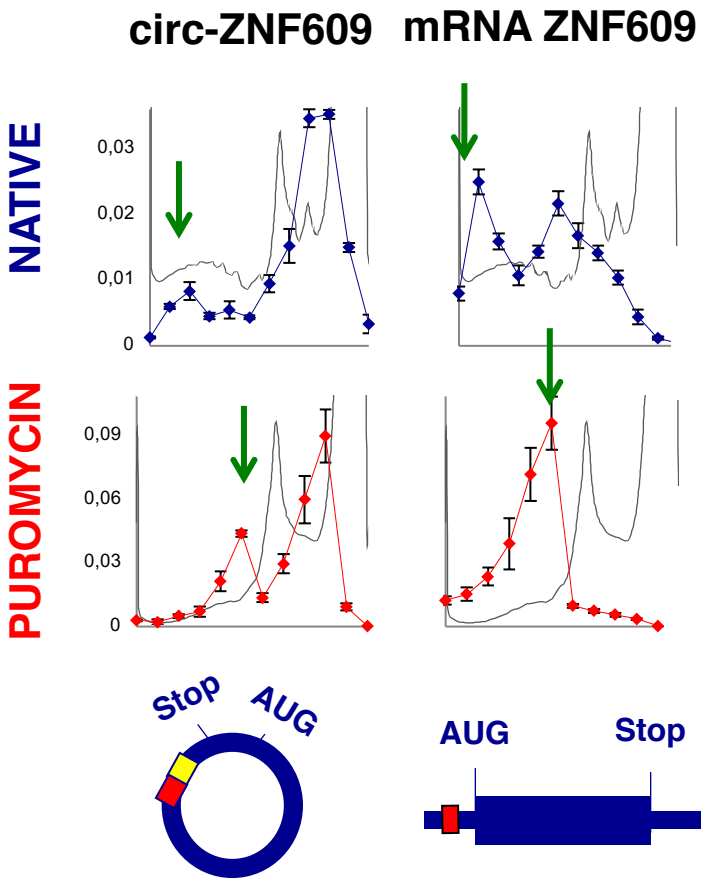
circRNAs can be translated

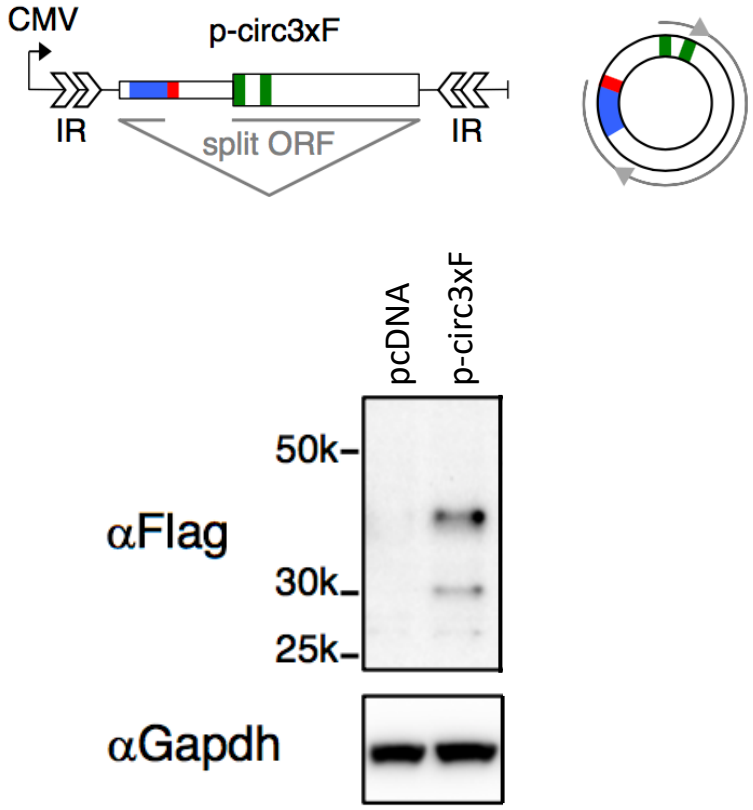






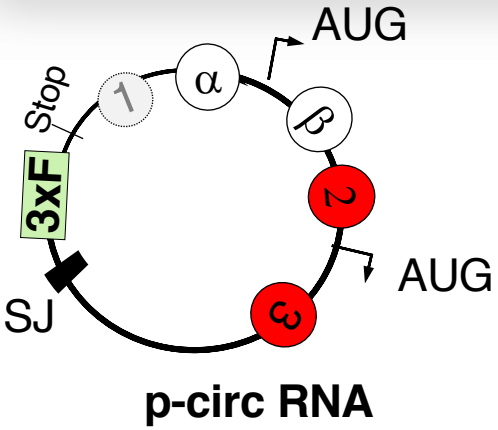
circ-ZNF609 can be translated



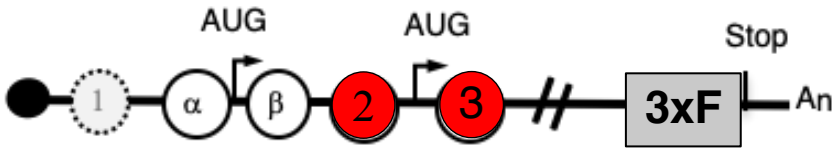


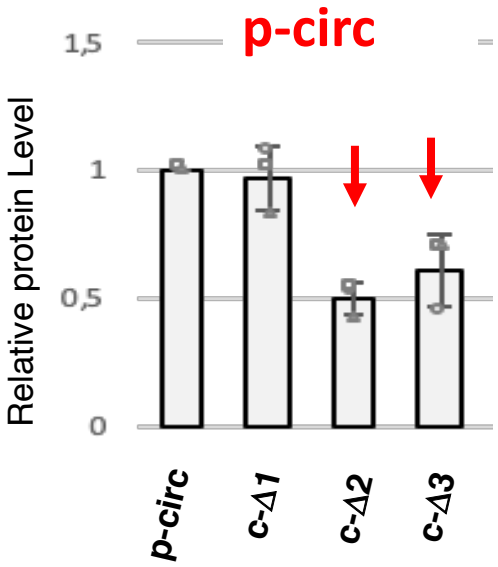
Legnini et al. Mol Cell 2017

m^{6}A modifications are required for efficient translation



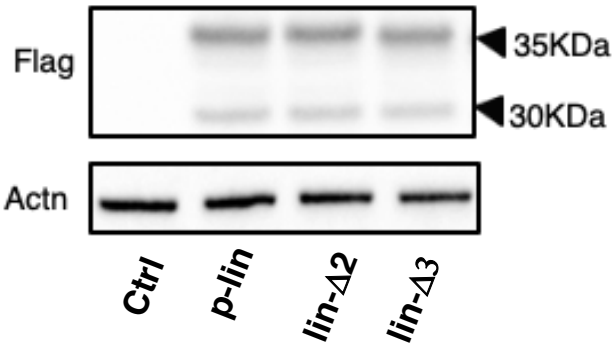
p-circ RNA



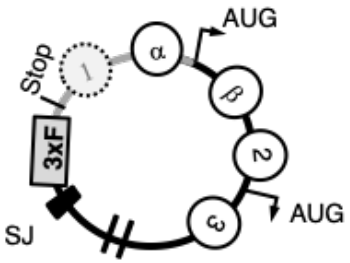


No change in RNA levels

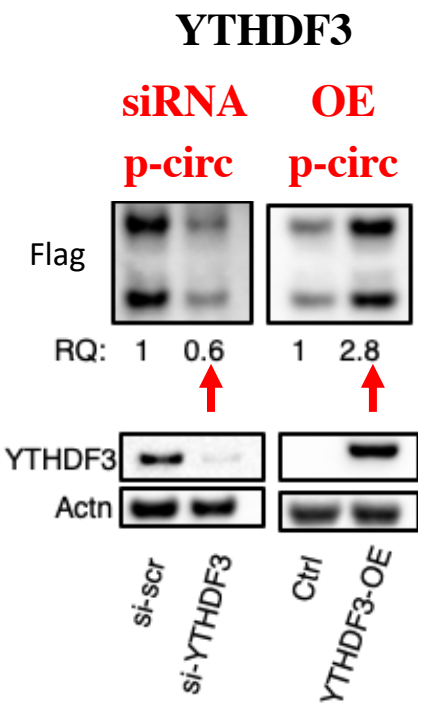
p-lin

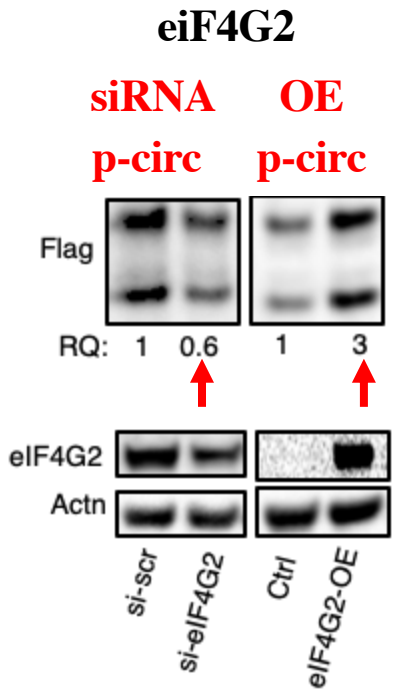


YTHDF3 and eiF4G2 - factors involved in cap-independent translation – are required for efficient translation of circ-ZNF609



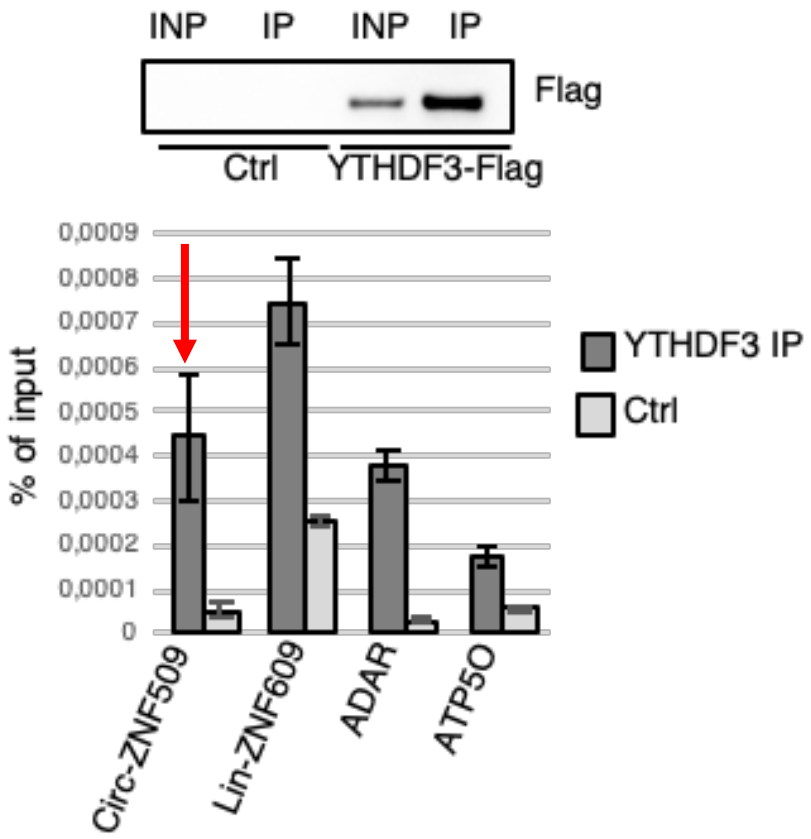
p-circ RNA

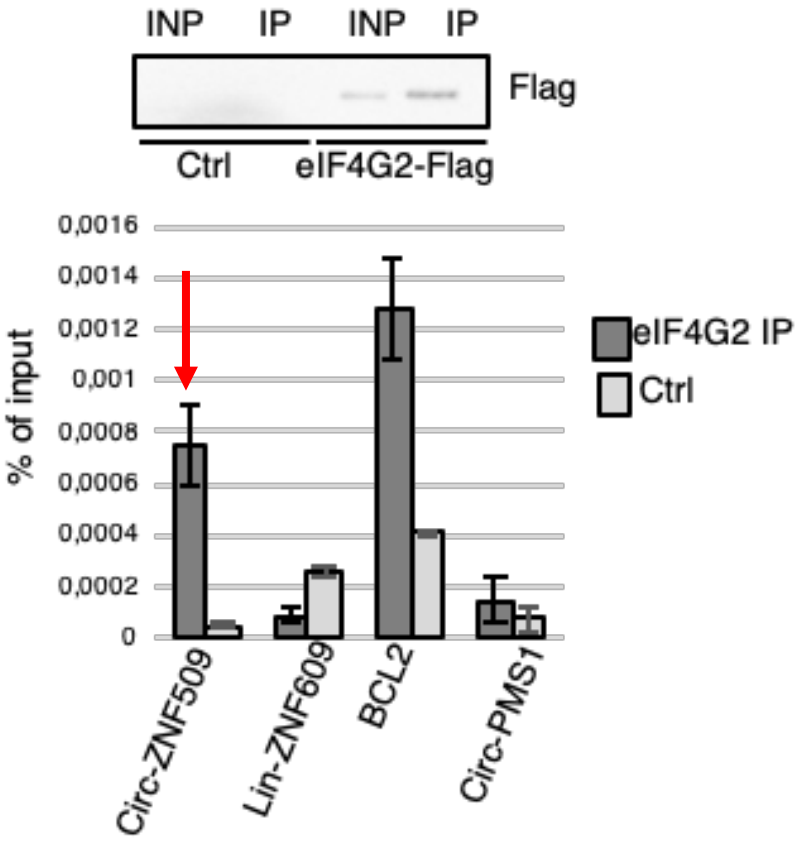




YTHDF3 and eiF4G2 interact with the endogenous circ-ZNF609

RIP experiments

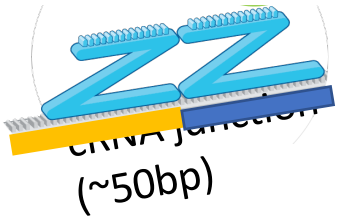


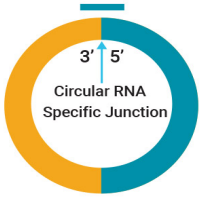


Yang et al. Cell Res. 2017

in situ analysis of circRNAs

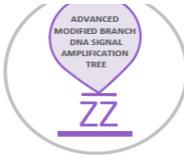
Hybridize to target

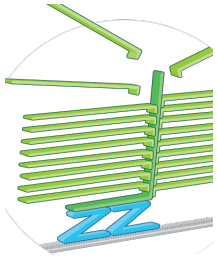




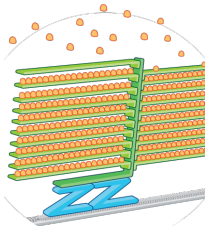
A highly sensitive-single molecule ***in situ*** detection system (BaseScope™ technology)

Amplify signal



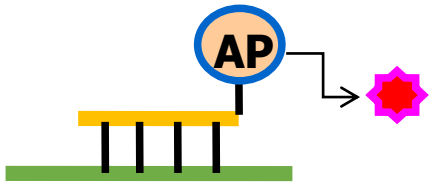


Amplifier probes



Labelled probes

Develop signal

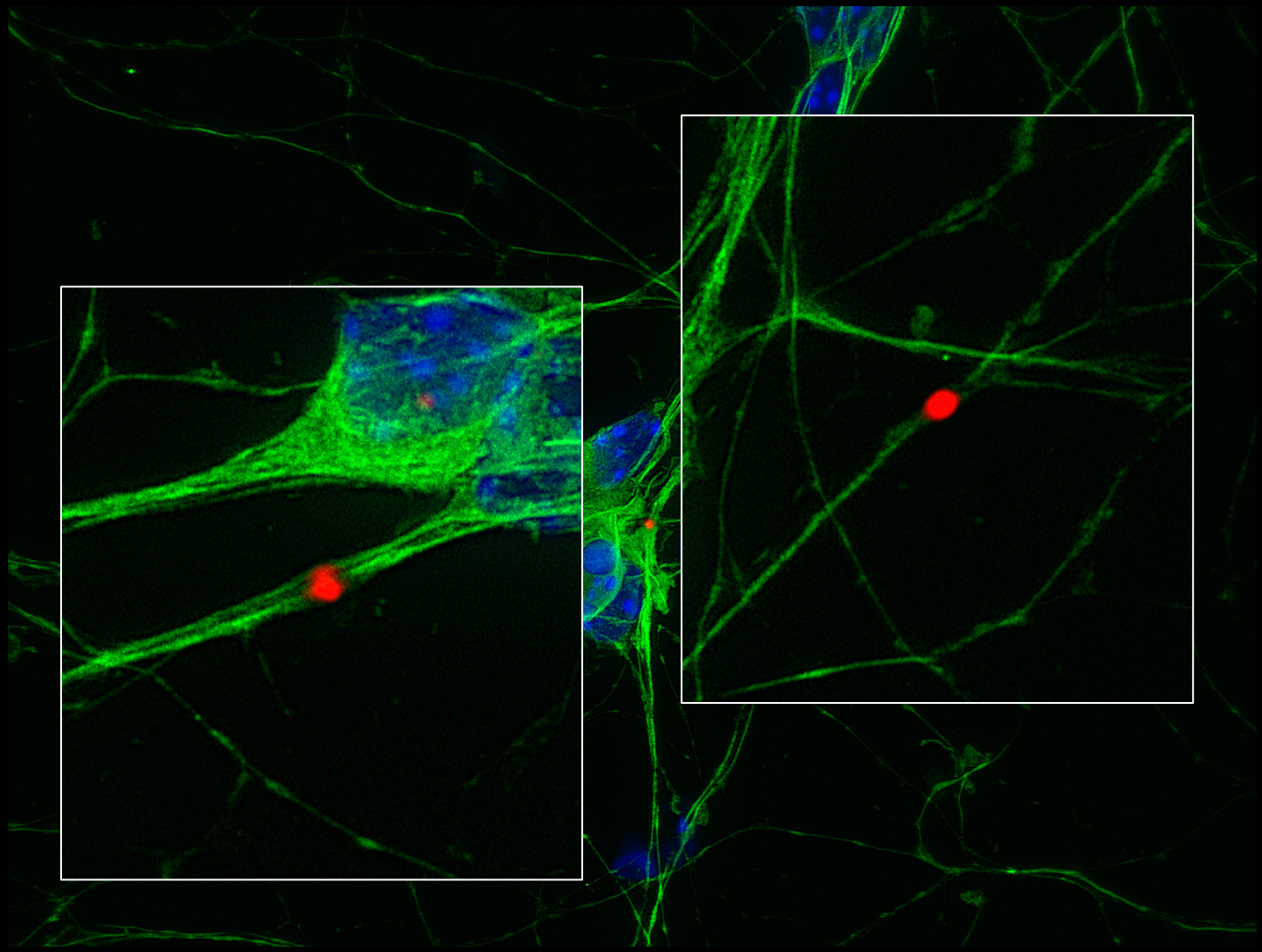


Chromogenic FastRED

T. Santini

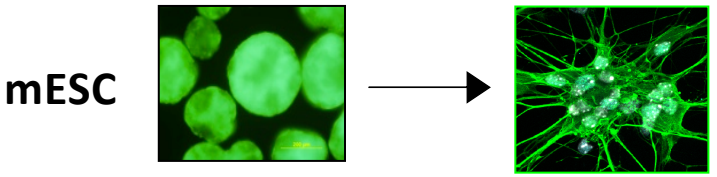
circ-31 localizes in neurites

TUB
C31
DAP
I

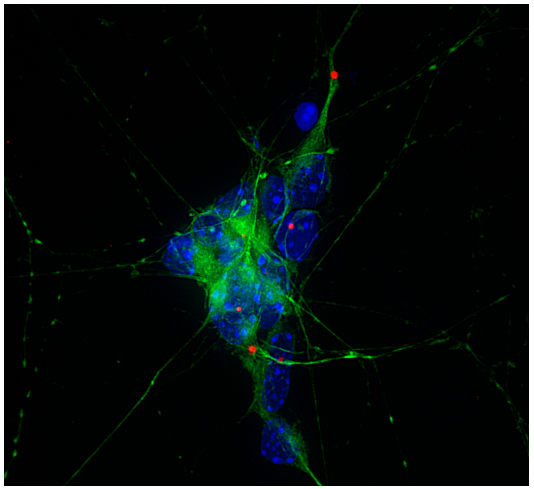


T. Santini

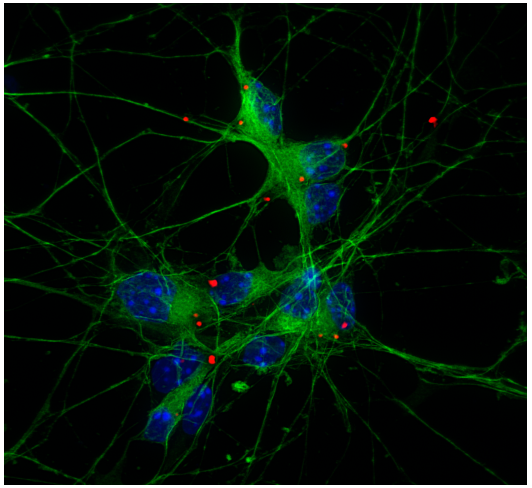
circ-31 moves along neurites



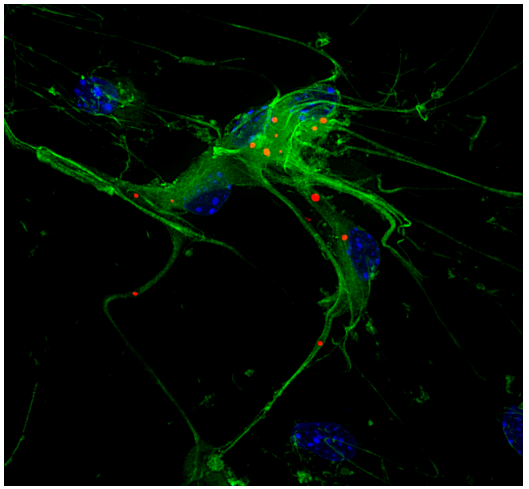
day 1

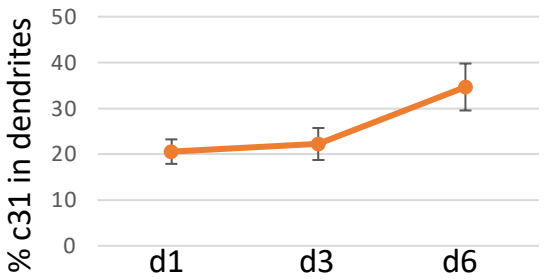


Day 3



Day 6



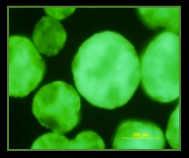


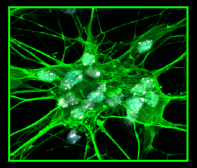
DAPI
Tubulin III
circ-31

D'Ambra E. and Santini T.

In ALS-MNs c-31 is trapped into FUS aggregates and does not translocate in the neurites

ALS^{FUS} mESC

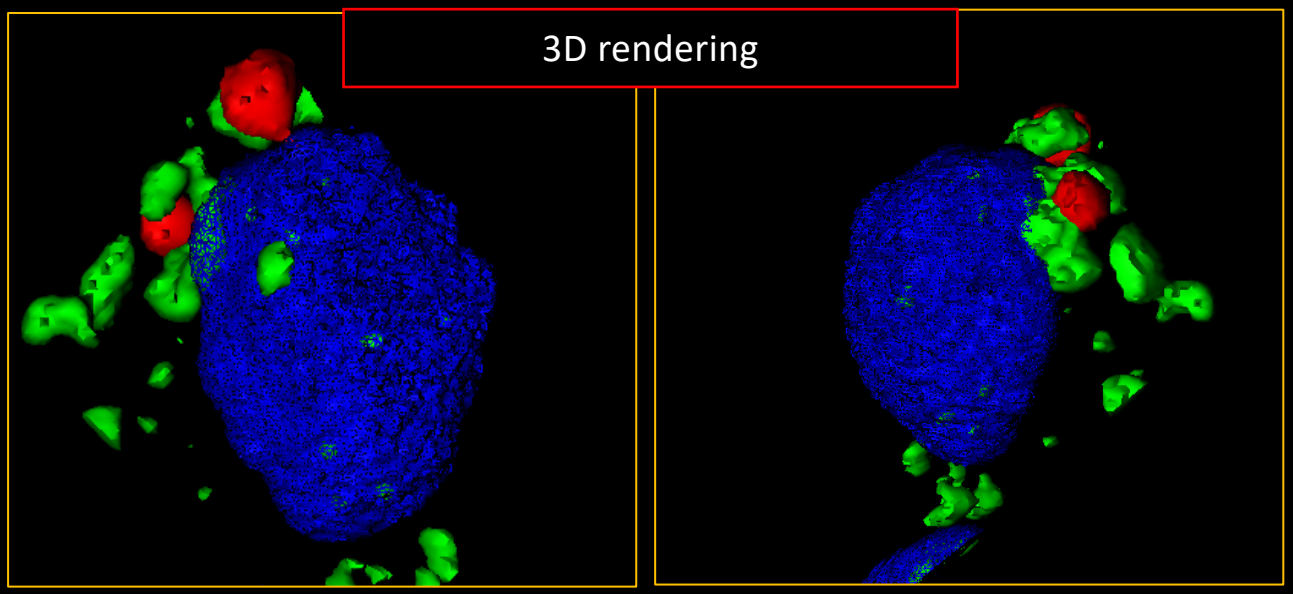




ALS^{FUS} MNs

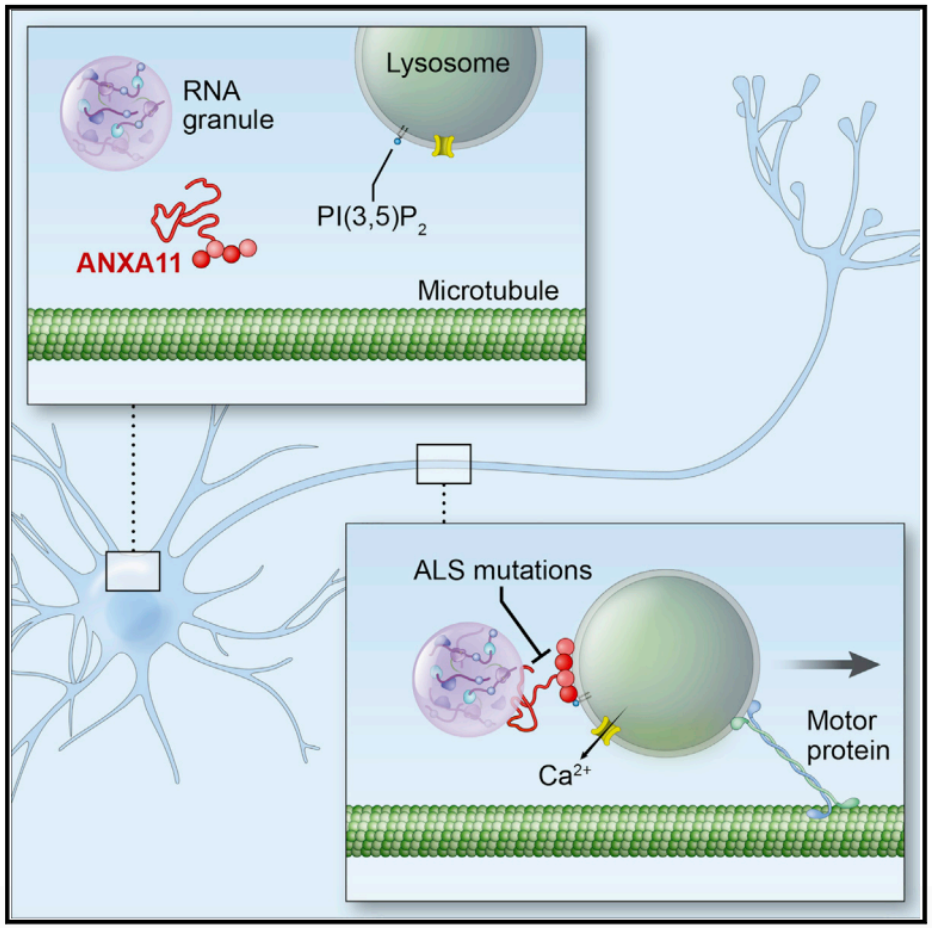
FUS
C31
DAPI

Trapping in FUS aggregates can impair trafficking of circRNA cargos along neurites
implication in ALS?



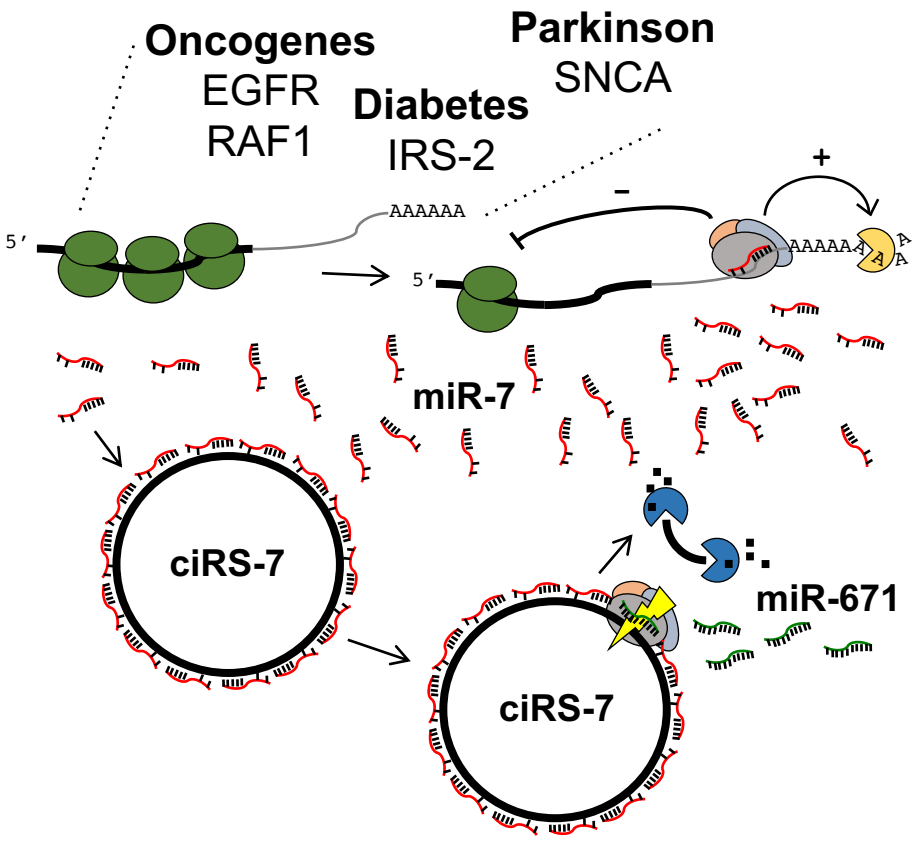
Is circ-31 involved in intracellular trafficking?

RNA granules 'hitchhike' on LAMP1-positive organelles (lysosomes) using annexin A11 as a tether. Cell. 2019, 179:147-16



c-31 interacts with several mRNAs and with Annexin A2

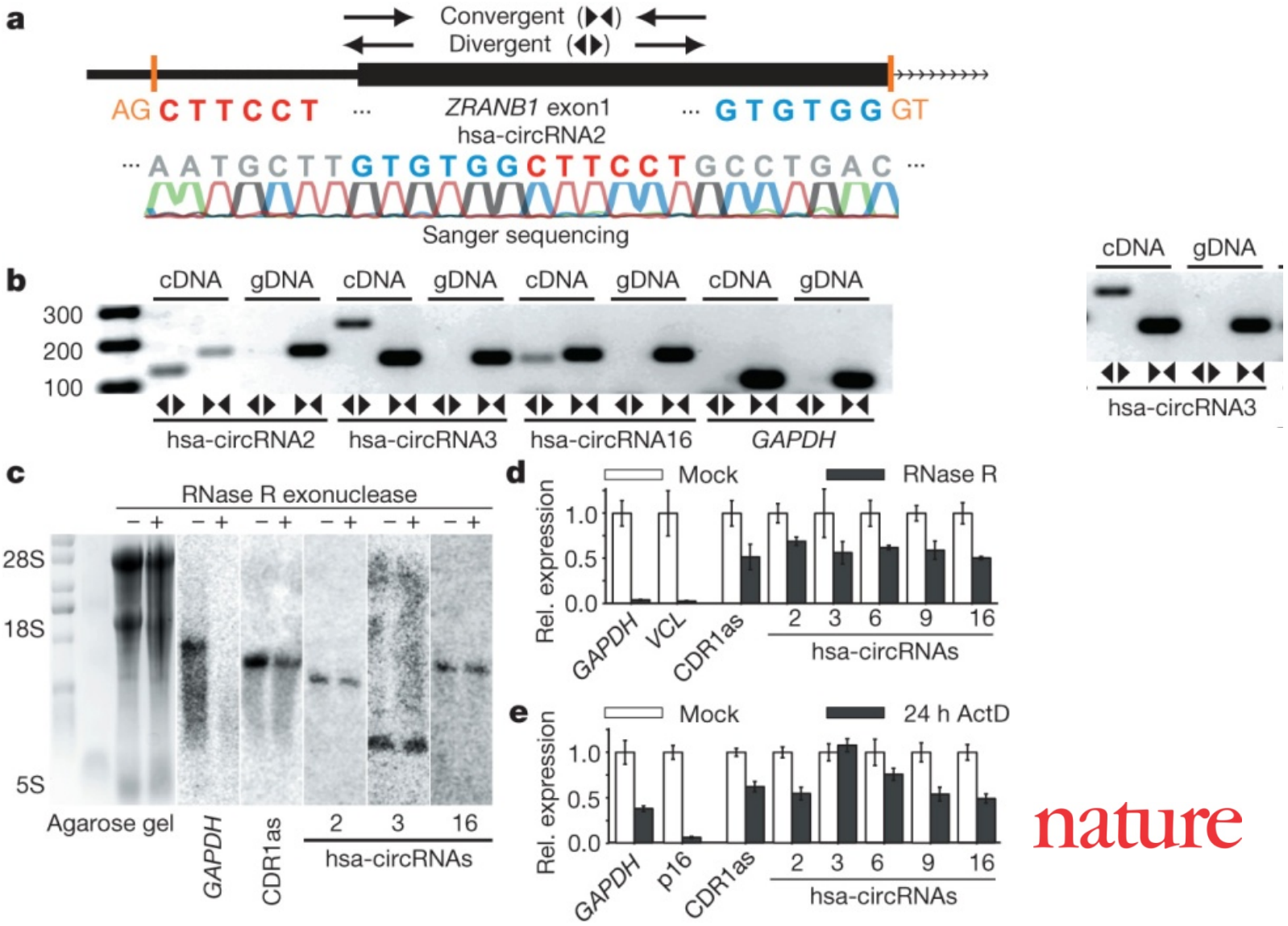
Circular RNA (circRNA) Sponge



Nature 495, 333–338 (21 March 2013)

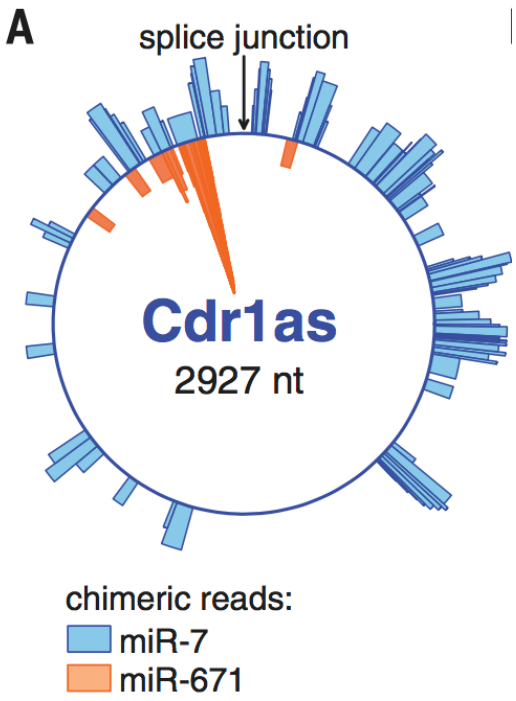
Circular RNAs are a large class of animal RNAs with regulatory potency

Sebastian Memczak ……. & Nikolaus Rajewsky



nature

Cdr1as



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 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.

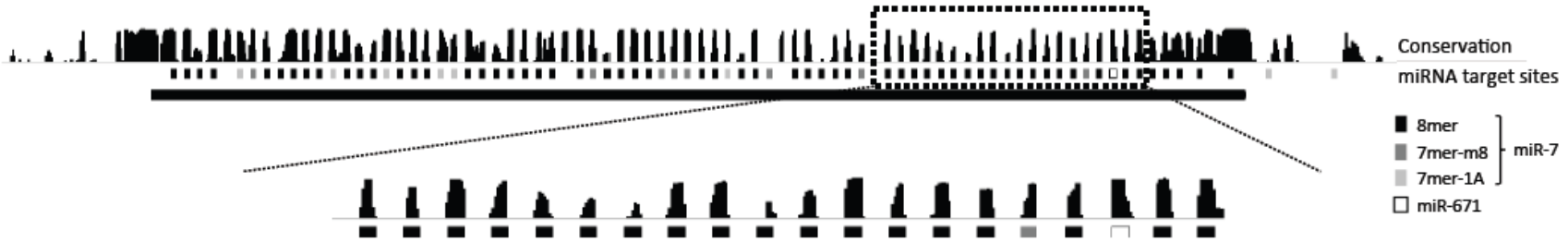
Adapted from Piwecka *et al.*, 2017

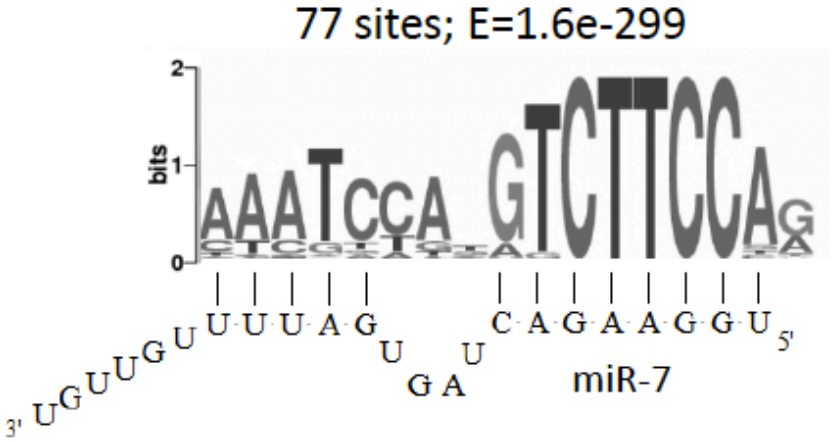
Nature. 2013 Mar 21;495(7441):384-8.

Natural RNA circles function as efficient microRNA sponges.

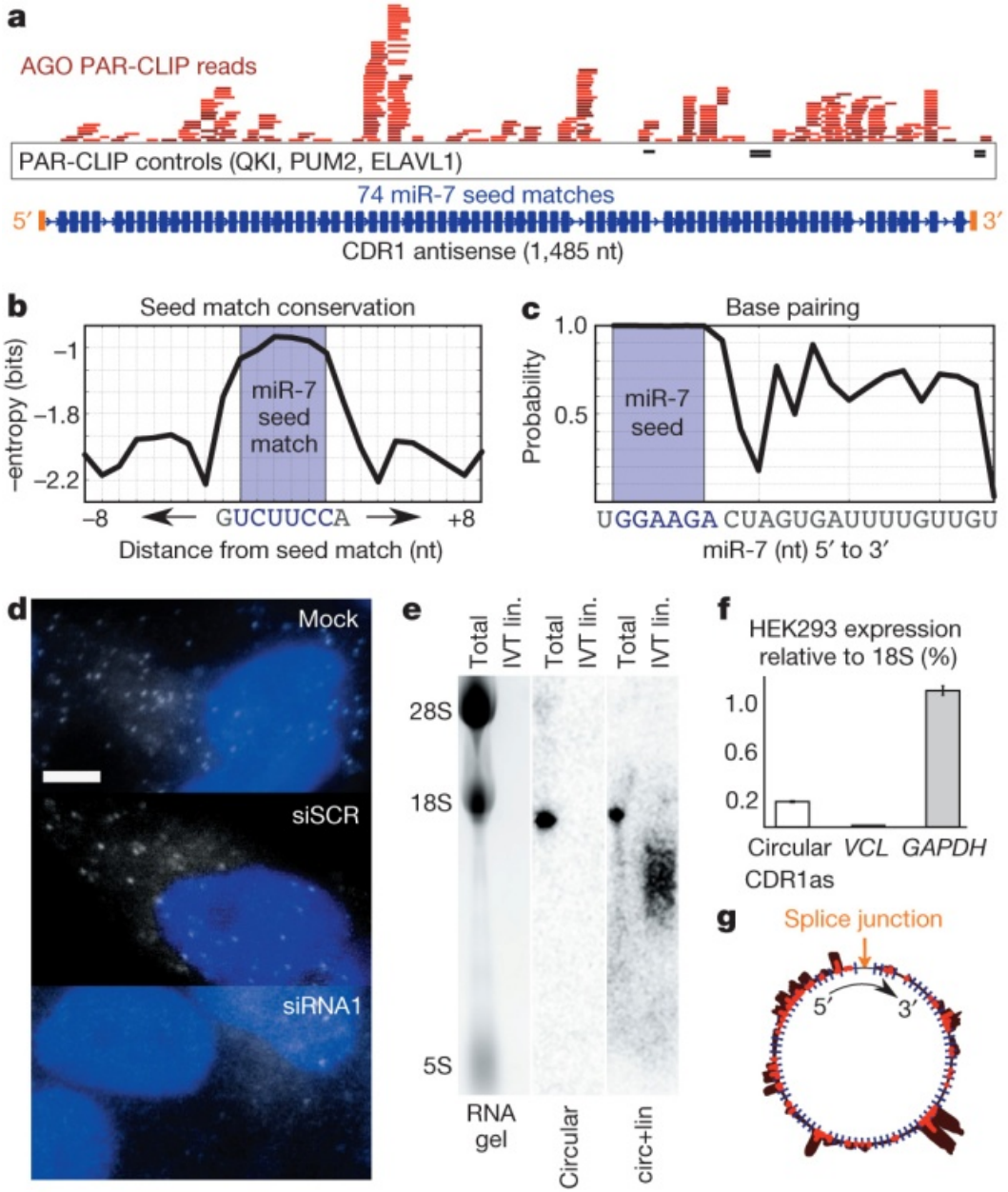
Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J.

Human: 77 miR-7 and 1 miR-671 sites





The circRNA CDR1as is bound by the miRNA effector protein AGO, and is cytoplasmic.

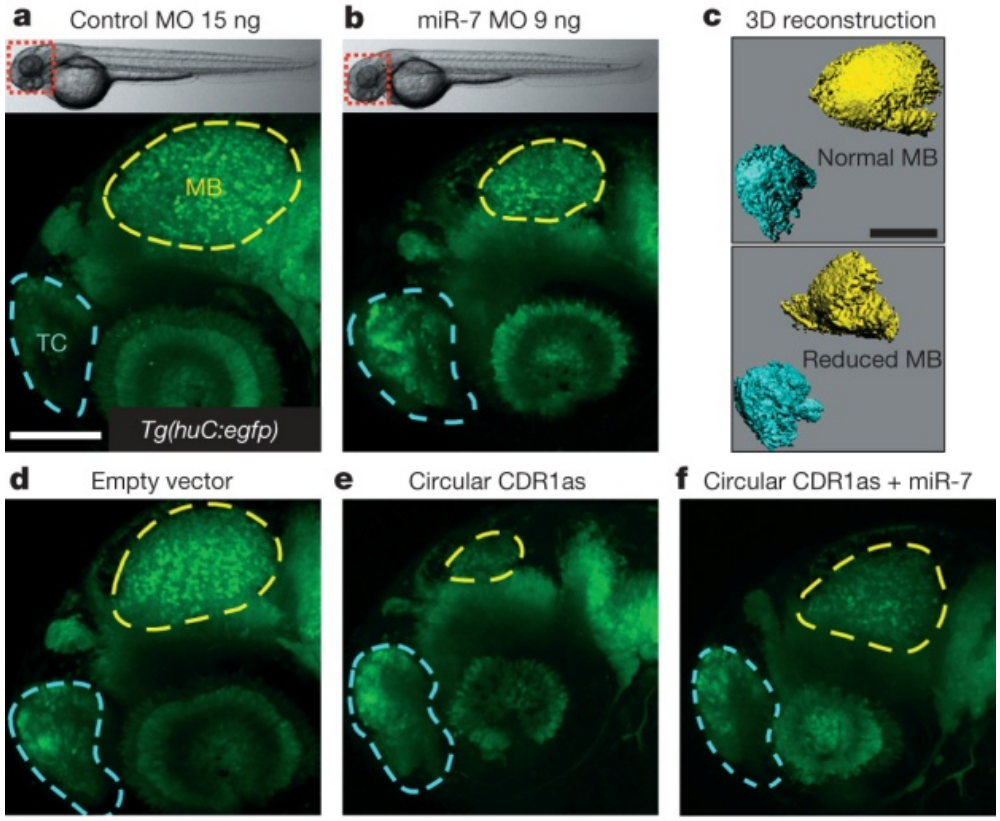


a, CDR1as is densely bound by AGO (red) but not by unrelated proteins (black). Blue boxes indicate miR-7 seed matches. nt, nucleotides. b, c, miR-7 sites display reduced nucleotide **variability across 32 vertebrate genomes** (b) and high base-pairing probability within seed matches (c). d, CDR1as RNA is cytoplasmic and disperse (white spots; single-molecule RNA FISH; maximum intensity merges of Z-stacks). siSCR, positive; siRNA1, negative control. Blue, nuclei (DAPI); scale bar, 5 µm (see also Supplementary Fig. 10 for uncropped images). e, Northern blotting detects circular but not linear CDR1as in HEK293 RNA. Total, HEK293 RNA; circular, head-to-tail probe; circ+lin, probe within splice sites; IVT lin., in vitro transcribed, linear CDR1as RNA. f, Circular CDR1as is highly expressed (qPCR, error bars indicate standard deviation). g, CDR1as. Blue, seed matches; dark red, AGO PAR-CLIP reads; bright red, crosslinked nucleotide conversions.

nature

Figure 2 | CircRNAs are stable transcripts with robust expression.
a, Human (hsa) ZRANB1 circRNA exemplifies the validation strategy. Convergent (divergent) primers detect total (circular) RNAs. Sanger sequencing confirms head-to-tail splicing. b, Divergent primers amplify circRNAs in cDNA but not genomic DNA (gDNA). GAPDH, linear control, size marker in base pairs. c, Northern blots of mock (2) and RNase R (1) treated HEK293 total RNA with head-to-tail specific probes for circRNAs. GAPDH, linear control. d, e, circRNAs are at least 10-fold more RNase R resistant than GAPDH mRNA (d) and stable after 24 h transcription block

In zebrafish, knockdown of miR-7 or expression of CDR1as causes midbrain defects.



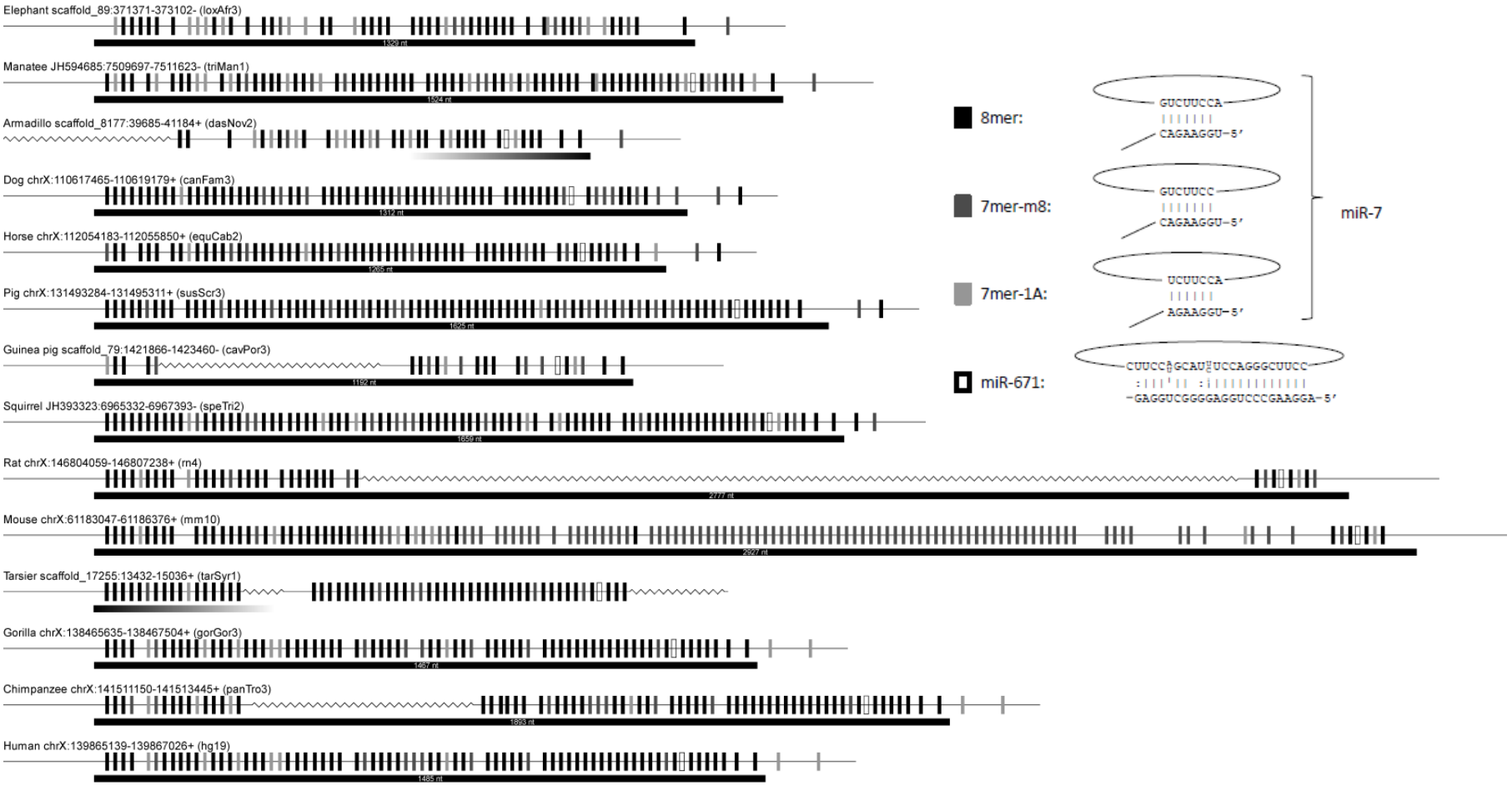
nature

zebrafish has lost the cdr1 locus, whereas miR-7 is conserved and highly expressed in the embryonic brain

a, b, Neuronal reporter (Tg(huC:egfp)) embryos (top, light microscopy) 48 h post fertilization (bottom, representative confocal z-stack projections; blue dashed line, telencephalon (TC) (control); yellow dashed line, midbrain (MB)). Embryos after injection of 9 ng miR-7 morpholino (MO) (b) display a reduction in midbrain size. Panel a shows a representative embryo injected with 15 ng control morpholino. c, Three-dimensional volumetric reconstructions. d, Empty vector control. e, Expression vector encoding human circular CDR1as. f, Rescue experiment with miR-7 precursor.

Conservation

Multiple miR-7 sites and one miR-671 site are pressent in all placental mammalian ciRS-7



circRNA funtions

- Adding an extra layer of regulation to post-transcriptional control of miRNA targets
- Can sharpen the loss of that miRNA activity over time
- Can act as a quality control mechanism, setting a threshold above which miRNA expression must rise to adequately repress the expression of critical target genes.
- uncouple the activity of an intron derived miRNA from the expression of its host gene
- Inhibit passenger strand activity
- Spatial control of miRNA in a cell
- Viral ciRSs could inhibit a host miRNA to alter gene expression program favorable for the virus replication

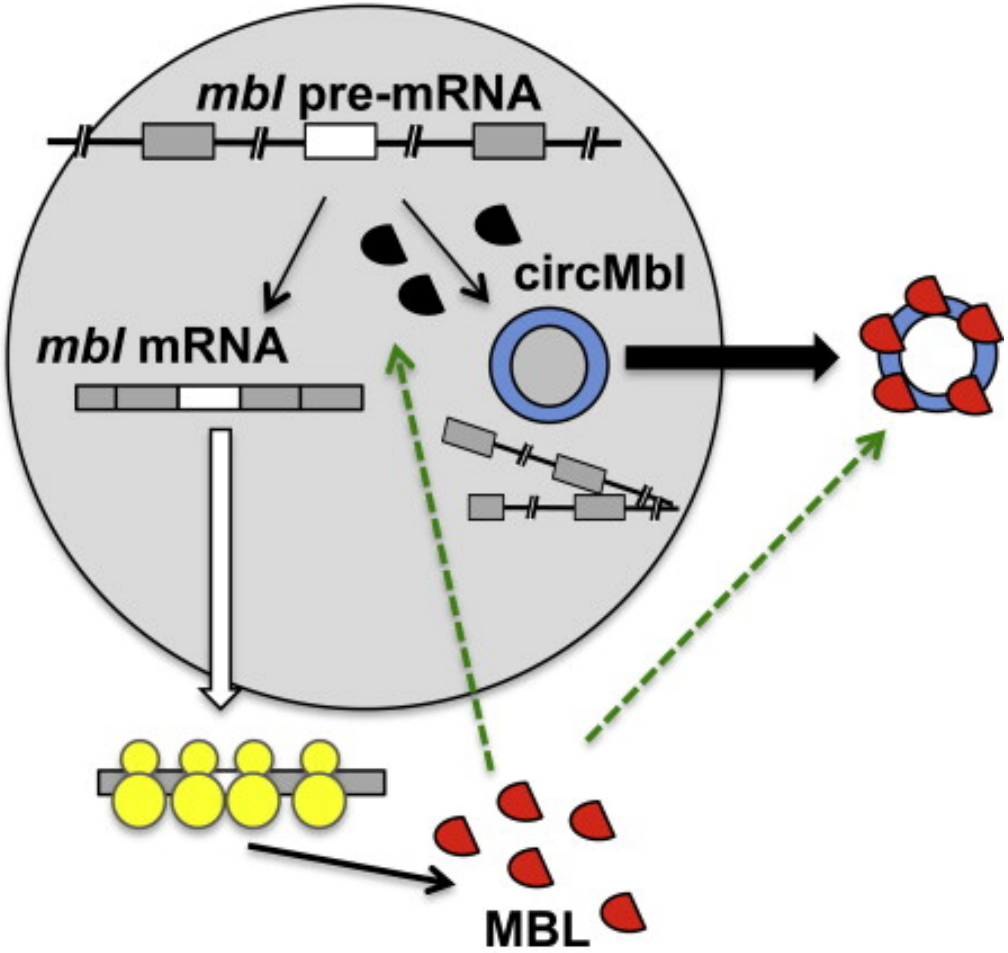
Mol Cell. 204, 56:55-66..

circRNA biogenesis competes with pre-mRNA splicing.

Ashwal-Fluss R…… and Kadener S.

Abstract

Circular RNAs (circRNAs) are widely expressed noncoding RNAs. However, their biogenesis and possible functions are poorly understood. Here, by studying circRNAs that we identified in neuronal tissues, we provide evidence that animal circRNAs are generated cotranscriptionally and that their production rate is mainly determined by intronic sequences. We demonstrate that circularization and splicing compete against each other. These mechanisms are tissue specific and conserved in animals. Interestingly, we observed that the second exon of the splicing factor muscleblind (MBL/MBNL1) is circularized in flies and humans. This circRNA (circMbl) and its flanking introns contain conserved muscleblind binding sites, which are strongly and specifically bound by MBL. Modulation of MBL levels strongly affects circMbl biosynthesis, and this effect is dependent on the MBL binding sites. Together, our data suggest that circRNAs can function in gene regulation by competing with linear splicing. Furthermore, we identified muscleblind as a factor involved in circRNA biogenesis.



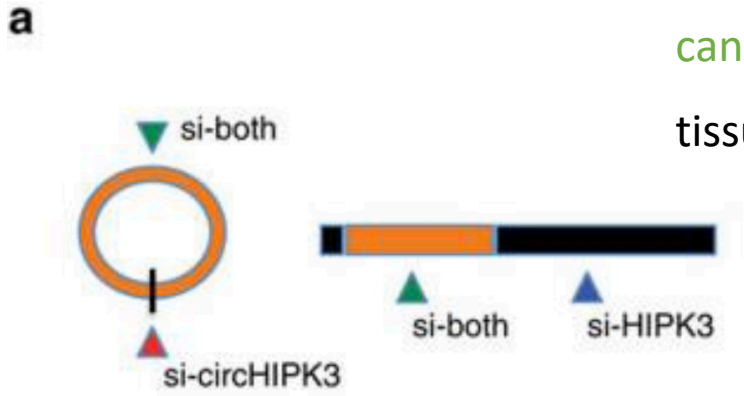
circRNA Biogenesis Competes with Pre-mRNA Splicing

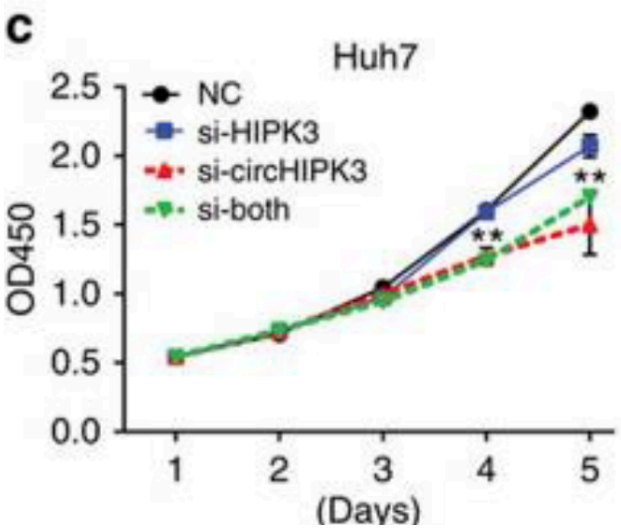
Molecular Cell, Volume 56, Issue 1, 2014, 55 - 66

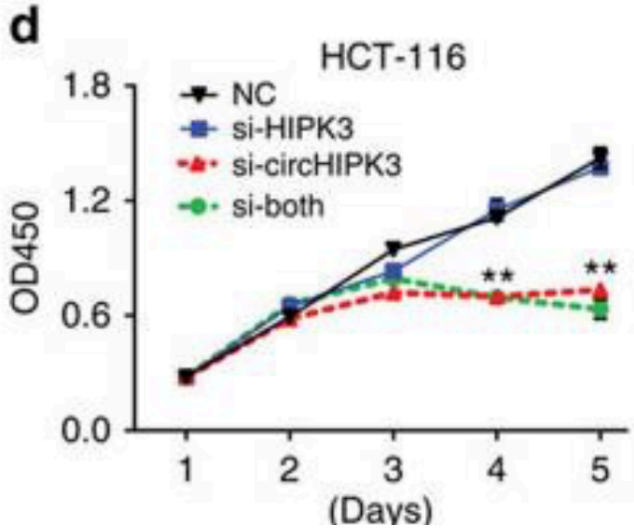
Reut Ashwal-Fluss , Markus Meyer , Nagarjuna Reddy Pamudurti , Andranik Ivanov , Osnat Bartok , Mor Hanan , Naveh ...

CircHIPK3 affects cell proliferation

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





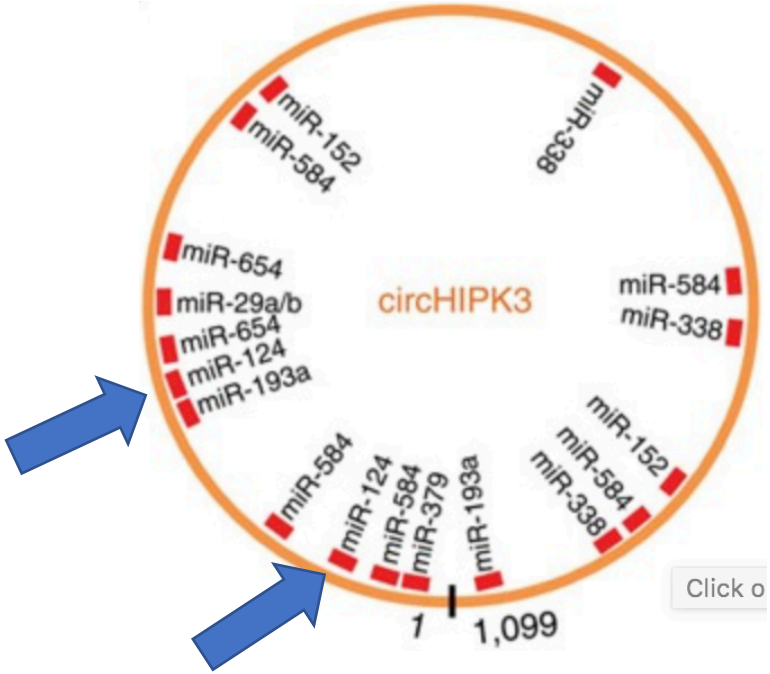


Zheng *et al.*, 2016

CircHIPK3: miR-124 sponge

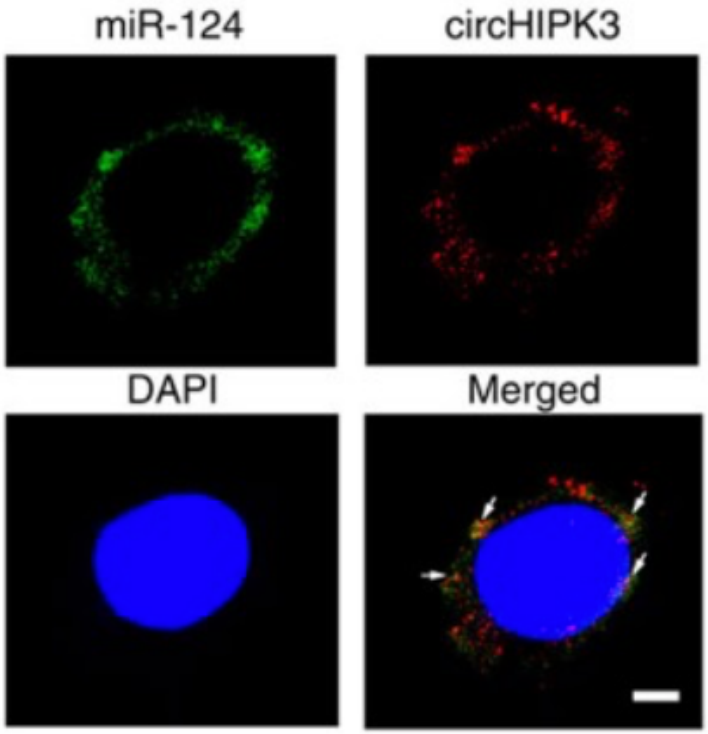
An AGO2-RIP was conducted and endogenous circHIPK3 was pulled-down and specifically enriched in the IP.

circHIPK3-associated miR-124 can inhibit luciferase activity.

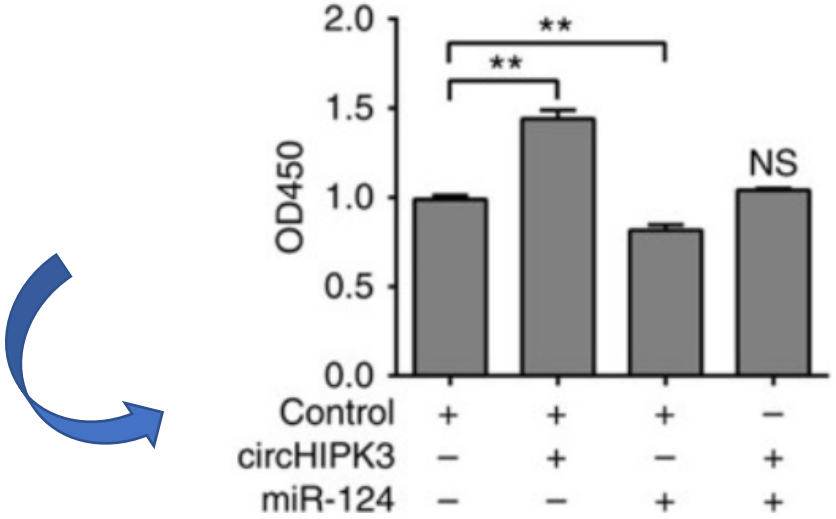


Zheng *et al.*, 2016

CircHIPK3: miR-124 sponge



- 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.

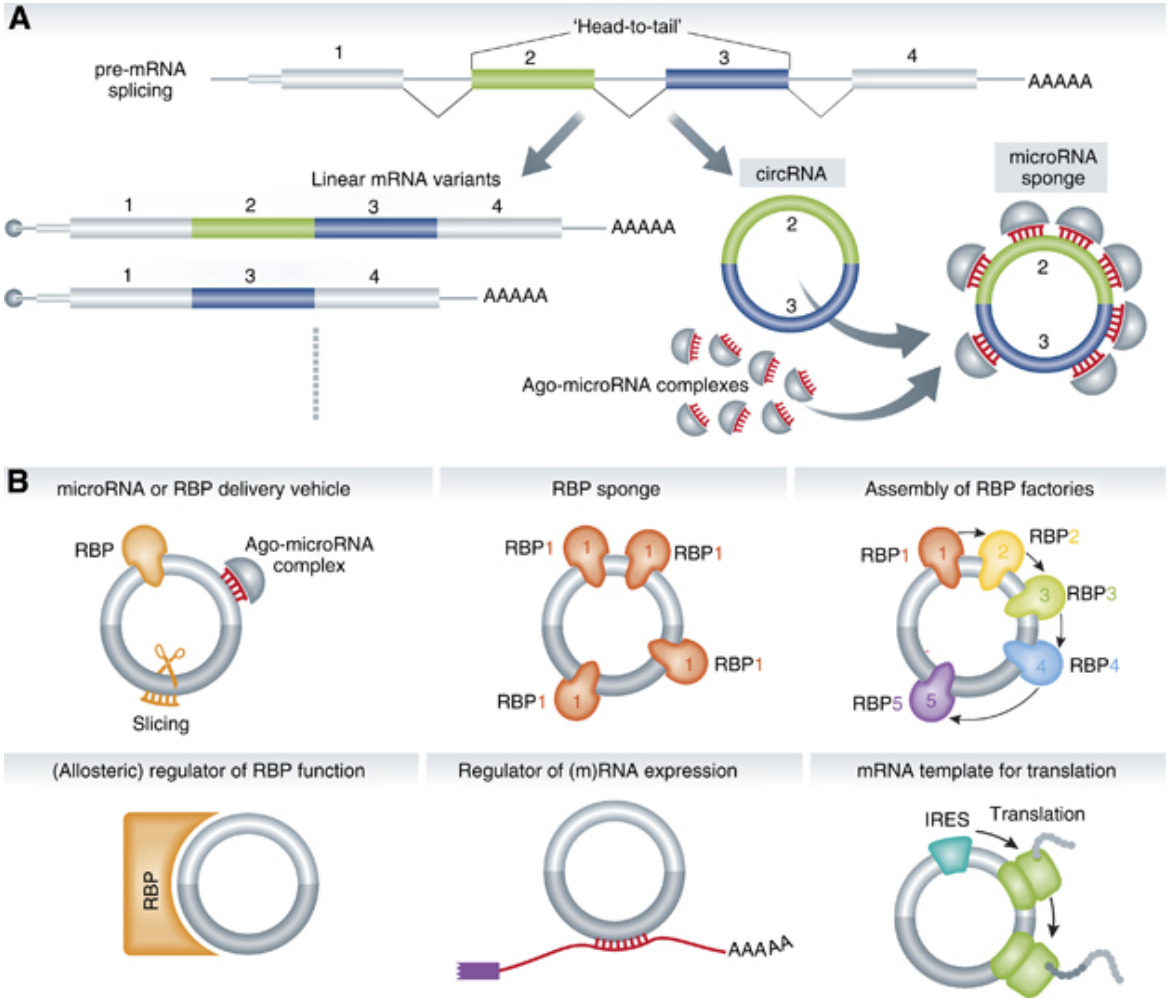


Zheng *et al.*, 2016

Circular RNAs: splicing's enigma variations

Matthias W Hentze and Thomas Preiss

The EMBO Journal (2013) 32, 923 - 925



Biogenesis and functions of circular RNAs. (A) A gene can be transcribed and spliced into linear and circular RNAs. Note the unique 'head-to-tail' splice junctions formed by an acceptor splice site at the 5′ end of an exon and a donor site at the 3′ end of a downstream exon. A demonstrated role for circRNAs is to act as a microRNA sponge. (B) Pictograms of additional plausible options for circRNA function.