# ALS and RNA binding proteins as therapeutic target

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# ALS (Amyotrophic Lateral Sclerosis)

- Most frequent Motor Neuron Disease (MND)
- Degeneration in motor neuron in the cerebral cortex and spinal cord -> muscle weakness
- ~10000 patients in Japan (2013)
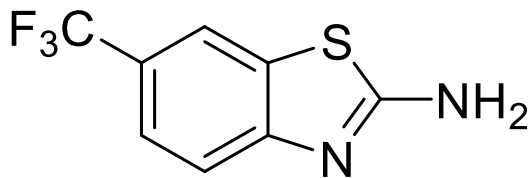- 90% of ALS is sporadic, 10% is familial.
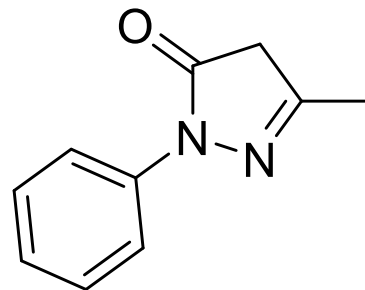


Lou Gehrig



ice bucket challenge

# Treatment of ALS

- Riluzole

  - Neuroprotective reagent, inhibits glutamate release

- Edaravone

  - Radical scavenger

- Both only slow progression by several weeks

  -> Other target is necessary.



riluzole



edaravone

# Characteristics of ALS

- RNA-binding proteins, such as FUS & TDP-43 are involved


- Excess Stress Granules are observed

# TDP-43  (TAR DNA binding Protein 43)

- Found in ubiquitin-positive aggregates

- Dominant inheritance in familial ALS10

- Shuttles nucleus and cytoplasm

- Localized to SGs under stressed condition

- Both WT and mutants aggregations are observed



Brain Research. *2012*, 1462, 16
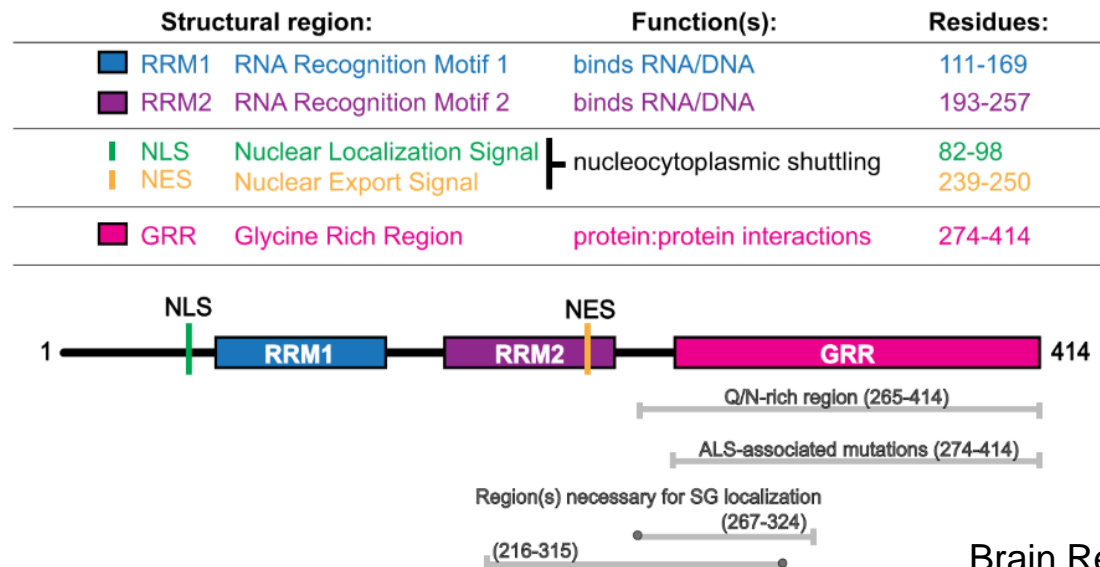
# FUS (Fused in Sarcoma)

- RNA binding protein
- Responsible gene of ALS6
- Shuttles nucleus and cytoplasm
- Localized to SGs under stressed condition
- Overexpression of WT FUS cause cell death
- FUS mutants aggregates
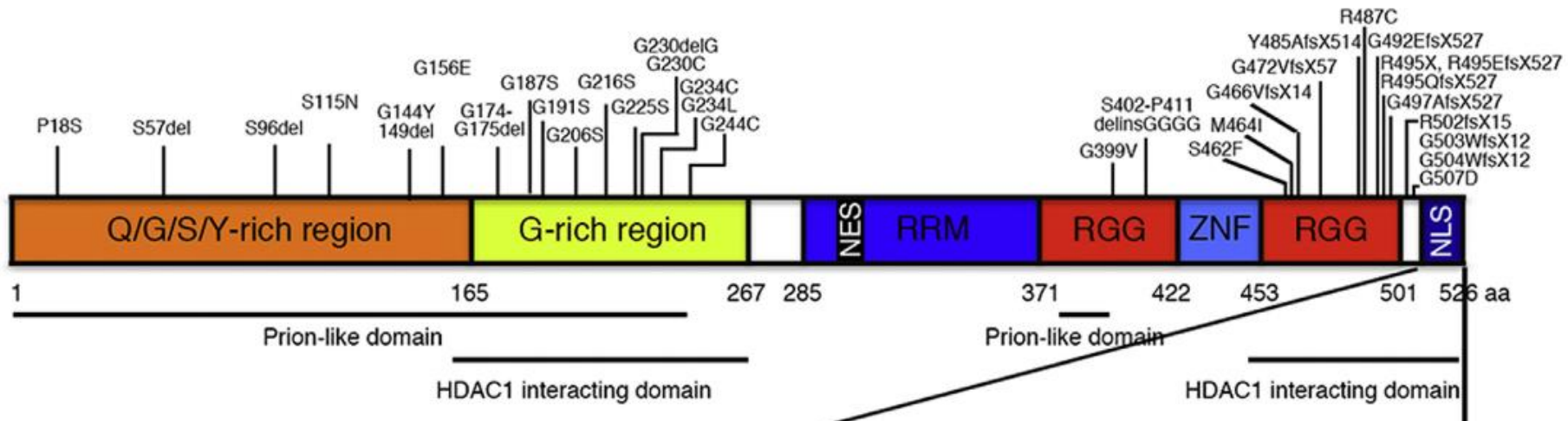
# Stress Granule (=SG)

- One of the membrane-less organelles in cells
- Liquid-Liquid phase separation (=LLPS)
- Interrupt translation of proteins under stress condition
- RNA-binding domain (RBD) and Prion-like domain (PLD) is important for localization
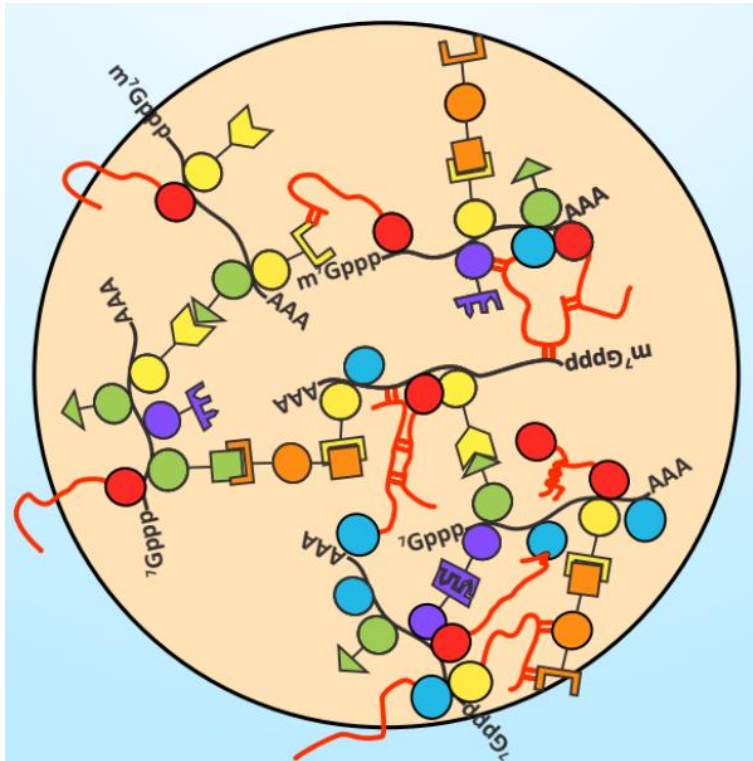


- mRNA
- Ribosome
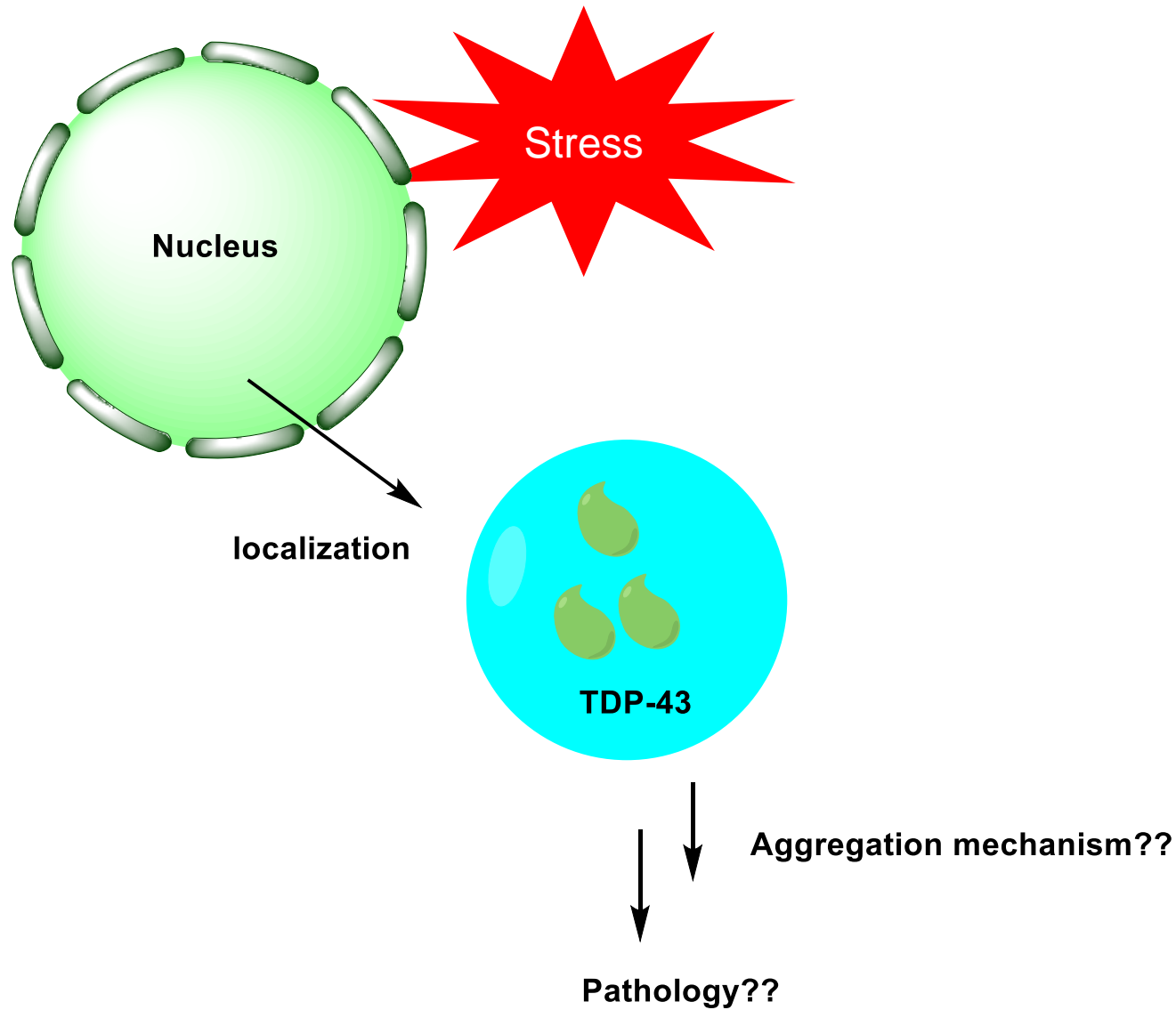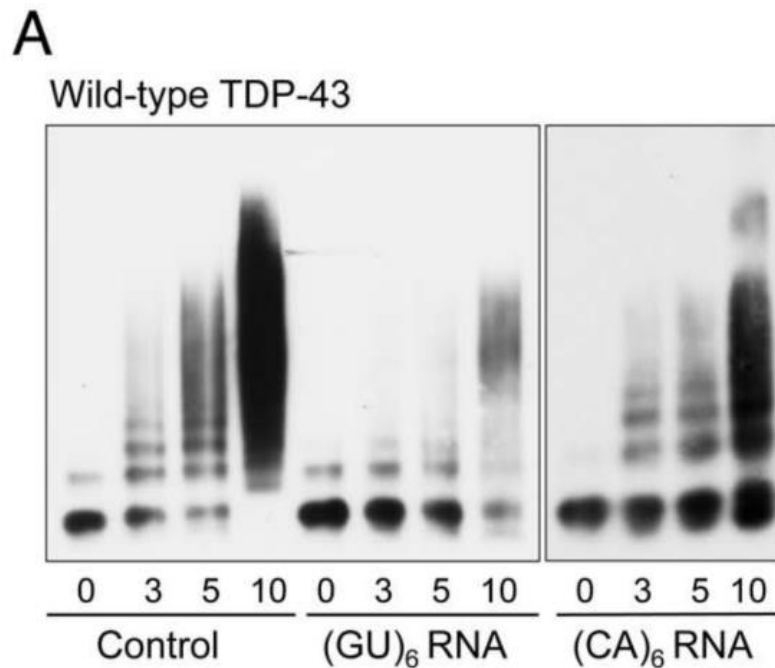- RNA binding proteins
- HDAC6
- RNA helicase

Cell Reports. 2018, 22, 1401

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# Questions for TDP-43 and ALS

# Binding to RNA impairs TDP-43 aggregation



A

Wild-type TDP-43

0 3 5 10    0 3 5 10    0 3 5 10
Control    (GU)$_6$ RNA    (CA)$_6$ RNA

- Strong affinity to GU repeats
- Addition of RNA impairs aggregation

**Figure 4 Specific binding of GU-rich RNA prevents TDP-43 aggregation.** SDD-AGE/immunoblot analyses of TDP-43 aggregation over time in the presence and absence of 2'-O-methyl, phosphorothiorate-modified RNA (4 μM RNA and 2 μM protein). (A) RNA composed of (GU)$_6$, (CA)$_6$ repeats, or control was added to rTDP-43 prior to triggering aggregation. (B) Aggregation of the RNA binding-deficient mutant F147/149L over time in the presence and absence of (GU)$_6$. All blots were probed with TDP-43 antibody and are representative of > 3 individual experiments.
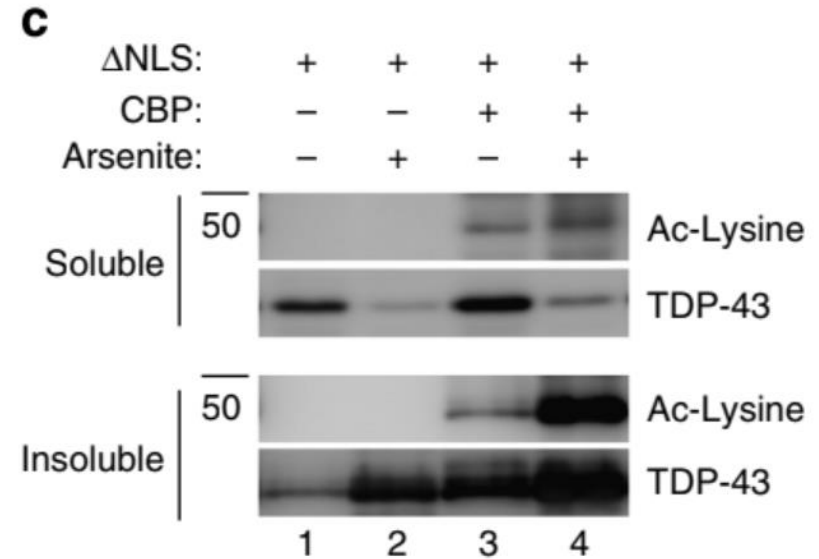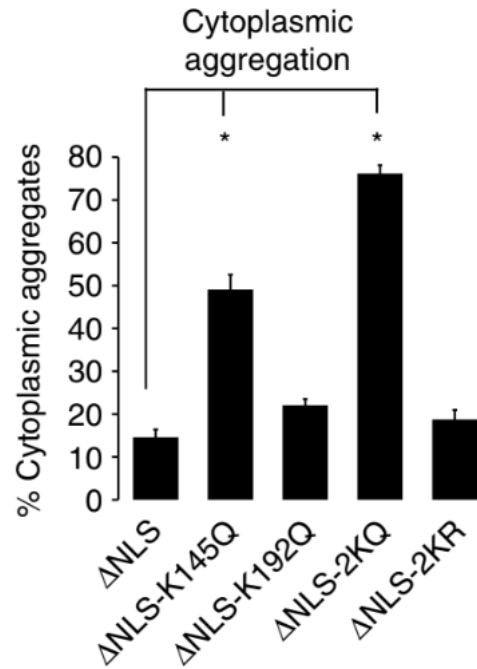
# TDP-43 is acetylated by CBP
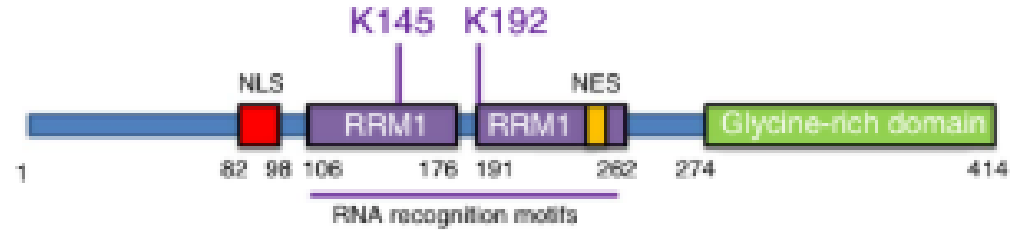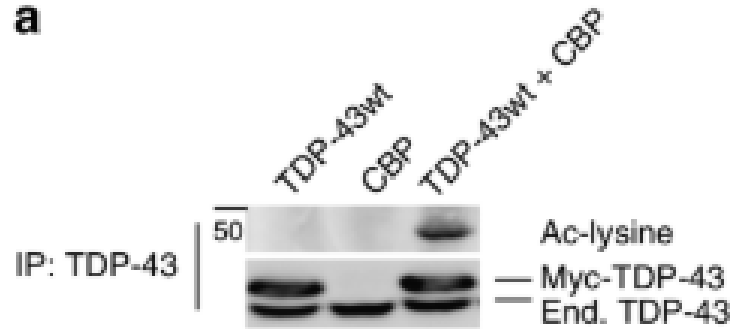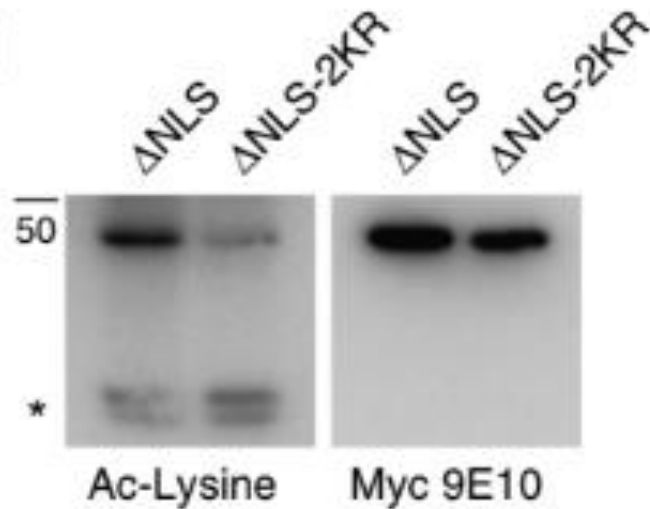


- K145 & K192 in RRM mainly acetylated.

# TDP-43 acetylation results in aggregation



of TDP-43 aggregates. Scale bar, 10 μm. (**b**) Nuclear stippling and cytoplasmic aggregation phenotypes were quantified for all TDP-43 acetylation-mimic and non-mimic mutants at residues Lys-145 and Lys- 192. Error bars indicate s.e.m., and the single asterisk indicates statistical significance with *P*-value <0.05 as measured by two-tailed unpaired *t*-test with unequal variance from *N* = 3 biological replicates. (**c**) Cells expressing acetylation-mimic

bars represent s.e.m. (**c**) Immunoblotting of soluble and insoluble fractions using anti-myc and anti-acetyl-lysine antibodies illustrates the accumulation of acetylated TDP-43 on co-transfection of CBP and exposure to 0.2 mM arsenite for 1 h. (**d**) Immunoprecipitation reaction using Ac-K145 followed by

- KQ mutant … acetylation mimic
- KQ mutant more likely to aggregate

# TDP-43 acetylation impairs RNA binding
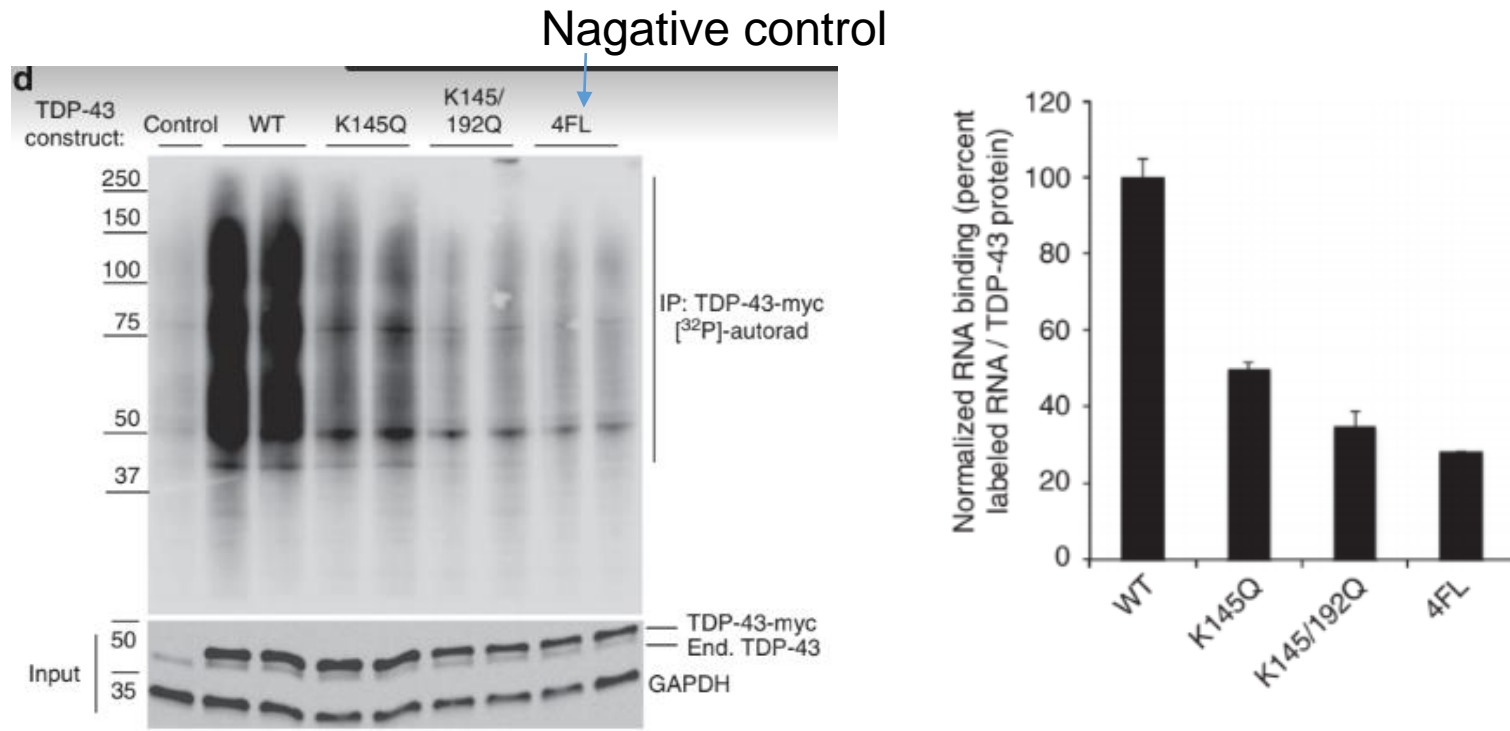


(N = 3), and error is displayed as s.e.m. (d) Crosslinking immunoprecipitation (CLIP) assay was performed on cells expressing wild-type TDP-43, K145Q, 2KQ (K145/192Q) or TDP-4FL plasmids. TDP-43-bound RNAs were immunoprecipitated using anti-myc (9E10), end-labelled with [$^{32}$P]-ATP and visualized using bis-tris gel immunoblotting followed by phosphorimaging analysis. Shown is a representative image containing duplicate samples for each construct that was obtained from N = 3 independent experiments. (e) TDP-43-associated RNAs were quantified using TYPHOON software and normalized to total TDP-43 protein inputs, as determined by immunoblotting with an anti-myc antibody shown in d. All mutants analysed (K145Q, K145Q/192Q, 4FL) showed significant reduction in RNA-binding compared with WT as assessed by Student's t-test (P-value ≤ 0.01) from N = 3 independent experiments. Error bars indicate s.d. of normalized intensity signals derived from experimental replicates.

- RNA binding was quantified by CLIP(Crosslinking immunoprecipitation) assay.
- KQ mutant showed low affinity to RNA.

# TDP-43 can be aggregated in droplet
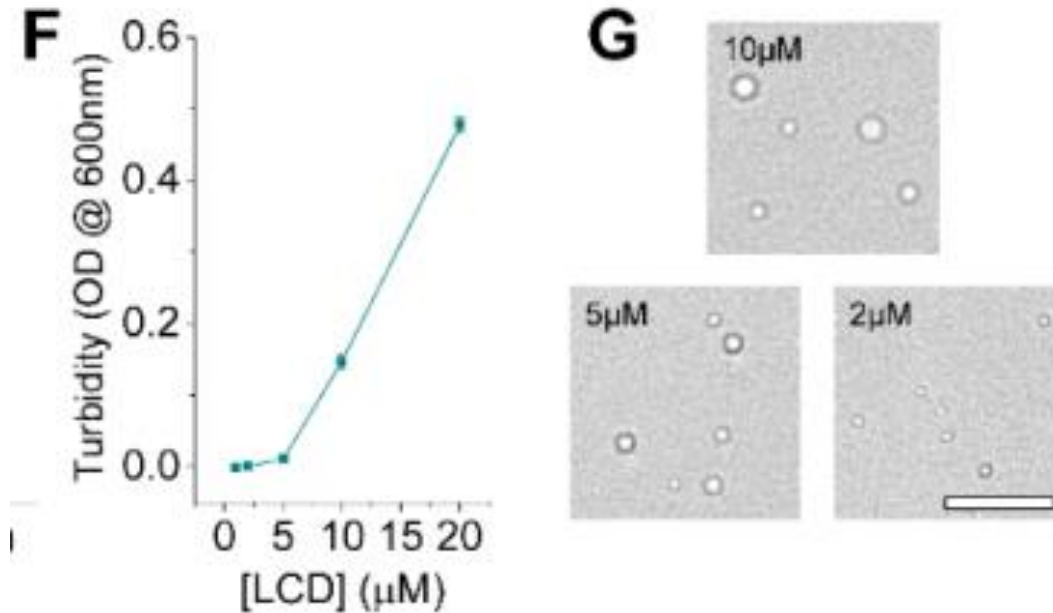


conditions: 10 mM KP pH 6, 25 °C. (F) LLPS as monitored by turbidity as a function of protein concentration. Buffer conditions: 10 mM KP pH 7.3, 150 mM NaCl, 10% PEG-10,000, 37 °C. (G) Representative bright field images corresponding to conditions described in panel F; scale bar: 5 μm. (H)



- TDP-43 LCD(Low Complexity Domain, = GRR) forms droplet under physiological condition.

# TDP-43 can be aggregated in droplet
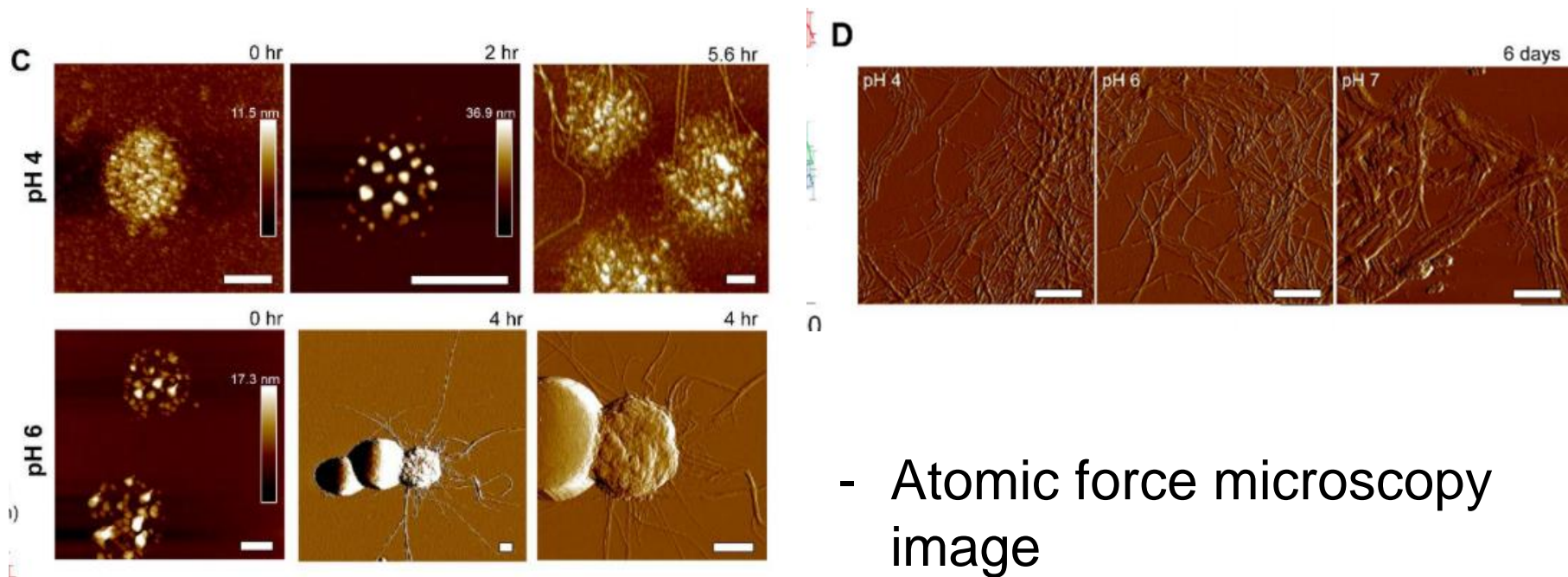


- Atomic force microscopy image
- Fibril was observed.
- High concentration of TDP-43 gets aggregated

represents an average of at least three droplets and error bars represent standard deviation. (C) Atomic force microscopy images of TDP-43 LCD droplets deposited and dried on mica at various time points during incubation at 25 °C under LLPS condition in a buffer containing 300 mM NaCl at pH 4 (upper images) or pH 6 (lower images). Protein concentration: 20 μM. White scale bars correspond to 400 nm. (D) Representative atomic force microscopy images of fibrils after prolonged (6 days) incubation of TDP-43 LCD under LLPS conditions at pH 4, 6, or 7 in the presence of 300 mM NaCl. Scale bars: 400 nm.
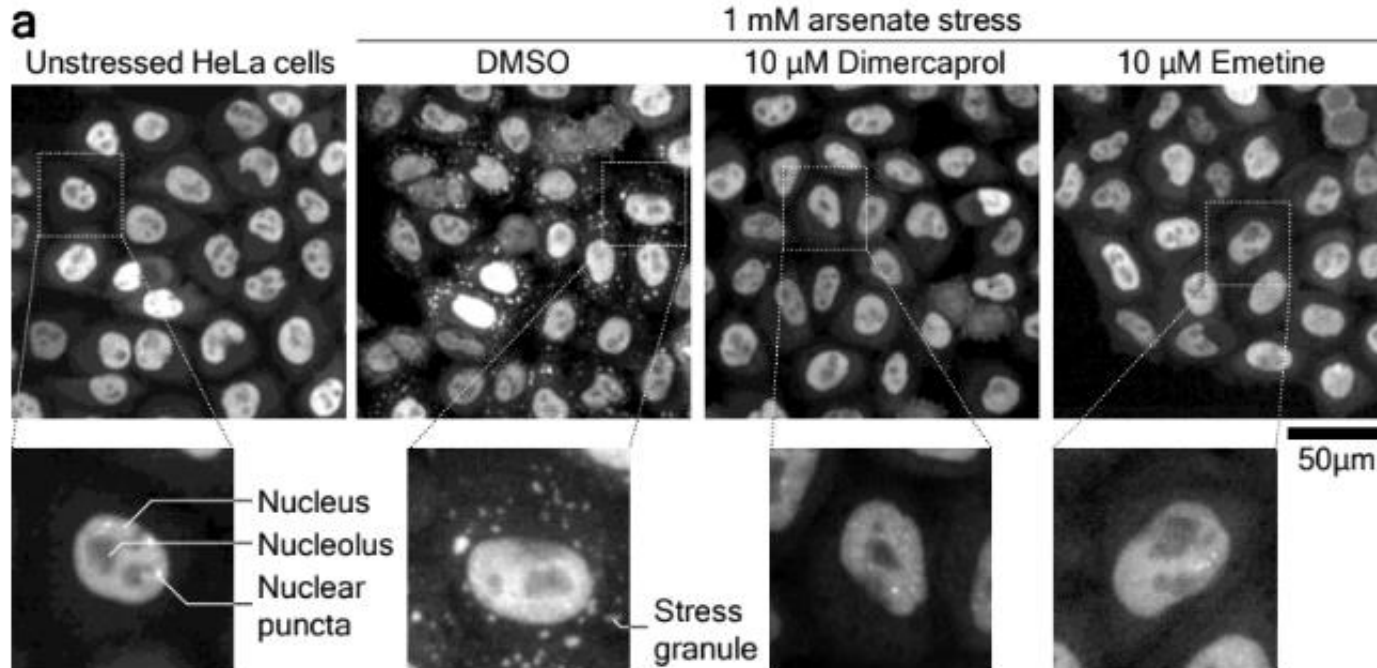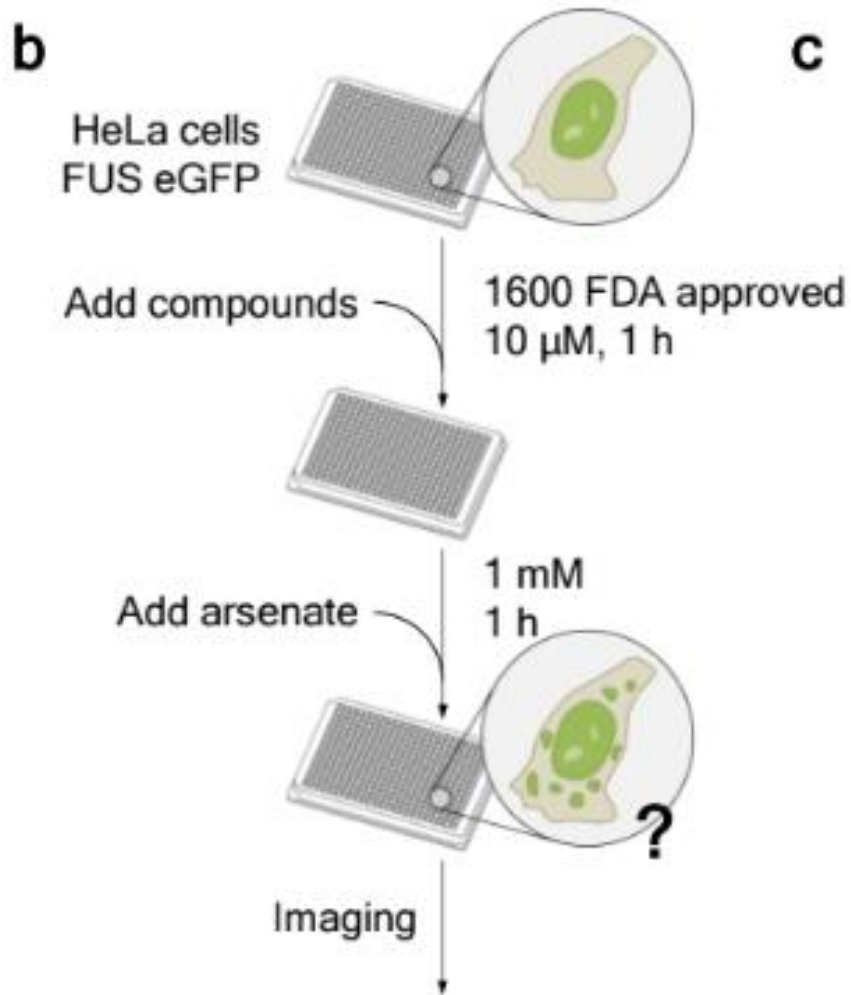
# TDP-43 aggregation summary
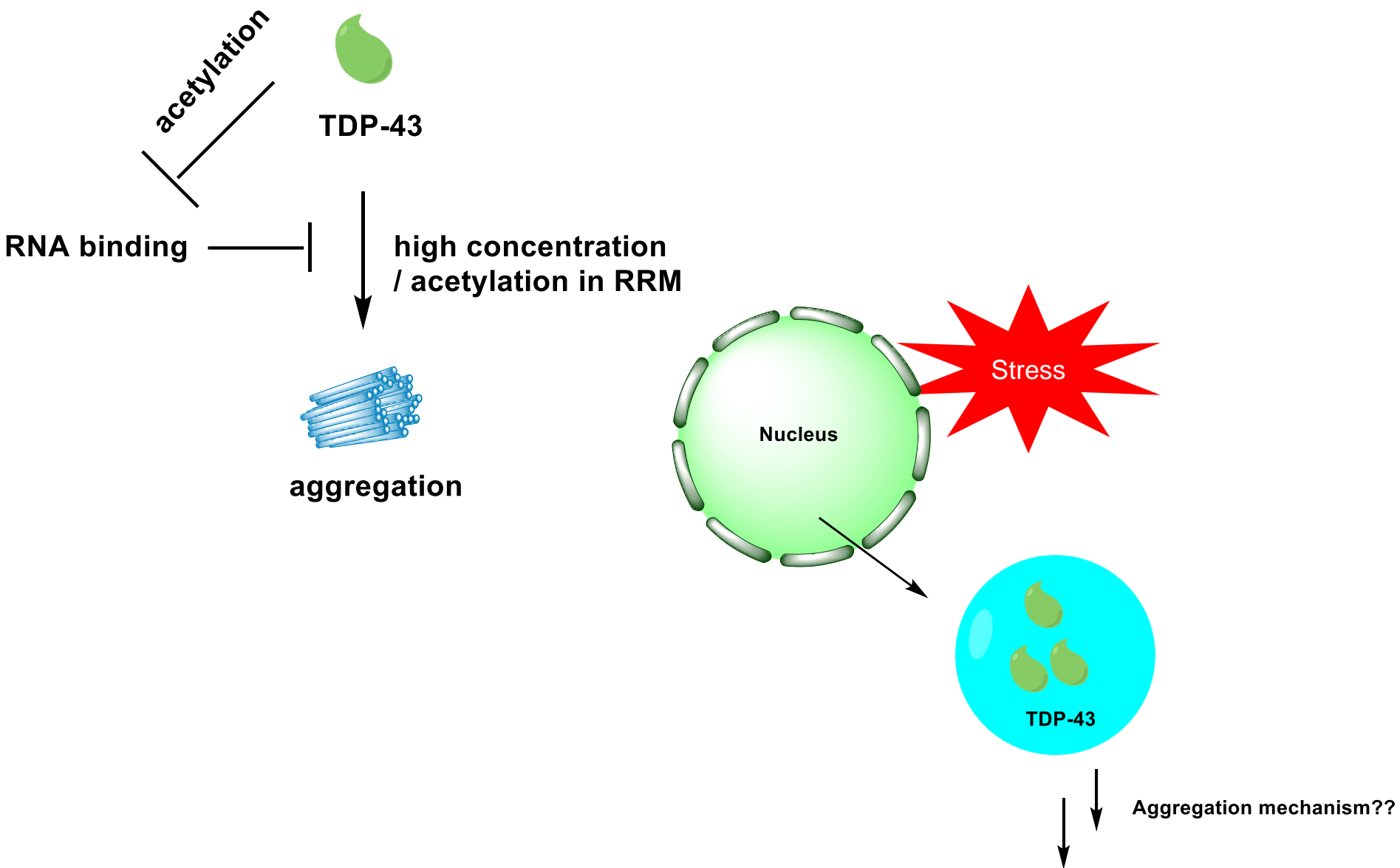
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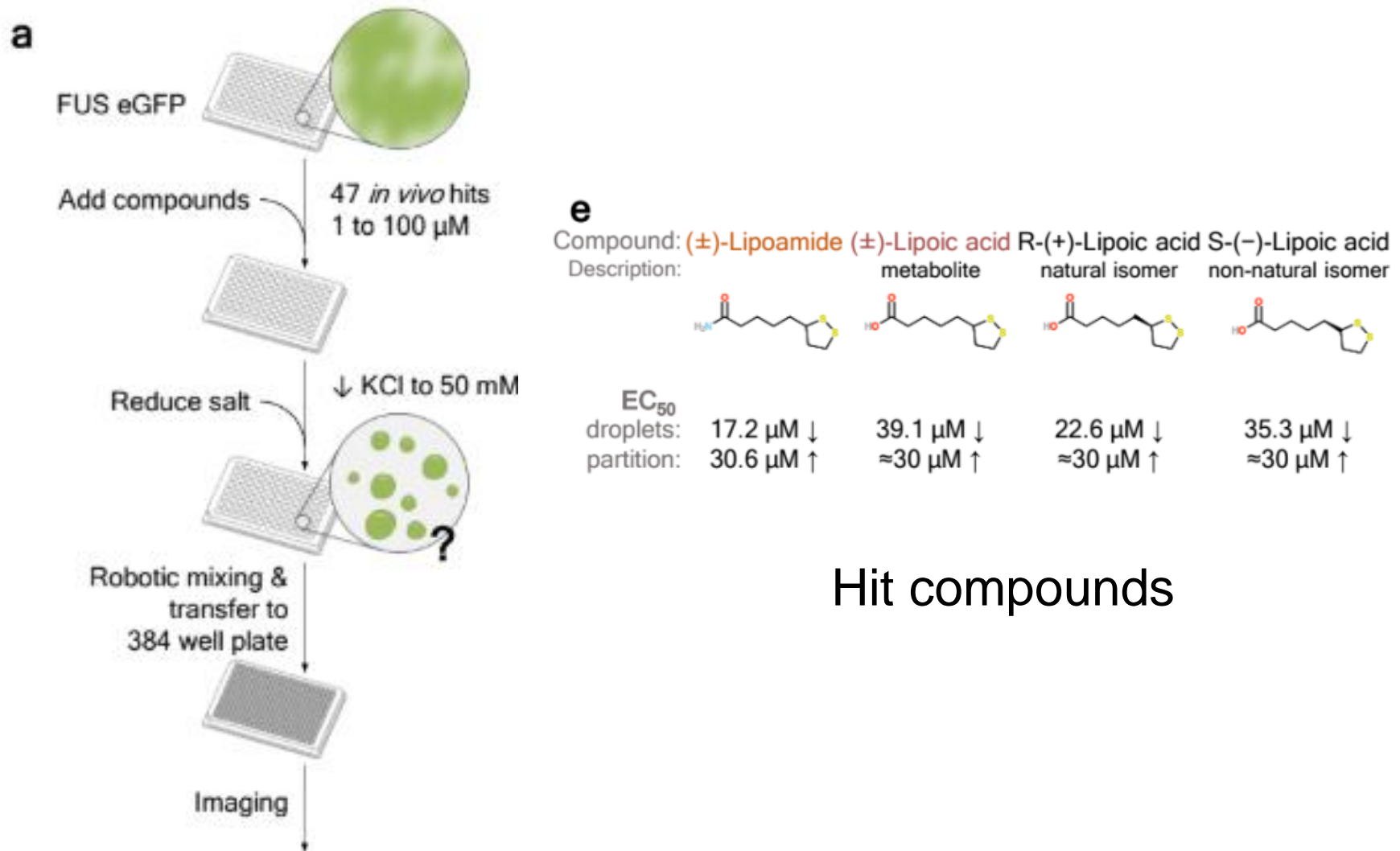- Stress Granule as ALS treatment target


- Summary

# FUS-GFP as SG marker



- Dimercaprol & emetine … reduce SGs
- FUS-GFP localizes in SGs under arsenate stress.
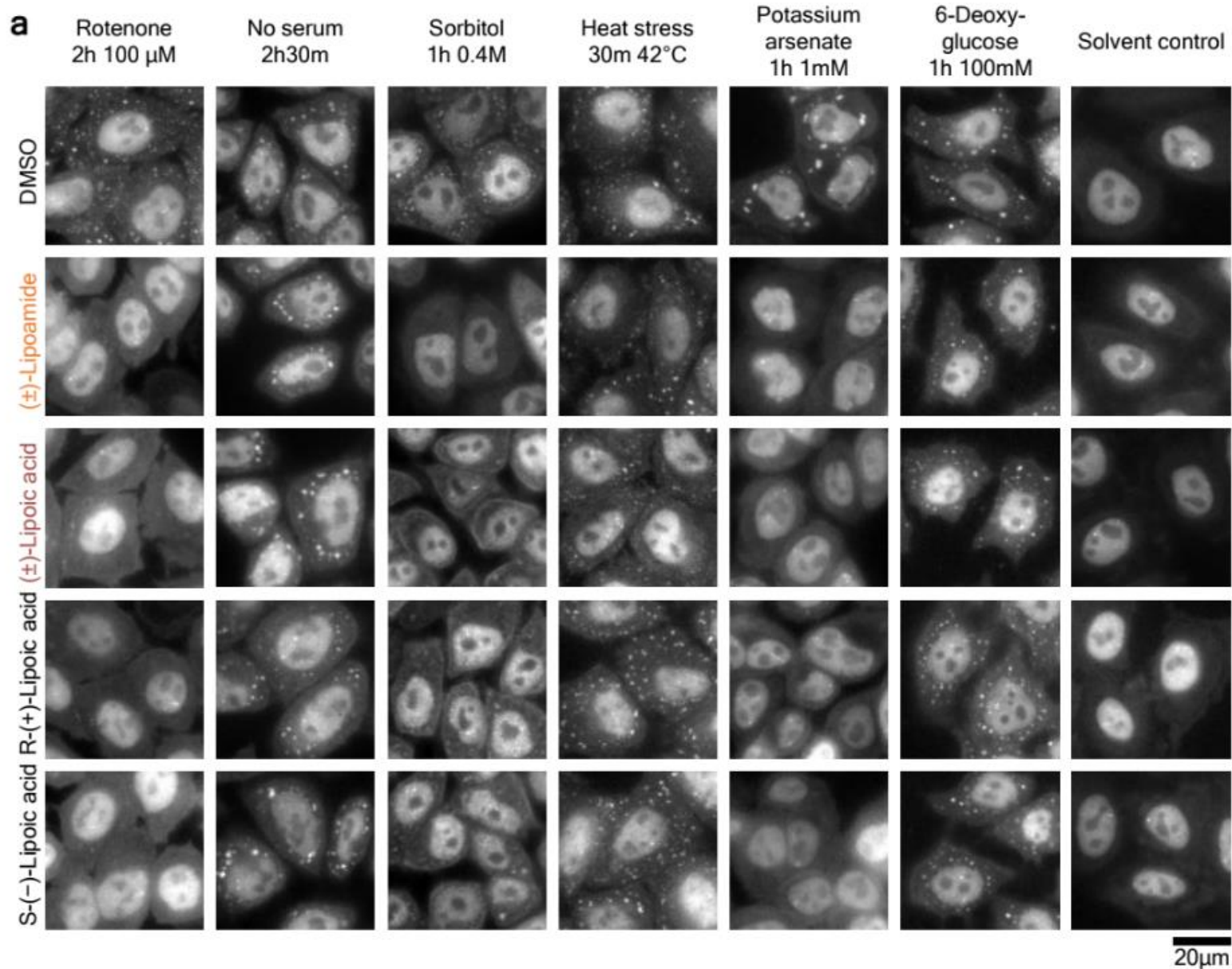-> Use of FUS-GFP as SG marker

# *In cell* screening of compounds
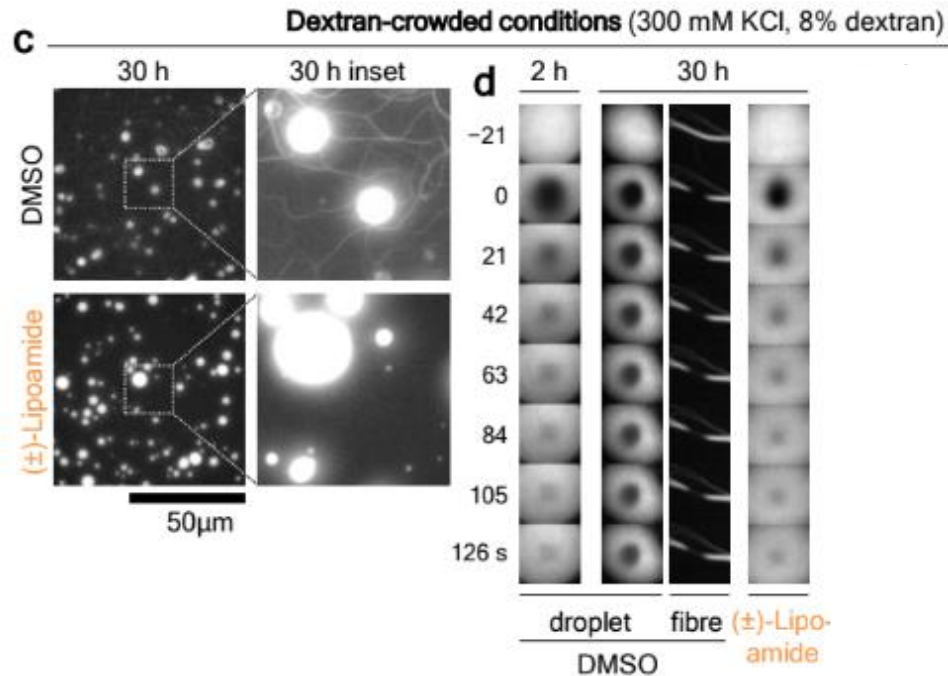


- 47 compounds hit

# *In vitro* screening of compounds



Figure 2. Screening for small compounds which affect FUS biomolecular condensate formation *in vitro.* a) Workflow for screening small molecules for effects on FUS liquid-liquid phase separation of purified FUS GFP *in vitro.* b) Ranked Z scores of change in condensate droplet number and signal

# Several SG decreased with compounds



**a** — Columns: Rotenone 2h 100 μM | No serum 2h30m | Sorbitol 1h 0.4M | Heat stress 30m 42°C | Potassium arsenate 1h 1mM | 6-Deoxy-glucose 1h 100mM | Solvent control

Rows: DMSO | (±)-Lipoamide | (±)-Lipoic acid | R-(+)-Lipoic acid | S-(−)-Lipoic acid

20μm

# Lipoamide prevent fibril formation



Dextran-crowded conditions (300 mM KCl, 8% dextran)

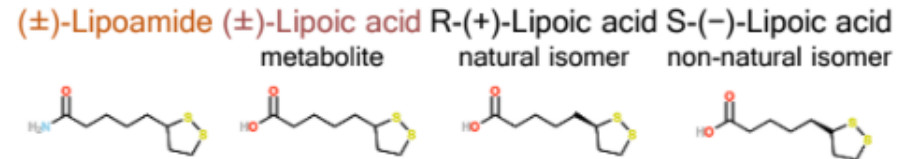- G156E FUS mutant … forms fibril
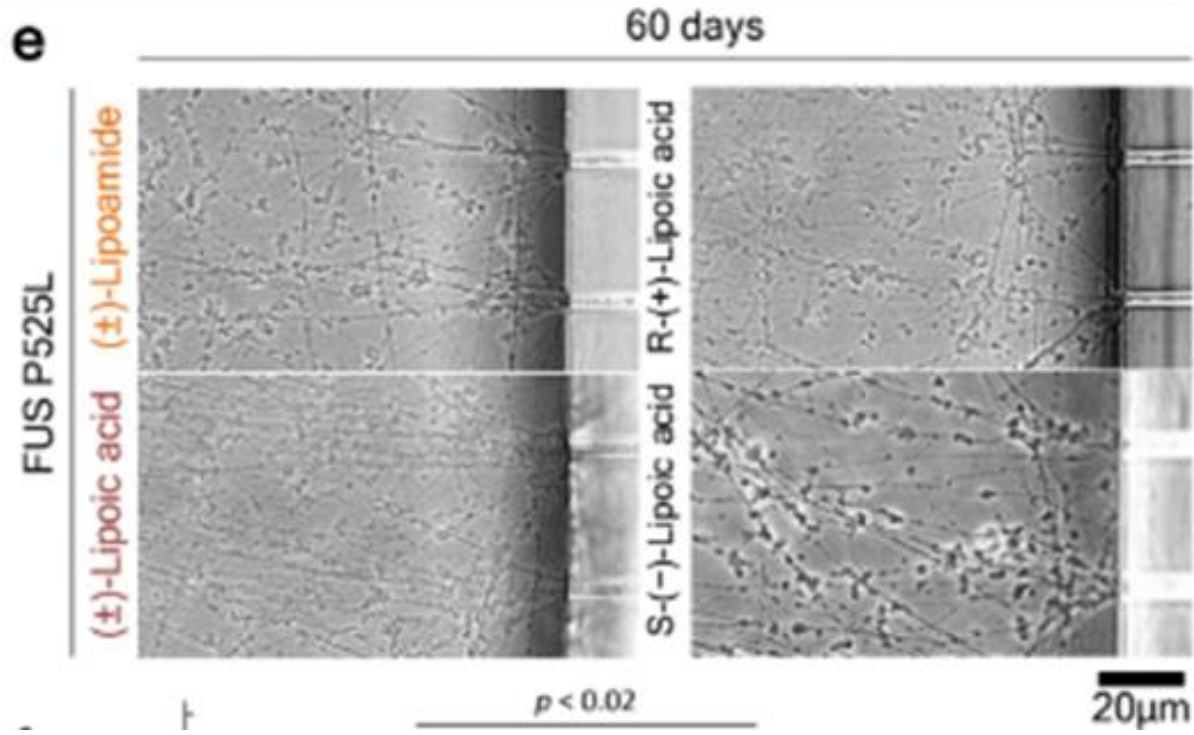- Lipoamide prevents fibril formation, too
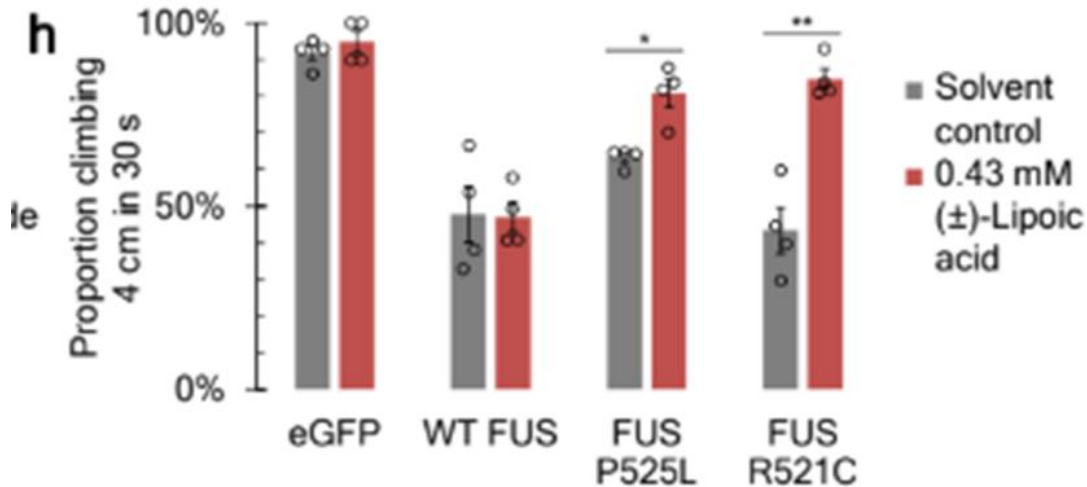- Lipoamide keeps droplet soft

# iPS-derived neuron as ALS model
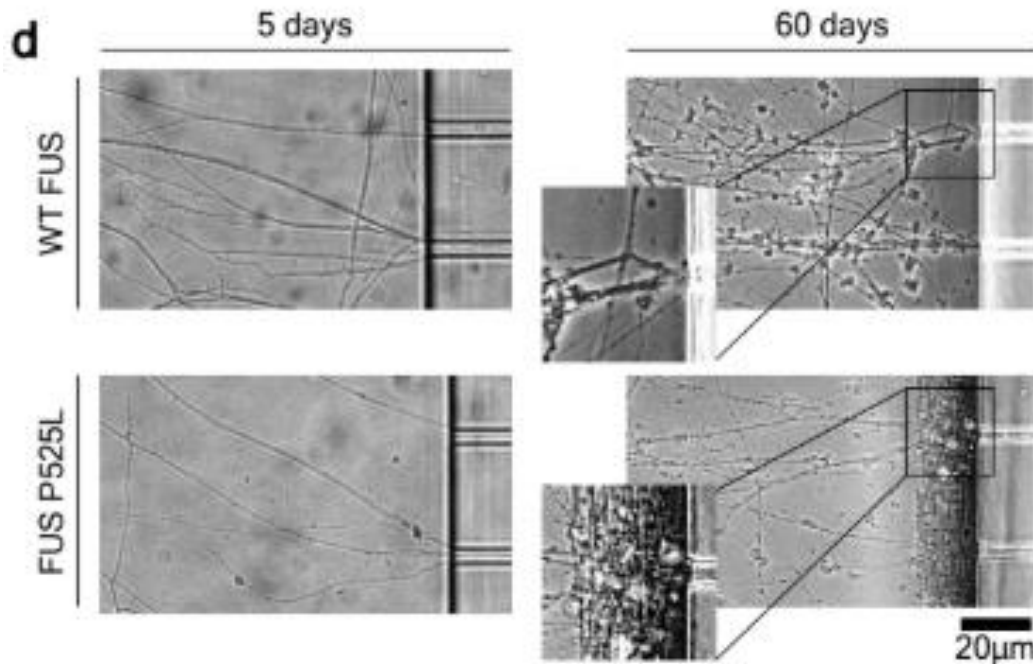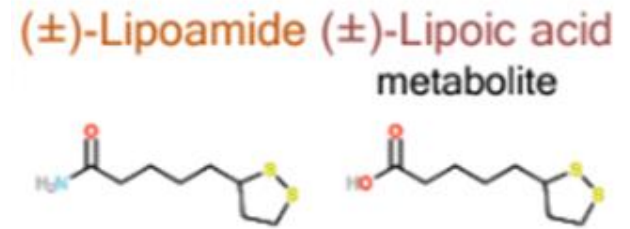


- FUS P525L mutant … ALS-associated NLS mutation
- iPS cells from ALS patient
- Material accumulation is observed in FUS P525L neuron

# Lipoamide reduces aggregation *in cell*



- Lipoamides prevented fibril

# Lipoamide recovers motor defects
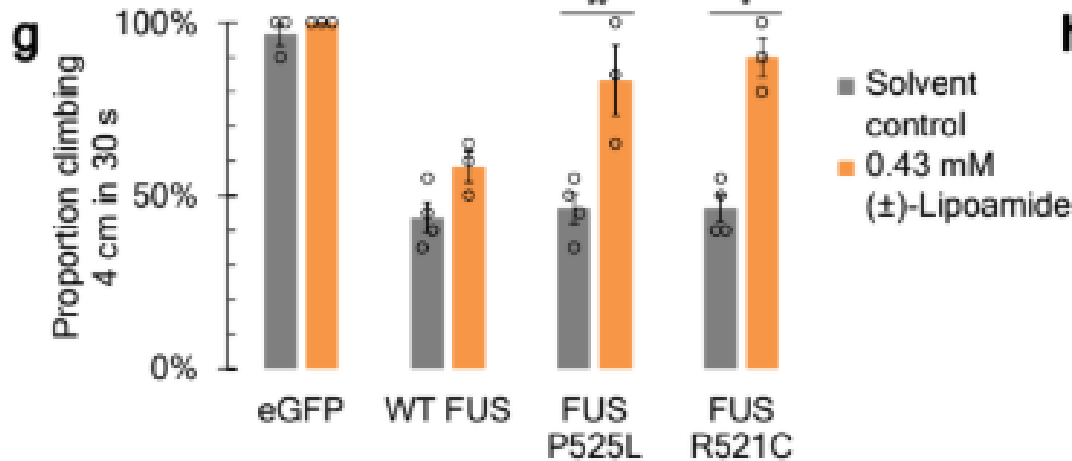


- *D. melanogaster*
- Expression of human FUS P525L/R521C
- Food supplementation with lipoamide
- Climbing ability recovered

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# Summary

- New target is necessary for ALS treatment

- TDP-43 / FUS aggregation is one of the reasons of ALS

- TDP-43 aggregation mechanism is still not uncleared

- Inhibition of SGs / protein aggregation may prevent ALS

# TDP-43 mutants

- Mutation in TDP-43 Glysine-rich domain in ALS10 patients.

- C-terminal and RRM is important for SG localization.

- TDP-43 aggregation is also found in the sporadic ALS patients



Reported mutations in TDP-43 in Familial ALS
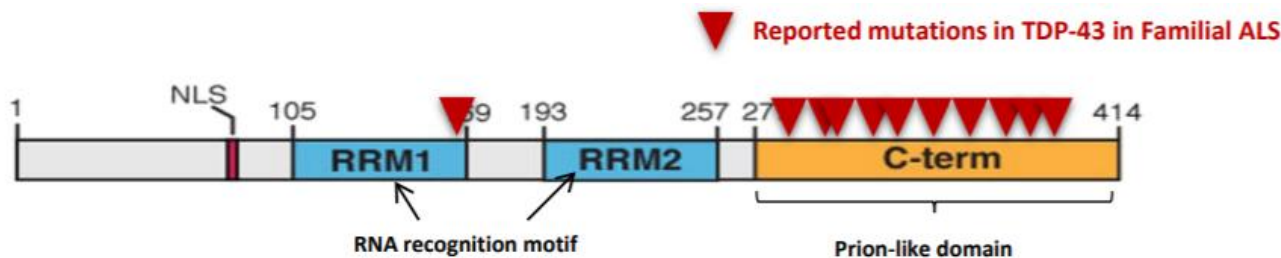
# Acetylation and stress granules
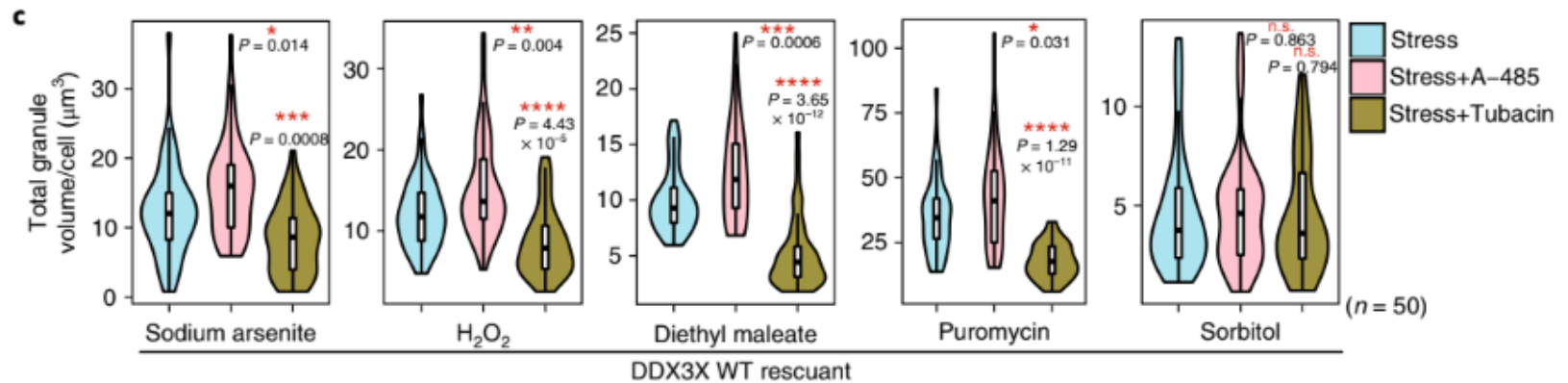


*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. **c**, DDX3X SG volume under different kinds of stress. After 3 h of treatment with 10 μM A-485 or 10 μM Tubacin, DDX3X WT rescuant was further treated with the indicated stresses (1 mM sodium arsenite for 1 h, 2 mM $H_2O_2$ for 1 h, 3 mM diethyl maleate for 1 h, 20 μg/ml puromycin for 3 h and 0.5 M sorbitol for 1 h), and localization of DDX3X was assessed by immunofluorescence microscopy. Total volume of DDX3X SGs per cell was quantified (n = 50 cells in total, from biologically independent experiments) and displayed in violin plot. P values were determined by Student's two-tailed t-test; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. **d**, Effects of CBP and HDAC6 inhibitors on SG formation.

- A-485 … HAT inhibitor
- Tubacin … HDAC6 inhibitor
- SG volume change by inhibitors

# SG formation process
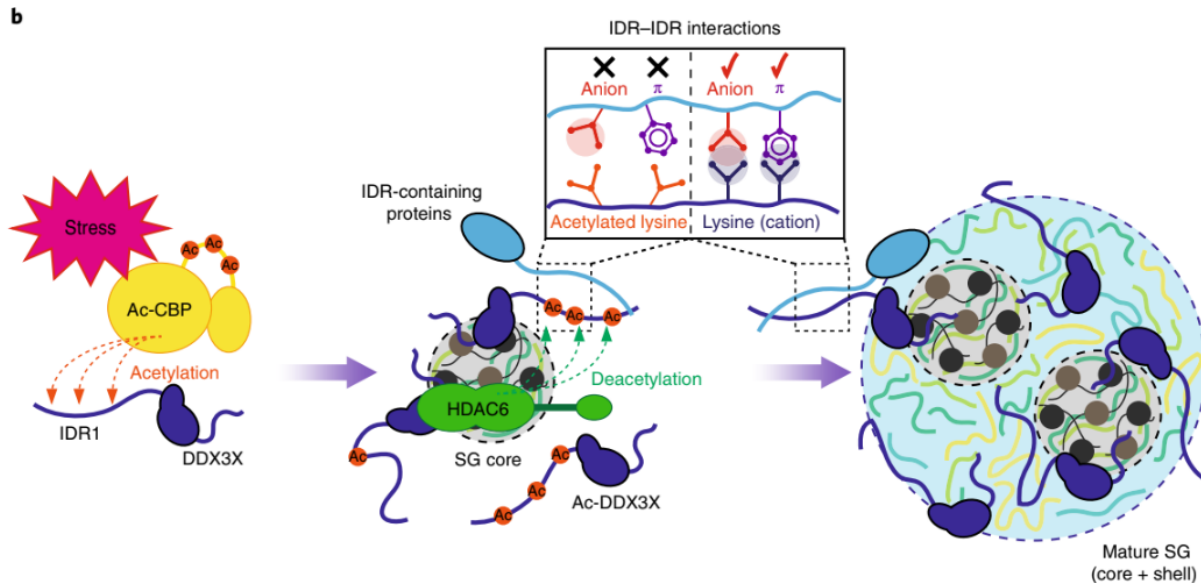


**Fig. 6 | SG maturation is promoted by deacetylation of DDX3X. a**, Simulation of SG growth curves for each construct with parameters in Supplementary Fig. 26a. The mean value of *r* for WT is arbitrarily set as 100, and the curves obtained with the best estimates of *T*, *k* and *r* are shown. The areas where each curve exists with three parameters (*T*, *k* and *r*) in the range of mean ± s.d. are also shown (WT: solid line ± gray zone, K118Q: dashed line ± orange zone and allQ: dotted line ± red zone). *T*, *k* and *r* represent the initiation time of SGs (*T*), their growth rate (*k*) and the ratio of SG area at steady state vs. *t* = 0. **b**, Proposed molecular model. Upon stress, CBP becomes activated and elicits acetylation of DDX3X-IDR1 among other proteins, thus impairing LLPS and leading to formation of small SGs. Though CBP does not localize to SGs, HDAC6 is recruited to SGs, where it deacetylates the IDR1. Positively charged lysine residues in the IDR1 contribute to cation–anion or cation–π interactions. This promotes LLPS of DDX3X and allows other IDR-containing proteins as interaction partners of DDX3X-IDR1 to engage in the formation of large mature SGs.

Stress
-> Incorporation of HDAC6 to SGs
-> Deacetylation of IDR Lysine
-> SG maturation