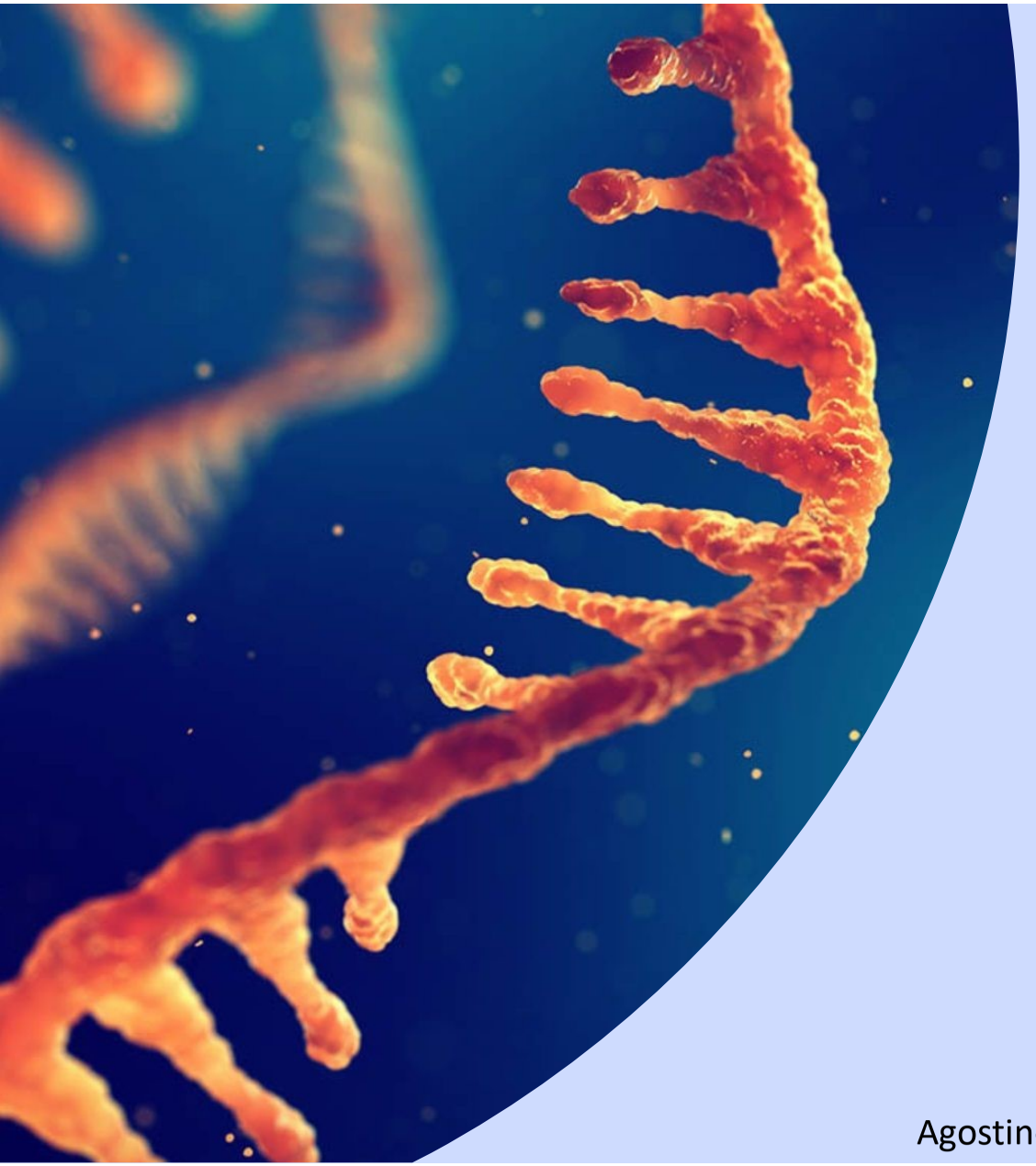


TRANSLATED lncRNAs

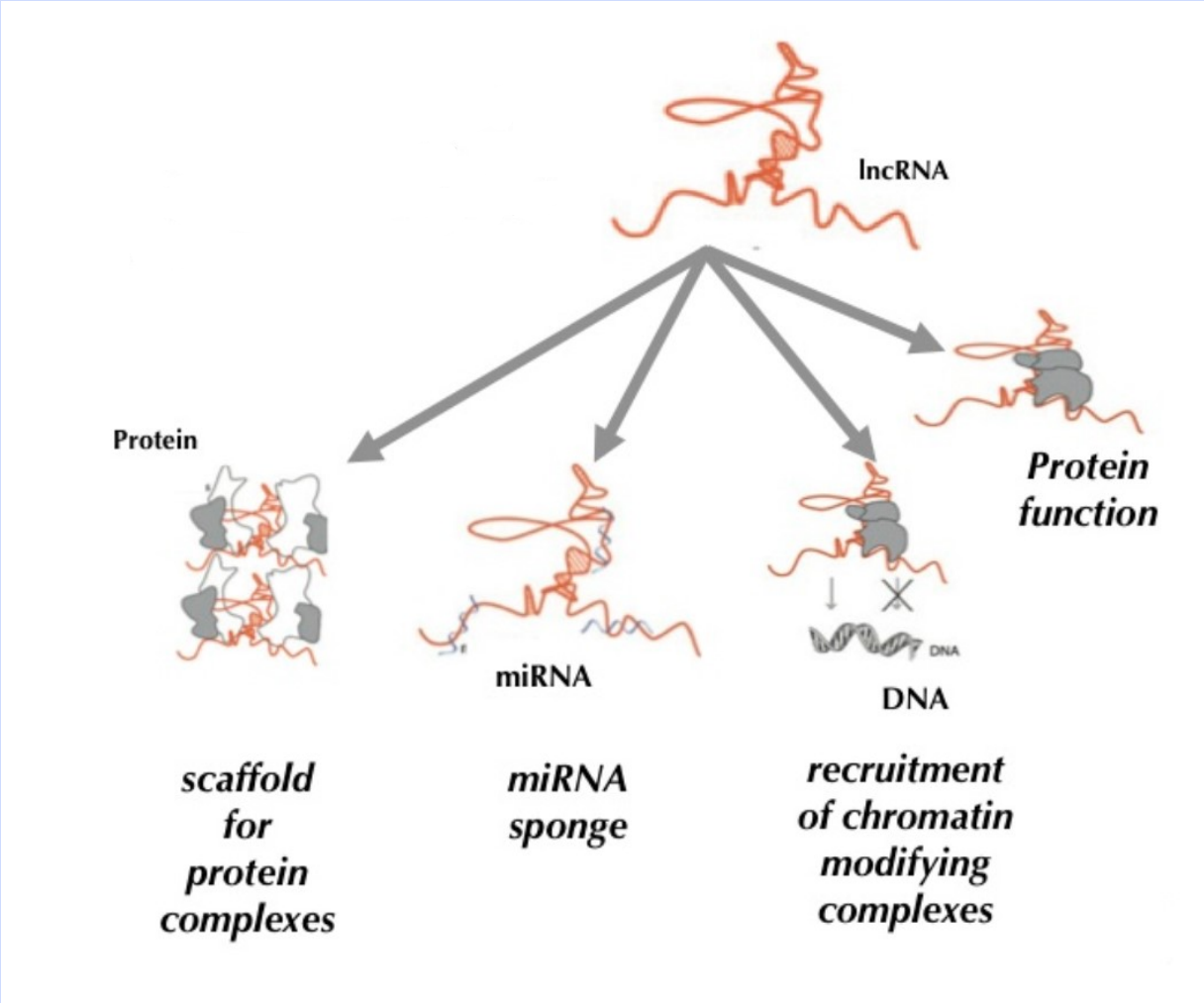
The peculiar case of SPAR and the regulation of mTORC1

Agostino Campanile, Alessia Pesaresi, Beatrice Alessandrini, Patrik Rossi

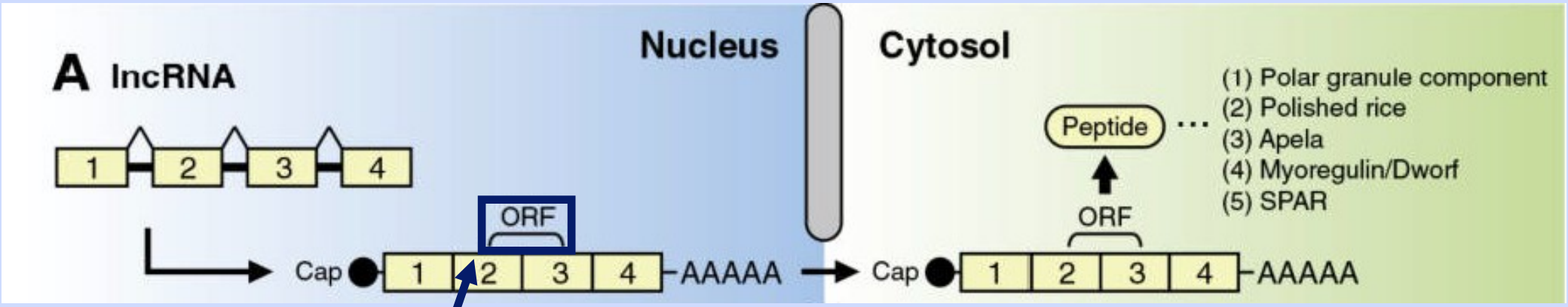
lncRNA

- Transcribed from genomic DNA (cDNA)
- Lots of classes ⟶ *Different biological involvements*
- > 200 nucleotides
- Costly poly-adenylated & can contain introns
- DON'T ENCODE FOR PROTEINS

FOR REAL?!



Also long non-coding RNA
are transcribed by RNA-pol II + spliced
+ capped
+ polyadenylated



Putative ncRNA contains only shorts ORFs (< 300 nt) that could encode for functional peptides with less than 100 amino-acids

= ***HIDDEN PEPTIDES***

HIDDEN PEPTIDES
how to identify them

(A) ORF prediction

Usually, only long ORFs encode for protein

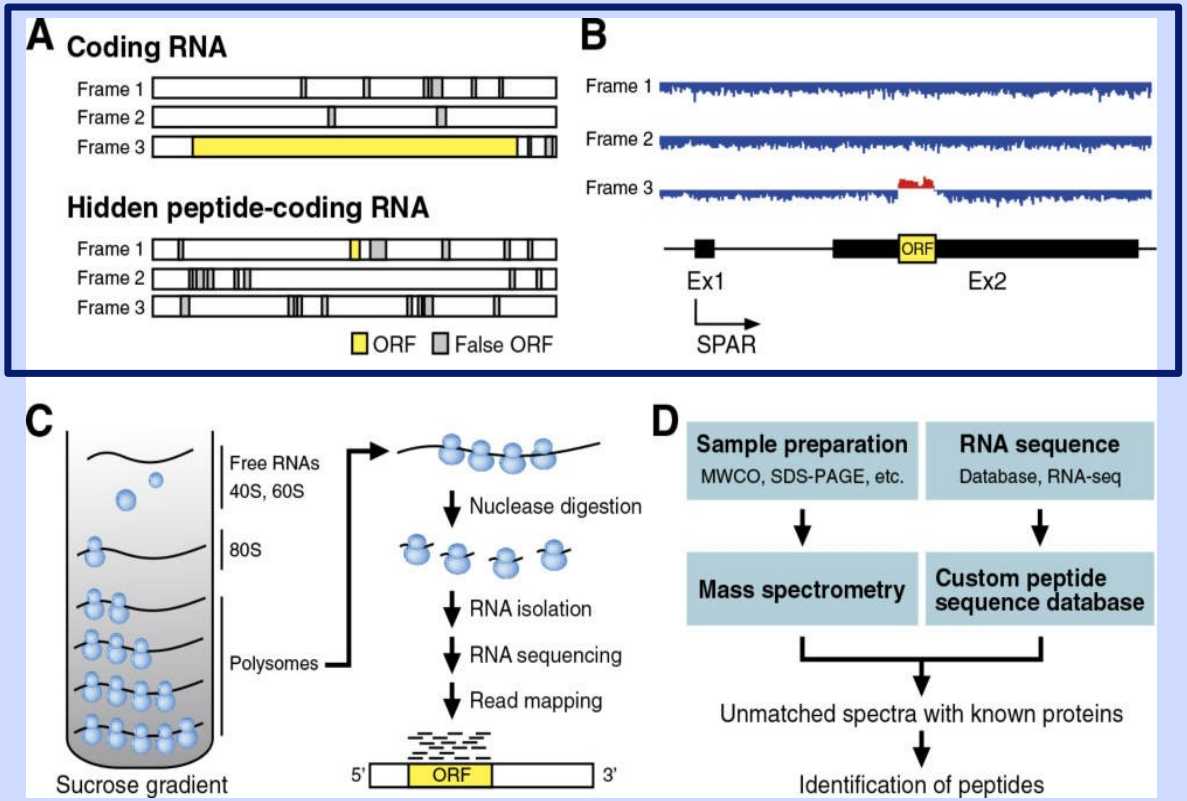
Putative lncRNA only contains short ORFs

➡ *Difficult to predict which ORF encodes*

(B) Computational Analysis

ORF sequences very well conserved

➡ *Prediction by comparison between species*



HIDDEN PEPTIDES
how to identify them

(C) Ribosome profiling

RNAs bound by ribosomes are in active translation, but also protected

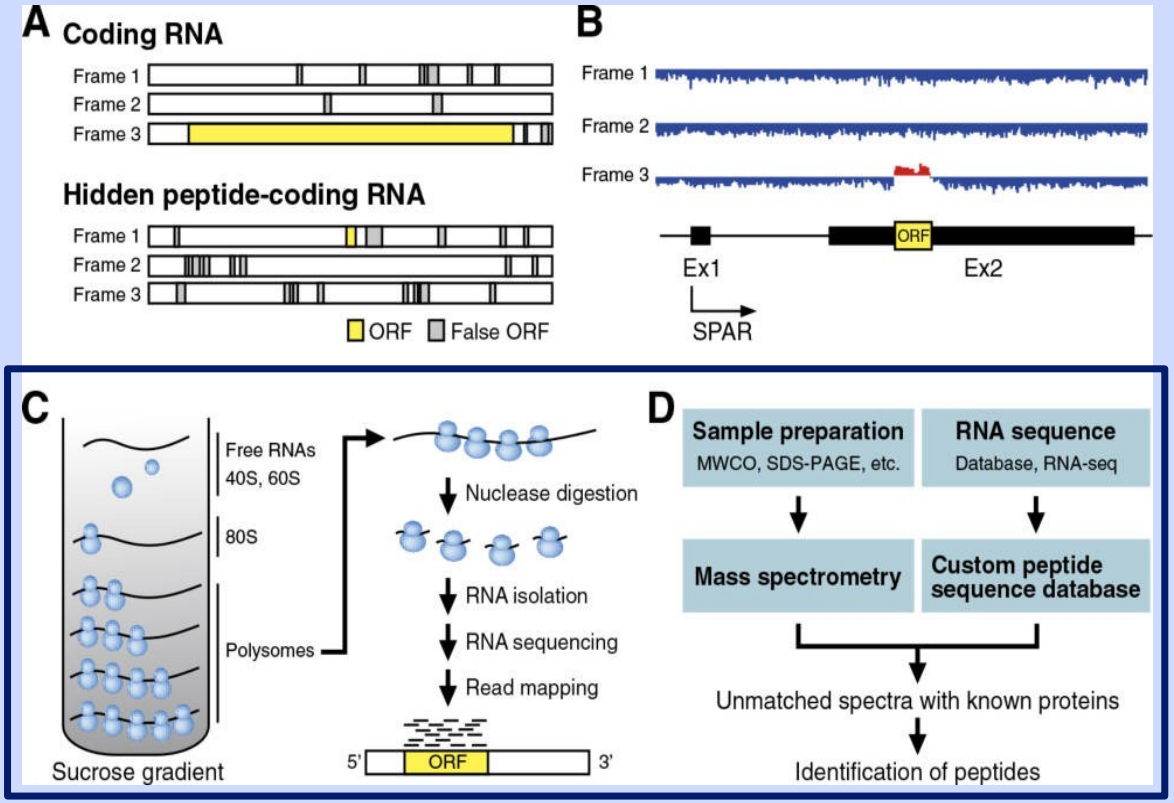
➡ *Treatment with nuclease to isolate these RNAs, then sequencing and mapping*

(D) Peptidomic approach

Peptides concentrated and then analyzed in MS

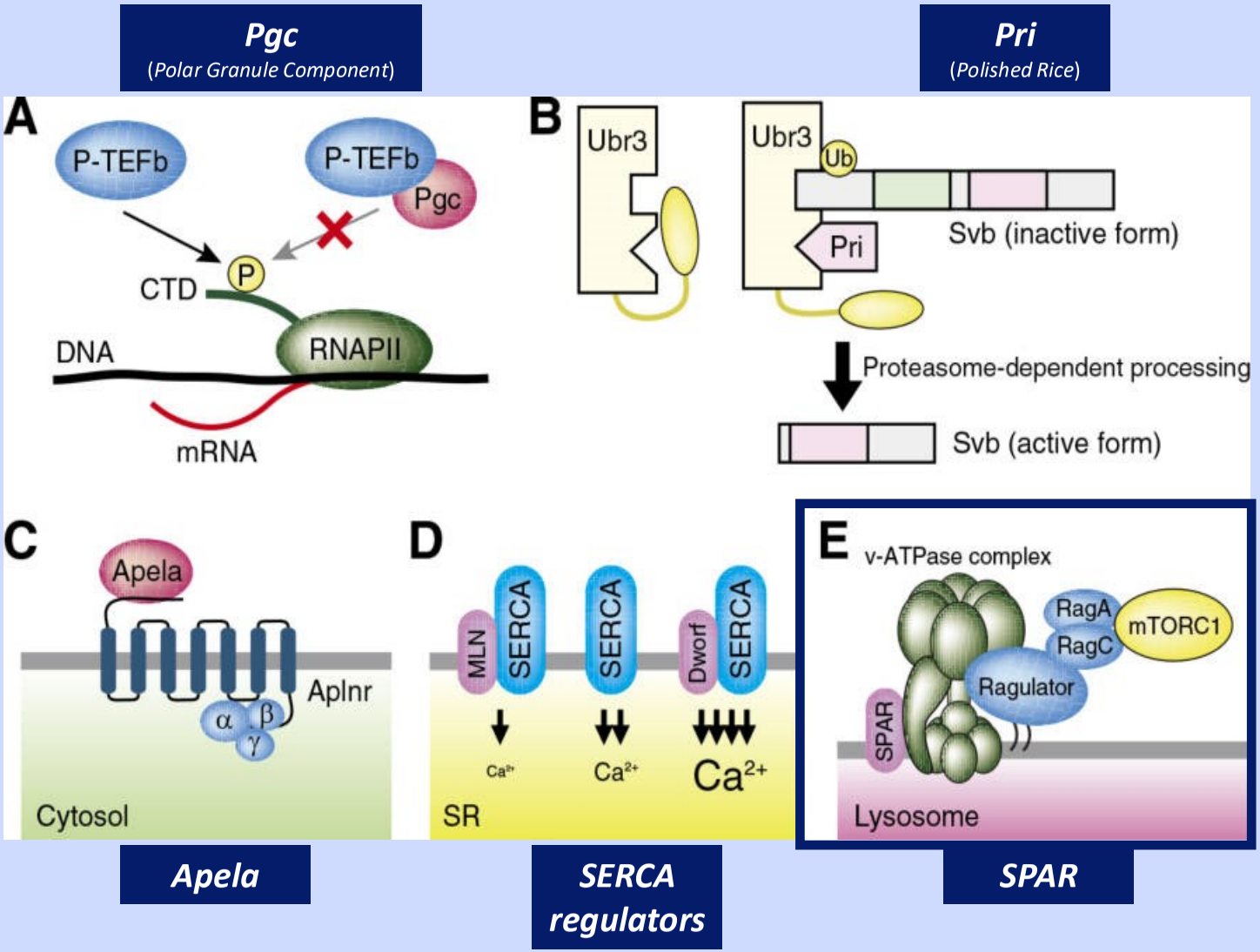
➡ *Comparison between MS/MS spectra and custom peptide sequence database*

➡ *Unmatched spectra correspond with hidden peptide*



HIDDEN PEPTIDES

Examples

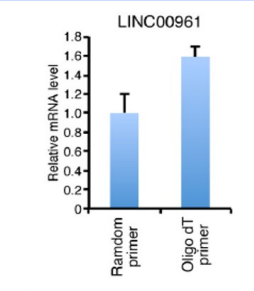


SPAR

90 aa (hidden) peptide encoded by LINC00961

Identified by

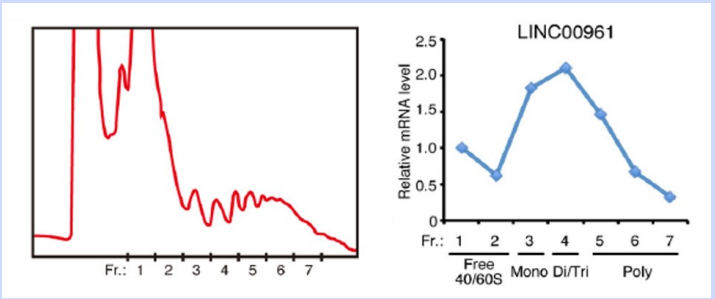
A



A. qPCR in Hela cells
(both random & oligo primers)

Poly-adenilation

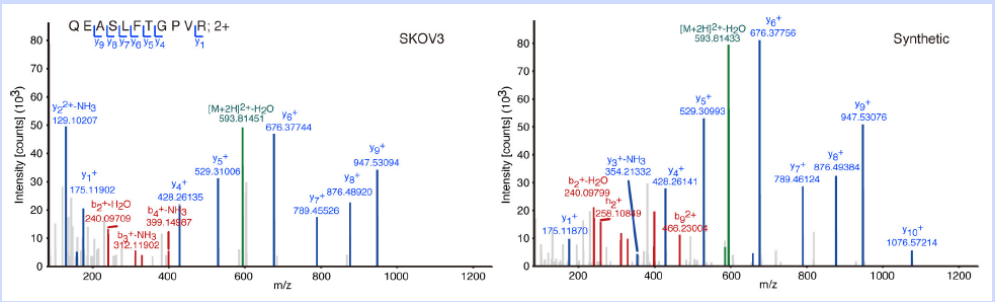
B



B. Representative ribosome profile

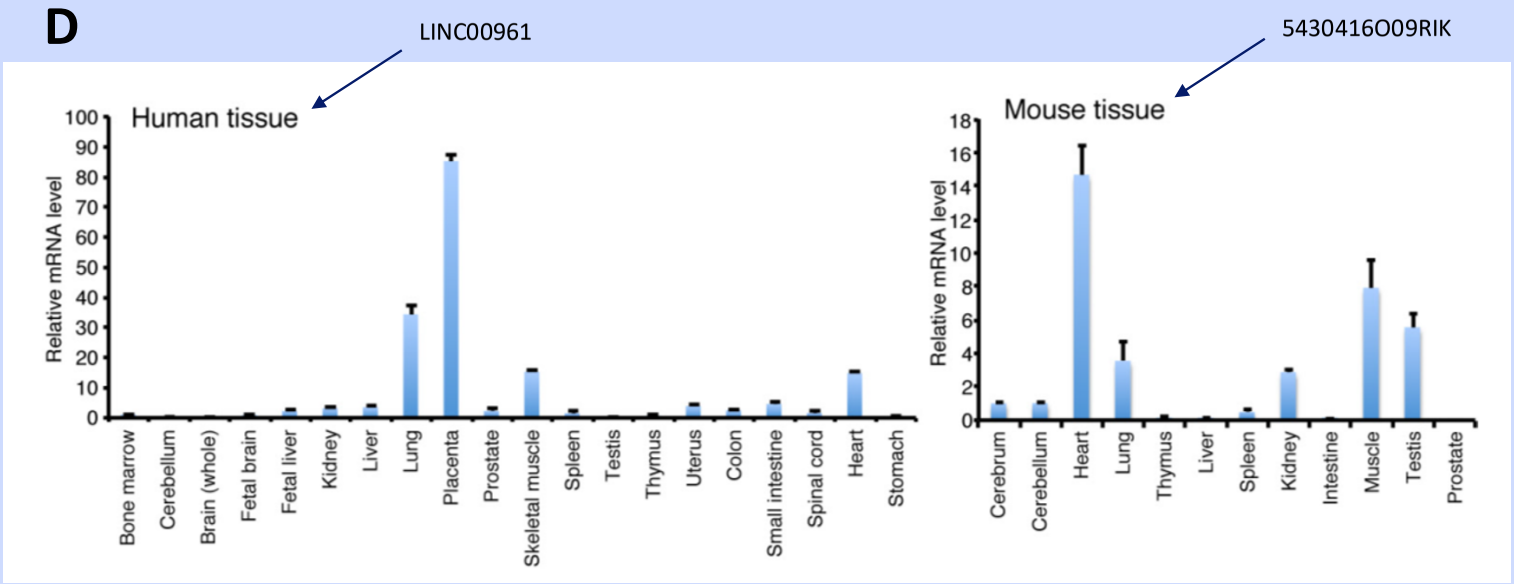
Located to actively translating ribosomes

C



C. Peptidomic Approach

Confirmation of spectral pattern of endogenous peptide



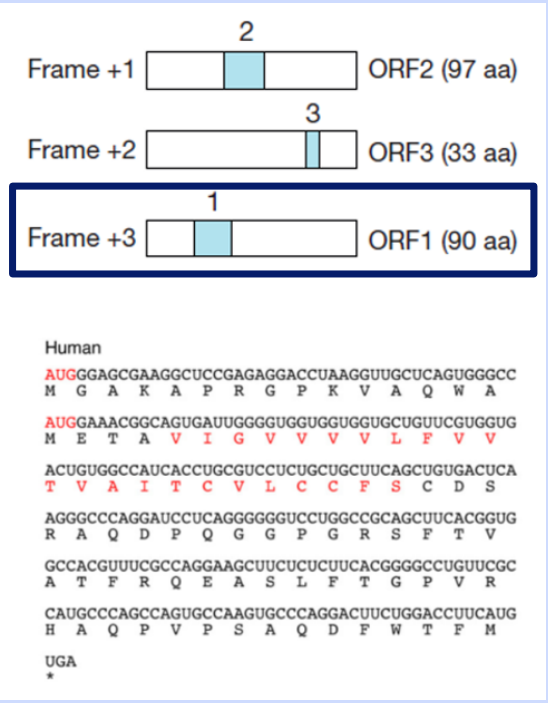
[**Data normalized relative to the value of cerebrum**]

D. qPCR in Human tissue & Mouse tissue → *High levels of mRNA expression in lung, heart and skeletal muscle*

3 ORFs found in LINC00961

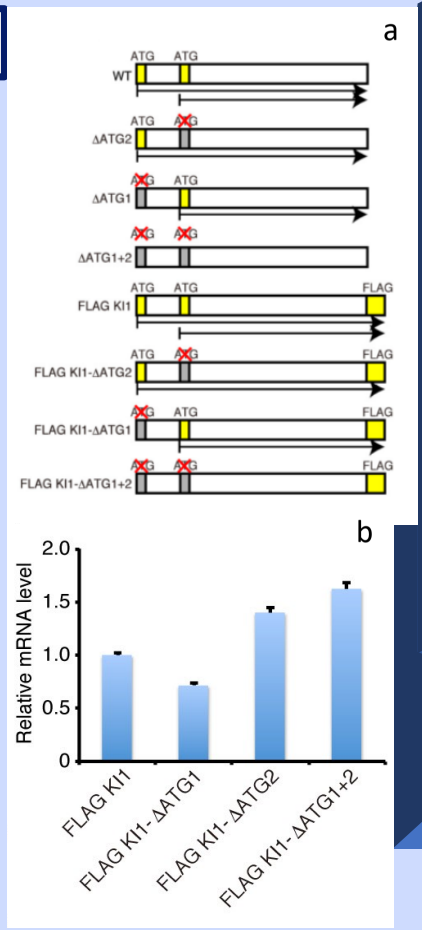
Flag Knock-In 1-3 at C-terminal to validate translation of each ORF (figure 3a)

1



2

3



IMMUNOBLOTTING: only ORF1 (sequence in figure 2) shows active translation
ORF2 and ORF3 don't!

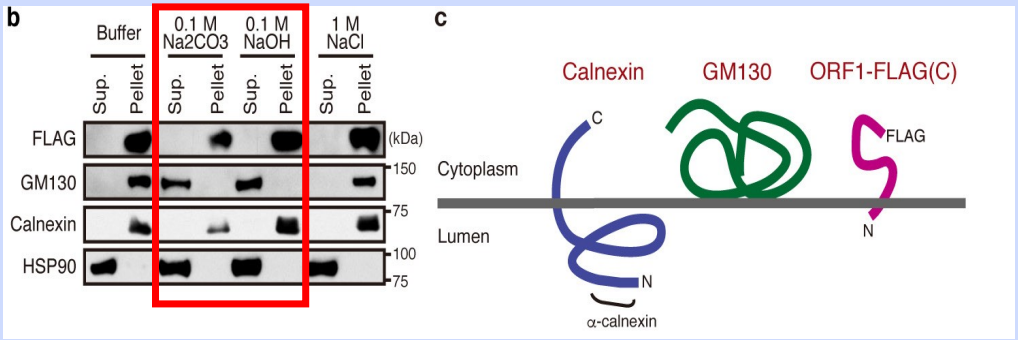
ORF1 has two in-frame ATG (ATG1 & ATG2) which can promote translation with same results in terms of (figure 3b):

- *Produced mRNA*
- *Polypeptide stability*

TRANSLATIONAL EFFICIENCY AFFECTS EXPRESSION

LINC00961 → Polypeptide

Associated with the membrane



Transmembrane protein or simply associated?

Homogenates treated with Na_2CO_3 (0.1M)/NaOH (0.1M)

- ⇨ ***Simple interaction with membrane disrupted*** (*GM130*)
- ⇨ ***Transmembrane interaction maintained*** (*Calnexin*)

LINC00961 polypeptide stays after treatment

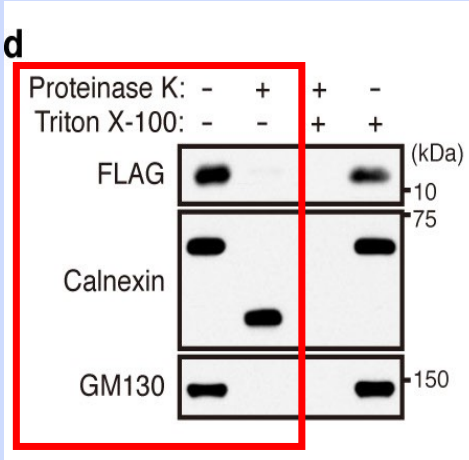
TRANSMEMBRANE PROTEIN

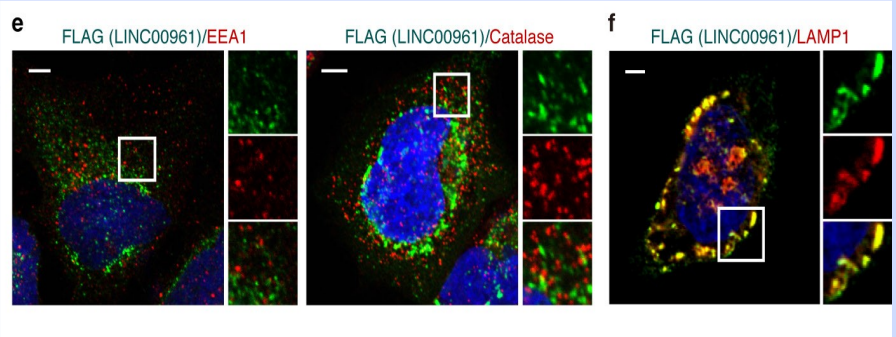
...more information about localization...

! Membrane fractions treated with **proteinase K** = cytosol proteins degradation

- Calnexin = *NO DIGESTION*
- GM130 & FLAG-tagged protein = **COMPLETE DIGESTION**

➔ C-TER IN THE CYTOSOL = TYPE I PROTEIN





! Immunofluorescence staining for Flag

- Co-localization with LAMP1 (late endosome & lysosome)
- Not with catalase (peroxisome) or EEA1 (early endosome)

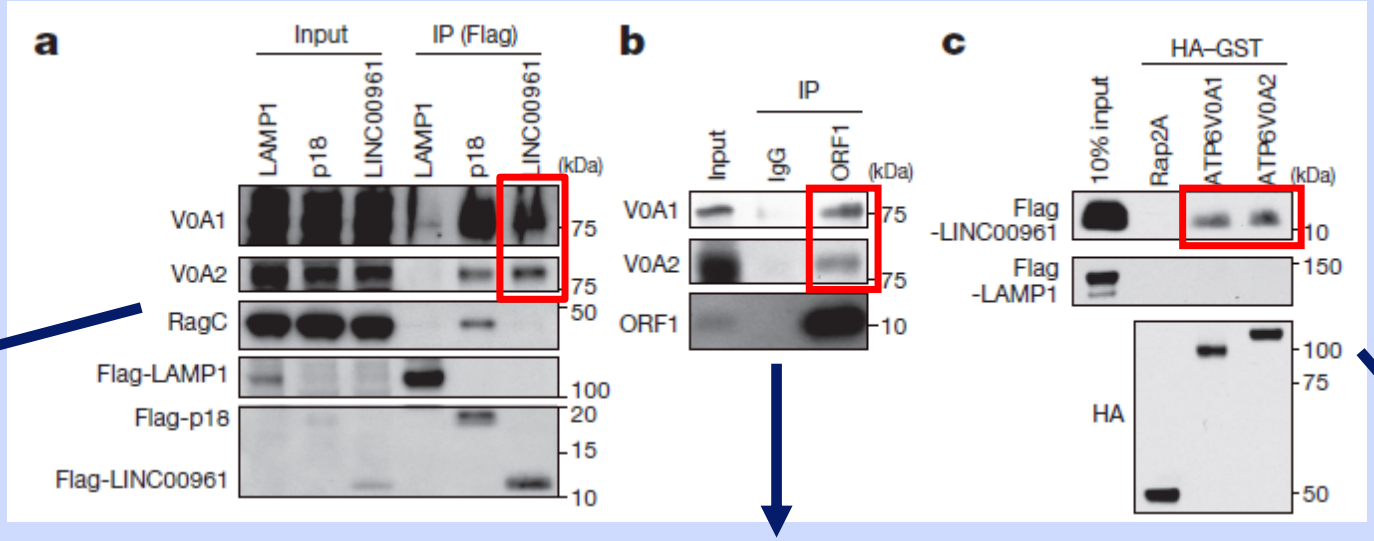
No lysosomal sorting signal in the sequence

TYPE I PROTEIN LOCALIZED ON LYSOSOME

Why is SPAR located to the lysosome?

MASS SPECTROMETRY TO IDENTIFY INTERACTOME → 4 subunits of the **V-ATPase complex**

- **V0A1**
- **V0A2**
- V0D1
- AP2



IP
Both V0A1 & V0A2 bound by SPAR

Ab against ORF1
Interaction with V0A1/V0A2 confirmed

In vitro Binding Assay
Direct interaction with ORF1

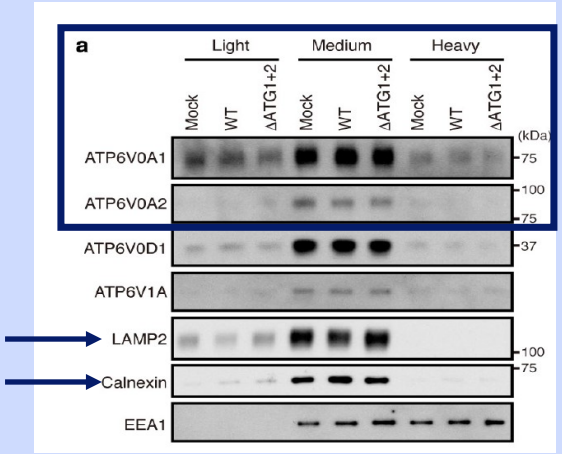
Link between V-ATPase & SPAR???

HEK293T cells, transduced with:

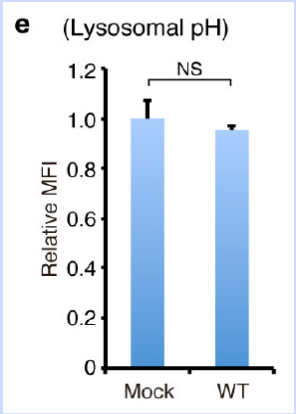
- Empty vector (*Mock*);
- Wild Type LINC00961 (*WT*);
- Mut LINC00961 (*ΔATG1+2*)

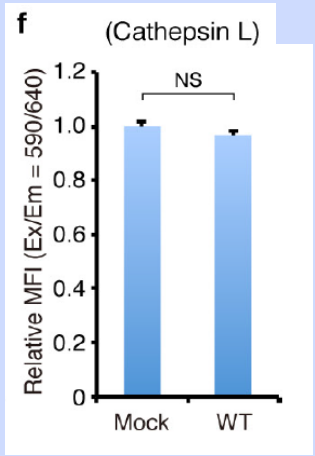
FRACTIONED
by sucrose gradient

- Light
- Medium
- Heavy

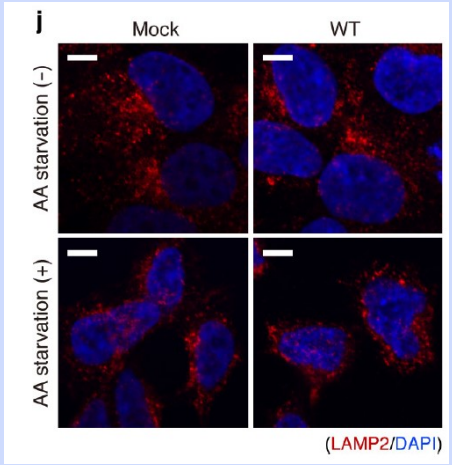


LOCALIZATION
no differences





FUNCTION
no differences



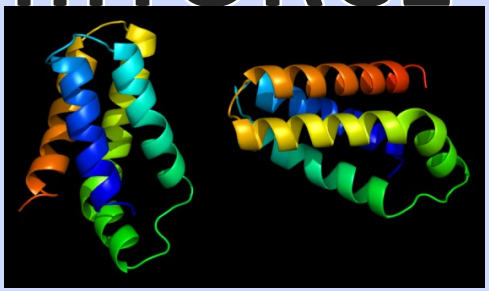
LYSOSOMAL MORPHOLOGY
No differences

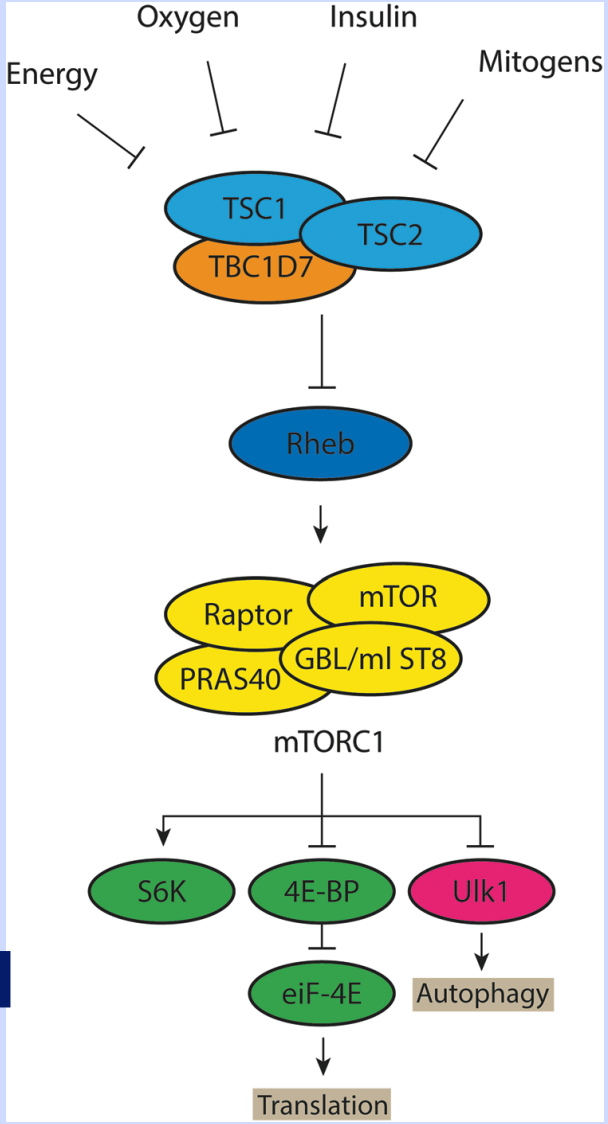
Link between V-ATPase & SPAR???

NO DIRECT LINKAGE BETWEEN V-ATPase & SPAR ACTIVITY

Why SPAR interact with V-ATPase complex?

V-ATPase ➔ Ragulator Complex ➔ mTORC1

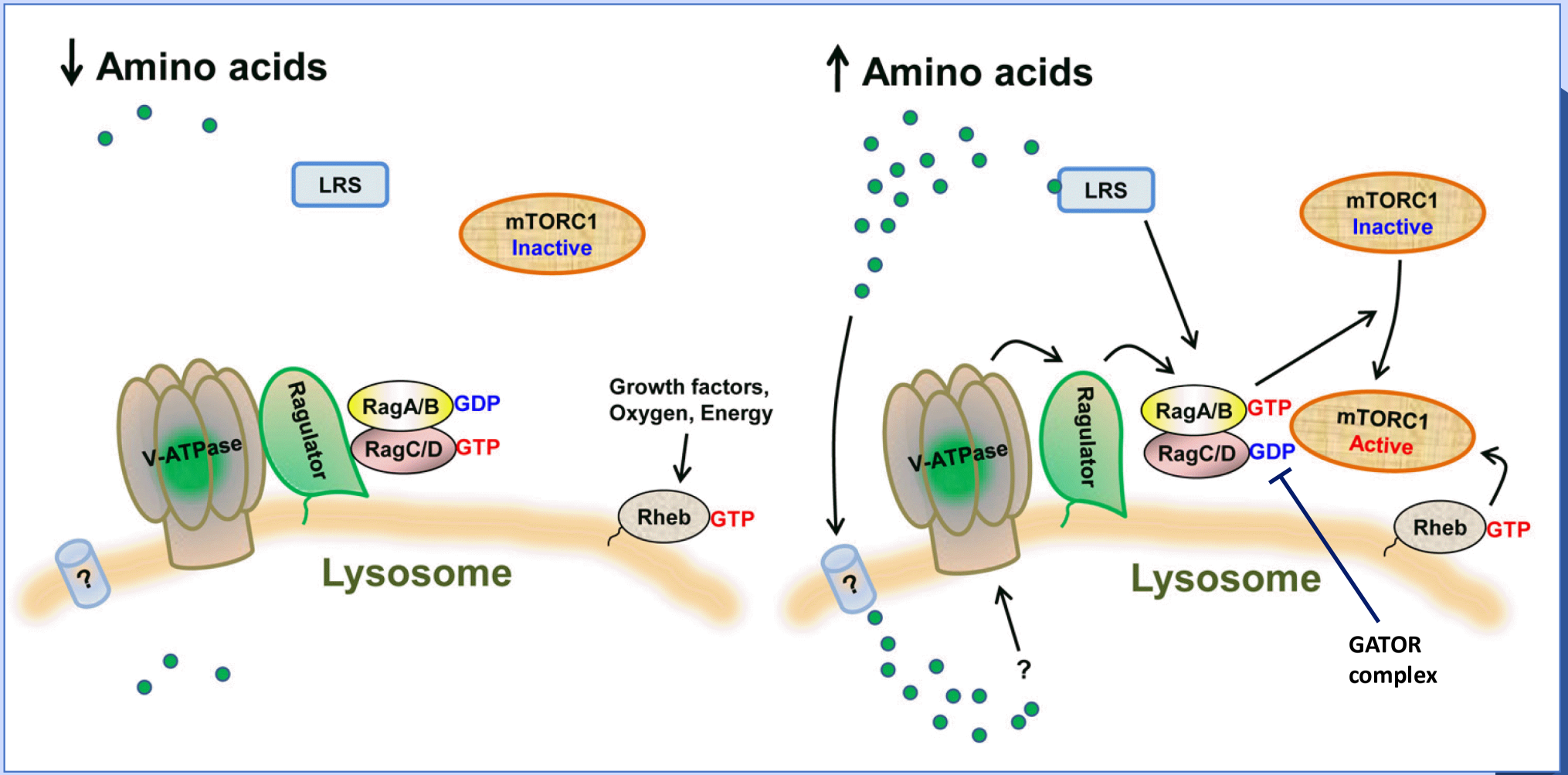


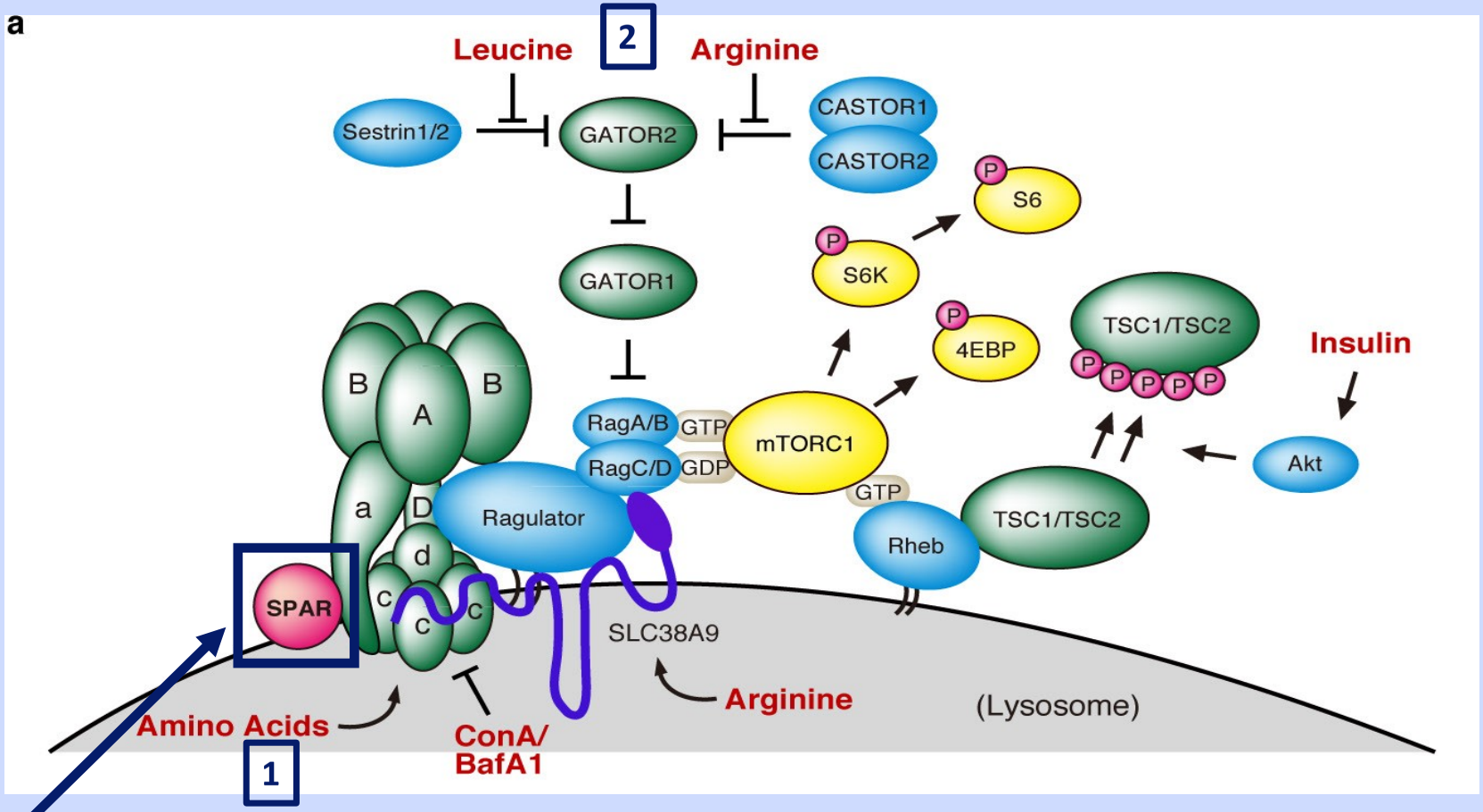


Amino-acid plays an important role in mTORC1 activation

PROMOTE GROWTH IN RESPONSE TO AVAILABILITY OF NUTRIENTS

Amino-acid impact on mTORC1 pathway





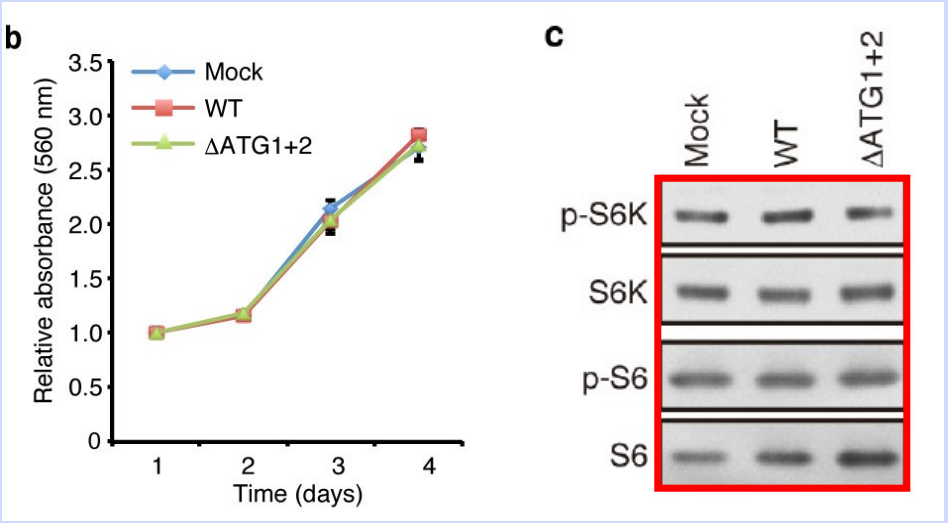
SPAR involvement in the mTORC1 activating pathway

HOW IT HAS BEEN DISCOVERED???

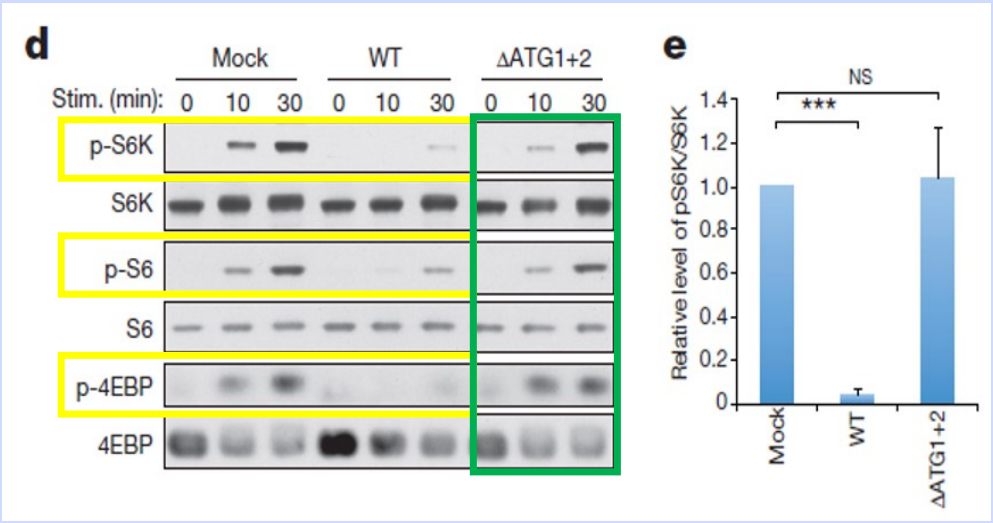
TESTS ON SPAR & mTORC1

1. **Proliferation**/Activity,
2. Localization, 3. Signalling

Normal aa levels



aa starvation (1h) & re-stimulation (10/30 min)



NO DIFFERENCES IN PROLIFERATION & mTORC1 ACTIVITY

↓↓↓ mTORC1 activity with WT

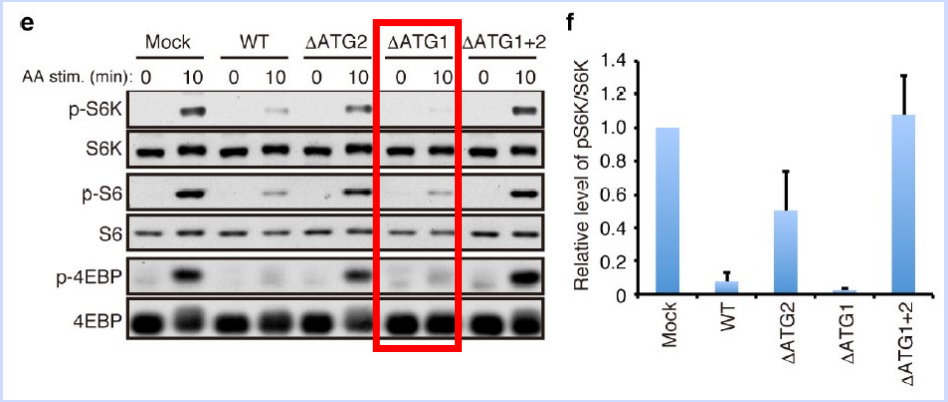
No differences with ΔATG1+2

TESTS ON SPAR & mTORC1

1. Proliferation/Activity,
2. Localization, 3. Signalling

aa starvation (1h) & re-stimulation (10 min)

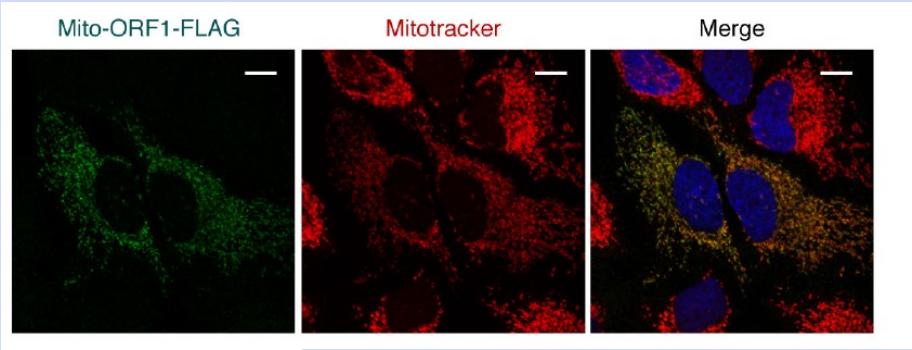
ΔATG1 = shorter (75 aa)
ΔATG2 = longer (90 aa)

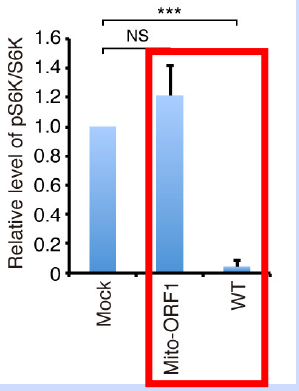


ΔATG1 inhibits more potently mTORC1 activity than WT or ΔATG2

ΔATG1 IS SUFFICIENT TO INHIBIT mTORC1

Localization & Activity





Lysosome or nothing!

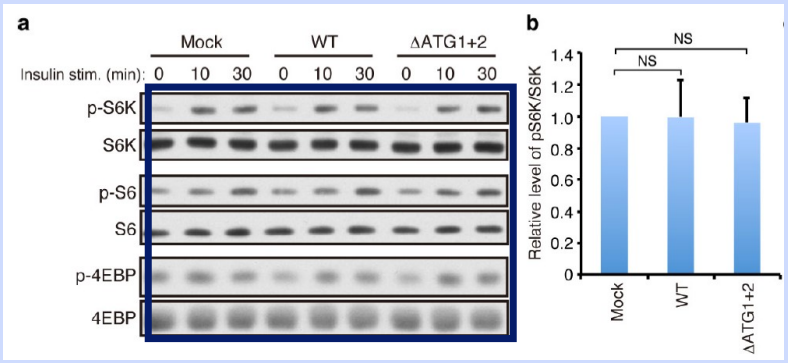
TESTS ON SPAR & mTORC1

1. Proliferation/Activity,
2. Localization, 3. **Signalling**

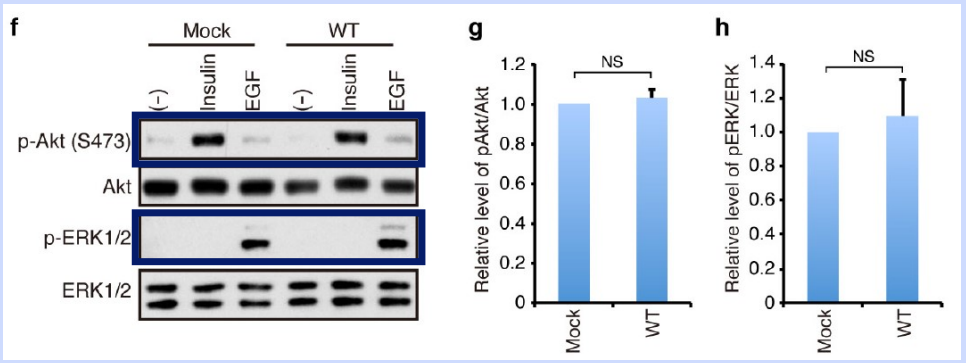
SPAR
=
***S**mall regulatory **P**olypeptide on **A**mino-acid **R**esponse*

SPAR blocks aa signalling = OK!

SPAR blocks other signalling pathways = ?? → NO!



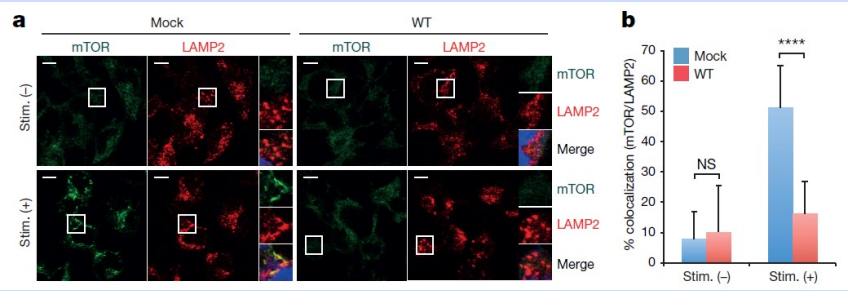
No Insulin-mediated activation!



Insulin and EGF stimulation for mTORC2 → No SPAR envolvement

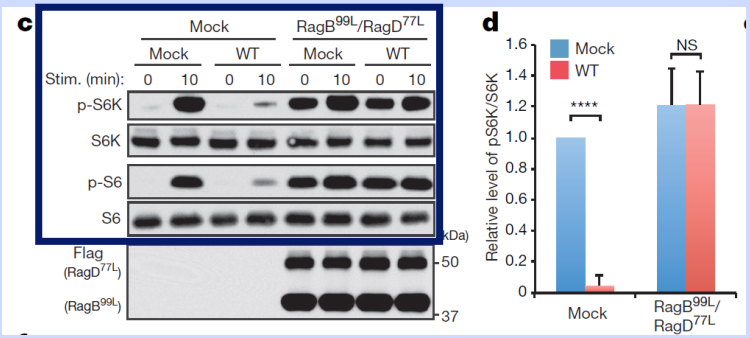
SPAR regulates mTORC1 ➔ WHICH POINT?

❑LOCALIZATION



➔ No co-localization when SPAR is expressed

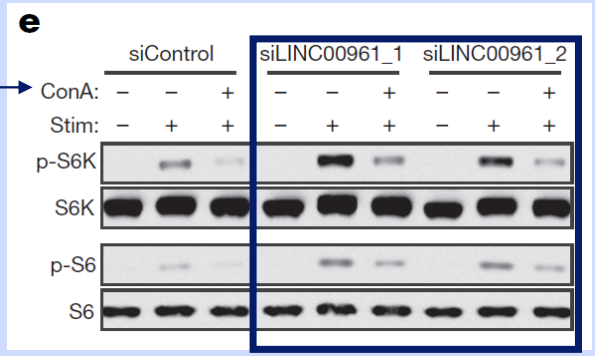
❑ RAG ROLE



➔ SPAR function upstream RAGs

❑V-ATPase role

Concanamycin
(V-ATPase inhibitor)



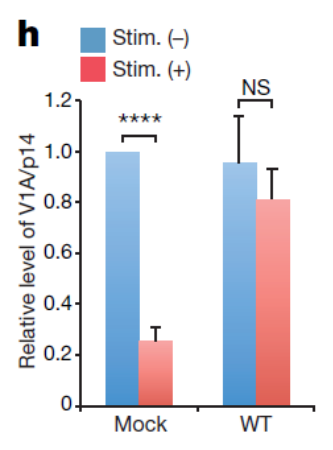
➔ SPAR works at v-ATPase level

SPAR regulates mTORC1 ➔ WHICH POINT?

SPAR INTERACTS AND REGULATES V-ATPase

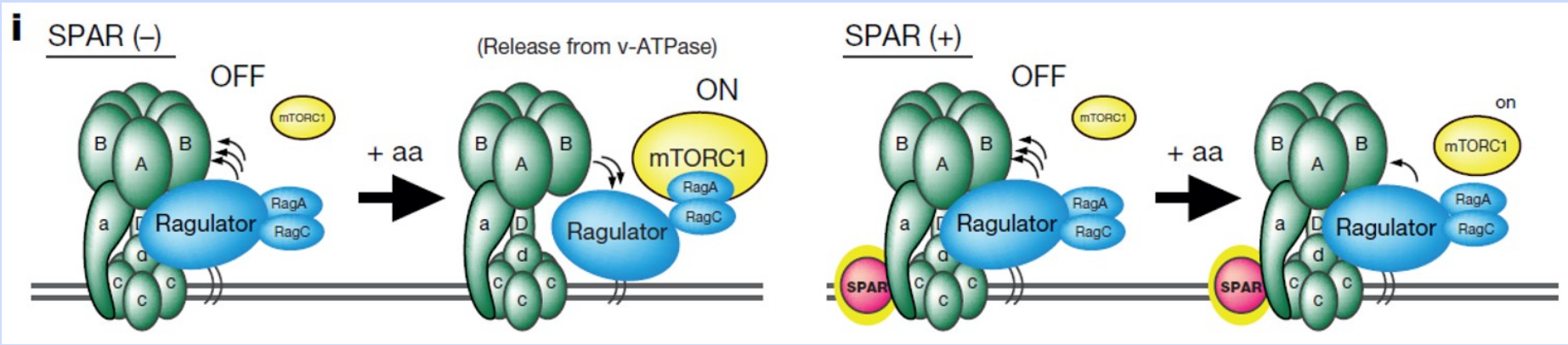
➡ HOW???

SPAR regulates mTORC1 ➔ WHICH POINT?



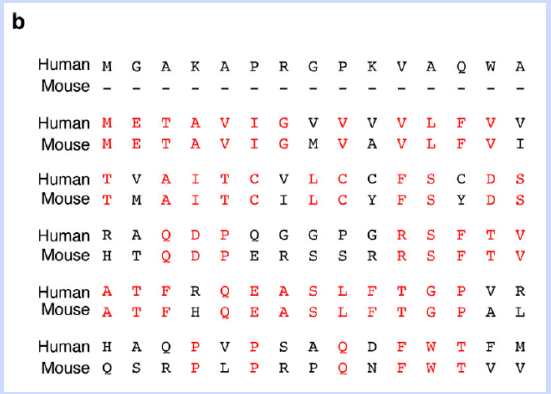
- AA Stimulation in Mock = ⬇⬇⬇ Interaction v-ATPase/RAG
- AA Stimulation in WT = No changes

➔ SPAR MAINTAIN v-ATPase-Ragulator-Rags SUPERCOMPLEX



Experiments in mice

IN VITRO



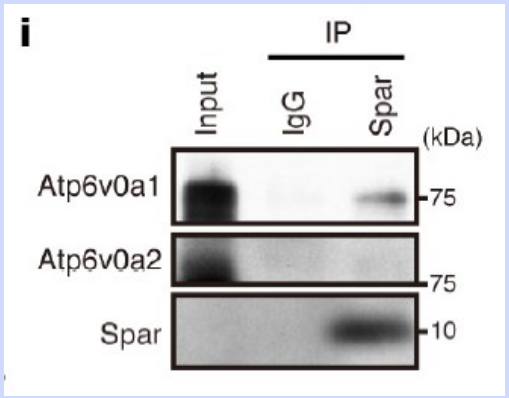
5430416O09RIK

65% homology w/ human

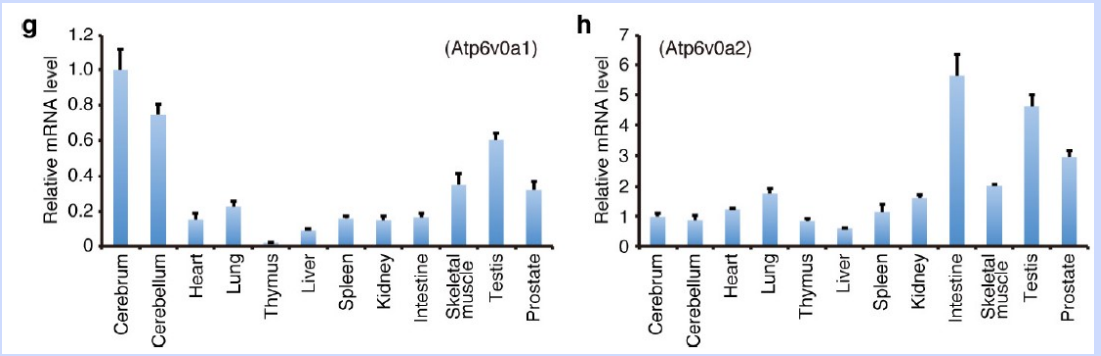
Functional conservation of SPAR

Expression in skeletal muscle

In Vivo???



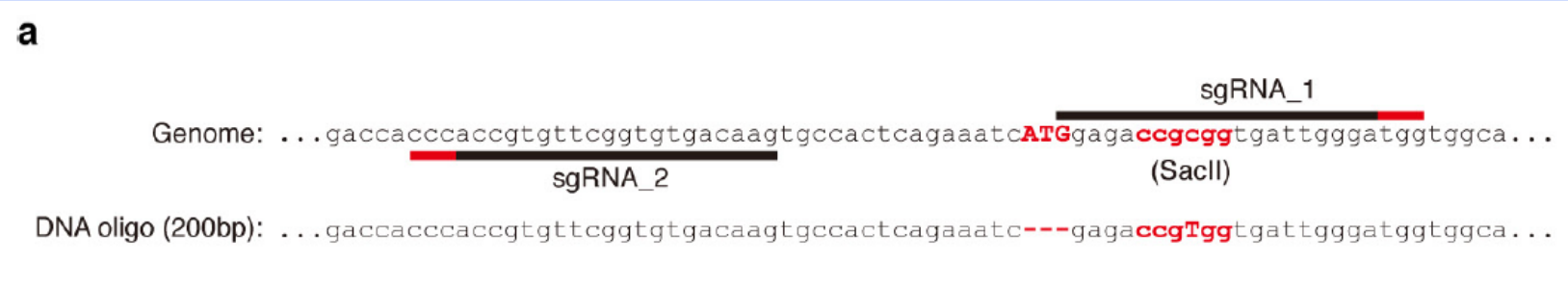
SPAR co-localized with v-ATPase complex in mice



V-ATPase complex expressed in skeletal muscle

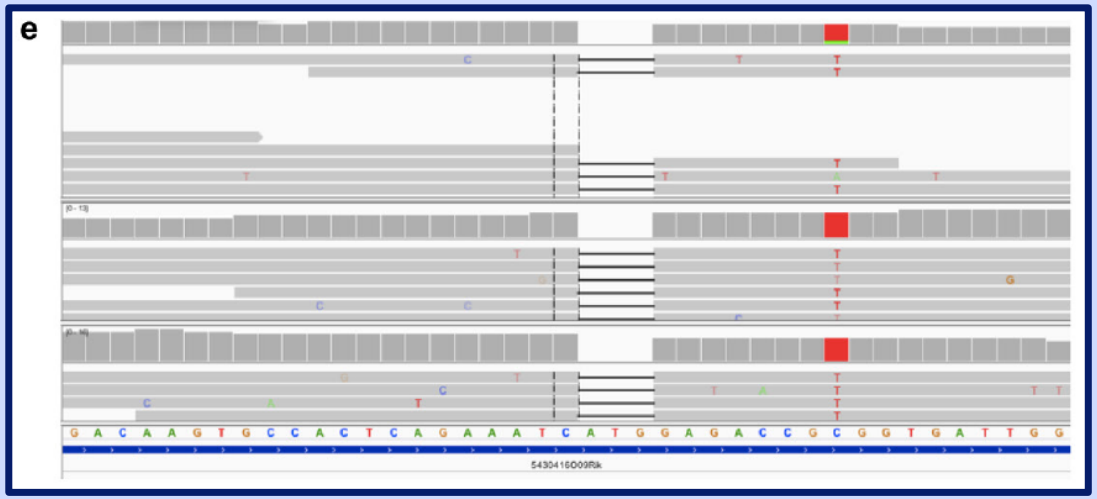
IN VIVO

Experiments in mice



CRISPR/CAS9 ⇨ **SPAR-deficient mice**

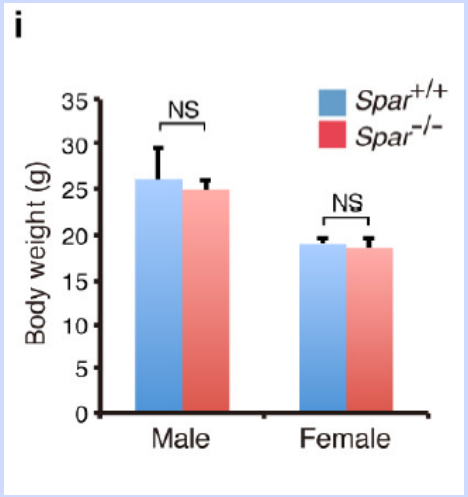
Genomic analysis for 3 genotypes



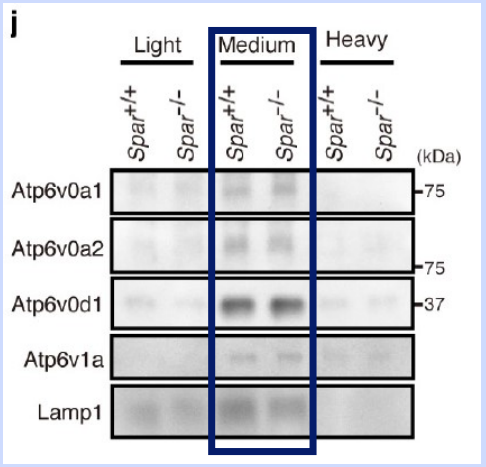
WGS ⇨ **no off-targets**

IN VIVO

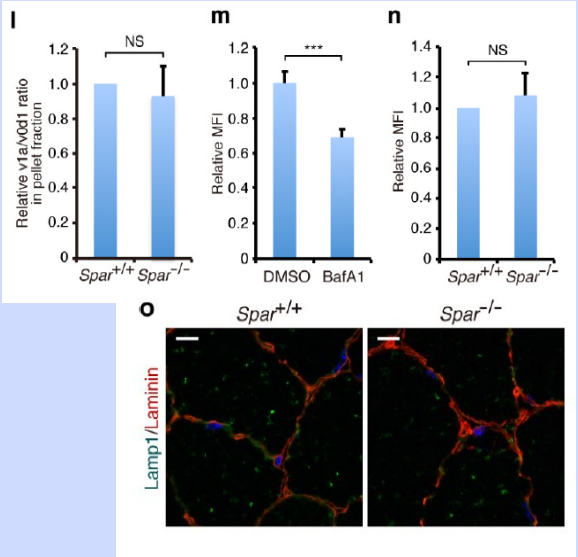
Experiments in mice



No morphologic differences

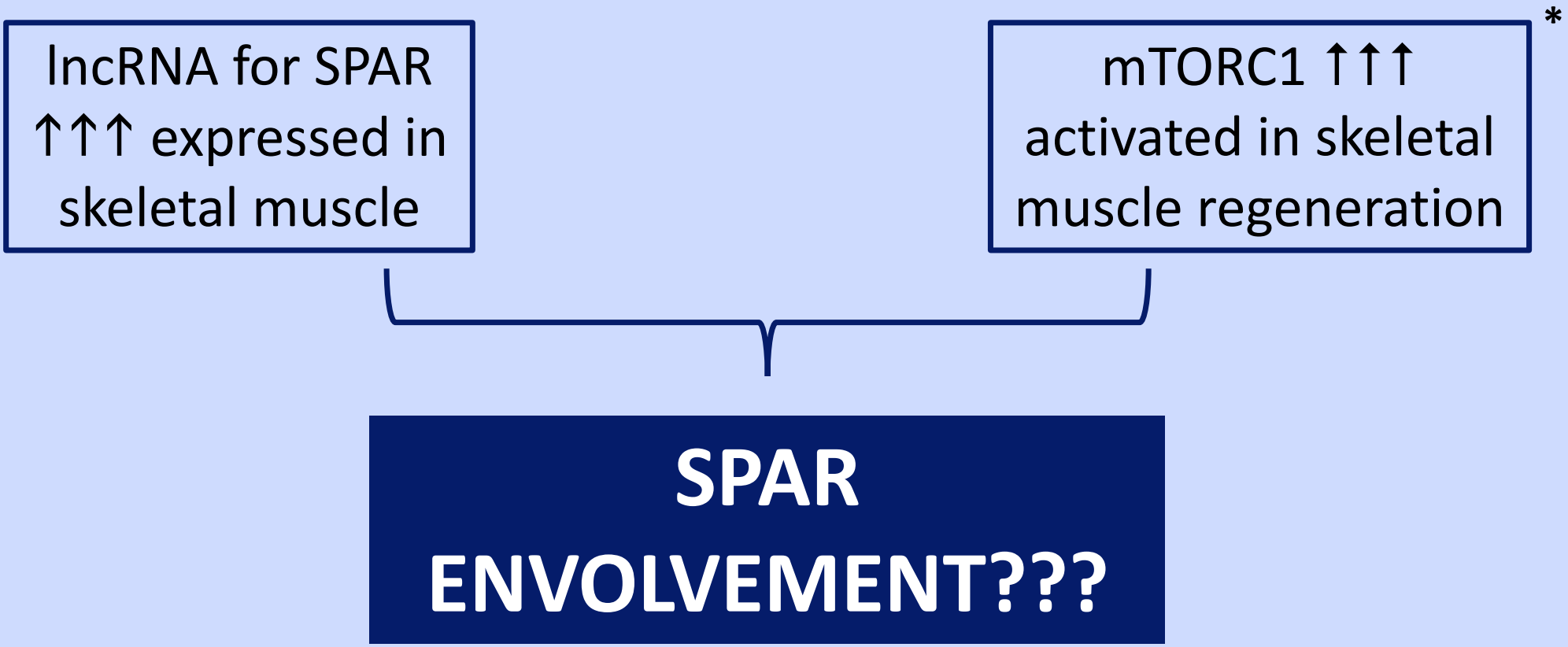


No differences in v-ATPase localization (Immunoblot)

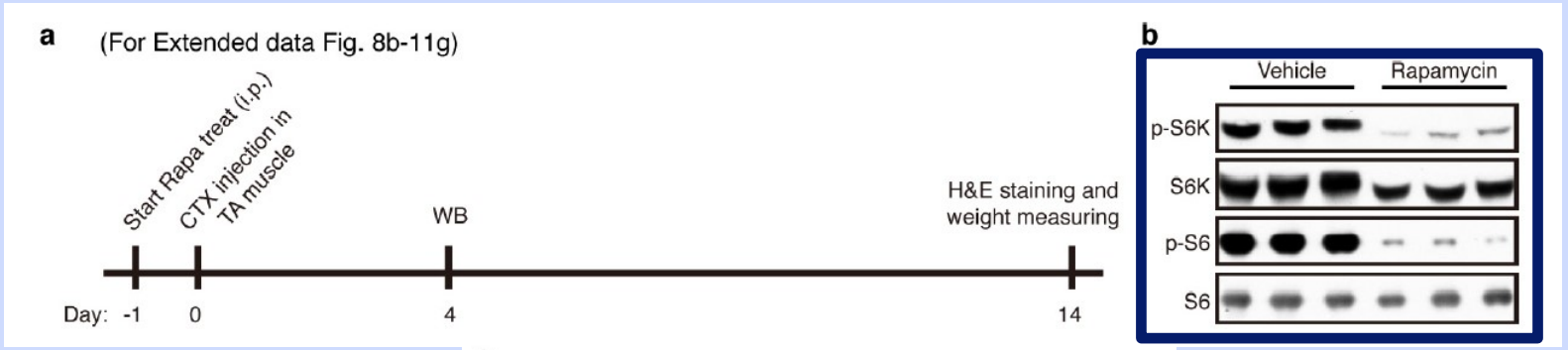


No differences in Lysosomal morphology

<u>LOCALIZATION</u>, <u>ASSEMBLY</u> & <u>LYSOSOMAL FUNCTION</u> OF v-ATPase **DON'T CHANGE**

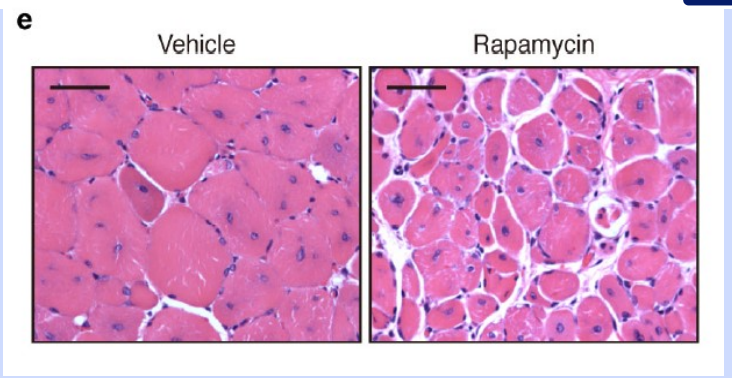


* Rapamycin stops muscle regeneration blocking mTORC1

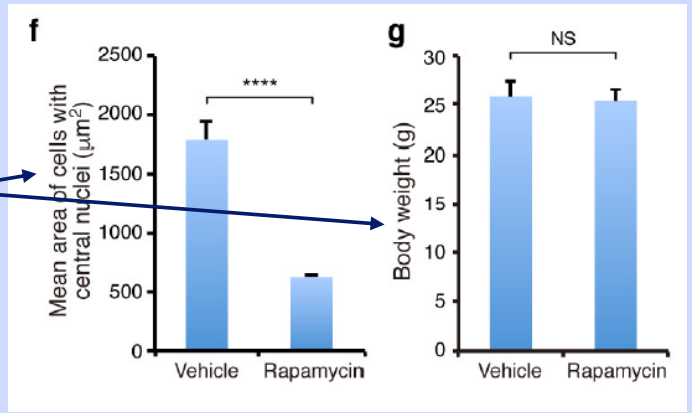


Rapamycin/CTX treatment

CTX = Cardiotoxin, snake toxin
⇨ *muscle injury*



Effect only in muscle

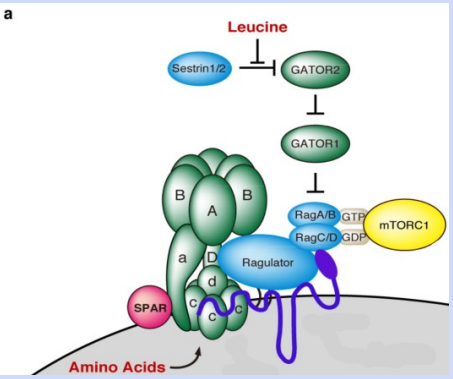


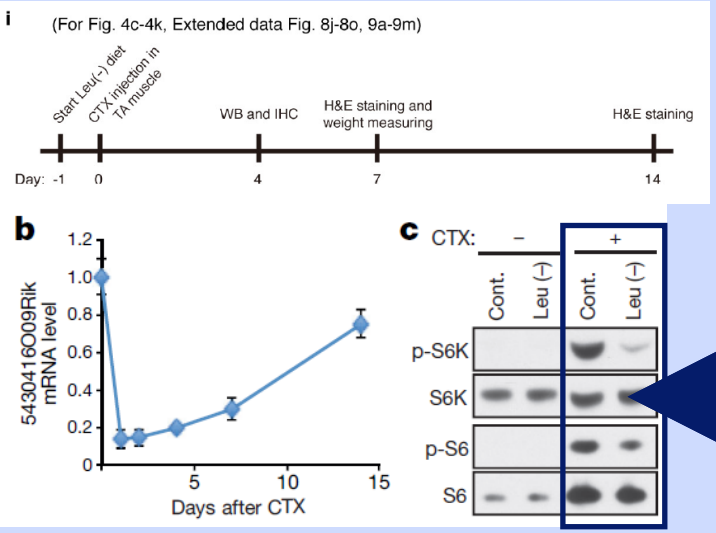
mTORC1 activity reduced by Rapamycin

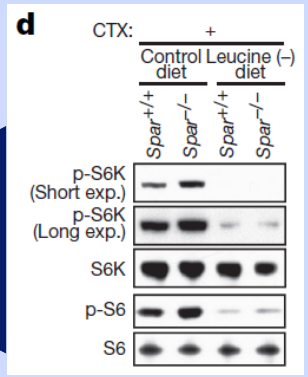
⇨ ***NO MUSCLE REGENERATION***

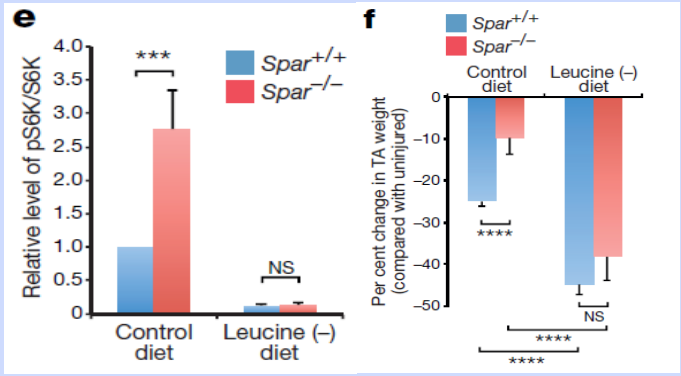
SPAR ENVOLVEMENT IN MUSCLE REGENERATION

Leucine deprivation as control







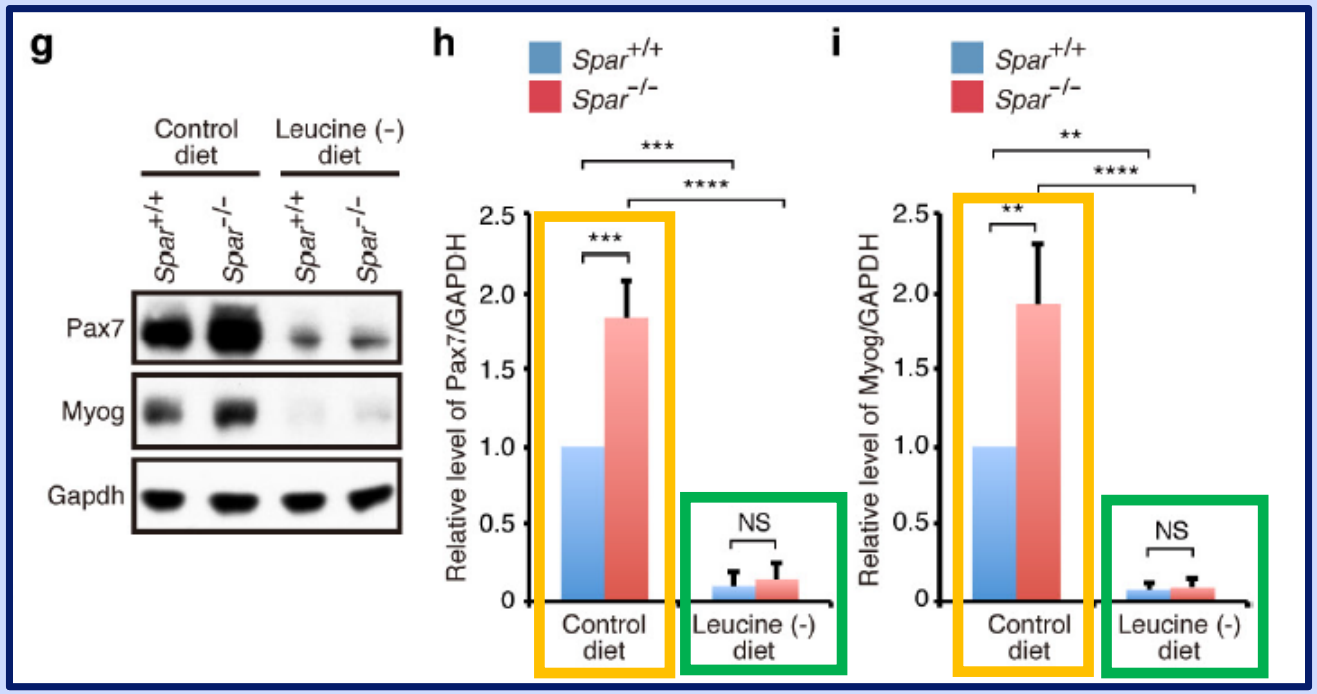


mTORC1 DOESN'T ACT PHYSIOLOGICALLY, WITH OR WITHOUT SPAR, IF THERE'S NO LEUCINE

ROLE OF SPAR & mTORC1 CONFIRMED

SPAR ENVOLVEMENT IN MUSCLE REGENERATION

Stem cell proliferation ↑
Differentiation ↑
Maturation ↑

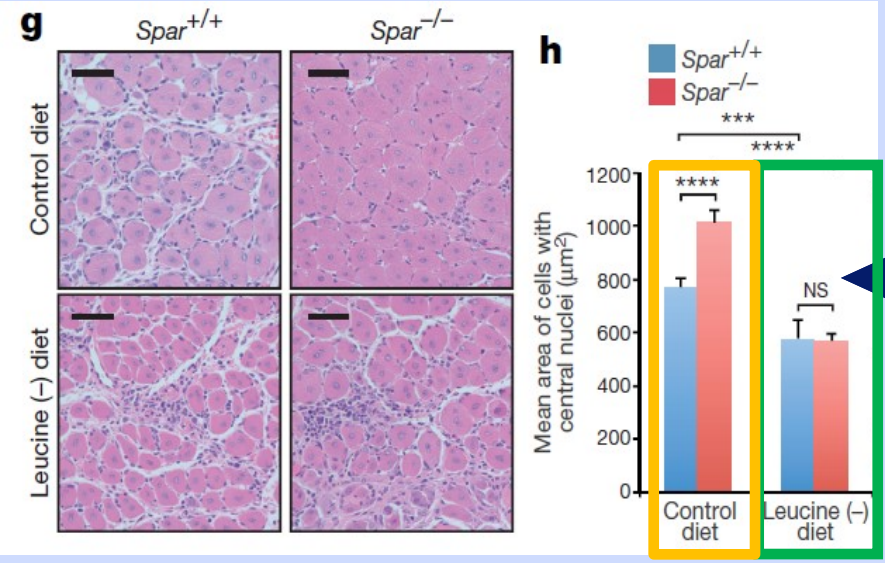


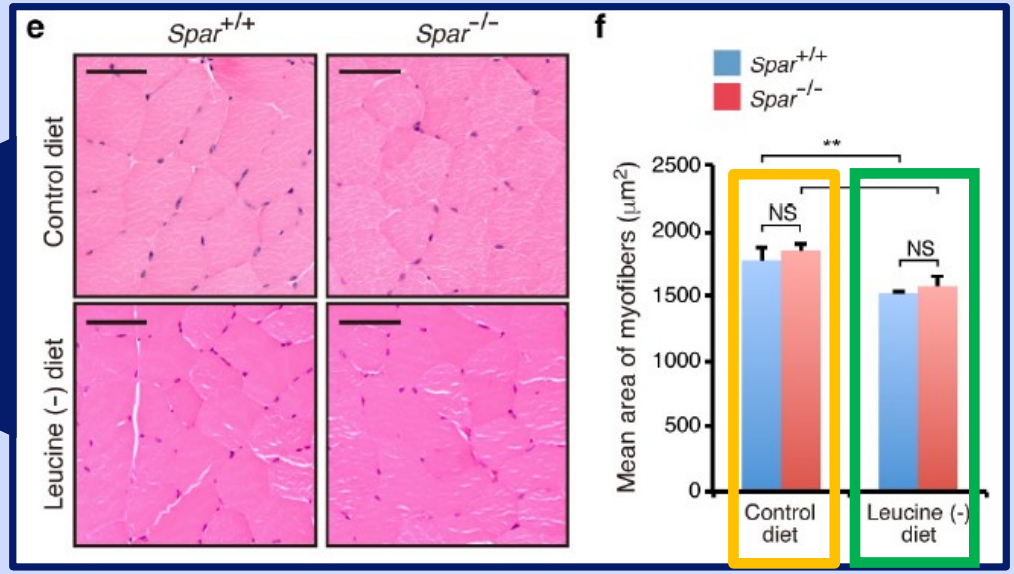
SPAR DEFICIENT CELLS (Inj) ⇨ Ctrl: ↑↑↑ Pax7/Myog

⇨ Leu-: ⊗ Pax7/Myog

SPAR ENVOLVEMENT IN MUSCLE REGENERATION

Stem cell proliferation ↑
Differentiation ↑
Maturation ↑



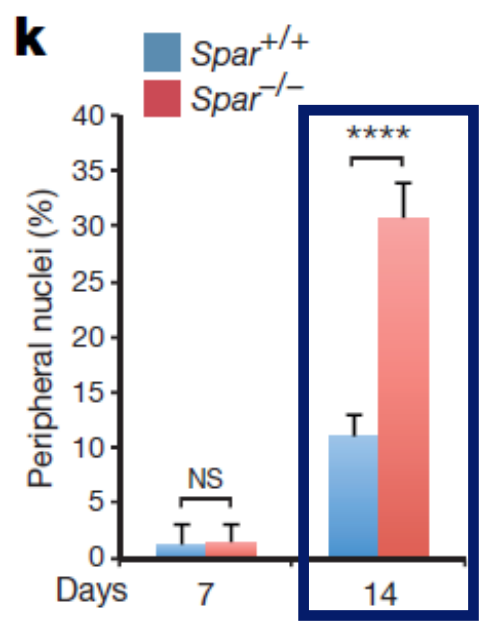


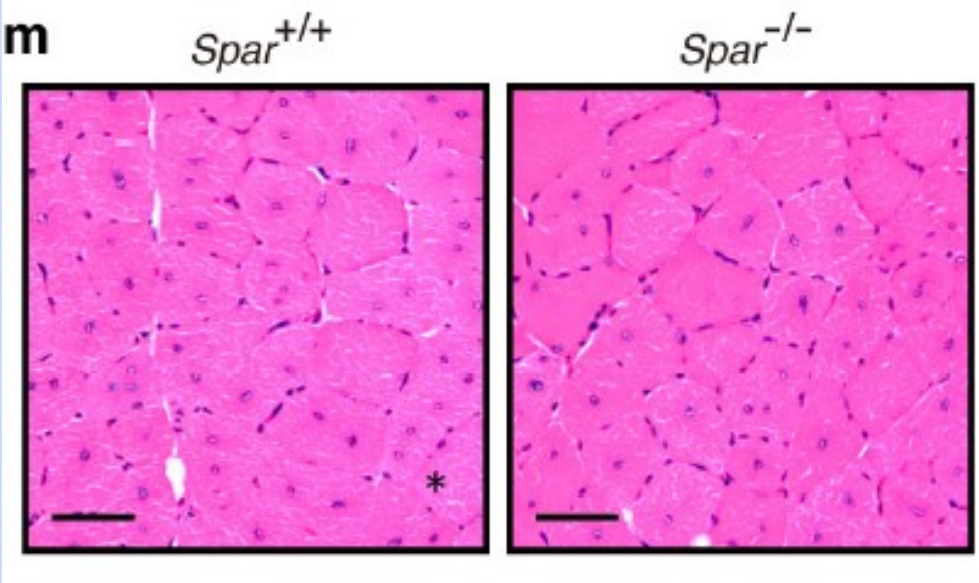
SPAR DEFICIENT MYOFIBRES ⇨ Ctrl: ↑↑↑ n°, = size

⇨ Leu-: = n°, = size

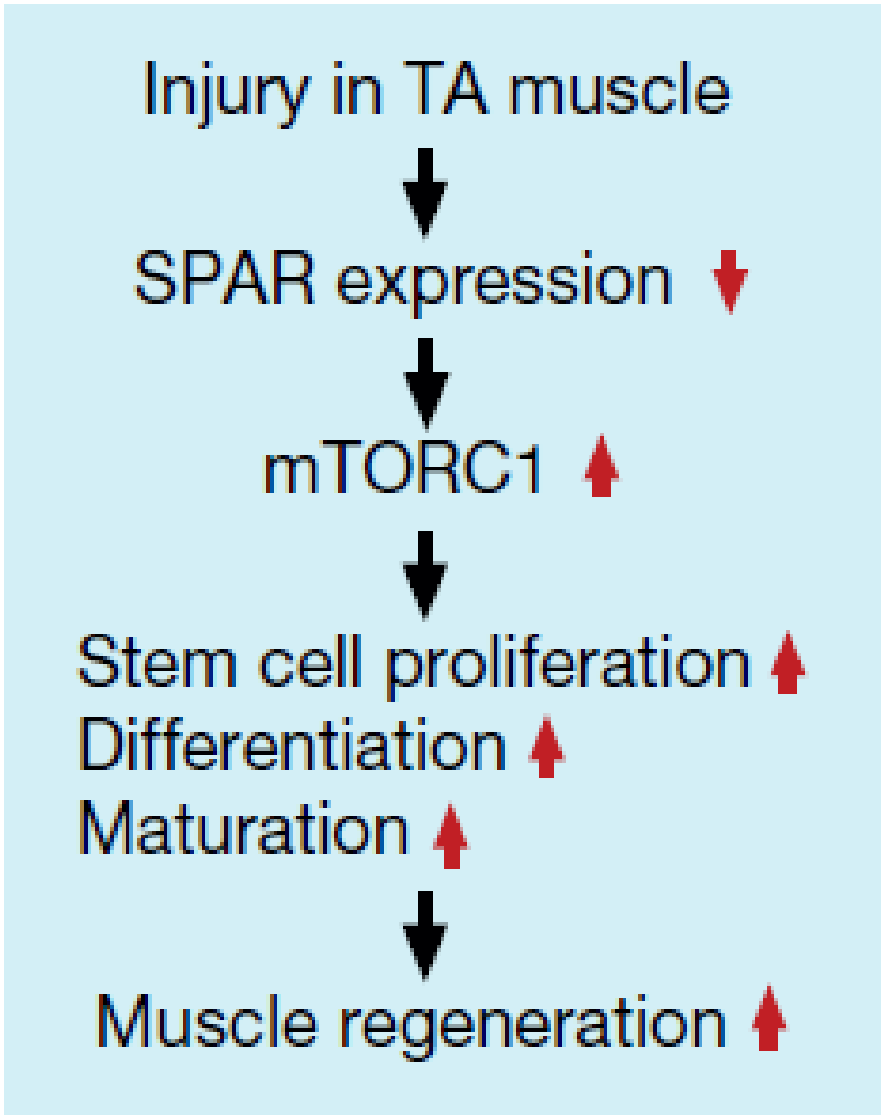
SPAR ENVOLVEMENT IN MUSCLE REGENERATION

Stem cell proliferation ↑
Differentiation ↑
Maturation ↑



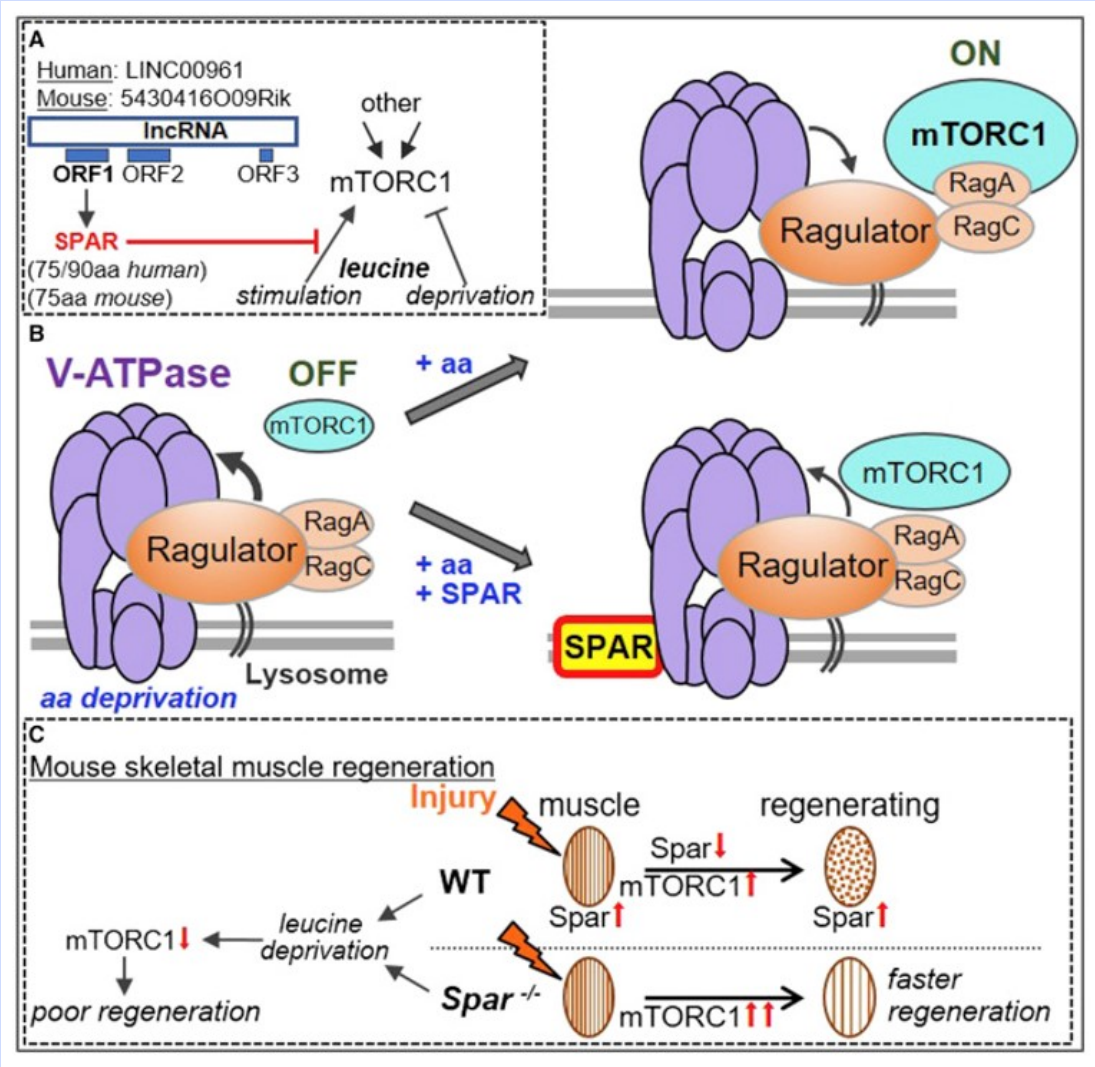


SPAR -/- ↑↑↑ MATURE CELLS AFTER 14 DAYS



To summarize…

Final proposed model for **mTORC1** and its (down)**regulation by SPAR**



References

LETTER

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mTORC1 and muscle regeneration are regulated by the LINC00961-encoded SPAR polypeptide

Akinobu Matsumoto[1], Alessandra Pasut[1]*, Masaki Matsumoto[2]*, Riu Yamashita[3]*, Jacqueline Fung[1], Emanuele Monteleone[1,4], Alan Saghatelian[5], Keiichi I. Nakayama[2], John G. Clohessy[1] & Pier Paolo Pandolfi[1]

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