

Serum microRNA-profiles indicate a role of Fragile-X-related proteins for ALS

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ENCALS

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Experimental Neurology

Ulm University Hospital

Weishaupt Lab

Identification of proteins associated with the GDCGG-motif

Discovery experiment: RNA pull-down assays:

5' biotinylated miRNAs:

Negative and positive control:

Cel-miR-39-3p: 5'-UCACCGGGUGUAAAUCAGCUUG-3'

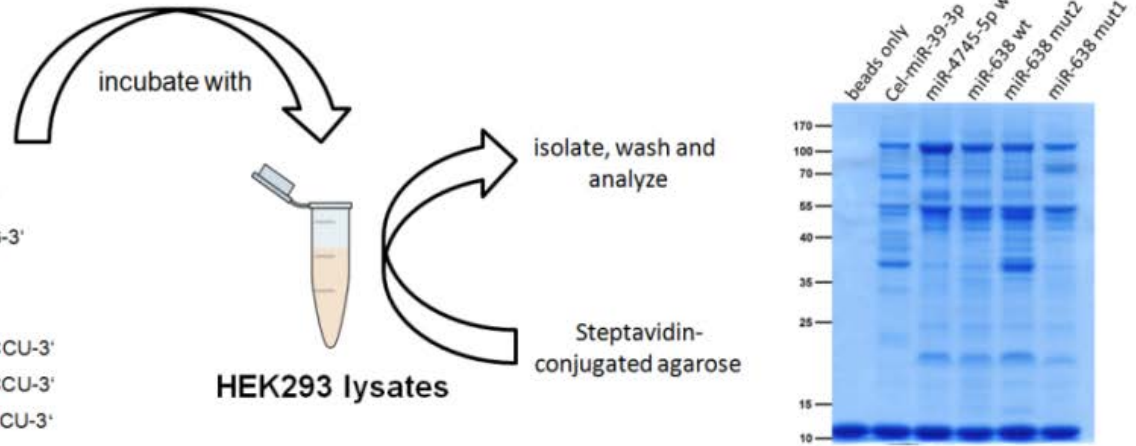
miR-4745-5p wt: 5'-UGAGUGGGGCUCCCGG**GACGG**CG-3'

Motif-specificity:

miR-638 wt: 5'-AGGGAUCGCG**GGCGGG**UG**GGCGG**CCU-3'

miR-638 mut1: 5'-AGGGAUCGCG**G**C**GGG**UG**G**C**GGG**CCU-3'

miR-638 mut2: 5'-AGGGAUCGCG**GAAA**GGUG**AAA**GCCU-3'



Mass spectrometric identification and quantification of pulled down proteins (n = 3-4)

37 proteins
closely associated
with the GDCGG motif
ranked by affinity

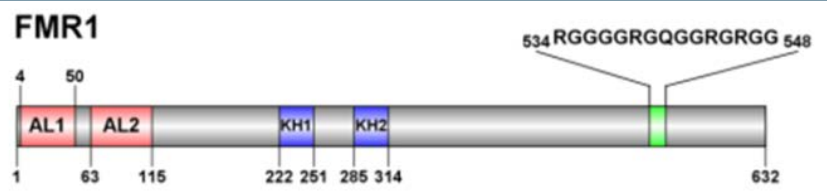
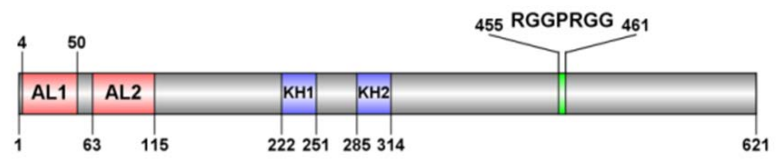
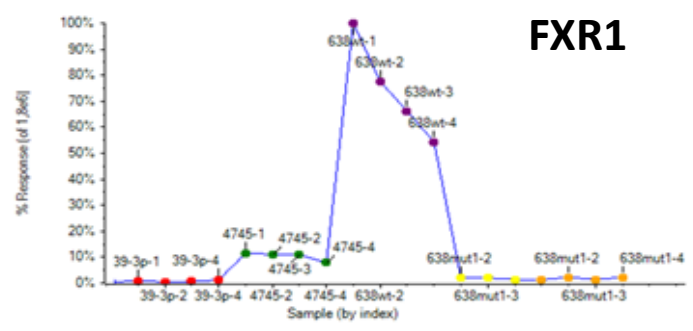
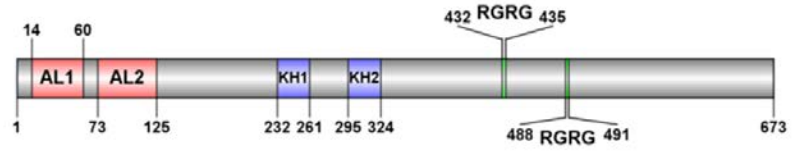
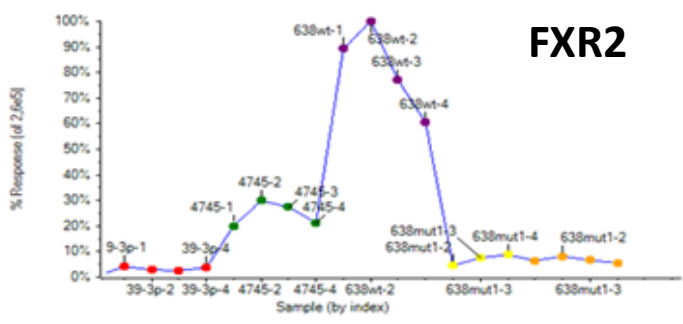
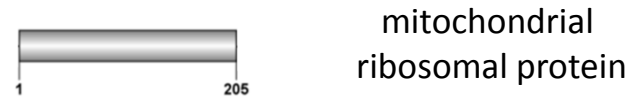
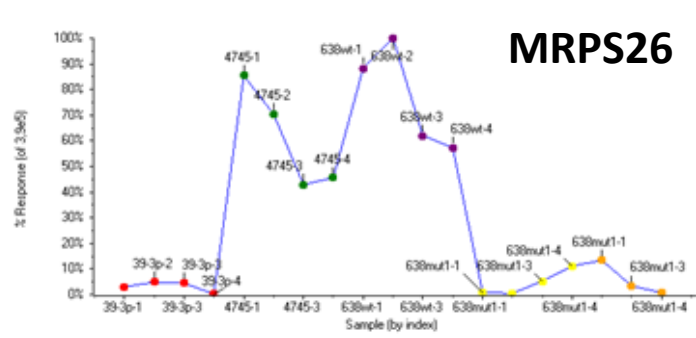
Gene Ontology analysis
of 37 candidate proteins

GO: term	p-value
RNA binding	7.9E-11
RNA polymerase III transcription factor activity	2.1E-4
RNA splicing factor activity	1.3E-3
snoRNA binding	1.3E-2
nucleotide binding	5.1E-2

Identification of proteins associated with the GDCGG-motif

Binding-profiles of Top 3 candidate proteins:

relative amount of pulled down protein



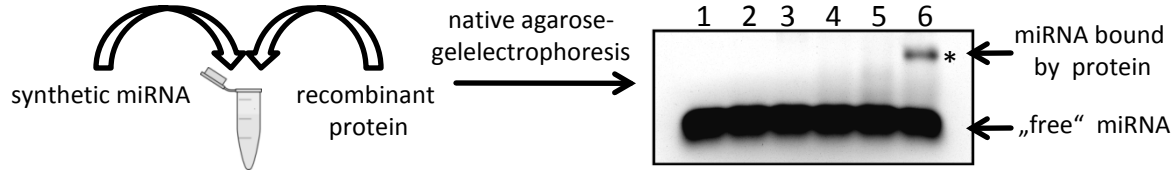
Fragile-X-Mental retardation protein

no motif **GACGG** **GCGGG** **GCCGG** **GAAAG**

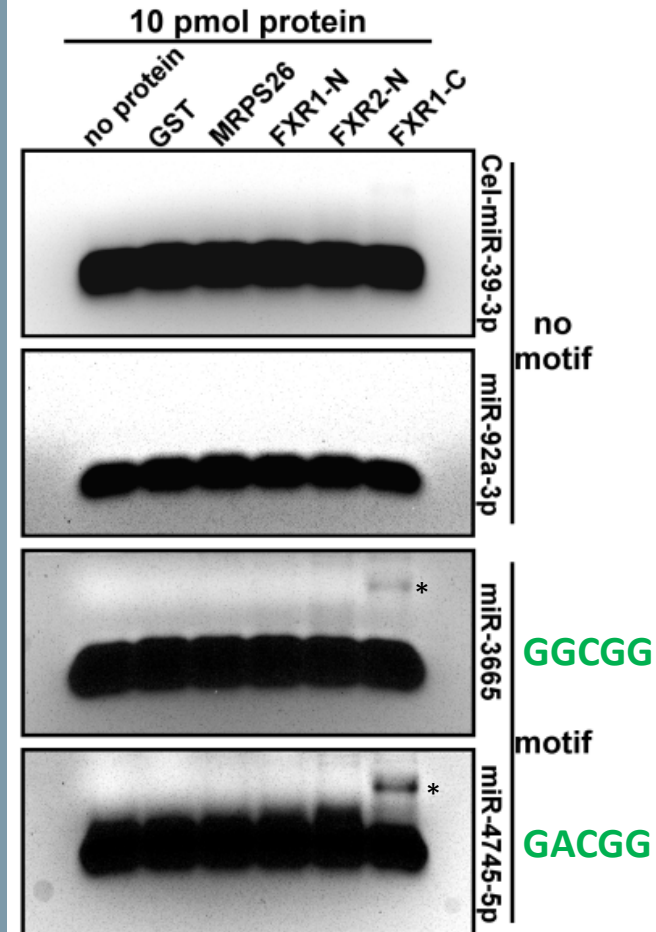
2x 2x 2x

Validation of candidate proteins: FXR1 directly interacts with GDCGG-miRNAs

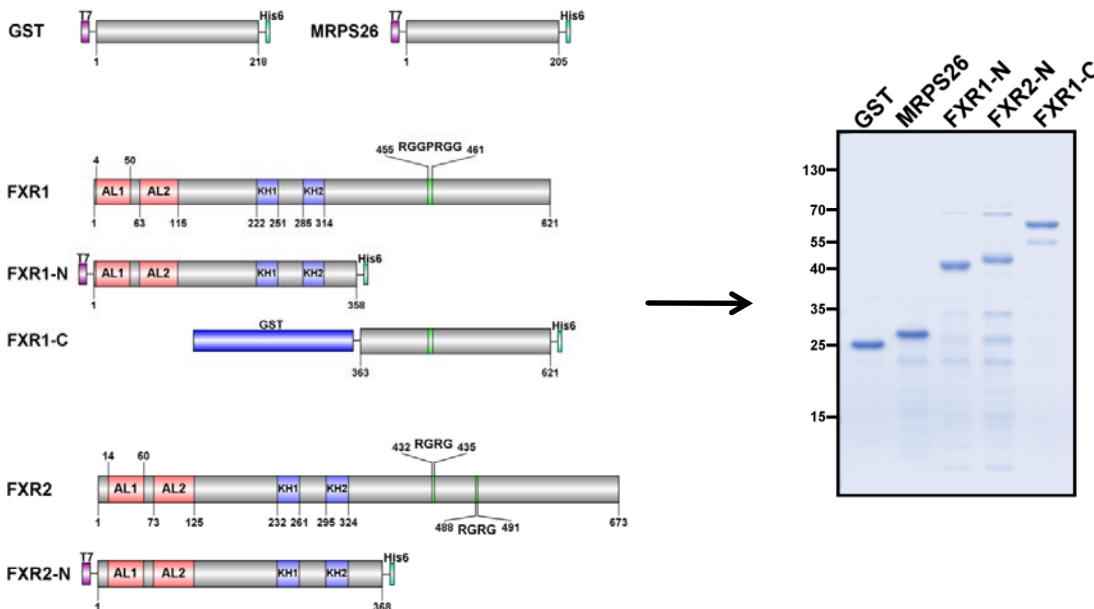
principle of band shift assays:



band shift assays:



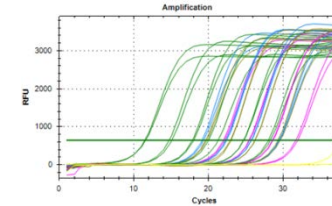
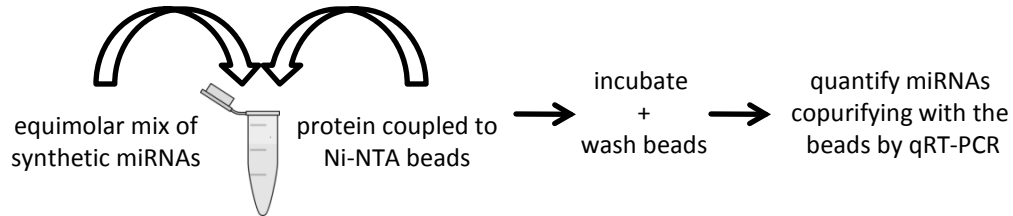
Purification of recombinant proteins from *E.coli*:



FXR2-C: no expression in *E.coli*

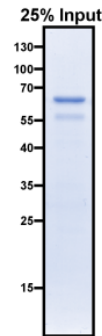
Validation of candidate proteins: FXR1 directly interacts with GDCGG-miRNAs

principle of competitive qRT-PCR enrichment assays:

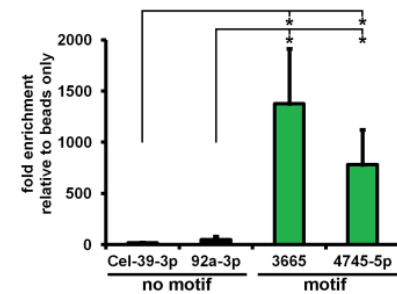


recombinant proteins from *E.coli*:

FXR1-C:

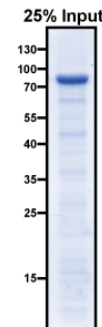
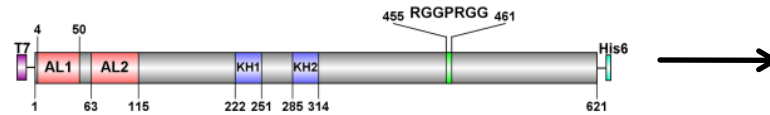


FXR1-C

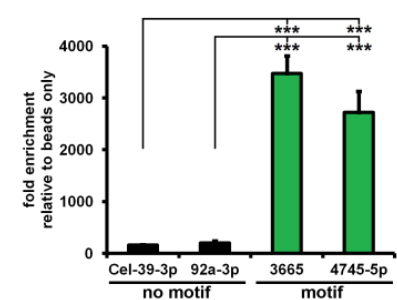


recombinant proteins from *E.coli*:

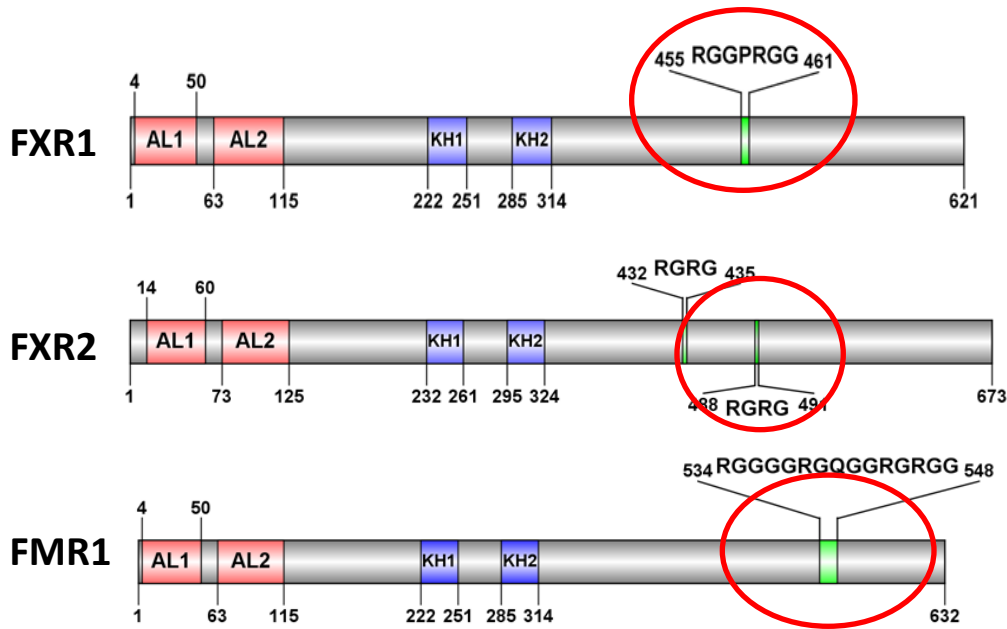
Full-length FXR1:



full-length FXR1



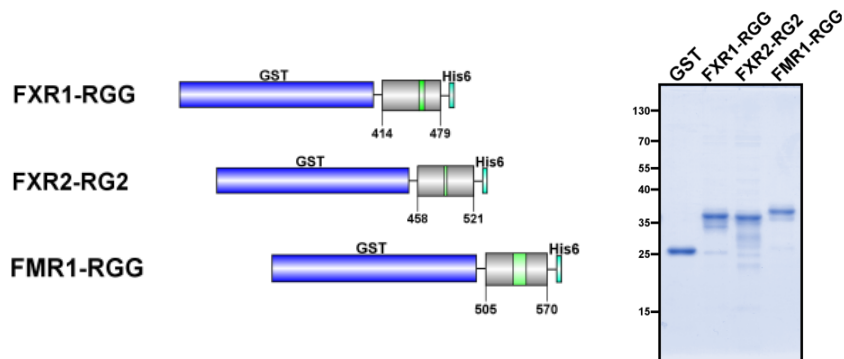
FXR1, FXR2 and FMR1 directly bind to GDCGG-miRNAs via its RGG/RG-motifs



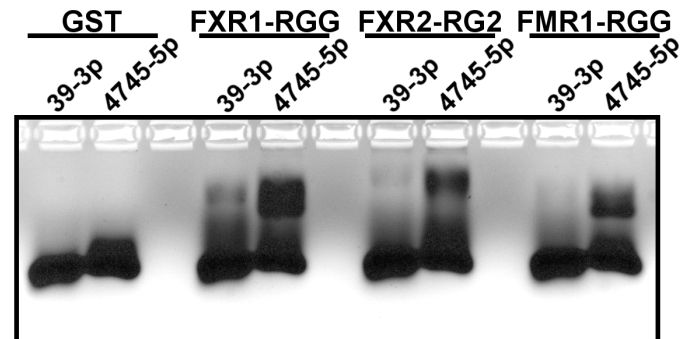
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FMRP 530-DGRRR*GGGR*GG*GG*GG*GGGRGRGGG-549 RGG
FXR1 437-DSRRRP-GGRGRSVSGGRGR-455 RGG
FXR2 479-ESRRRPTGGRGRGPPAPRP-498 RG2
    
```

recombinant proteins from *E.coli*:



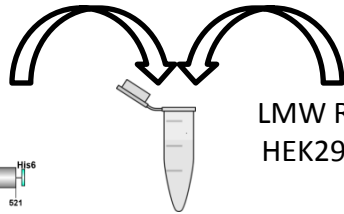
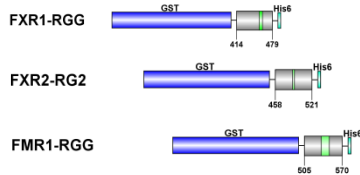
band shift assay:



39-3p: no motif
 4745-5p: **GACGG**

FXR1 may be the most important player in ALS

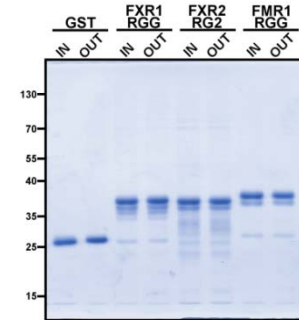
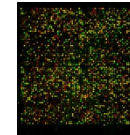
recombinant proteins from *E.coli*:



LMW RNA of HEK293 cells

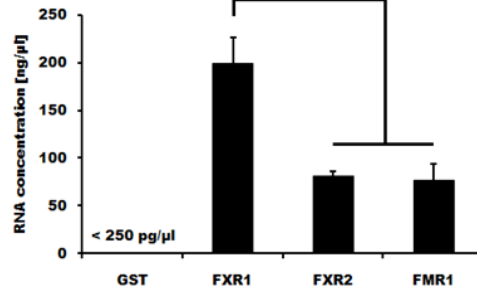
incubate + wash beads

analyze copurifying miRNAs by miRNA microarray

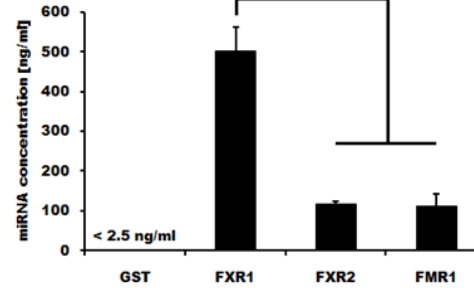


FXR1 has the highest affinity for RNA/miRNA:

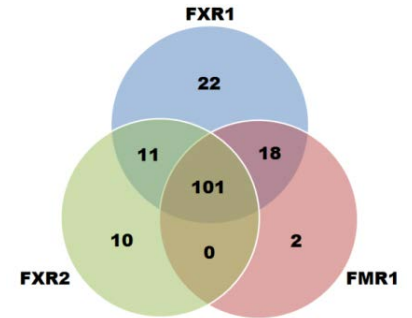
total RNA



miRNA



miRNAs copurifying with FXR1/FXR2/FMR1 largely overlap:

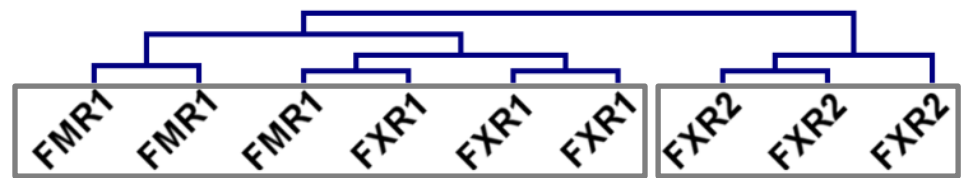


determination of RNA yield copurifying with FXR1/FXR2/FMR1:

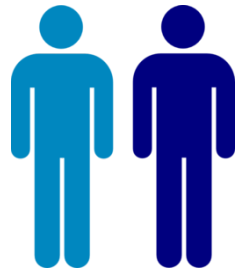
FXR1 and FMR1 show similar specificity for miRNAs with the GDCGG motif:

miRNAs with motif significantly enriched? (<i>p</i> -value Fisher's exact test)	
FMR1	3.83E-9
FXR1	5.09E-9
FXR2	5.38E-6

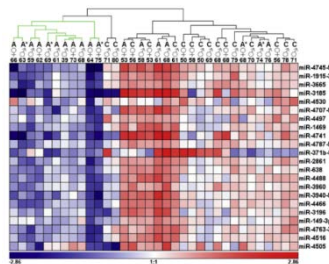
hierarchical cluster analysis of miRNAs enriched by all 3 proteins:



Summary and outlook



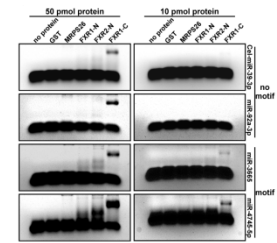
observation in
ALS patients and
preclinical mutation carrier



deregulated
serum miRNAs



consensus nucleotide motif
in downregulated miRNAs



identification and validation
of specific binding proteins
(FMR1/FXR1/FXR2)



- Expression analysis of FMR1/FXR1/FXR2 in ALS *post mortem* tissue
- Neuropathological staining of FMR1/FXR1/FXR2
- Localization studies of FMR1/FXR1/FXR2 in iPSC derived motor neurons carrying ALS mutations

- does downregulation/mislocalization of FXR-proteins cause the extracellular ALS-related miRNA profiles?
- connection of FXR-proteins and serum miRNAs (e.g. transport or secretion)?
- connection of FXR-proteins and ALS-related proteins?
- biological function of miRNA-binding by FXR-proteins?

Thank you!



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Dr. Kathrin Müller
Dr. David Brenner
Anika Marie Helferich
Sarah Brockmann
Clara Bruno
Kirsten Sieverding
Tselmen Daria
Alina Zerr
Nadine Todt
Elena Jasovski
Aline Sage



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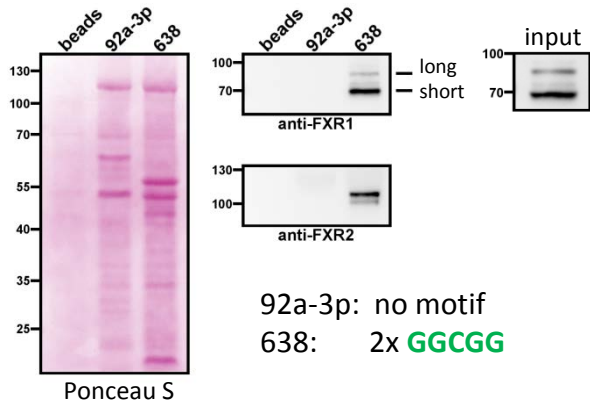
Prof. Dr. Albert C. Ludolph
Neurology,
Ulm University Hospital

charcot  stiftung
FÜR ALS-FORSCHUNG

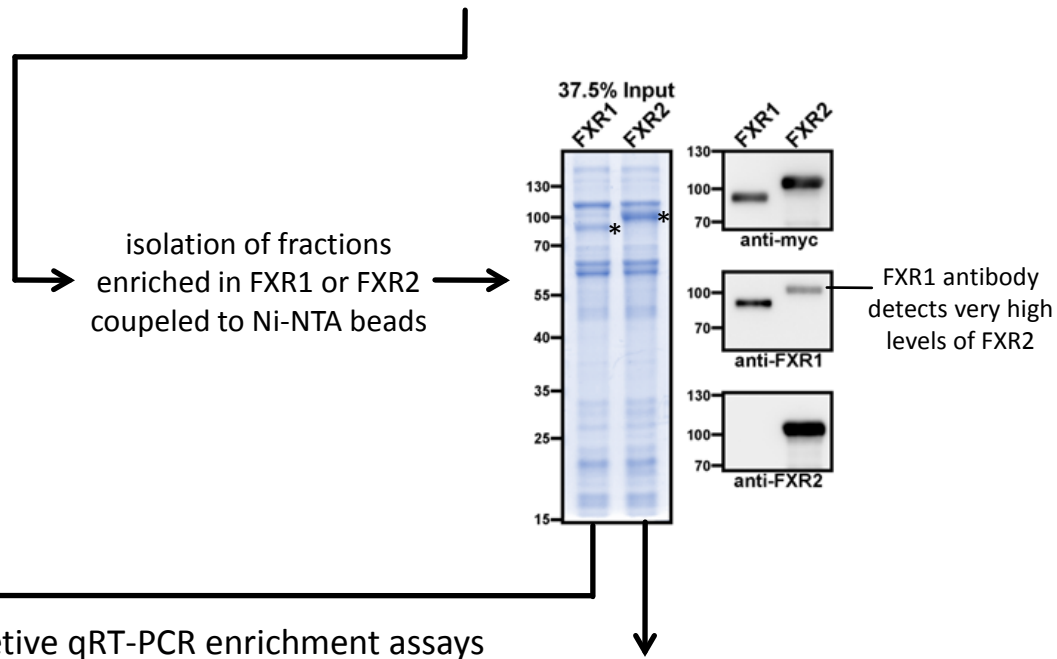
FXR1 and FXR2 expressed in human cell lines also bind to GDCGG-miRNAs

miRNA pulldown in lysates of HEK293 cells:

→ interaction with endogenous FXR1 and FXR2

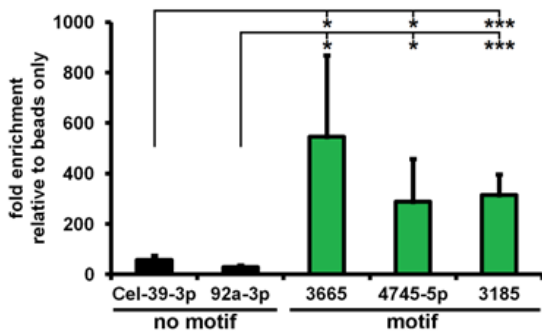


overexpression of FXR1 or FXR2 in HEK293 cells



competitive qRT-PCR enrichment assays

FXR1



FXR2

