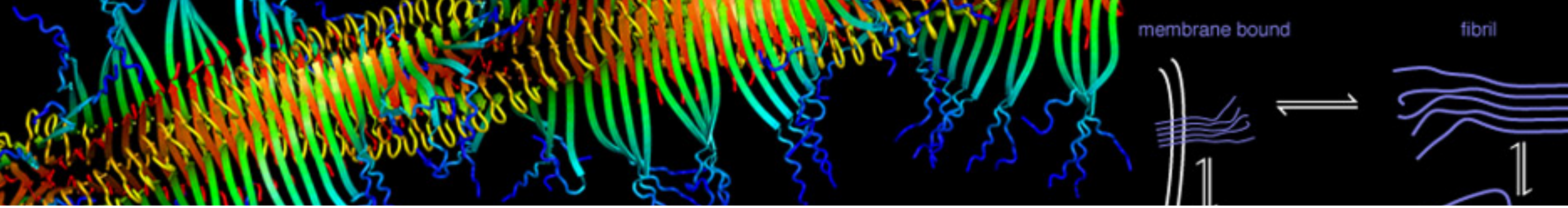
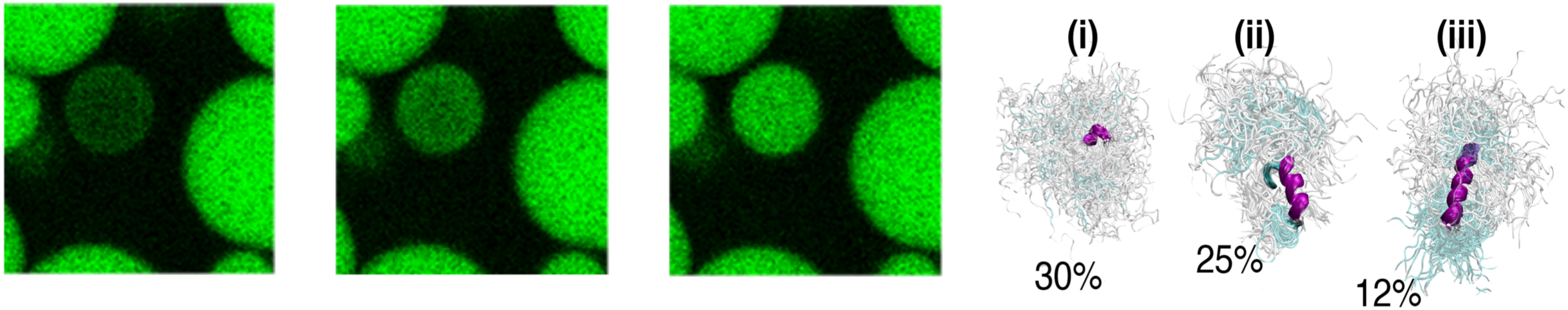



# Seeing the disordered structure of liquid-liquid phase separating RNA-binding proteins

**Nicolas Lux Fawzi**

Department of Molecular Pharmacology, Physiology and Biotechnology

Brown University

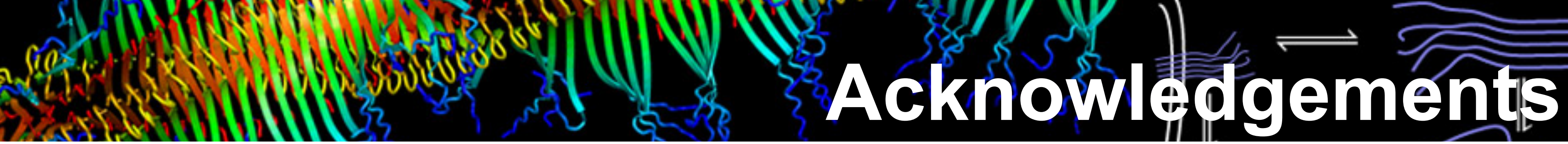



BROWN

*Order and Disorder in Biology and Human Disease*

*Rutgers University*

*February 26, 2019*



# Acknowledgements

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BROWN

C·O·B·R·E SKELETAL HEALTH AND REPAIR

NSF

***Collaborators:***
Jeanne Stachowiak - *UT Austin*
Sapun Parekh - *MPI Polymer/UT Austin*
Jeetain Mittal and Greg Dignon - *Lehigh*
Frank Shewmaker - *USUHS*
Yuh Min Chook and Mike Rosen - *UT Southwestern*
Robert Cole and Robert O'Meally - *Johns Hopkins*
Tanja Mittag and Erik Martin - *St Jude*
Yuna Ayala - *St Louis University*
H Broder Schmidt and Rajat Rohatgi - *Stanford*
Tony Hyman and Richard Wheeler - *MPI Dresden*

HFSP HUMAN FRONTIER SCIENCE PROGRAM
FUNDING FRONTIER RESEARCH INTO COMPLEX BIOLOGICAL SYSTEMS
Supported by

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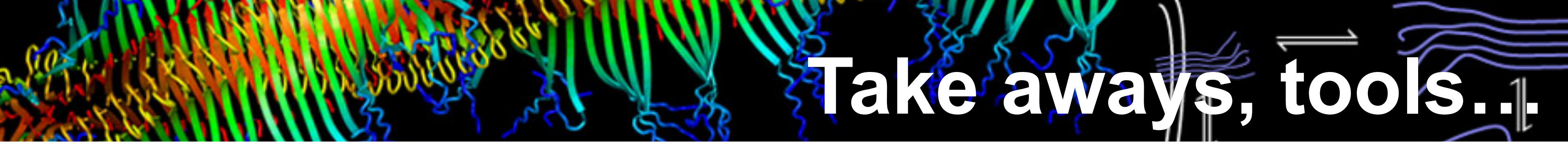
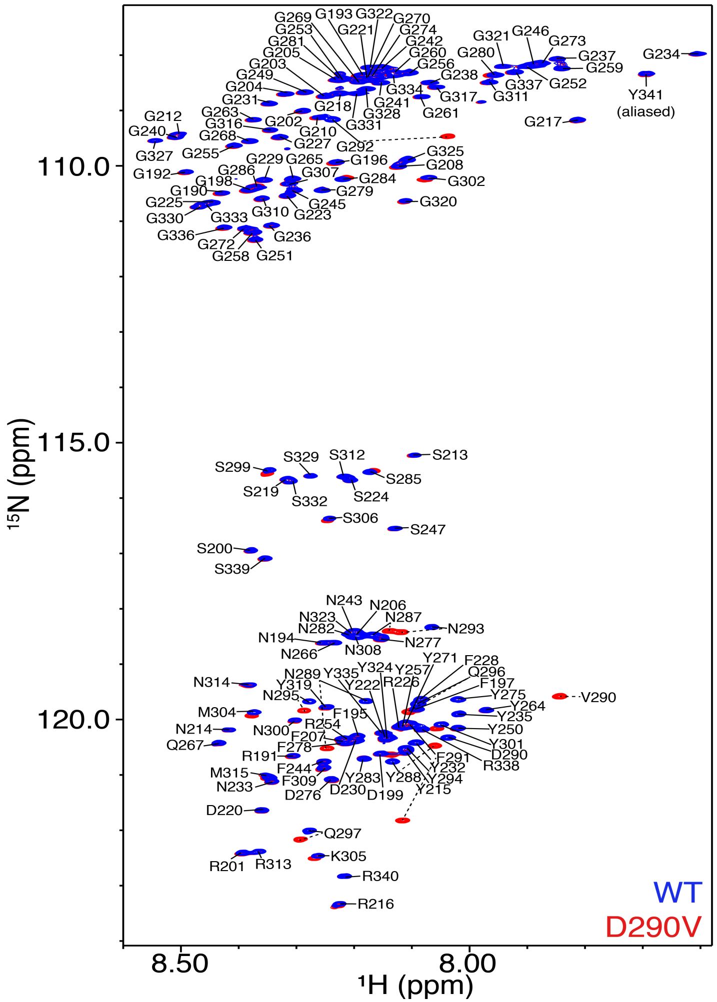


# Take aways, tools…

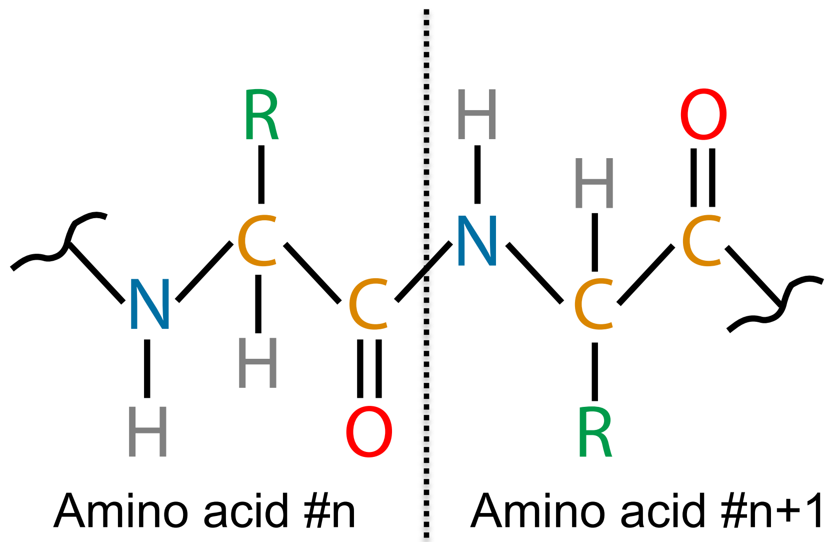
- **NMR "sees" disorder domains at atomic scale**
  - Atomic details of interactions, structure, effect of mutations and post-translational modifications

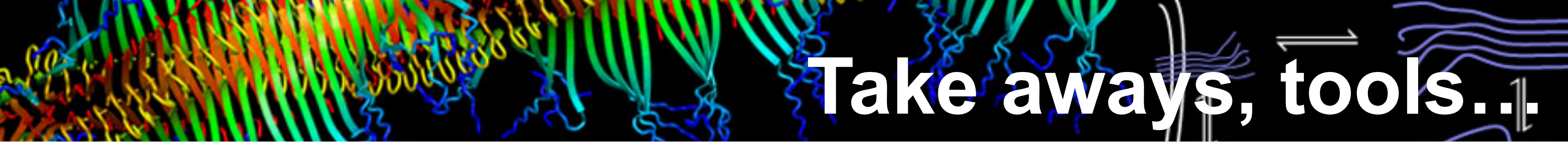



Felix Bloch (Nobel prize winner in NMR) recalling Nobel prize winning physicist Niels Bohr's description of NMR.

*You know, what these people do is really very clever. They put little spies in the molecule and send radio signals to them, and they have to radio back what they are seeing.*

From J. Mattson and M. Simon, 1996.





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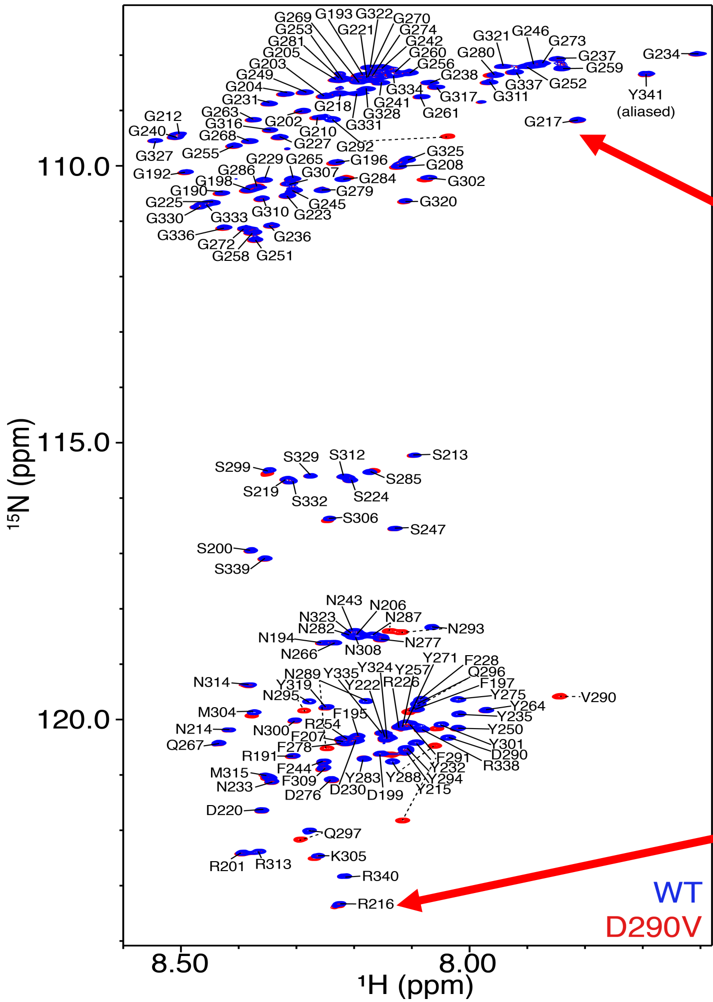


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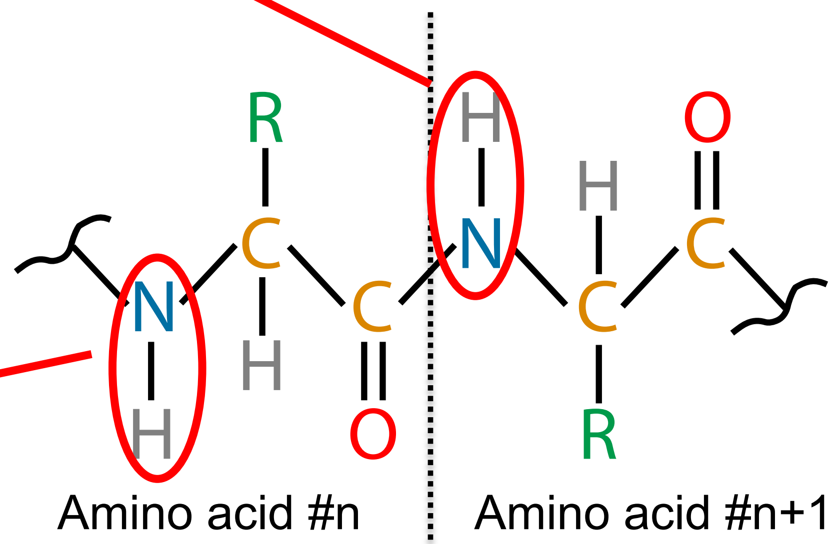

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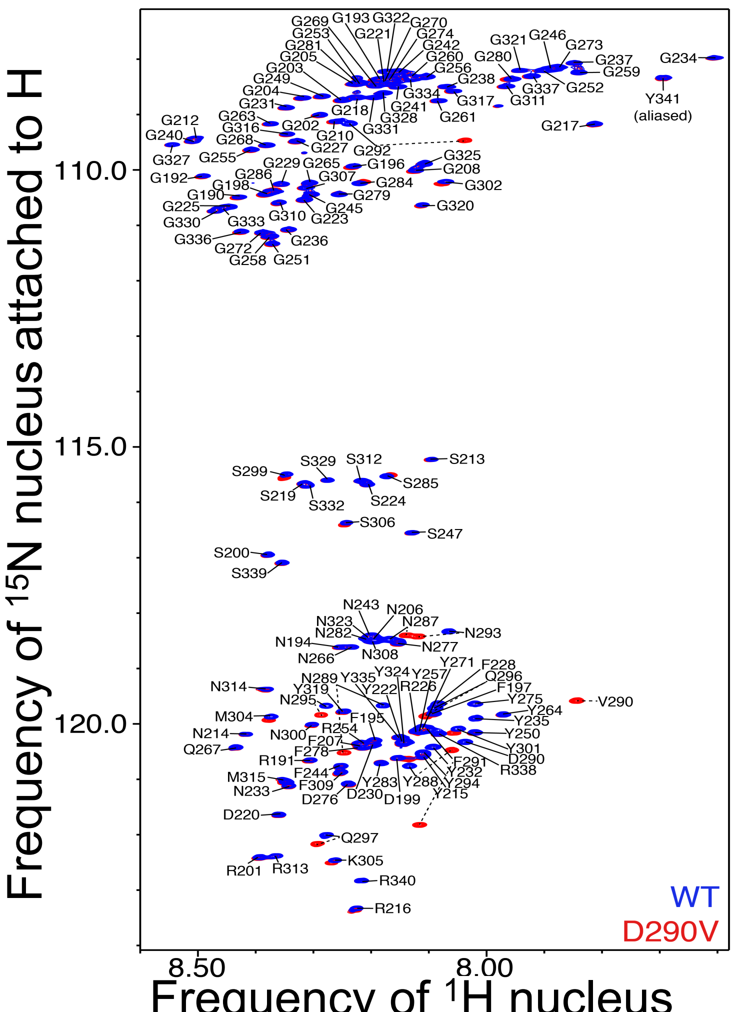
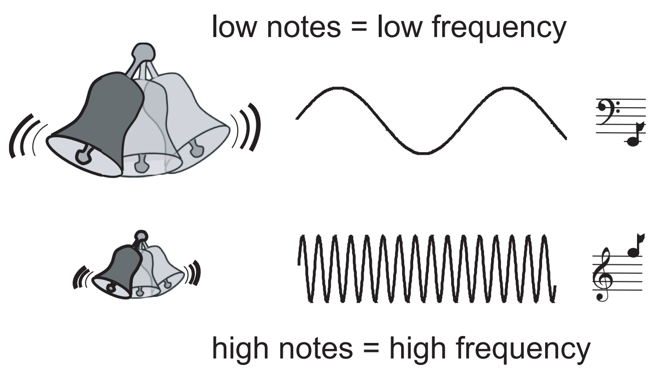
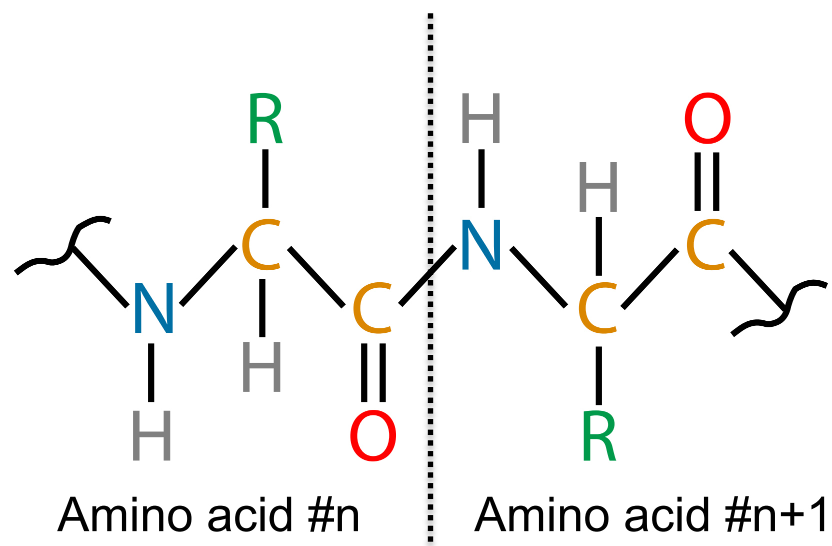
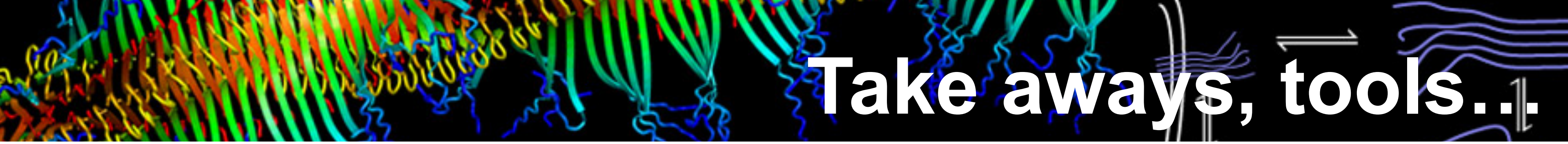



All the nitrogen nuclei are <u>distinct</u> low notes

All the hydrogen nuclei are <u>distinct</u> high notes





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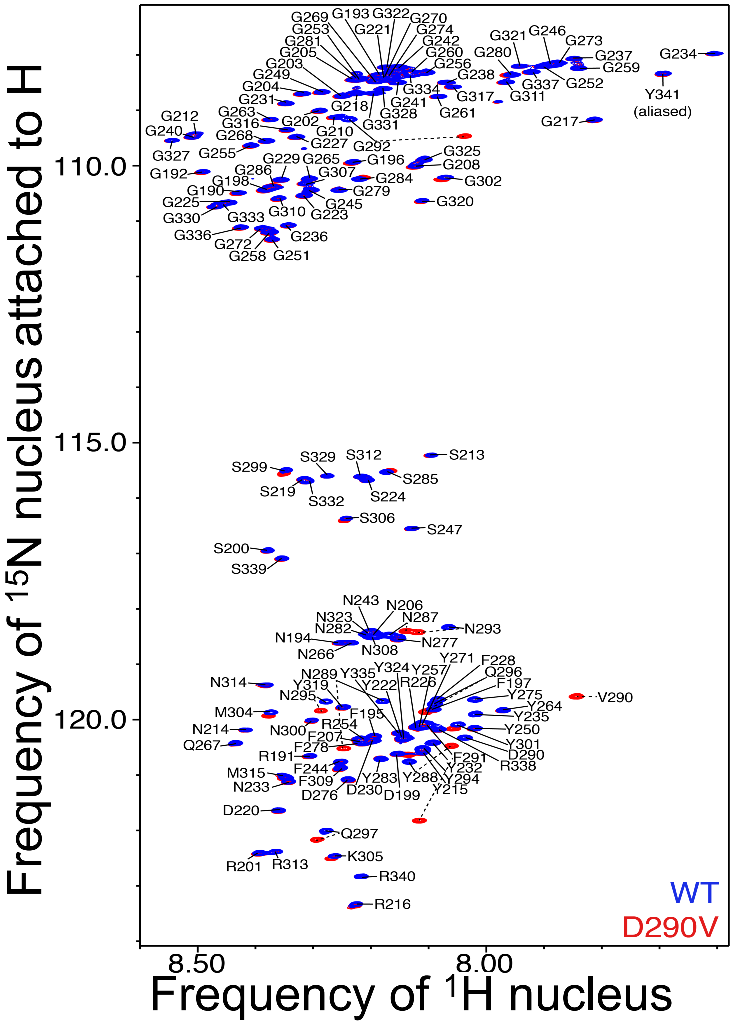
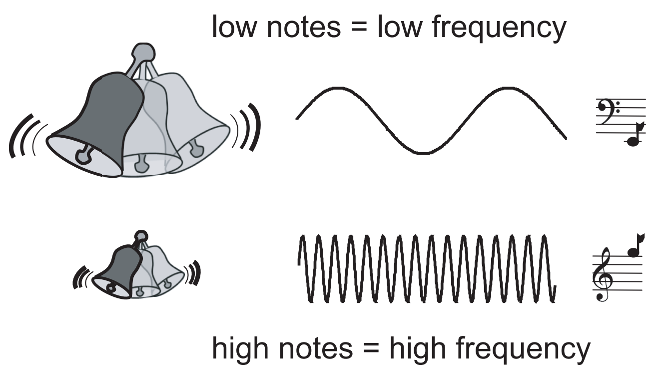
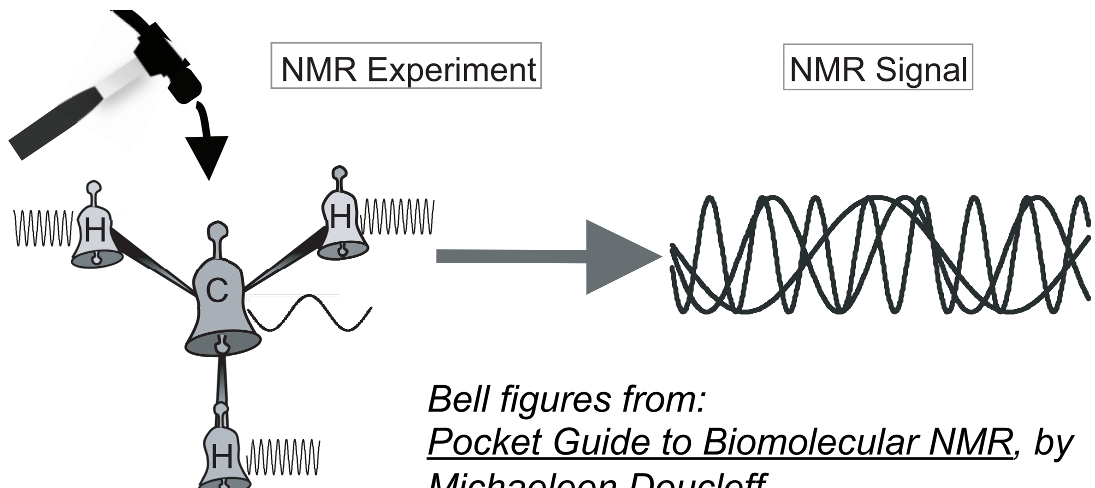


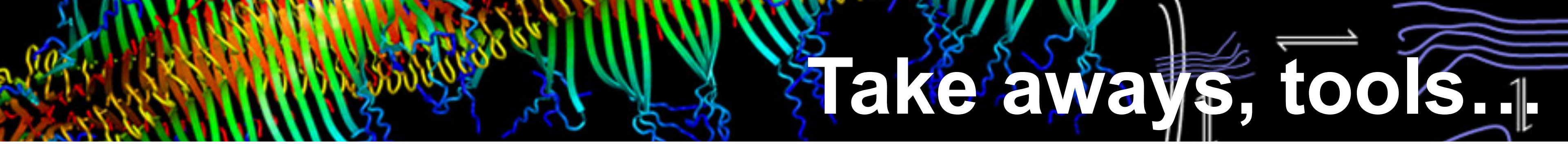

All the nitrogen nuclei are <u>distinct</u> low notes

All the hydrogen nuclei are <u>distinct</u> high notes

NMR experiment ≈ ring all the “bells” and listen for the “notes” that come back.




*Bell figures from:*
*<u>Pocket Guide to Biomolecular NMR</u>, by Michaeleen Doucleff*



# Take aways, tools…

- **NMR "sees" disorder domains at atomic scale**
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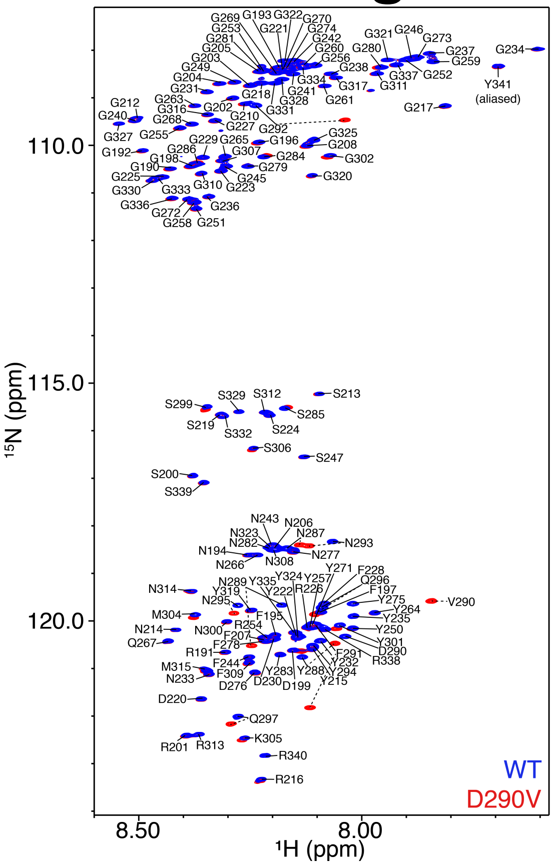
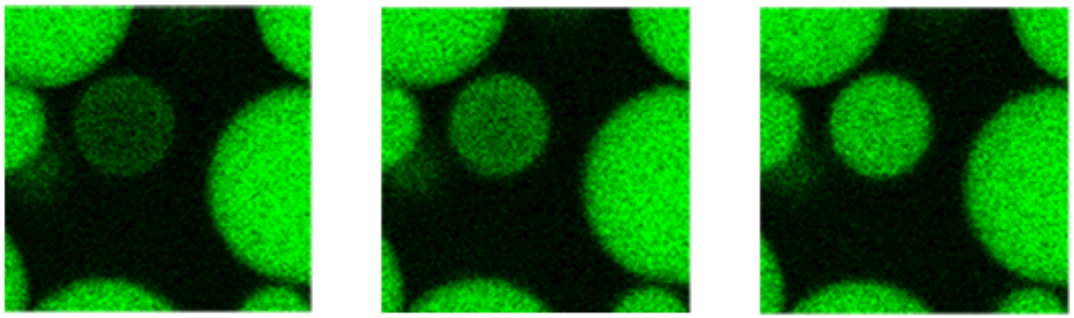
- **Combining with microscopy to see micron-scale behavior**

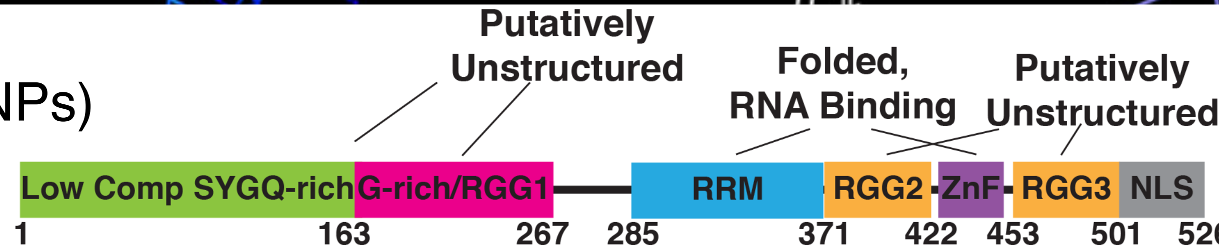


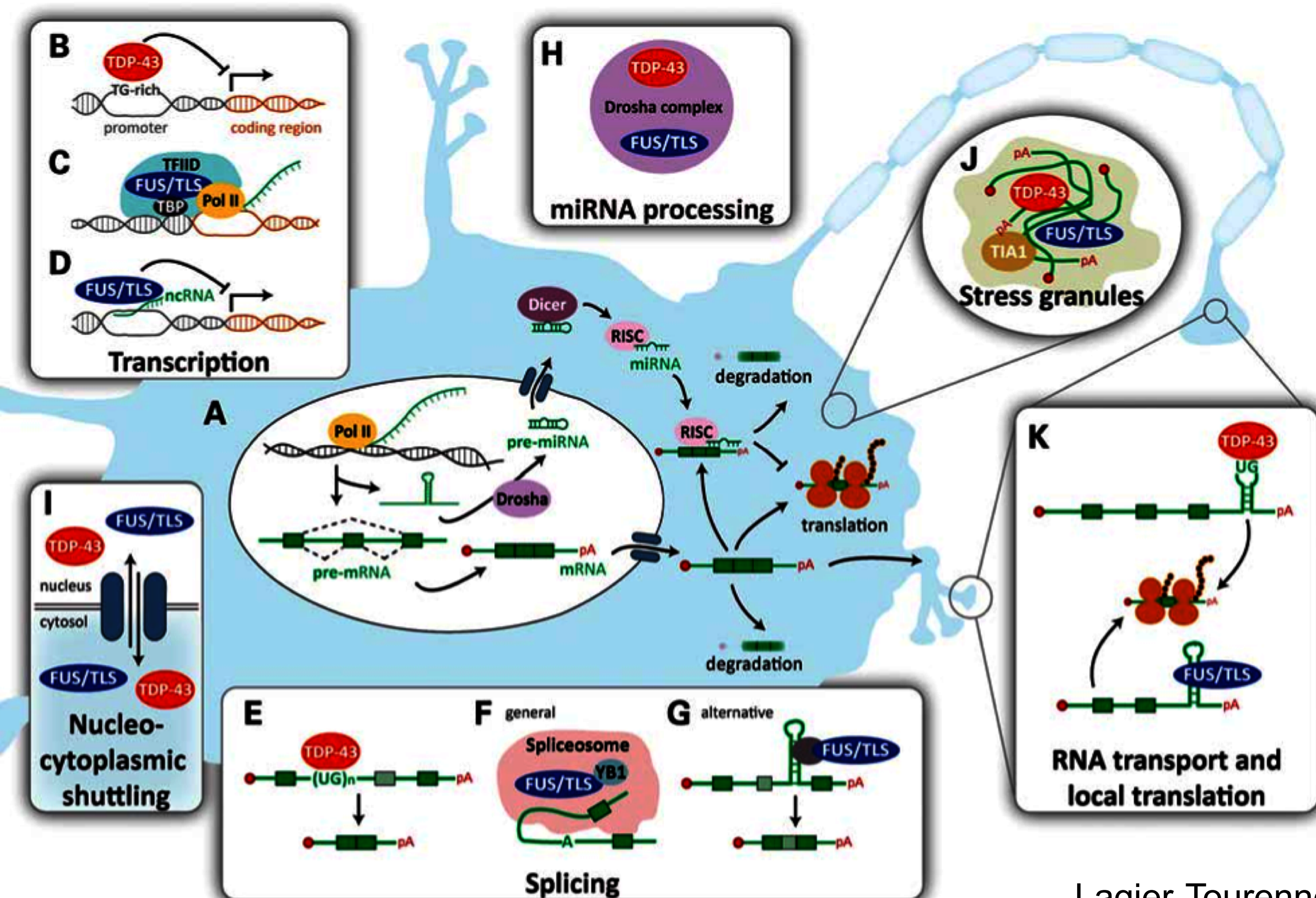

+



# RNA-binding protein - function

Heterogeneous nuclear ribonucleoprotein (hnRNPs)

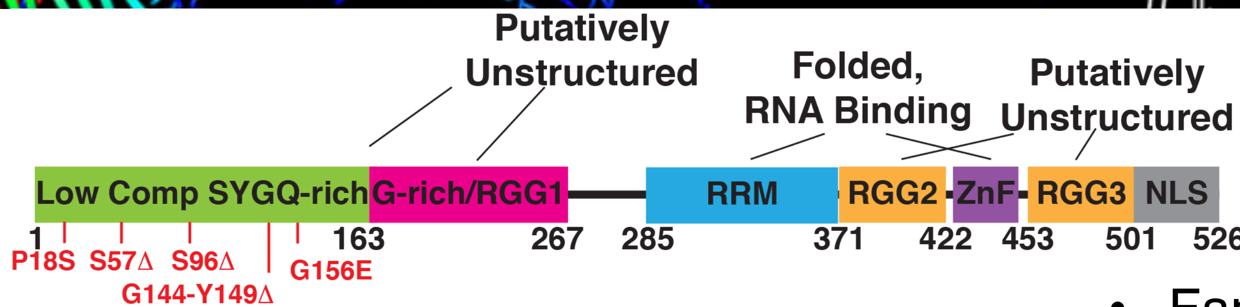



- Family of RNA-binding proteins
- Folded RNA binding domains
- Regions/domains of “low complexity” = disordered
- Nuclear/cytoplasm shuttling
- Ubiquitously expressed and in high cellular concentration

Lagier-Tourenne *et al.*, *Human Mol. Gen.*, 2010

# Fused in Sarcoma (FUS)




*Mutations associated with ALS*

- Mutations in NLS and Low complexity (LC aka SYGQ-rich) regions are associated with amyotrophic lateral sclerosis, leading to protein aggregation

- Family of RNA-binding proteins
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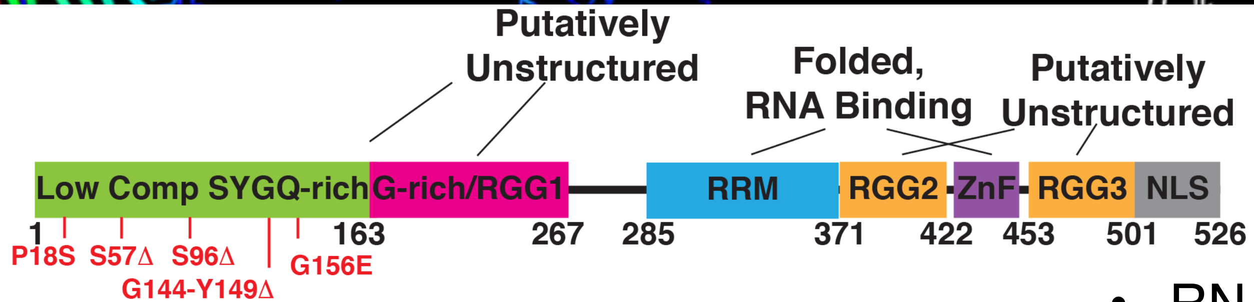
# Fused in Sarcoma (FUS)

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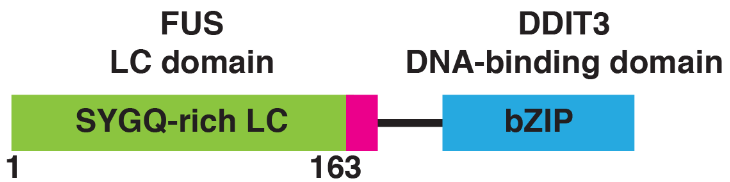
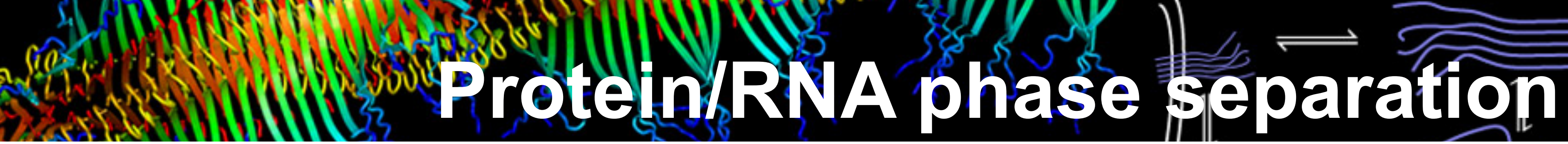



***Fusion associated with sarcoma***

- Fusions of FUS LC (and paralogs TAF15 and EWS) with any of a dozen DNA-binding domains → sarcoma and leukemia

- RNA binding domains
- Low complexity putatively disordered domains
- Nuclear/cytoplasm shuttling
- Cellular [FUS]

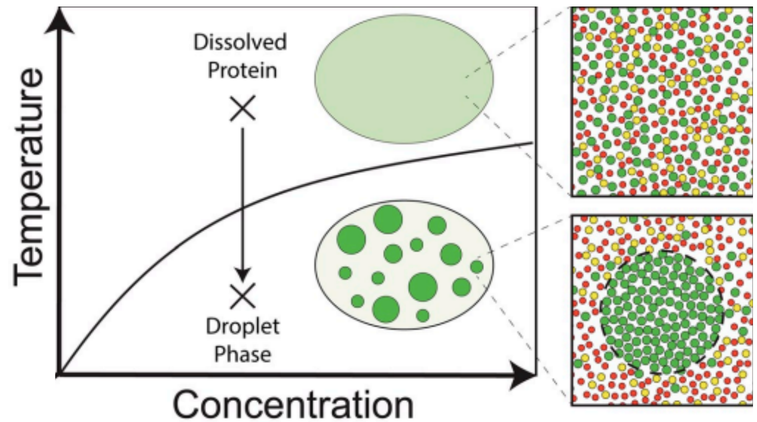
  > 2 μM



# Protein/RNA phase separation

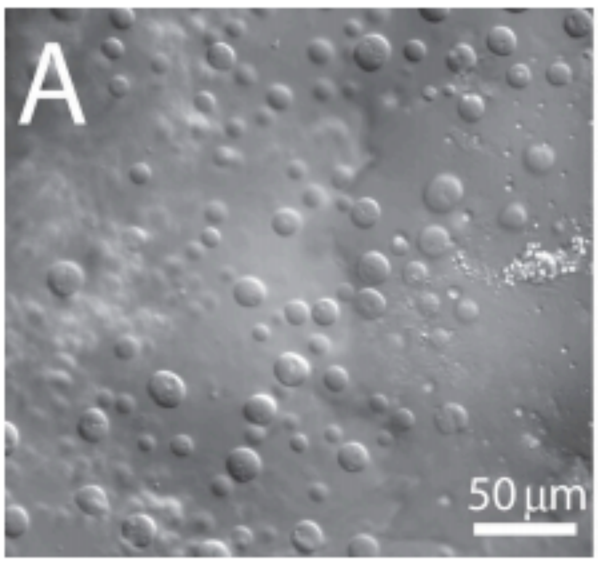
**Membraneless organelles**

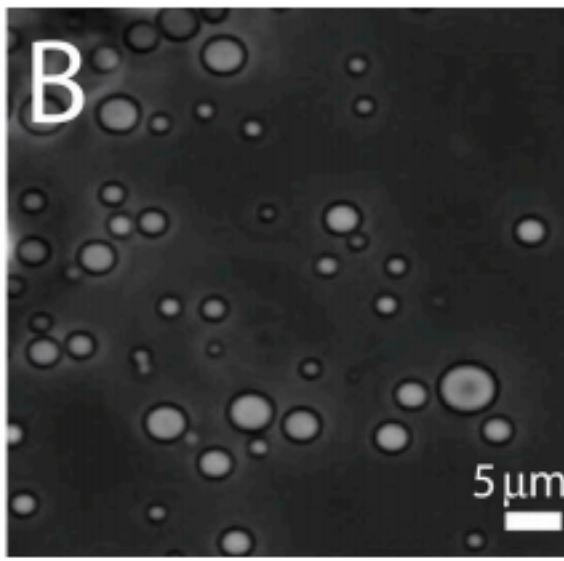
- Non-membrane bound granules or “droplets”
- Nuclear: Nucleoli, Gems, Cajal bodies
- Cytoplasm: Stress granule, processing body
- Flow and fuse and retain spherical shape




JCB Review: Brangwynne JCB (2013)

Molliex et al. Cell (2015)



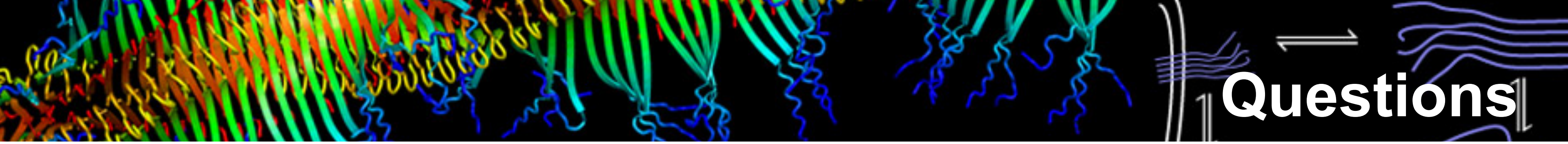




Stress Granules have Liquid Droplet Properties

**Nucleoli (and other RNP granules) within the nucleus of an X. laevis oocyte**

**In vitro myelin basic protein**

**Stress granules in cells**



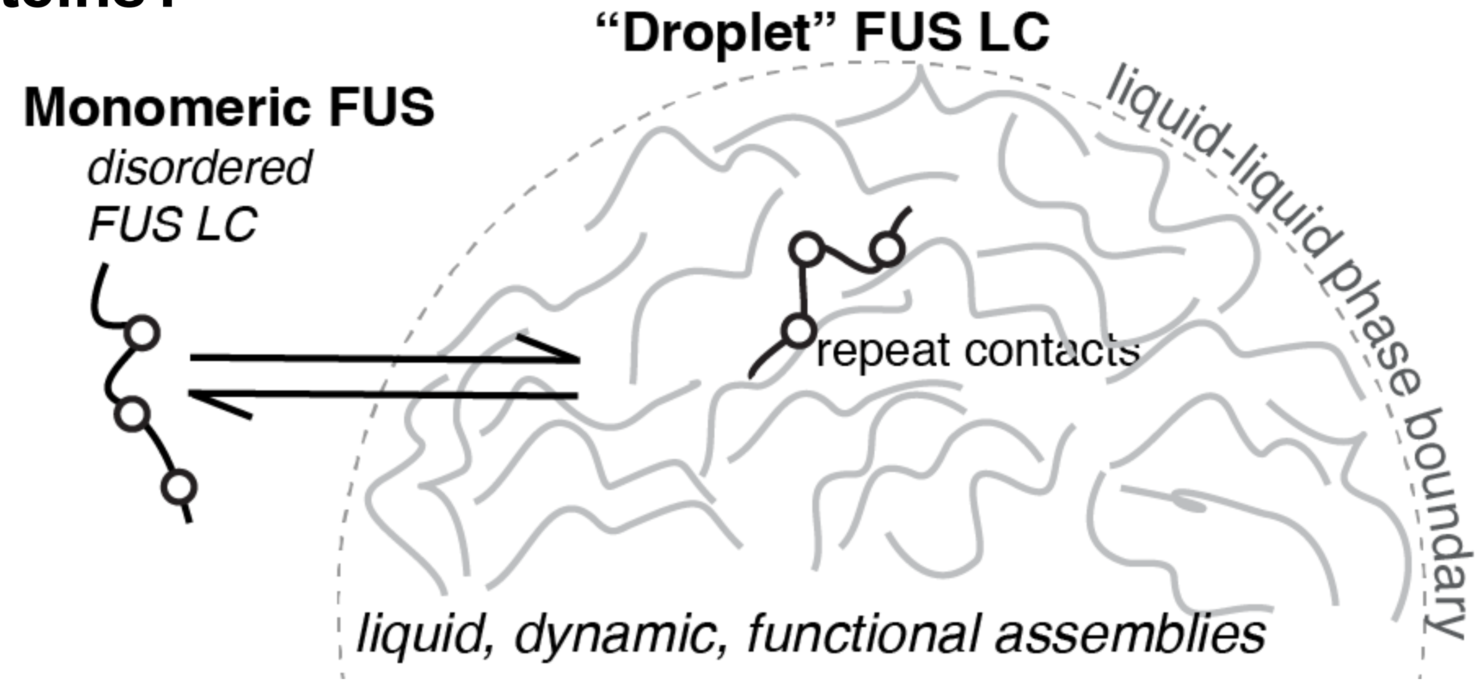
# Questions

**What is the structure of the low complexity domain of FUS?**

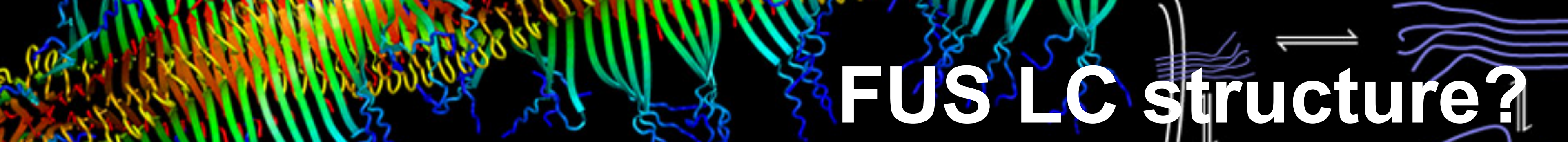
as a monomer in free solution?

upon liquid-liquid phase separation?

**Does FUS phase separation recruit other low complexity proteins?**




**What regulates biological phase separation?**



# FUS LC structure?

M A S N D Y T Q Q A T Q
S Y G A Y P T Q P G Q
G Y S Q Q S S Q P Y G Q Q
S Y S
G Y S Q S T D T S
G Y G Q S
S Y S
S Y G Q S Q N T
G Y G T Q S T P Q
G Y G S T G
G Y G S S Q S S Q S
S Y G Q Q S S Y P
G Y G Q Q P A P S S T S G
S Y G S S S Q S S
S Y G Q P Q S G
S Y S Q Q P S Y G G Q Q Q
S Y G Q Q Q S Y N P P Q
G Y G Q Q N Q Y N S

**163 residues, unusual sequence**

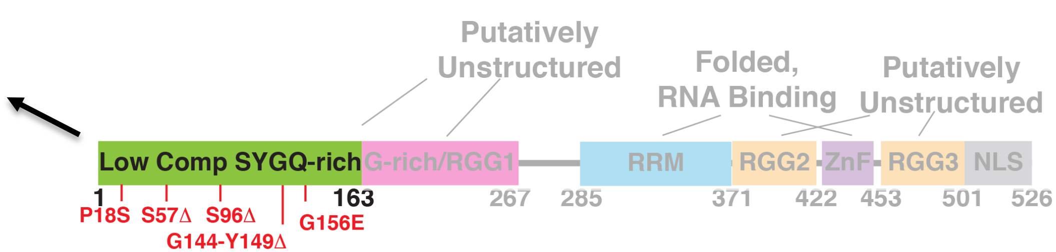
Rich in amino acids:

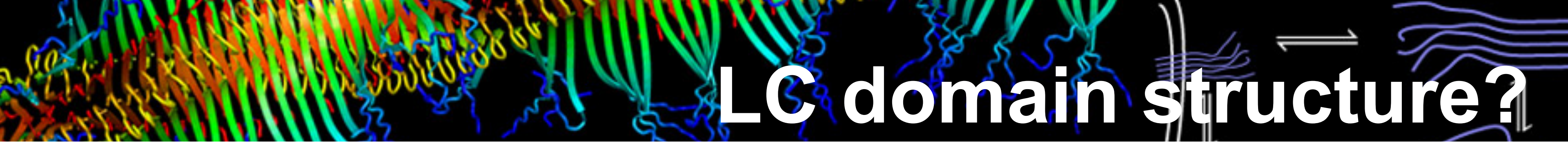
- serine (S)
- tyrosine (Y)
- glycine (G)
- glutamine (Q)

Lacking other amino acids:

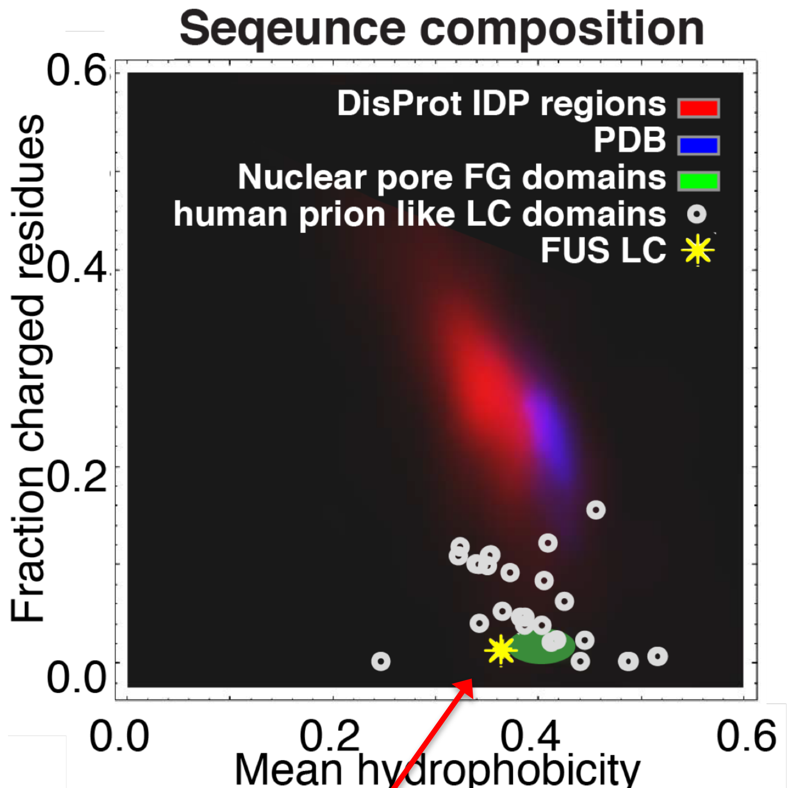
- Positively charged (none present)
- Negatively charged (only 2)
- Aliphatic (leucine, isoleucine, alanine almost entirely absent)

**What structure does FUS LC form?**





# LC domain structure?




Adapted from: Schmidt and Görlich, *Elife* 2015

**"Prion-like" Low complexity (LC) domains: unusual sequences**

Rich in amino acids:

- serine (S)
- tyrosine (Y)
- glycine (G)
- glutamine (Q)

Lacking other amino acids:

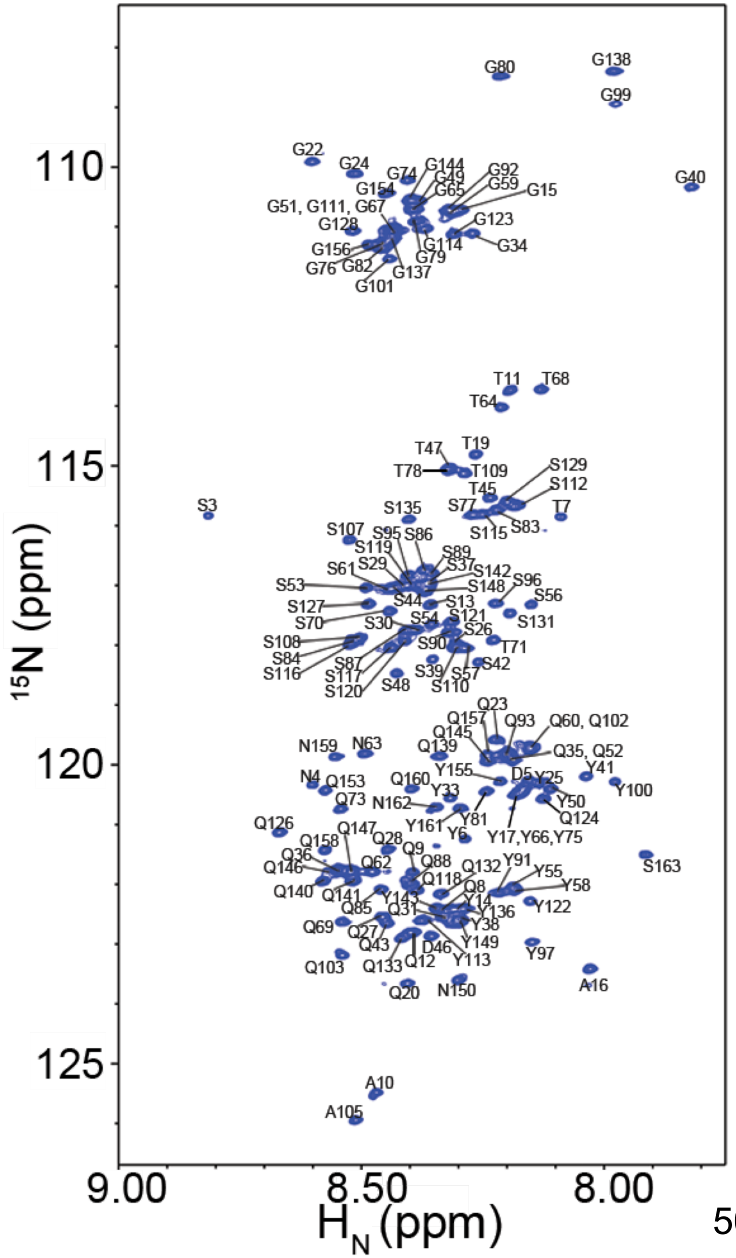
- Positively charged (none present)
- Negatively charged (only 2)
- Aliphatic (leucine, isoleucine, alanine almost entirely absent)

**What structure does FUS LC form?**

***Prion-like domains:***

- **occupy an unusual part of protein sequence universe**
- **resemble FG Nups in nuclear pore domains? (But no Phe)**

# FUS LC is unstructured




**NMR of dispersed FUS LC**

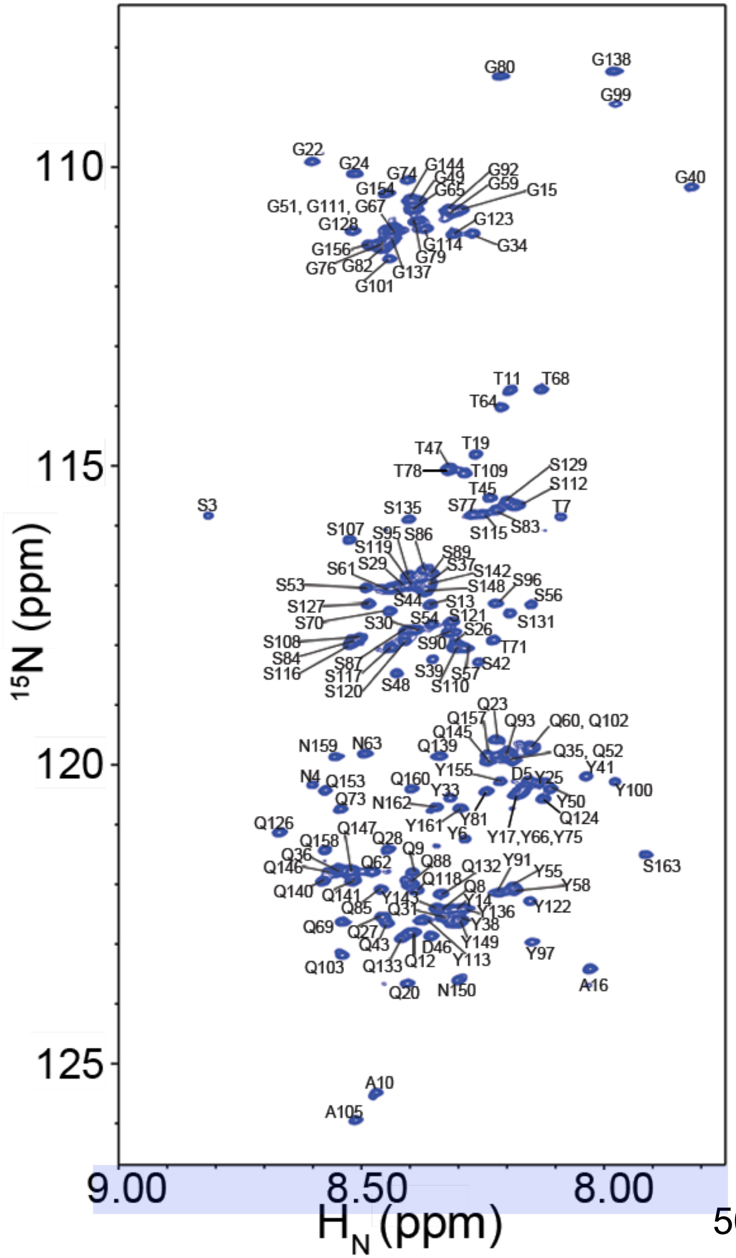
- Atomic resolution NMR
- Narrow range of amide $^1$H shifts characteristic of disordered protein

Each dot here is a peak that represents a signal from a single NH backbone position in the FUS LC protein sequence

From the distribution of peak positions this we see that **FUS LC is unstructured**

50 µM FUS LC in 50 mM MES/BisTris 150mM NaCl pH 5.5 @ 25 °C

# FUS LC is unstructured




*FUS LC structural work by Kathleen Burke*

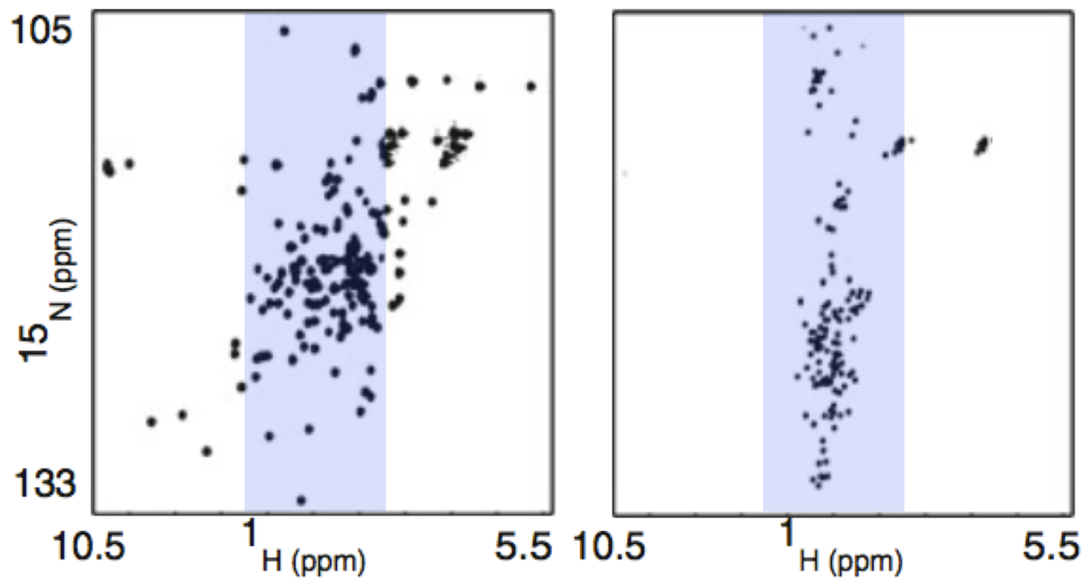
Burke KA et al. *Mol Cell* (2015)

## NMR of dispersed FUS LC

- characteristic of disordered protein

***Reference proteins:***

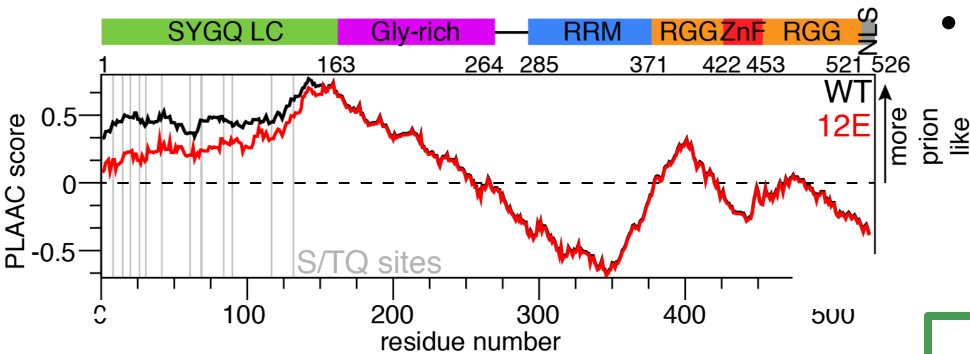
Folded Disordered



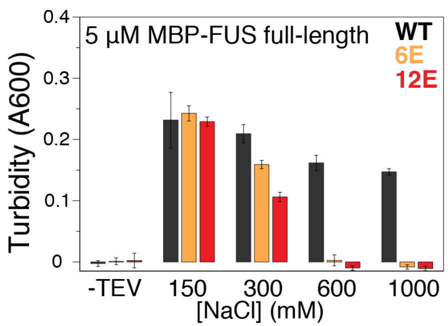

*Pocket Guide to Biomolecular NMR, by Michaeleen Doucleff*

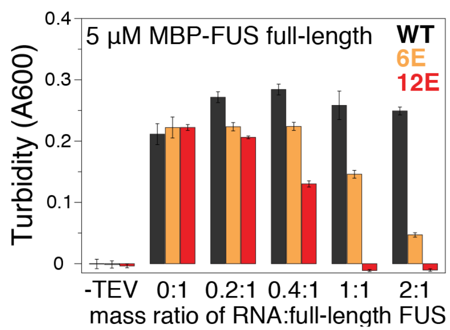
50 μM FUS LC in 50 mM MES/BisTris 150mM NaCl pH 5.5 @ 25 °C

# IDR phosphorylation modifies assembly



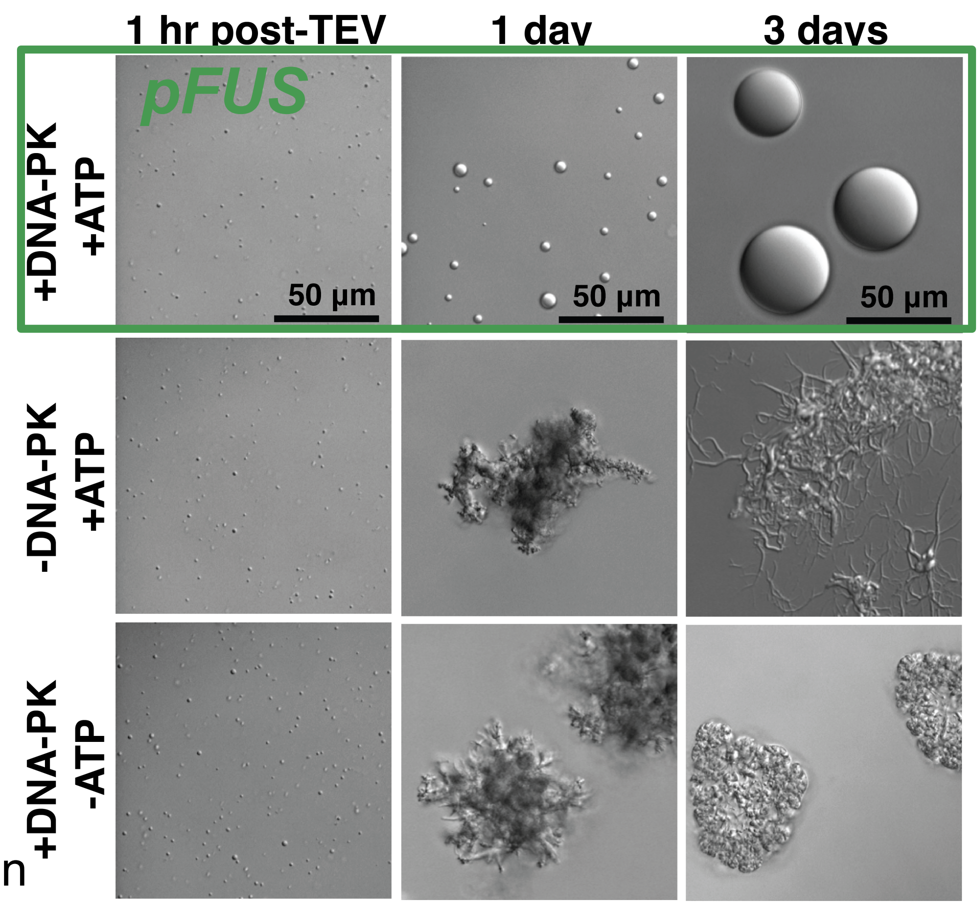

FUS LC phosphorylation/ phosphomimetics reduce “prion-like” character of FUS LC domain



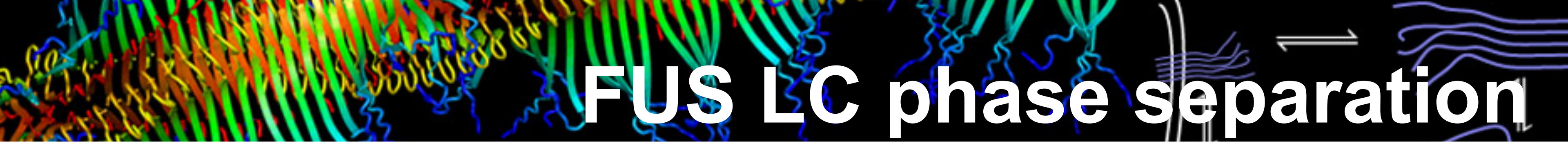

Phosphomimetics disrupt phase separation **when RNA present or with high salt**

- Disrupts conversion of phase separated **full-length FUS** into aggregates (following cleavage of MBP solubility tag with TEV protease)

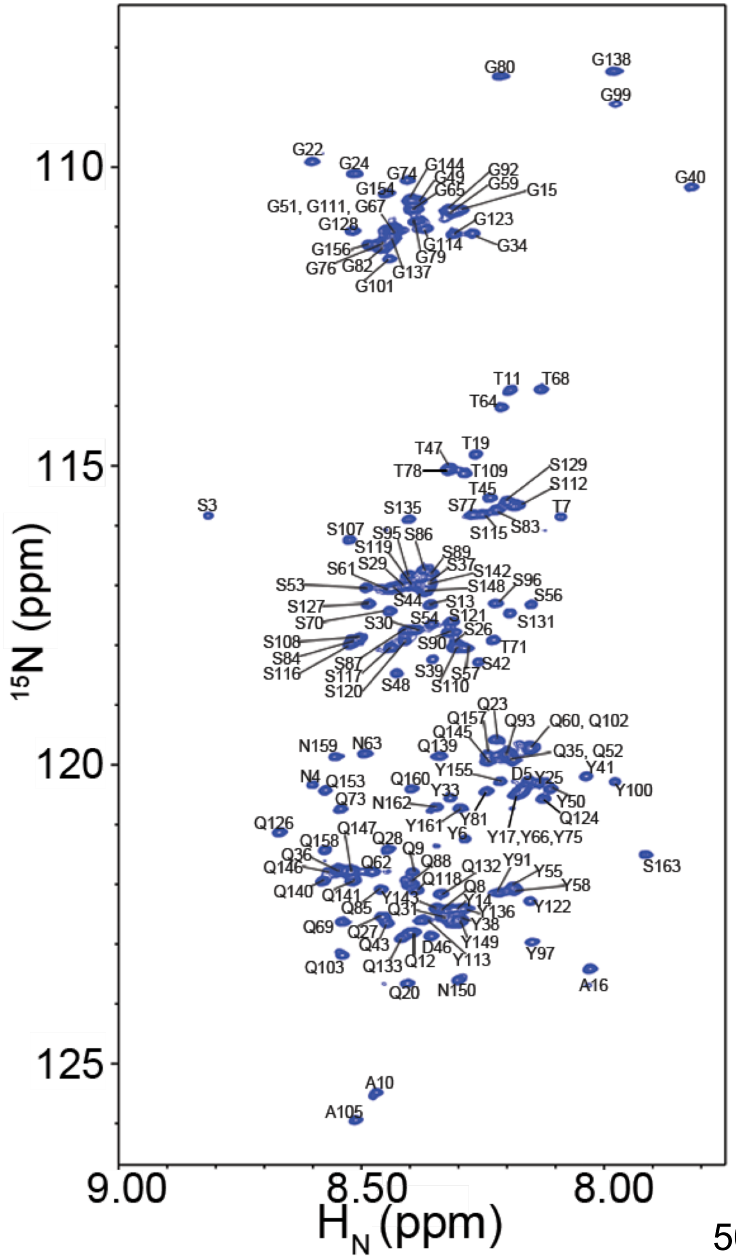


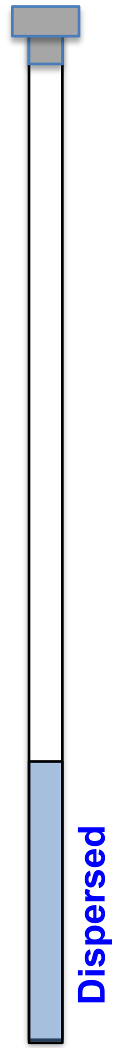

Monahan Z* Ryan VH* et al *EMBO Journal* (2017)

# FUS LC phase separation



**What is the structure of FUS LC inside a "droplet"?**




50 µM FUS LC in 50 mM MES/BisTris 150mM NaCl pH 5.5 @ 25 °C

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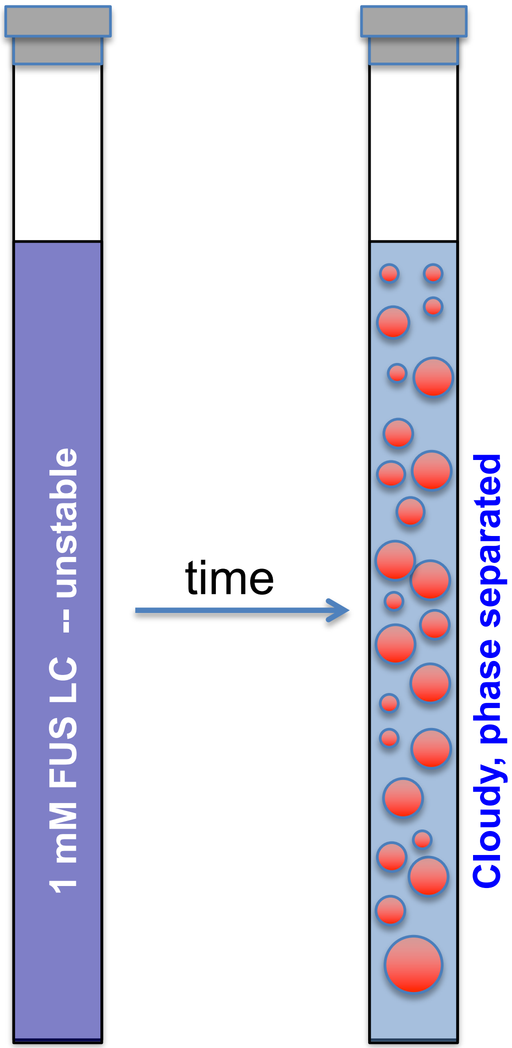

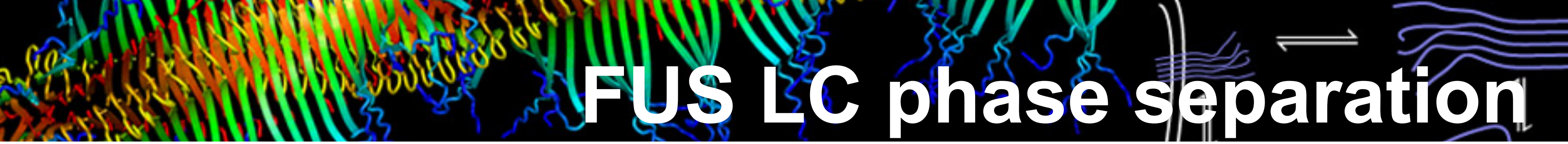

# FUS LC phase separation

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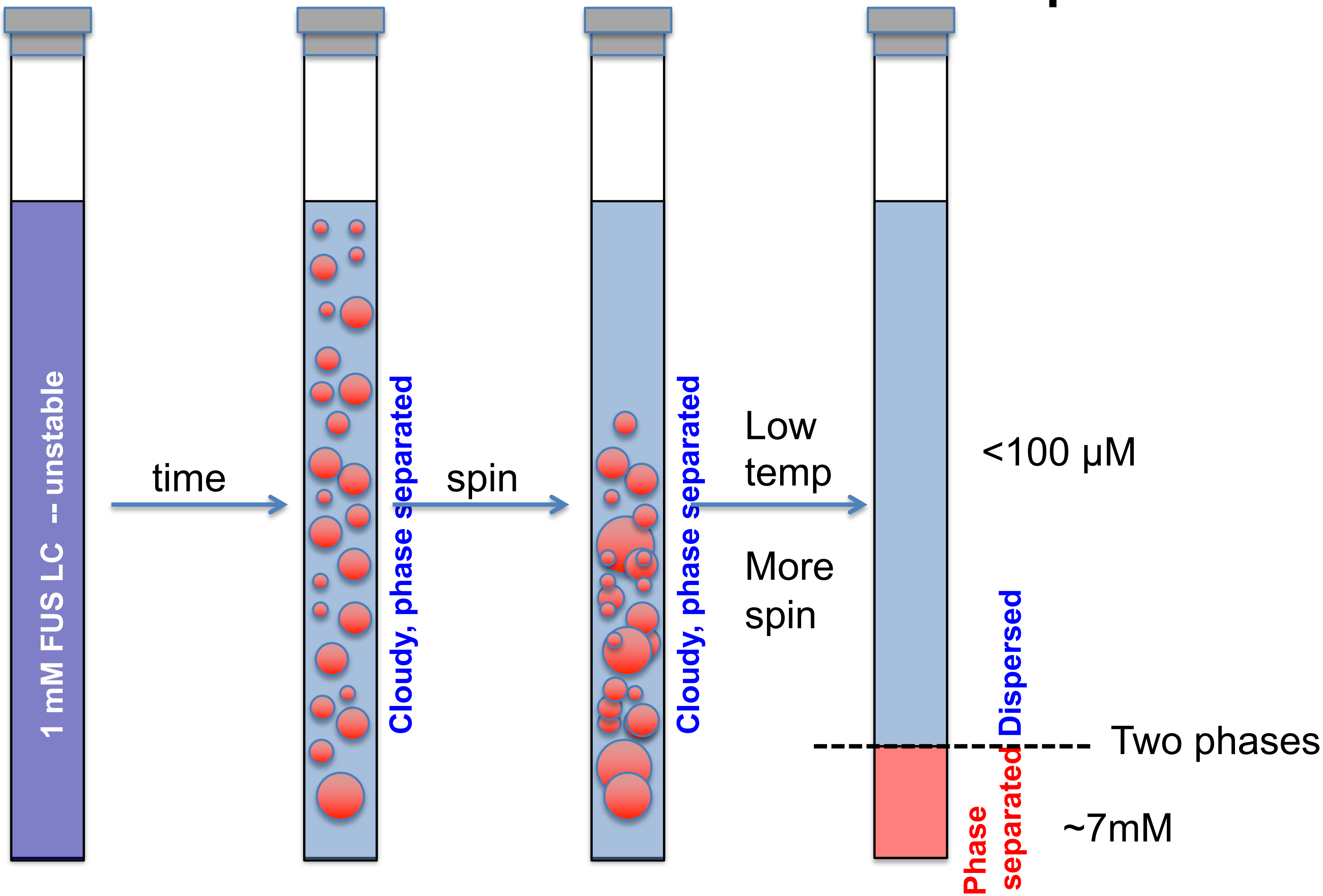
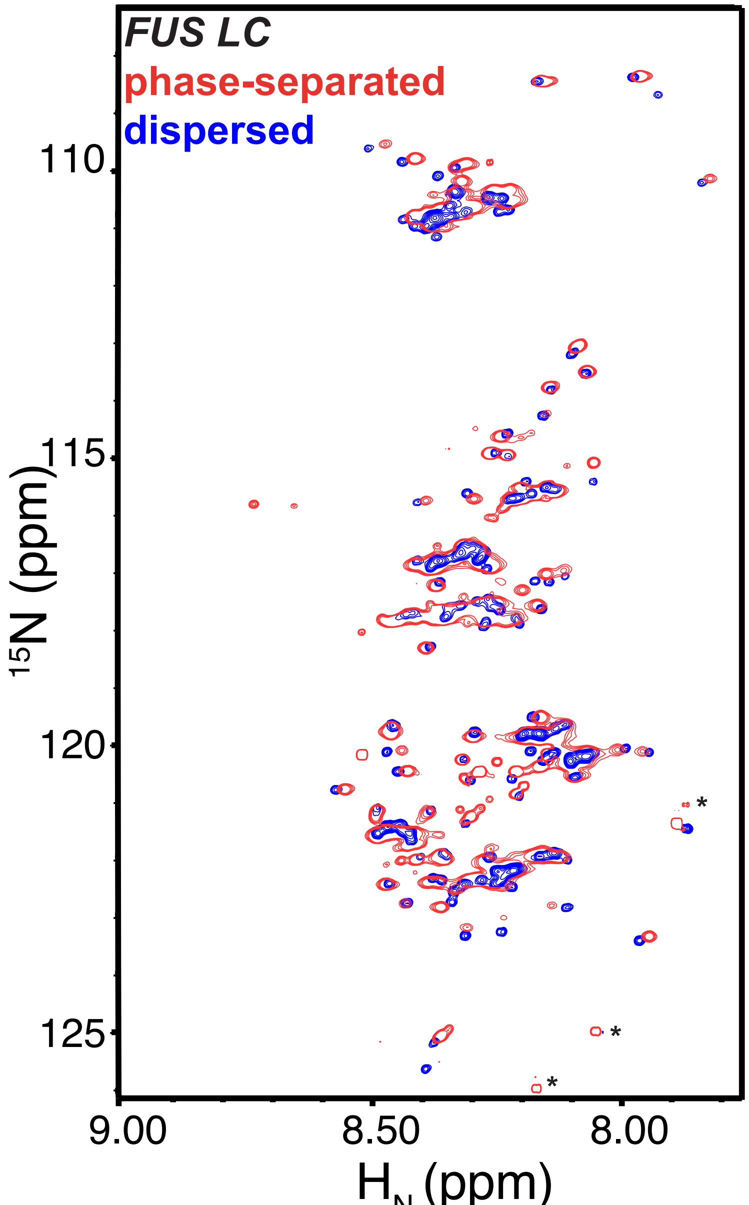
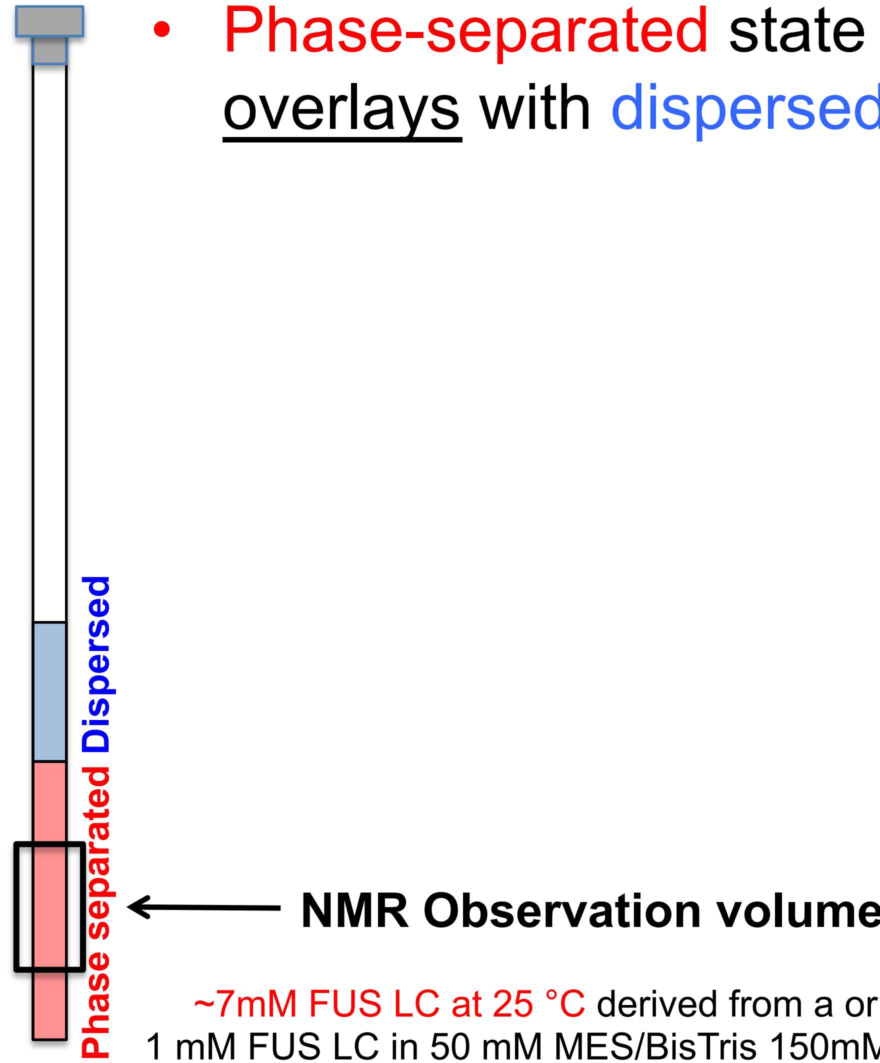


# Atomic details of FUS LC droplets

**FUS LC primarily disordered in liquid phase separated state**

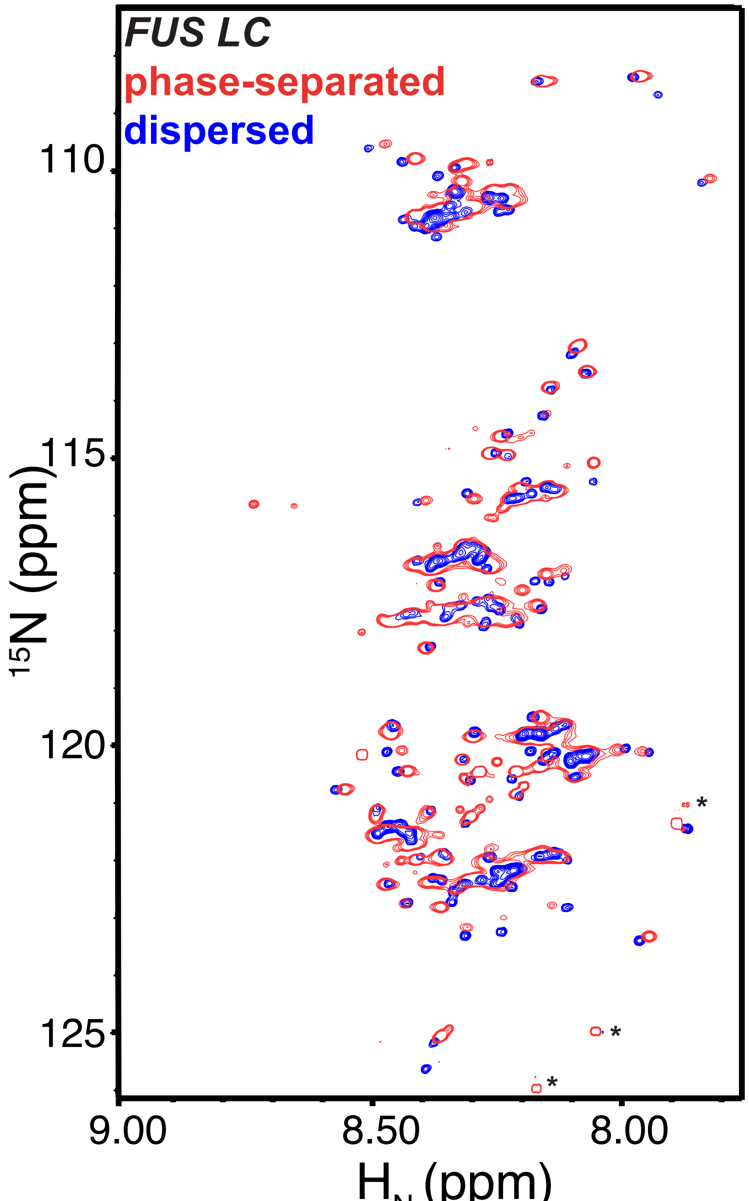
- Phase-separated state overlays with dispersed

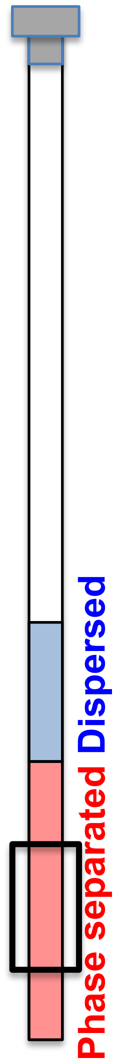



~7mM FUS LC at 25 °C derived from a original sample: 1 mM FUS LC in 50 mM MES/BisTris 150mM NaCl pH 5.5

# Atomic details of FUS LC droplets



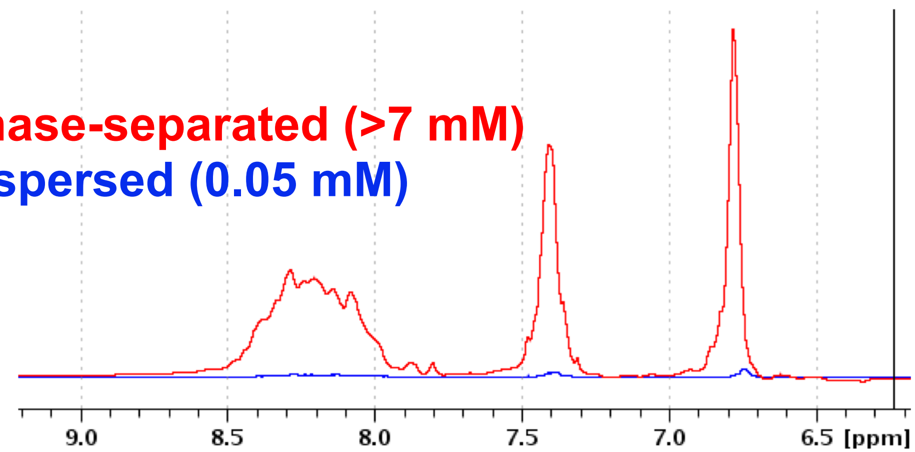




## FUS LC primarily disordered in liquid phase separated state

- Phase-separated state overlays with dispersed

**Is this a major or minor state?**
*Raw intensity data*

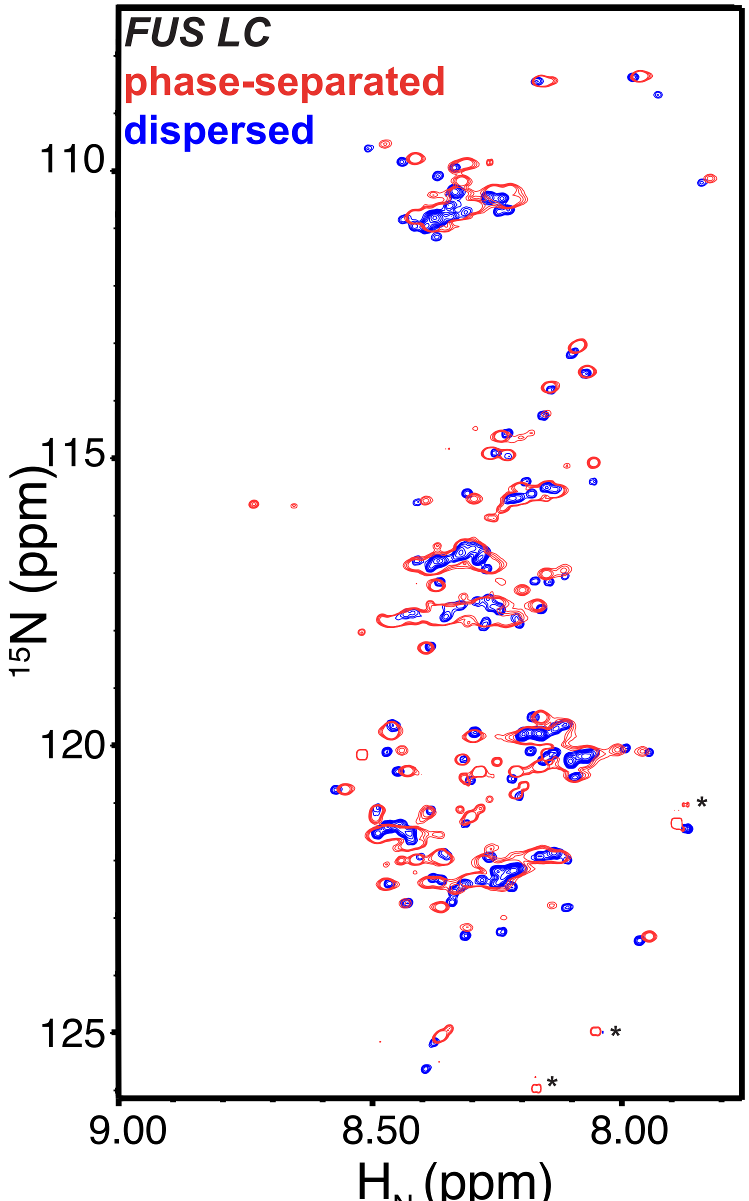
**phase-separated (>7 mM)**
**dispersed (0.05 mM)**

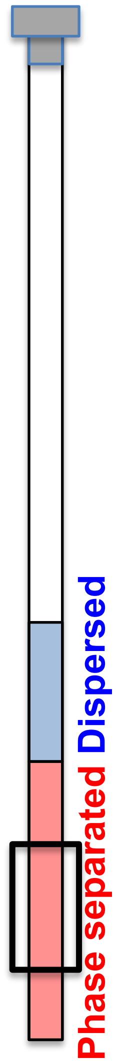



**NMR Observation volume**

~7mM FUS LC at 25 °C derived from a original sample:
1 mM FUS LC in 50 mM MES/BisTris 150mM NaCl pH 5.5

# Atomic details of FUS LC droplets

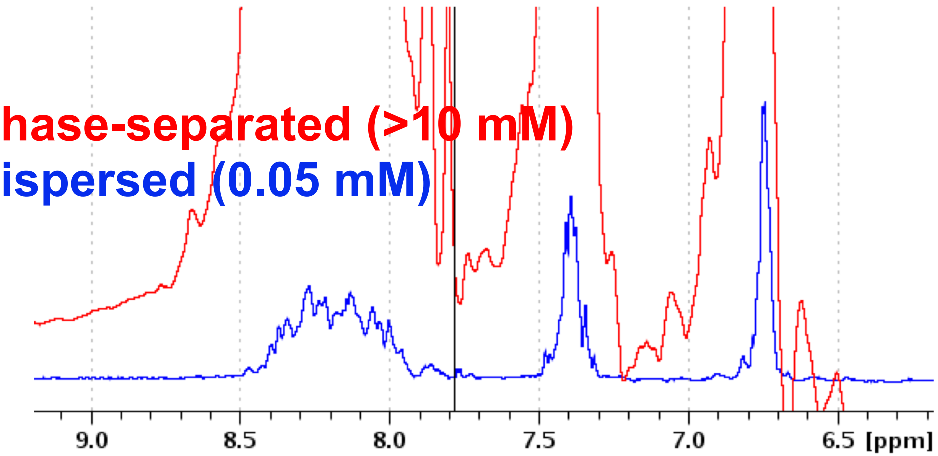



## FUS LC primarily disordered in liquid phase separated state

- Phase-separated state overlays with dispersed

**Is this a major or minor state?**
*Raw intensity data*

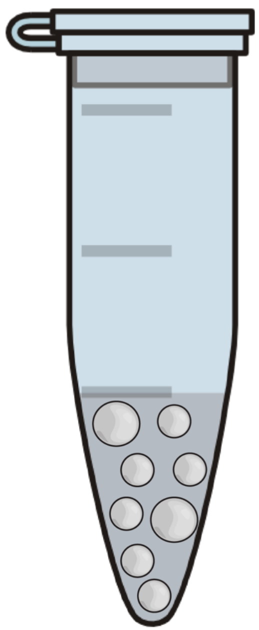



***100x signal in phase-separated vs control***

**NMR Observation volume**

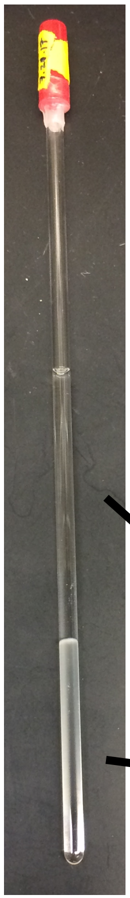
~7mM FUS LC at 25 °C derived from a original sample:
1 mM FUS LC in 50 mM MES/BisTris 150mM NaCl pH 5.5

# Using NMR spectroscopy to see within the phase separated state

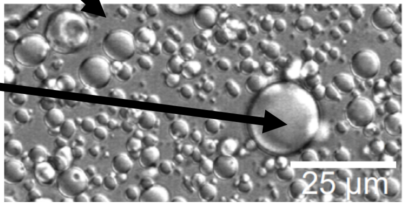


Phase separated FUS LC

Cool + Slow centrifugation in NMR tube



Dilute phase



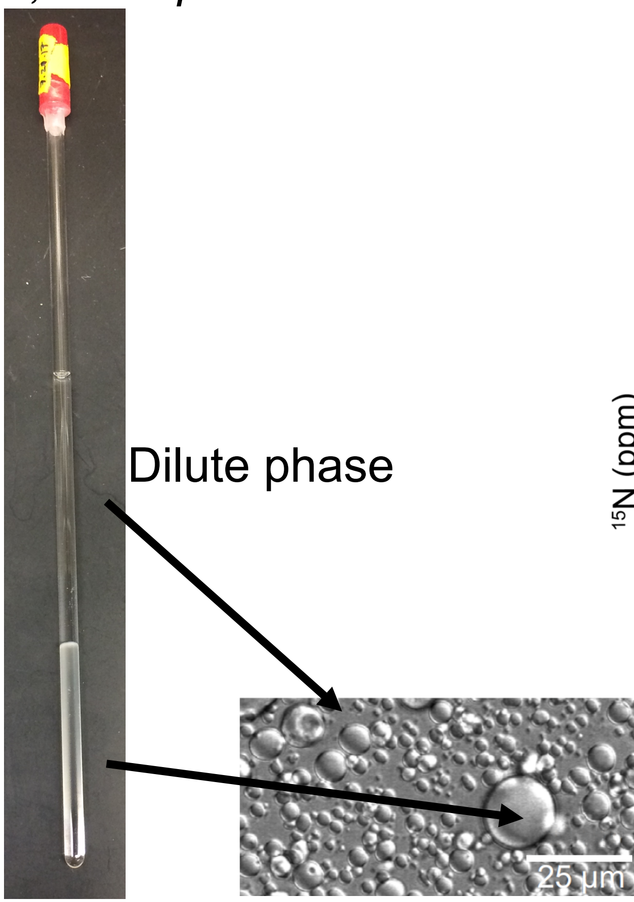

Concentrated phase

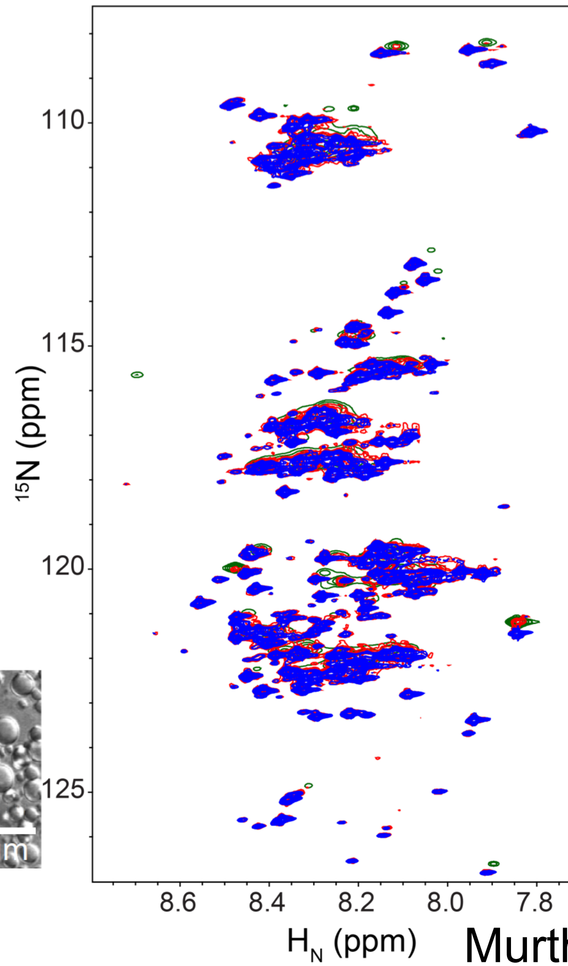
*FUS data from Anastasia Murthy*

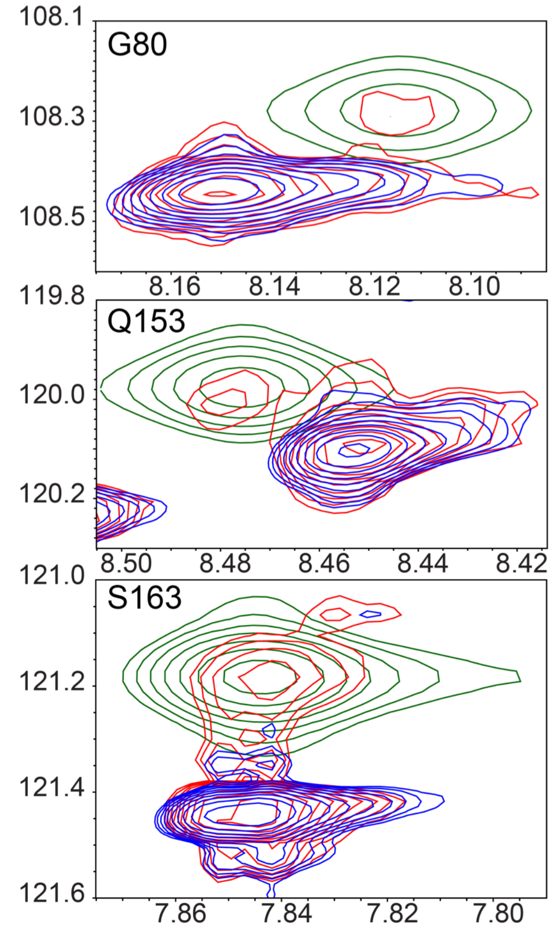
# Using NMR spectroscopy to see within the phase separated state

*The chemical environment of spontaneous droplets is similar to the large, monophasic condensed droplet.*

$\text{FUS LC}_{\text{Dispersed}}$ + $\text{FUS LC}_{\text{Condensed}}$
$\text{FUS LC}_{\text{Dispersed}}$
$\text{FUS LC}_{\text{Condensed}}$



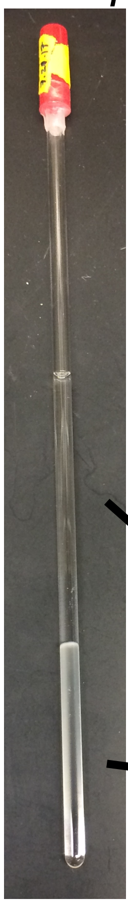



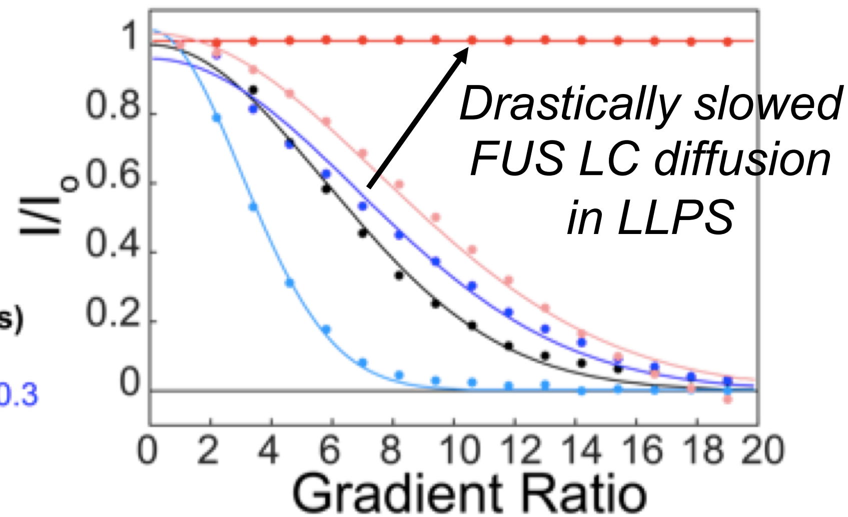

Murthy AM et al. in preparation

# Using NMR spectroscopy to see within the phase separated state

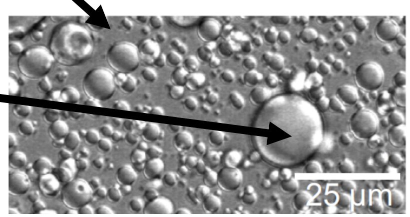
*FUS LC diffusion in spontaneous droplets is similar to the large, monophasic condensed droplet.*



**Diffusion coefficient (μm²/s)**
Lysozyme 104 ± 10.1
Dispersed FUS LC 85.1 ± 10.3
$Buffer_{Dispersed}$ 440.0 ± 40.9
Condensed FUS LC n.a.
$Buffer_{Condensed}$ 69.4 ± 7.47



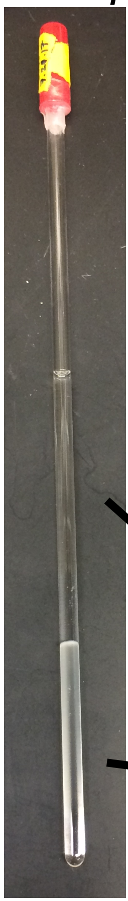

Dilute phase



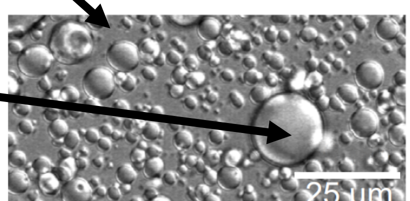

Concentrated phase

# Using NMR spectroscopy to see within the phase separated state

*FUS LC diffusion in spontaneous droplets is similar to the large, monophasic condensed droplet.*



Dilute phase




Concentrated phase

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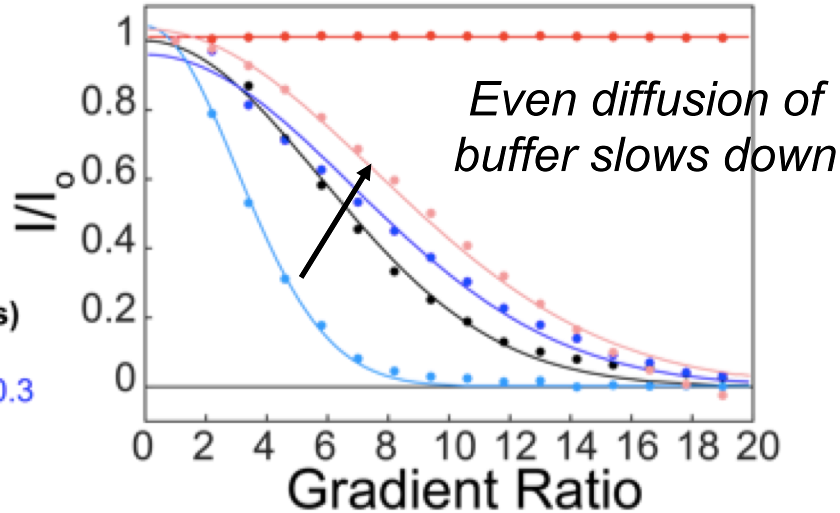
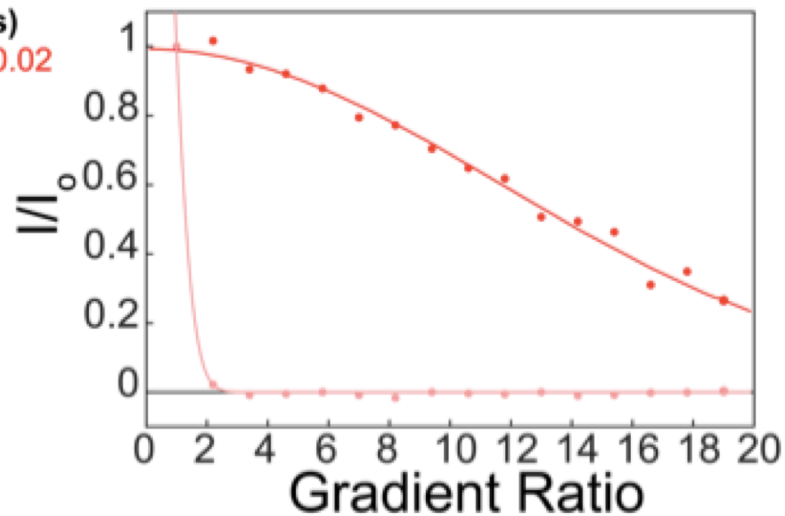
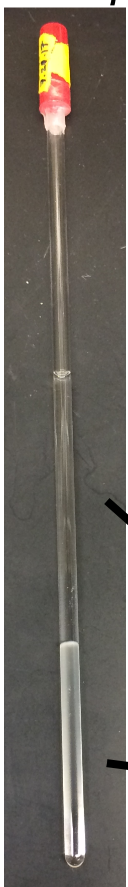



**Diffusion coefficient (µm²/s)**
Condensed FUS LC 0.17 ± 0.02
$Buffer_{Condensed}$ 48.0 ± 9.97

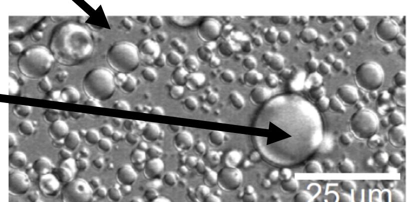


# Using NMR spectroscopy to see within the phase separated state

*FUS LC diffusion in spontaneous droplets is similar to the large, monophasic condensed droplet.*



Dilute phase




Concentrated phase

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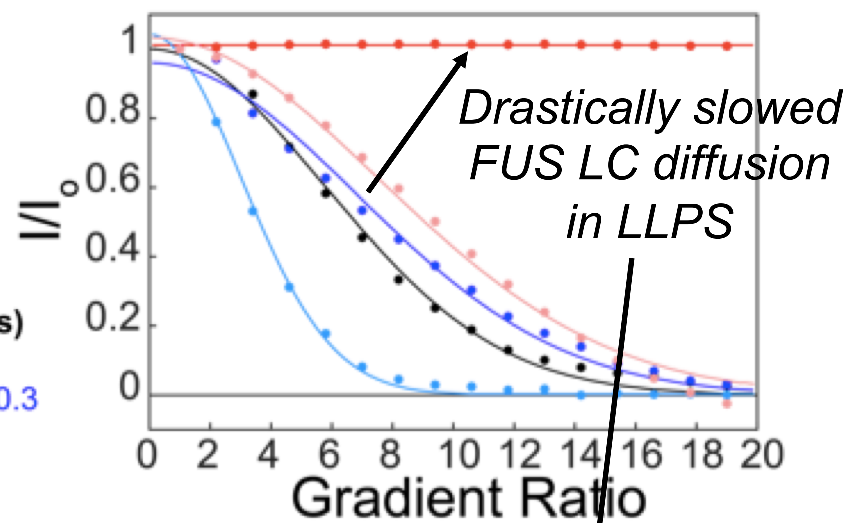
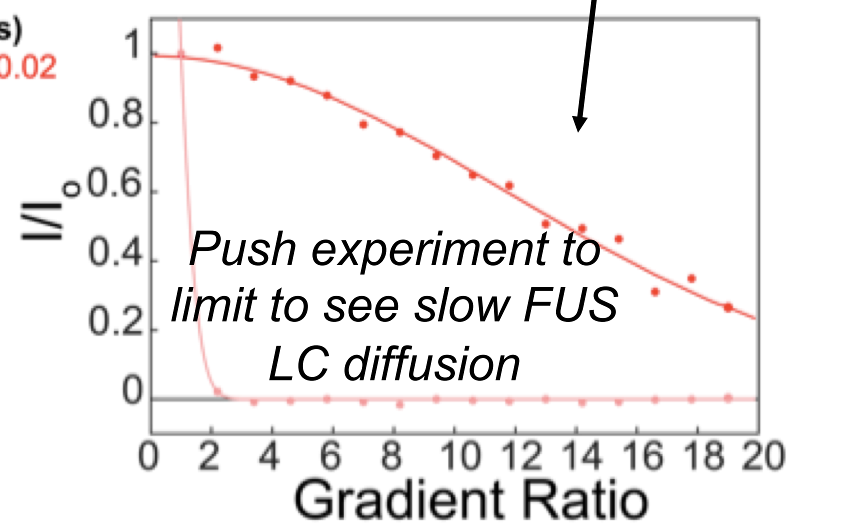
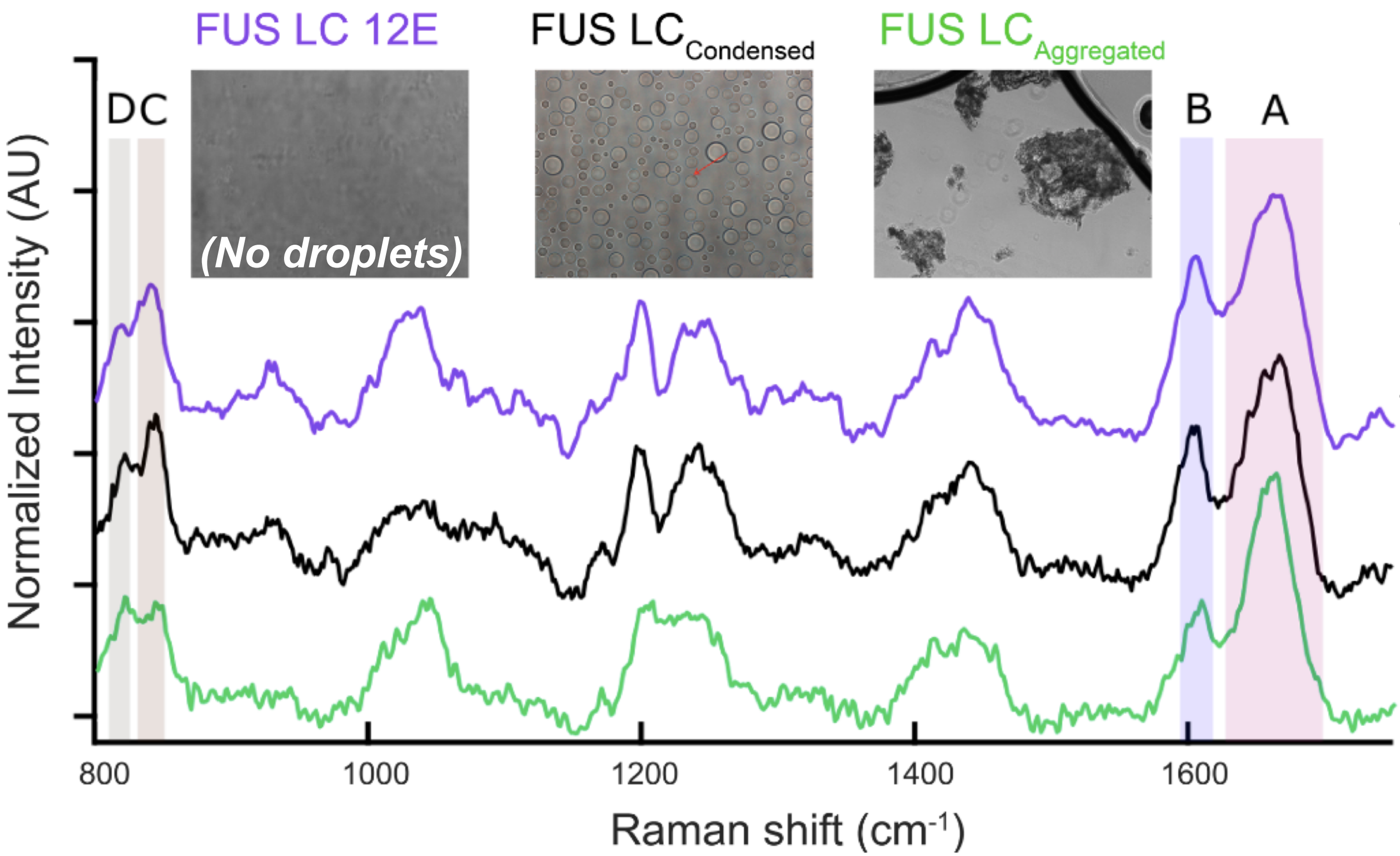


# Using CARS hyperspectral imaging to see within the phase separated state

*FUS LC vibrational spectrum in droplets resembles a non-phase separated form (FUS LC 12E) characterized by NMR in detail, more so than an aggregated form.*

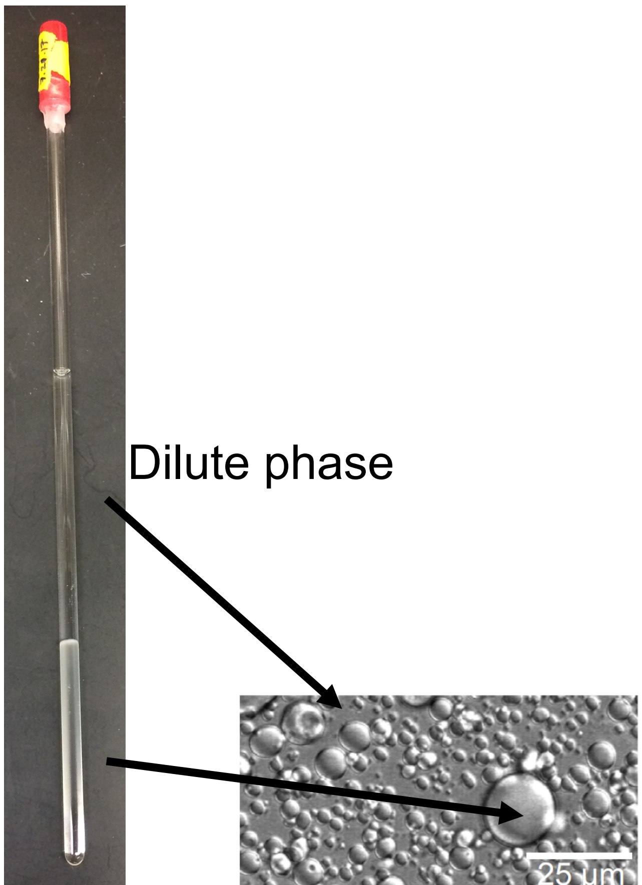



*Yelena Kan and Sapun Parekh (MPI Polymer and UT Austin)*

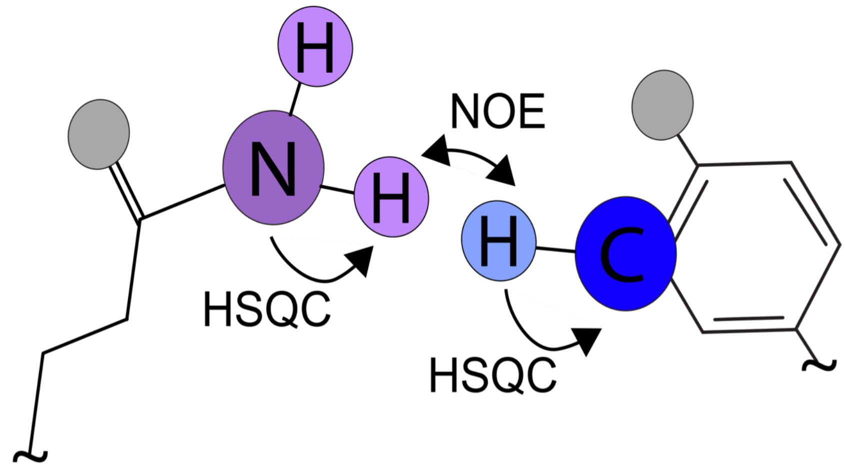
*Major state of FUS LC residues is structurally disordered*

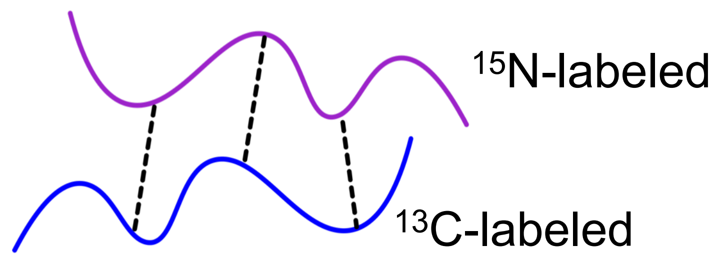
# Using NMR spectroscopy to see within the phase separated state

NMR experiments (HSQC-NOESY-HSQC) to observe interactions between amide hydrogen and carbon-attached hydrogen




Concentrated phase

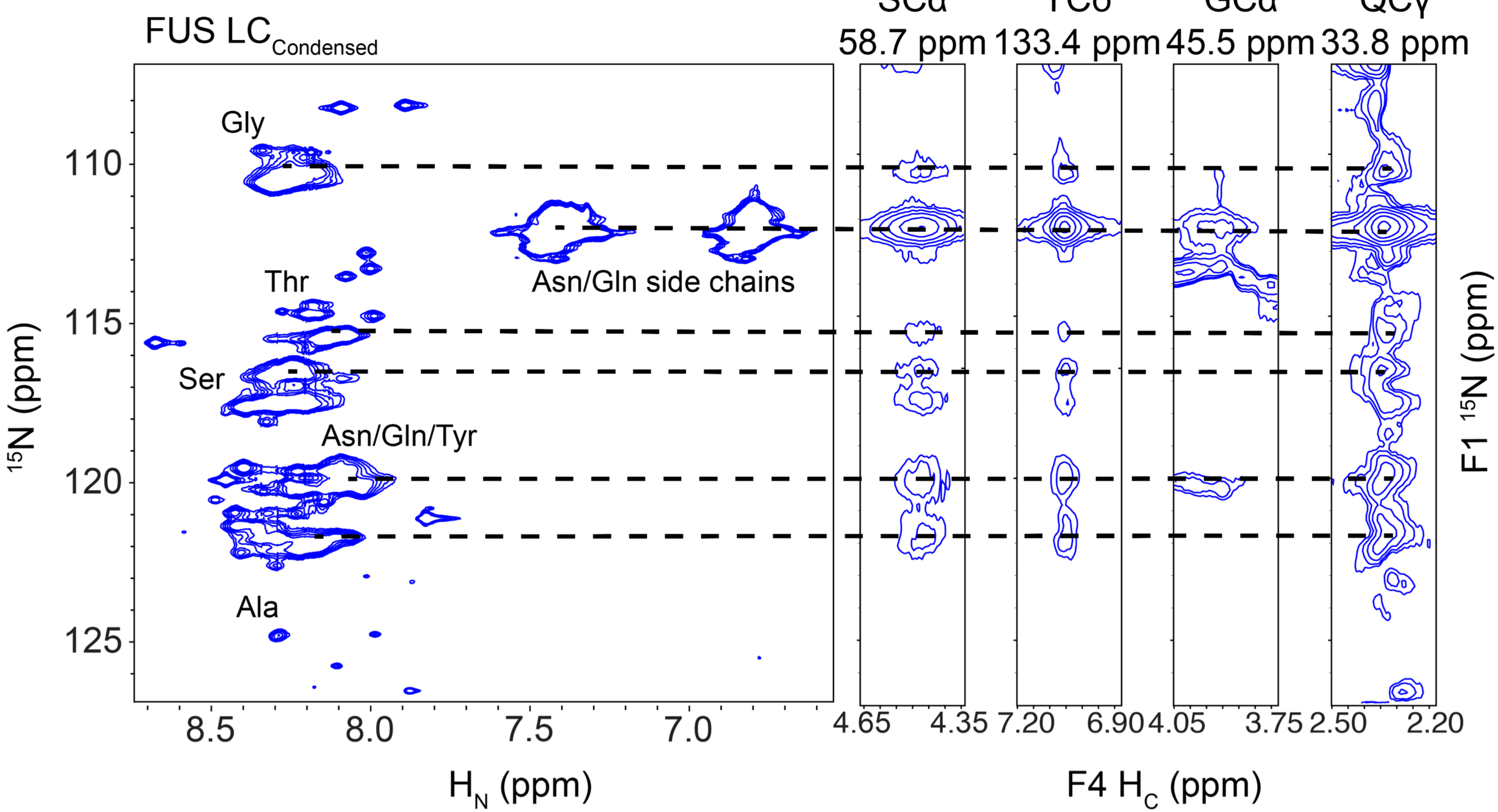



Inter-molecular interactions between FUS LC monomers within phase separated state

# Non-specific intermolecular contacts between all residue types underlie FUS LC LLPS

*Intermolecular contacts:*
*NOEs from $^{15}$N-labeled to $^{13}$C-labeled residues*



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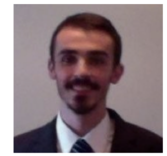
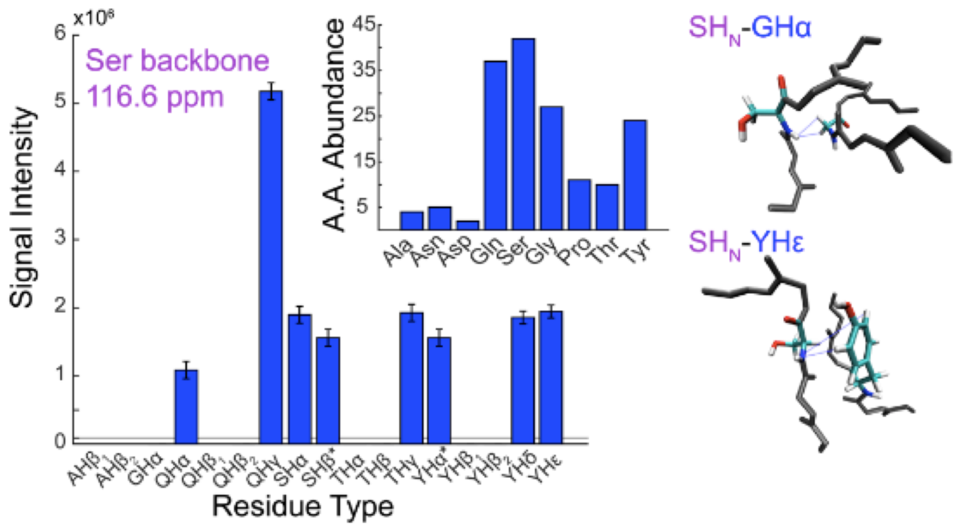
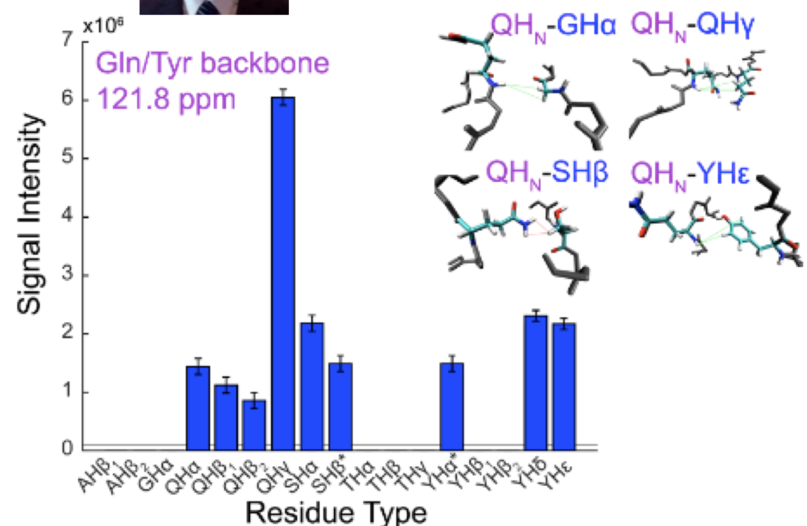
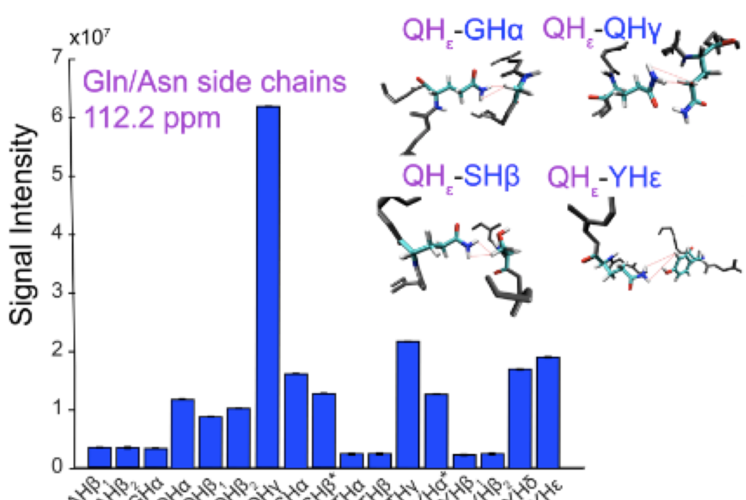
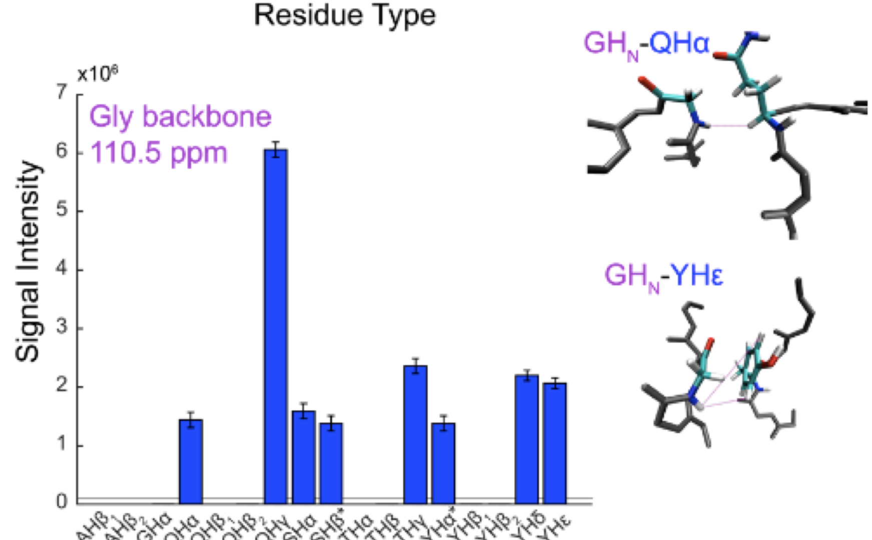


*MD simulations by Greg Dignon*

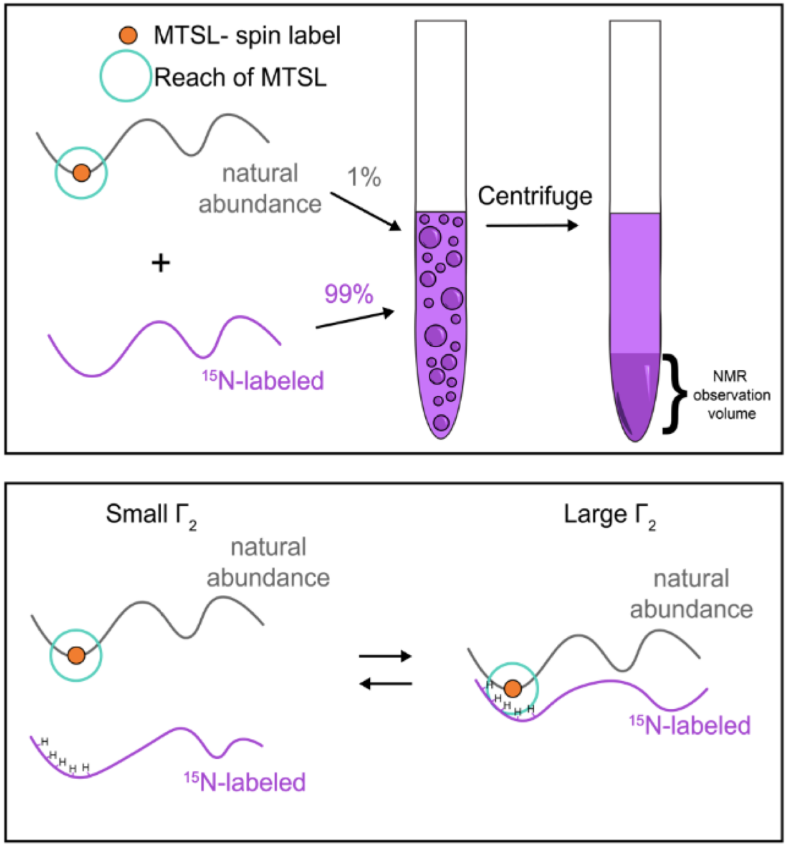


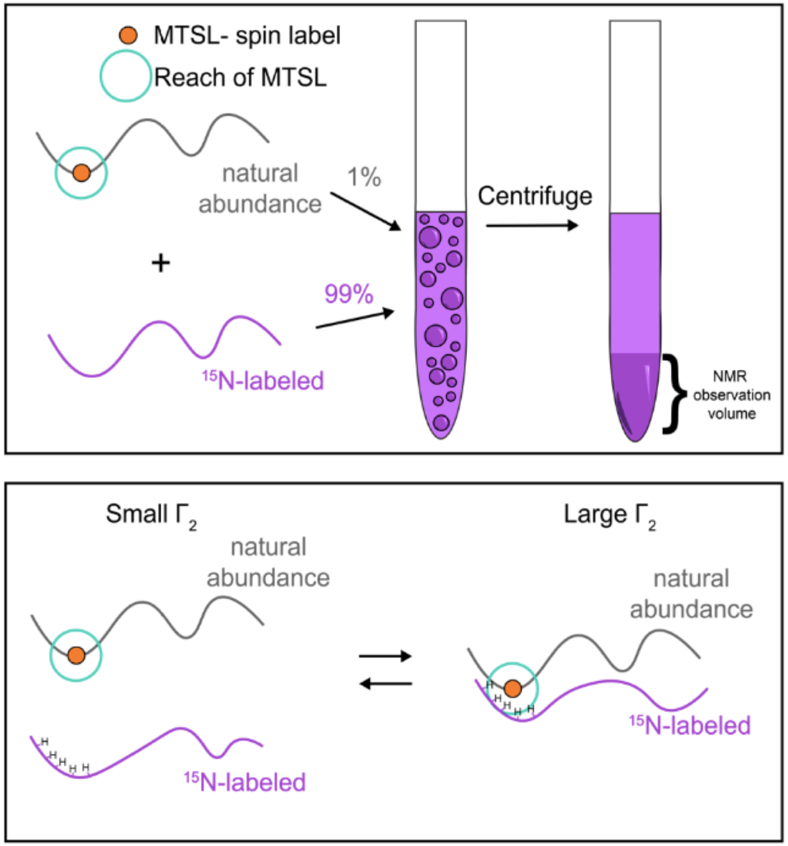



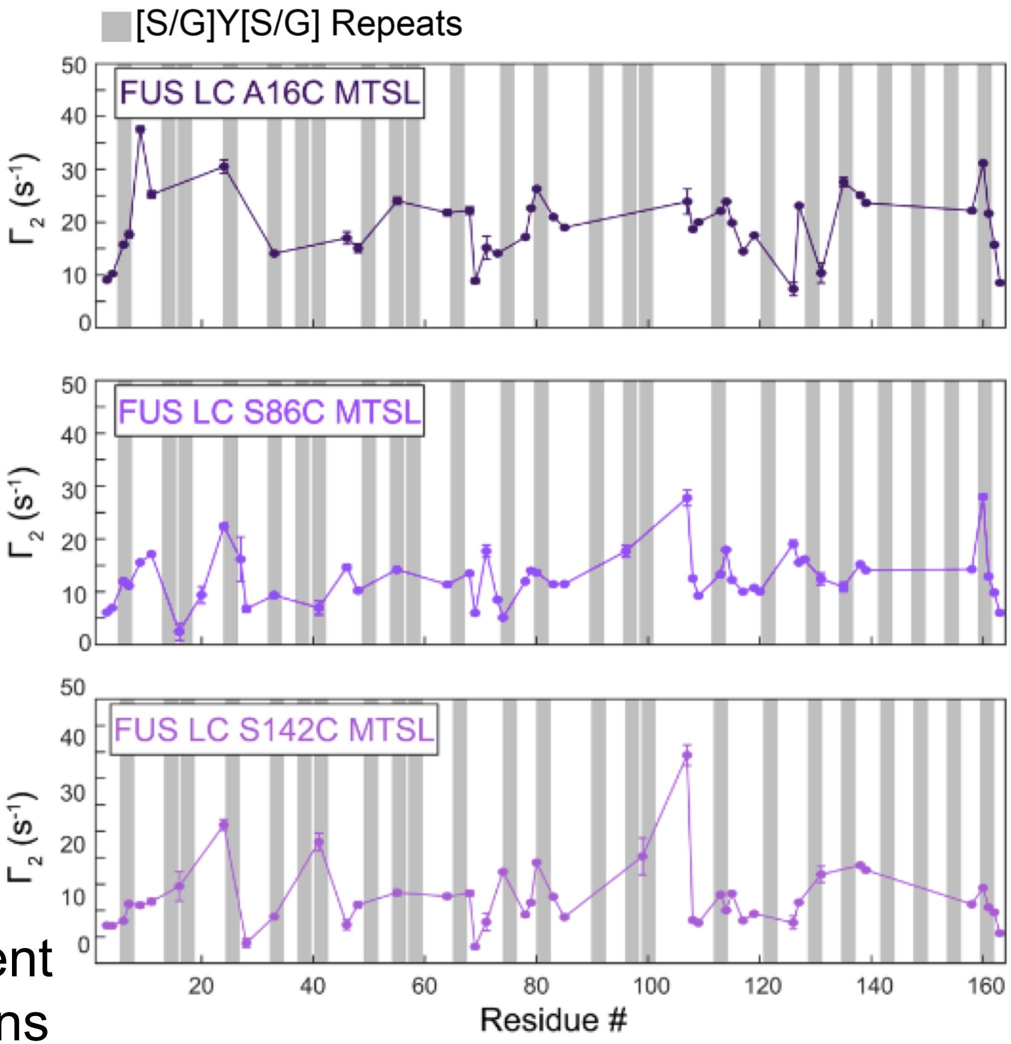
# Interactions that stabilize LLPS are not localized to a particular region of FUS LC




Paramagnetic relaxation enhancement NMR to observe transient, interactions in the condensed phase

# Interactions that stabilize LLPS are not localized to a particular region of FUS LC

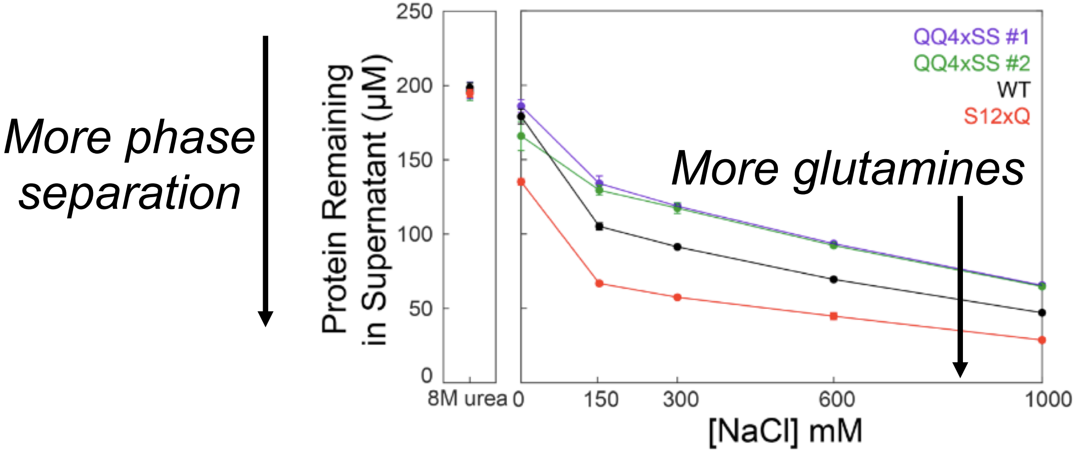


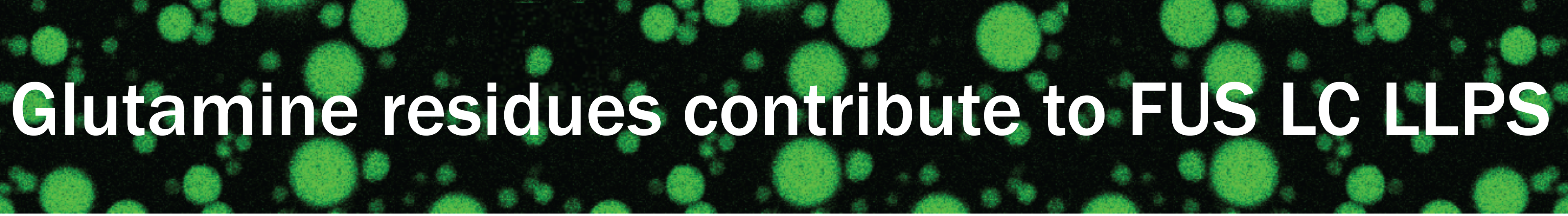




Paramagnetic relaxation enhancement NMR to observe transient, interactions in the condensed phase

# Glutamine residues contribute to FUS LC LLPS

*Glutamine substitutions modulate FUS LC LLPS*

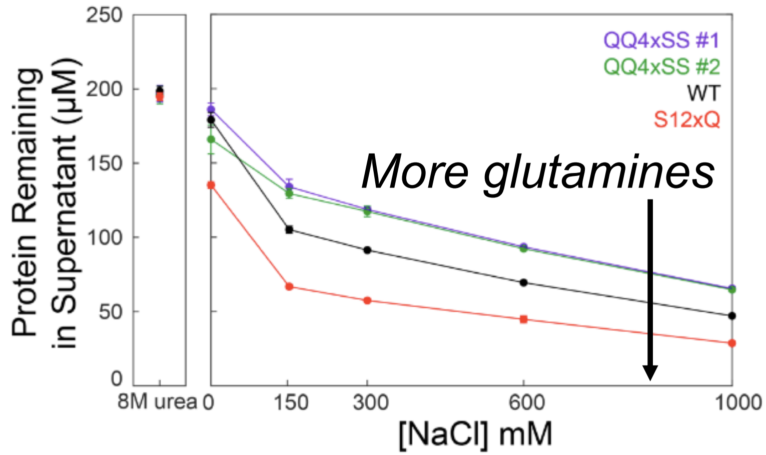


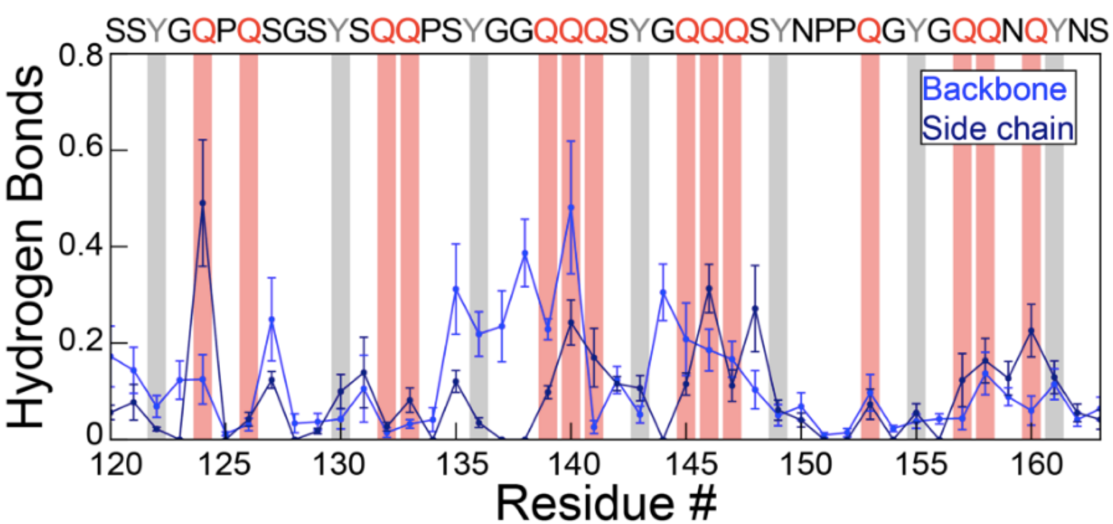


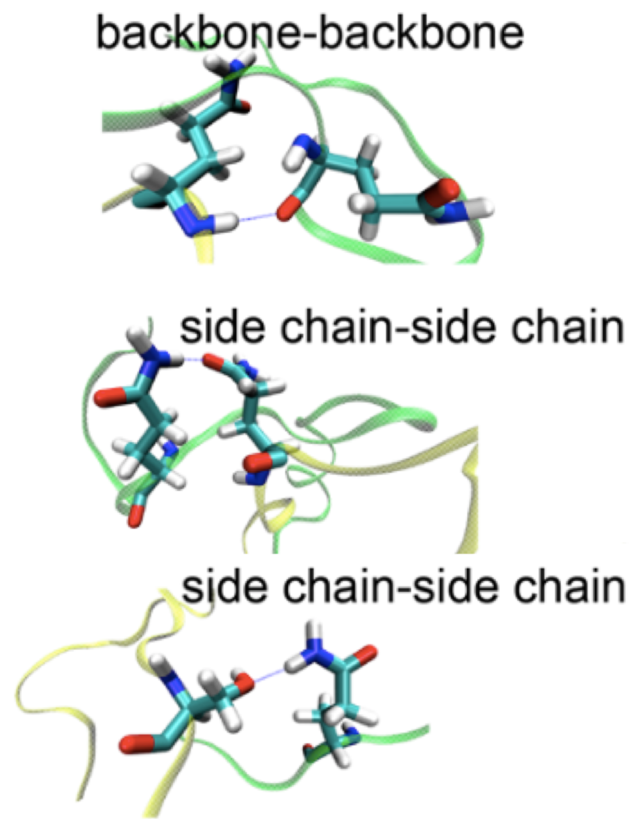
# Glutamine residues contribute to FUS LC LLPS

*Glutamine substitutions modulate FUS LC LLPS*

*More phase separation*



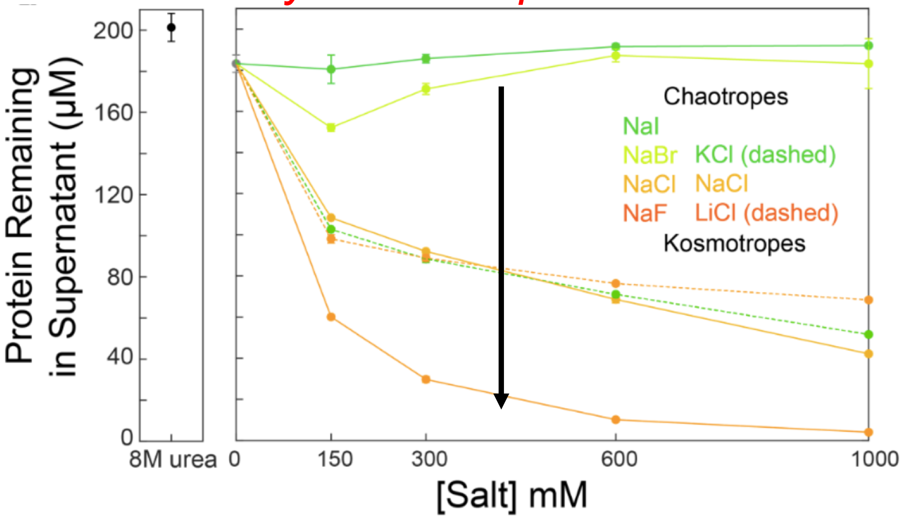



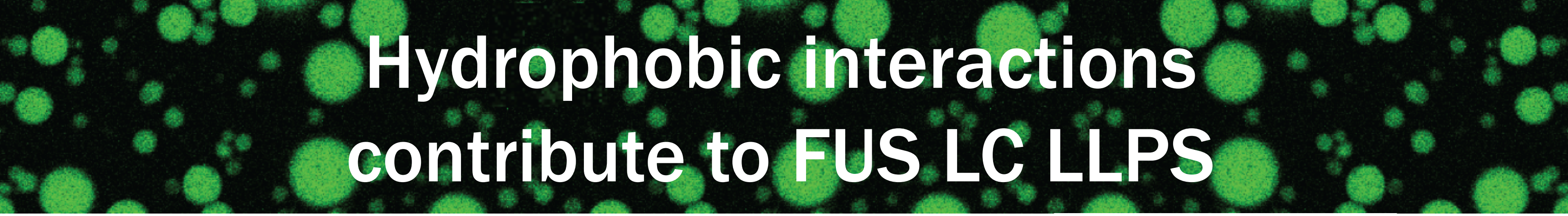

*Glutamine contacts stabilized by intermolecular hydrogen bonds in FUS LC fragment molecular simulation*

# Hydrophobic interactions contribute to FUS LC LLPS

*FUS LC LLPS induced by kosmotropic salts*

*More phase separation*





# Hydrophobic interactions contribute to FUS LC LLPS

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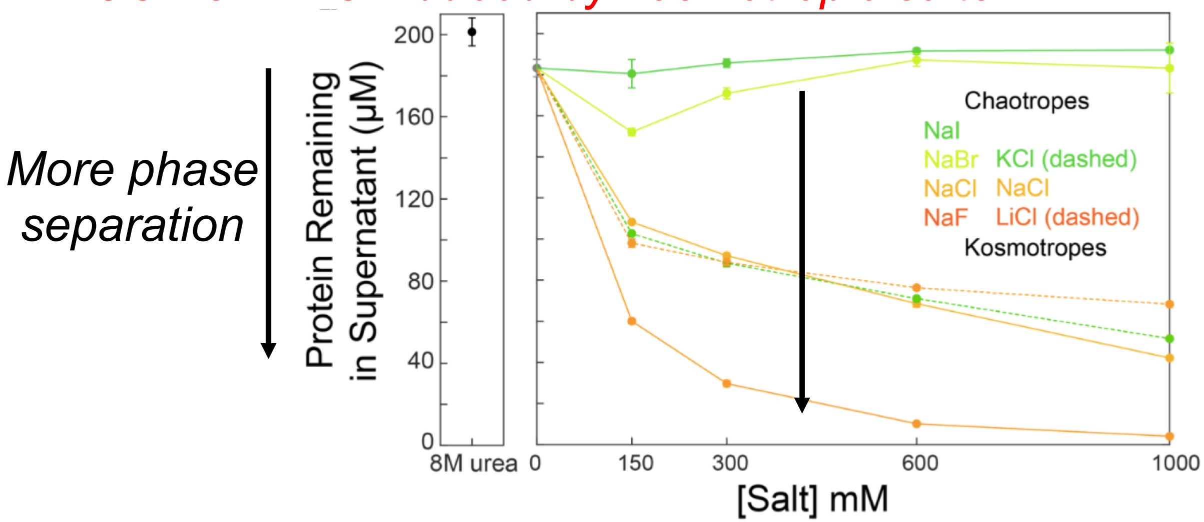
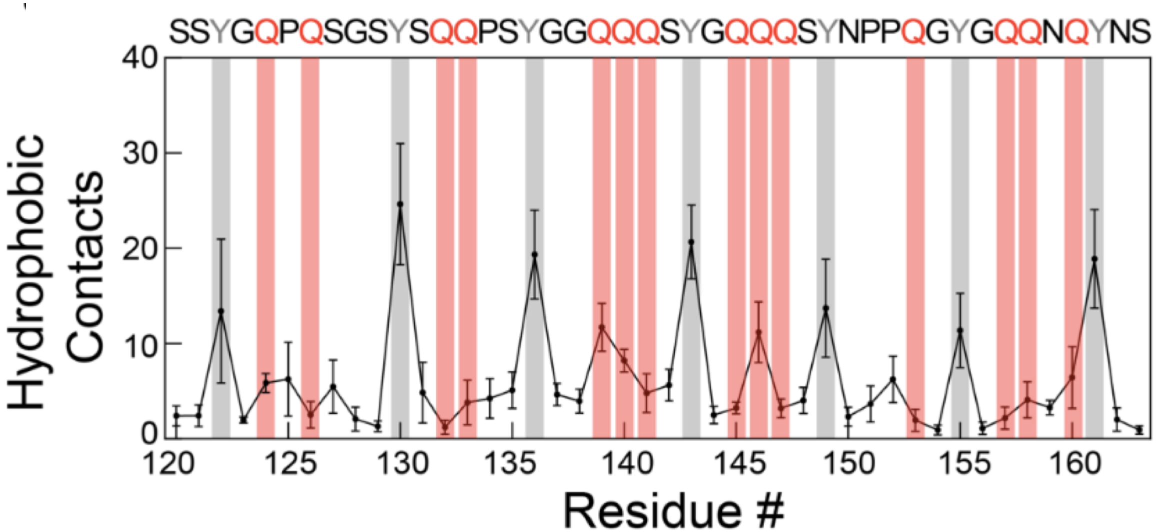
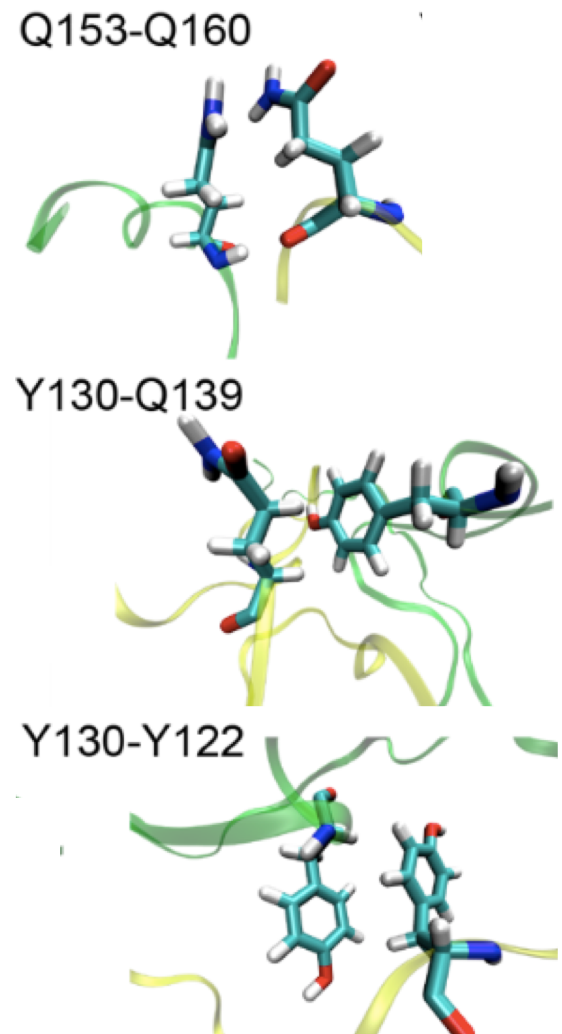


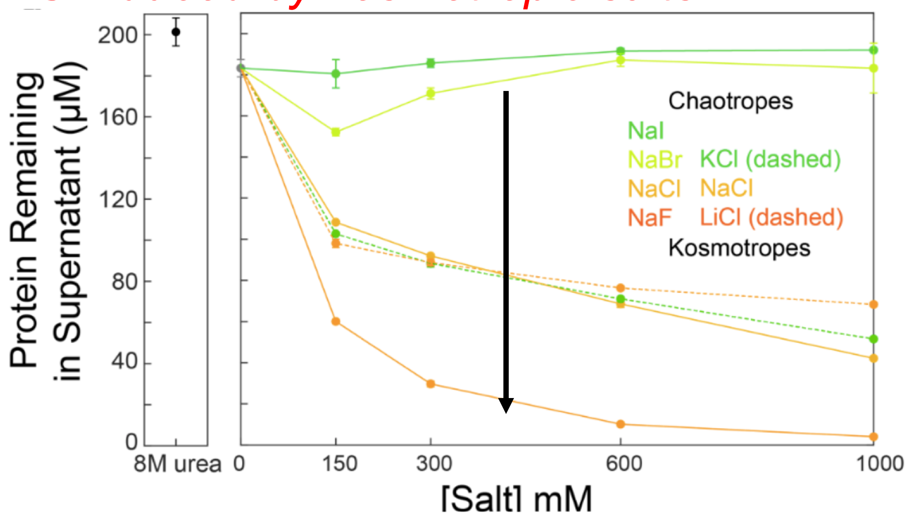



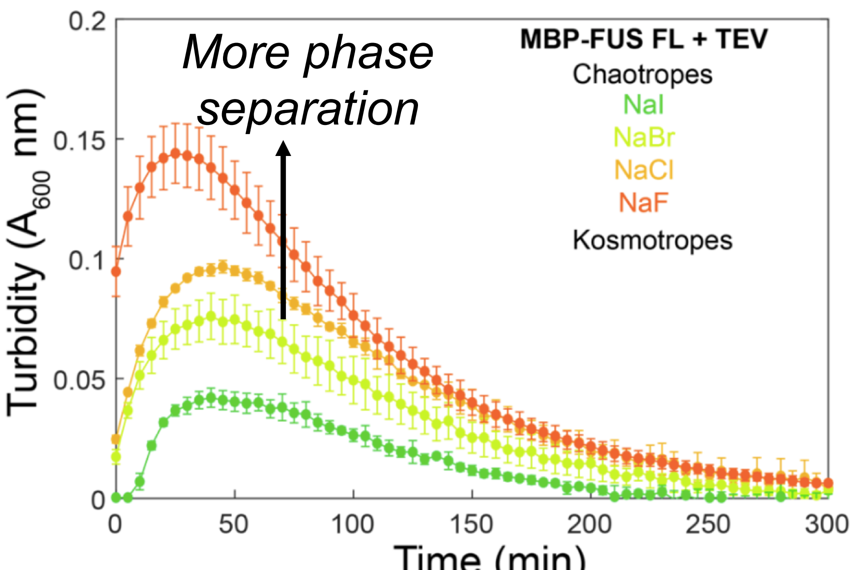

*Hydrophobic contacts mediated by glutamine and tyrosine*

# Hydrophobic interactions contribute to FUS LC LLPS

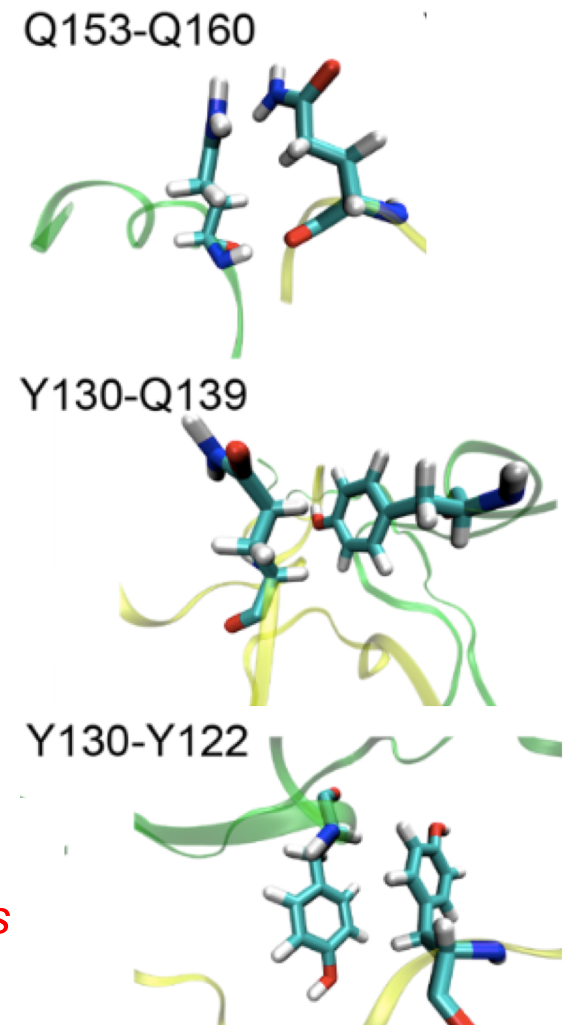
*FUS LC LLPS induced by kosmotropic salts*

*More phase separation*



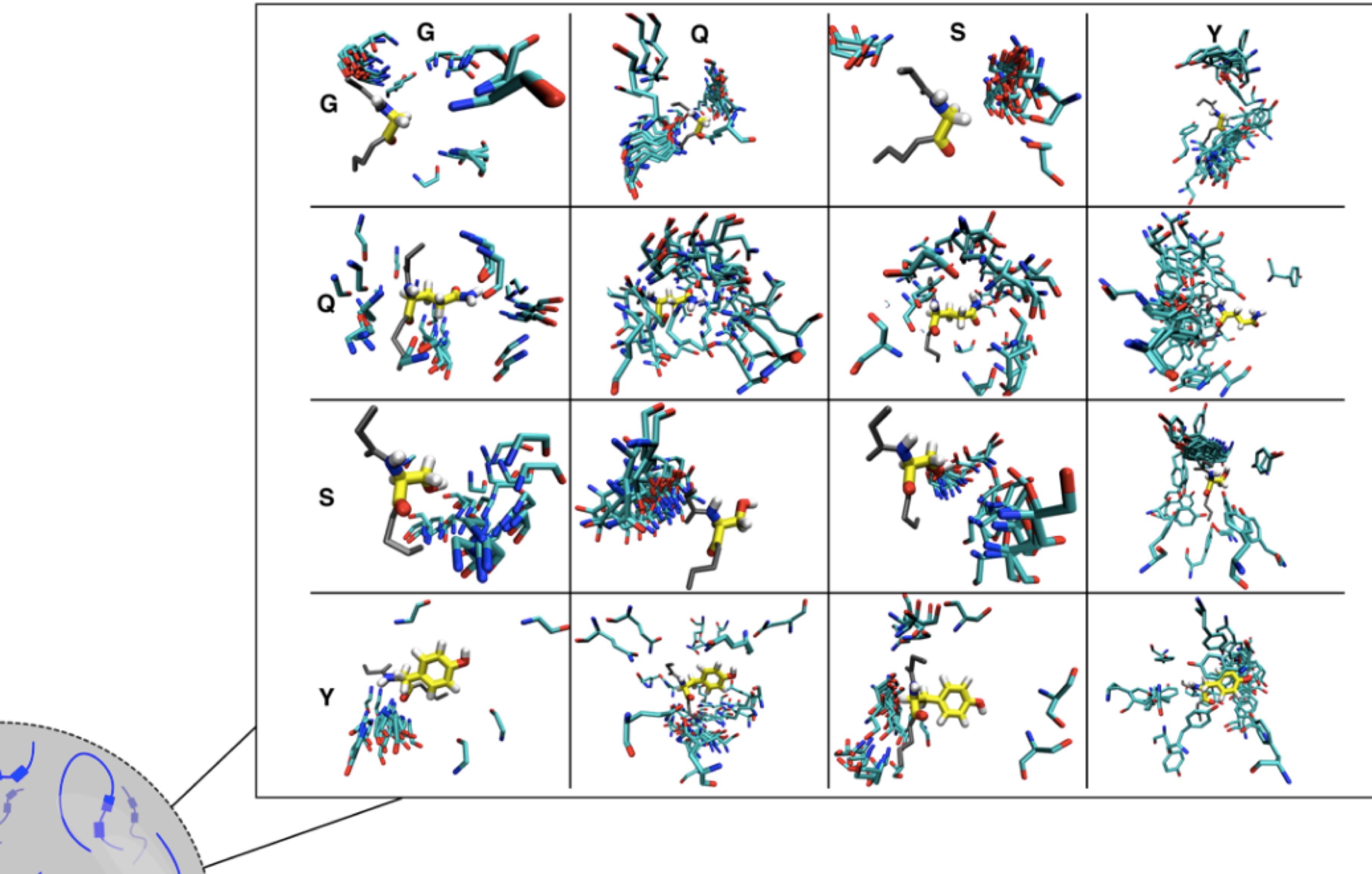



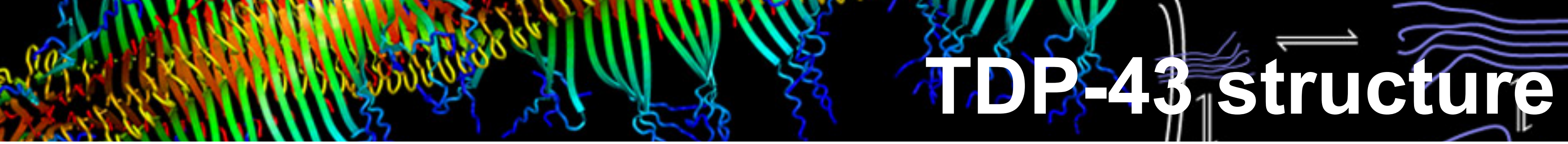

*Full length FUS LLPS also induced by kosmotropic salts*




*Hydrophobic contacts mediated by glutamine and tyrosine*

# Dynamic contacts mediating FUS LC LLPS



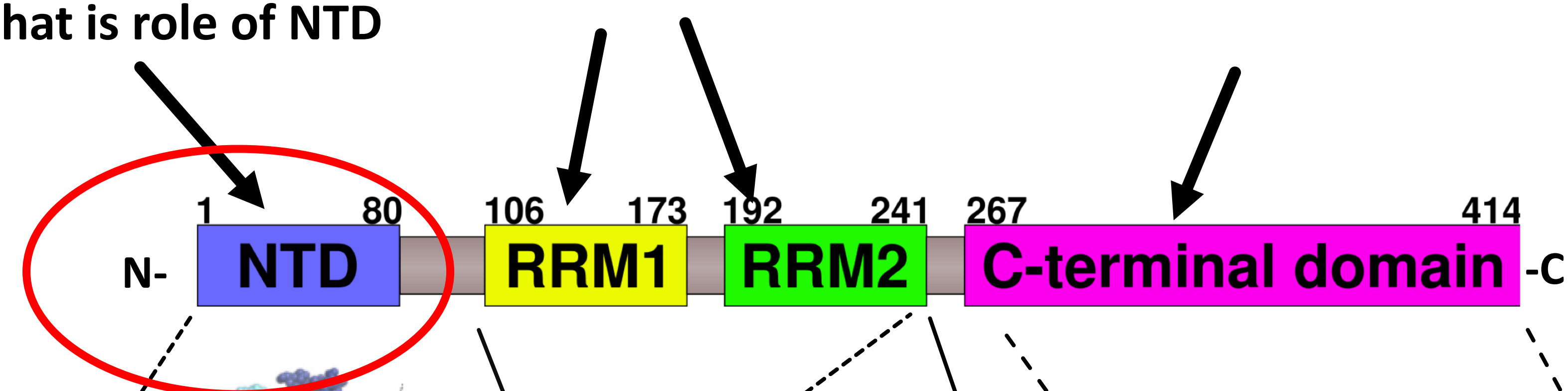


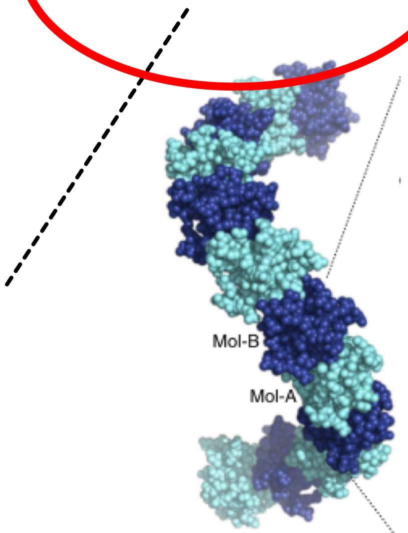
# TDP-43 structure

**Mediates aggregation in ~90% of ALS**
**Site of 50 out of 52 known ALS mutations**

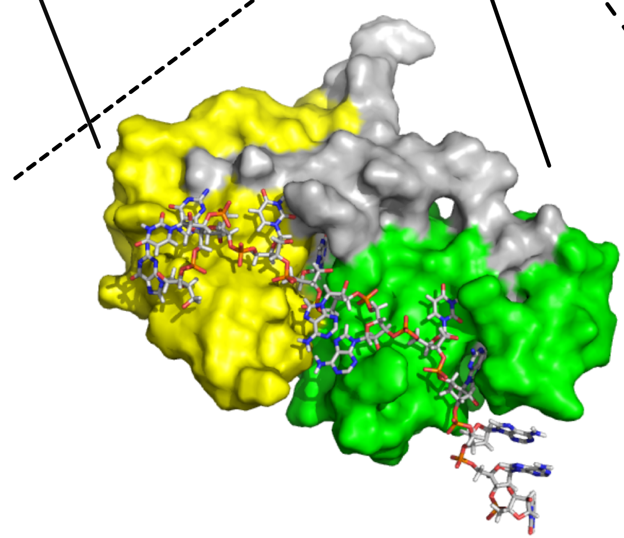
**Multimerization**
**What is role of NTD**

**RNA-binding**

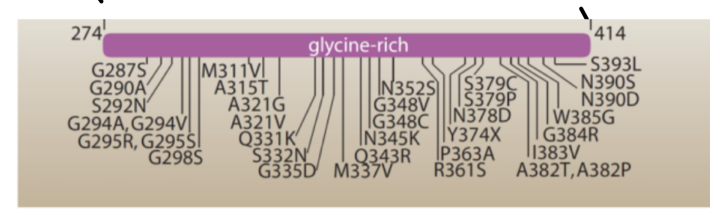


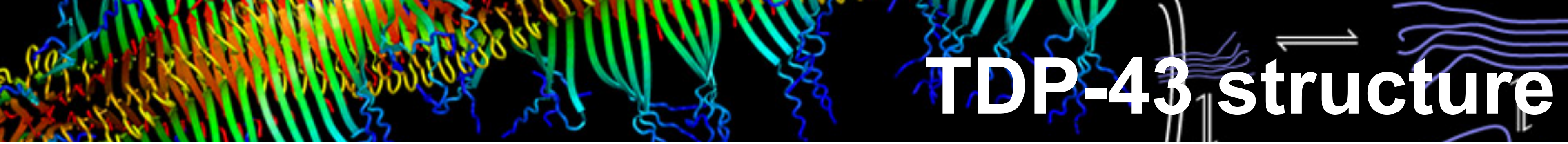

Afroz *et al*., *Nature Communications*, 2017.



Lukavsky *et al*., *Nat. Struc. Mol. Bio.*, 2014.

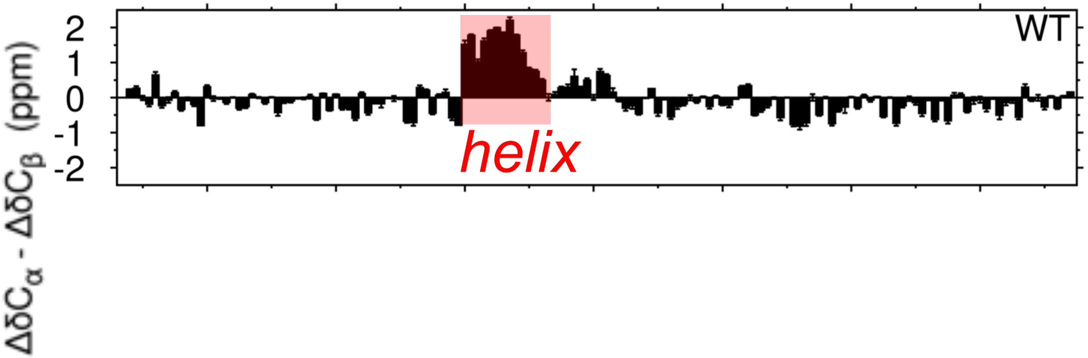


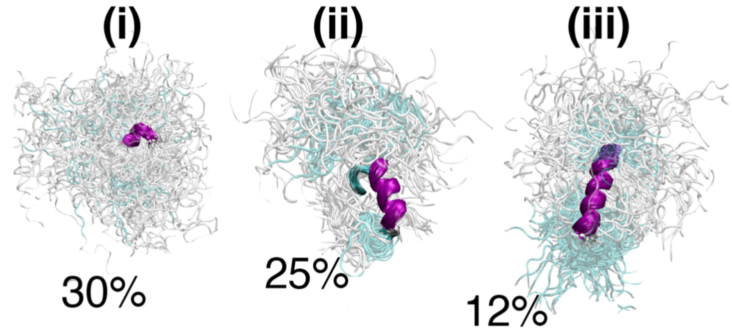

Ling et al., *Neuron*, 2013.



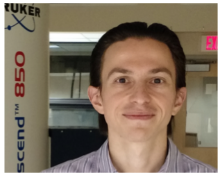
# TDP-43 structure

## TDP-43 CTD contains **helical** region



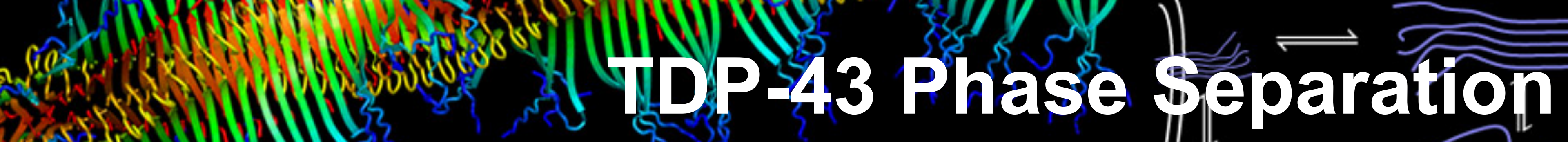




```
                                                      319                    341
Human TDP-43      268 RQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMGGGMNFGAFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSGS-GFNGGFGSSMDSKSSGWGM 414
Zebrafish TAR     275 QMMERAGRFG---NGFGGQ-GFAGSRSNMGGGGGGSSSSLG---NFGNFNLNPAMMAAAQAALQSSWGMMGMLA-QQNQSGTSGTSTSGTSSSRDQAQTYSSANSNY-GSSS-AALGWGTGSNSGAASAGFNSSFGSSMESKSSGWGM 412
DNA binding protein                                   319                    341
```



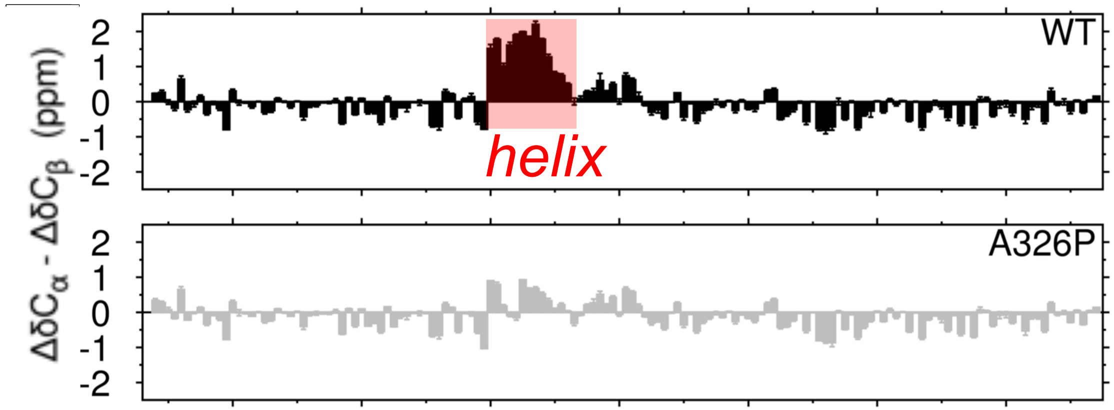
*TDP-43 CTD work by*
*Alex Conicella, Gul Zerze, Greg Dignon*

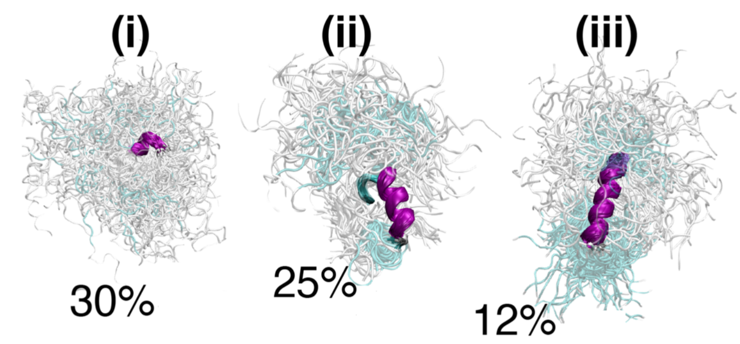
Conicella et al., *Structure.* (2016)



# TDP-43 Phase Separation

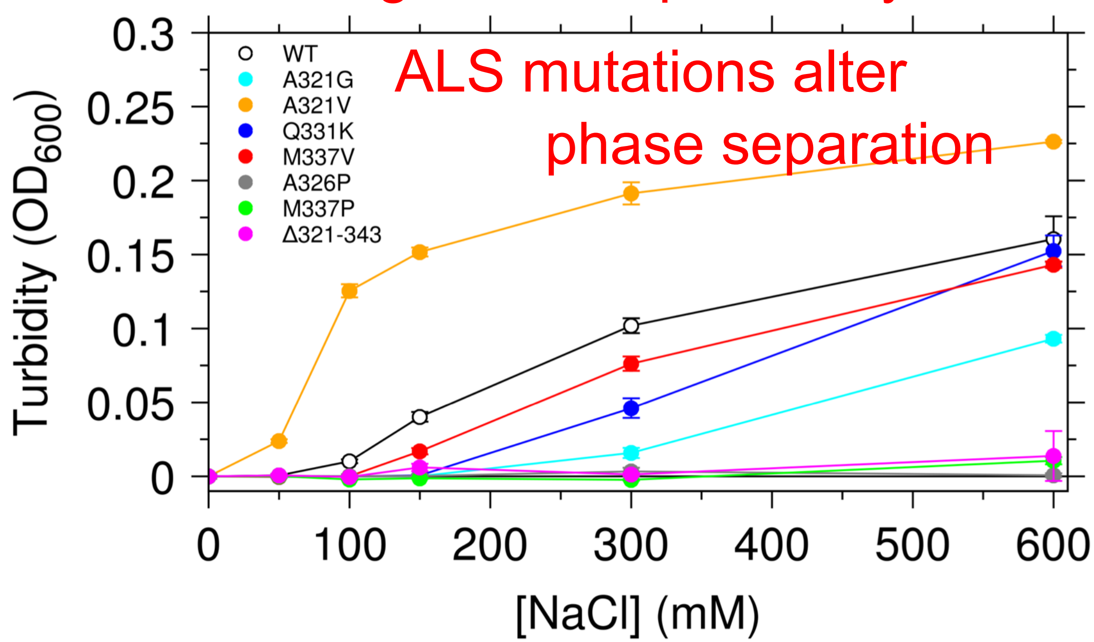
TDP-43 CTD contains **helical** region



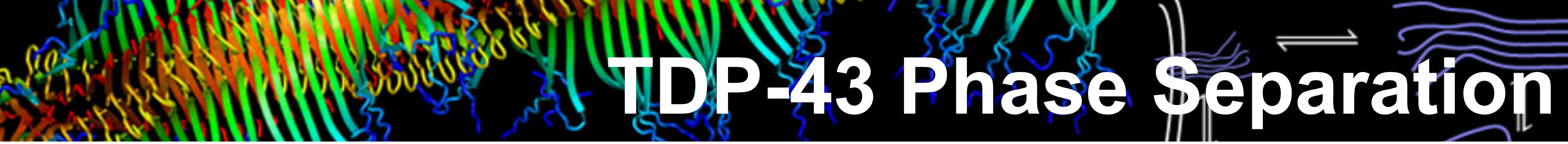

Requires helical region…

Helical region is cooperatively formed

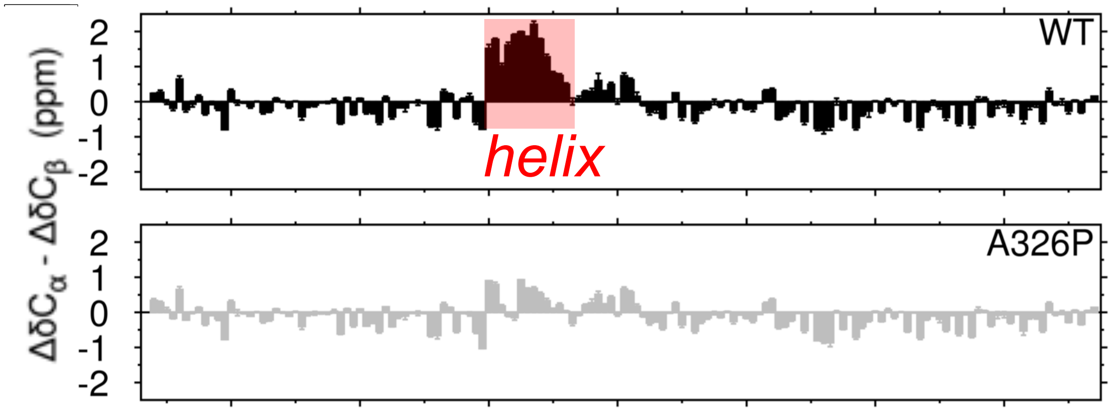


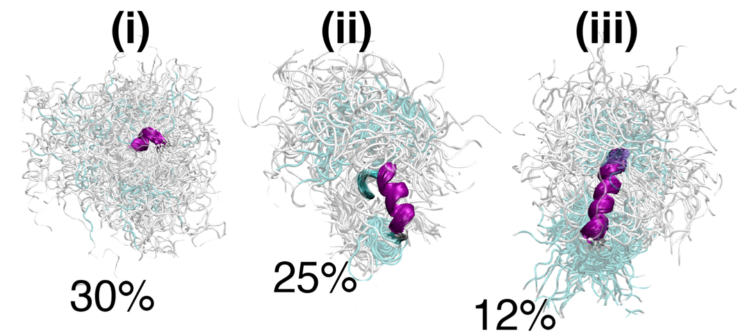

ALS mutations alter phase separation

# TDP-43 Phase Separation



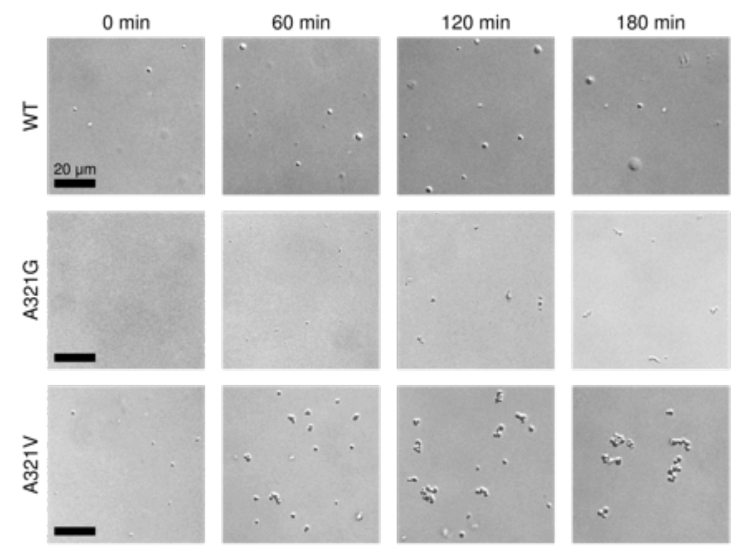
TDP-43 CTD contains **helical** region



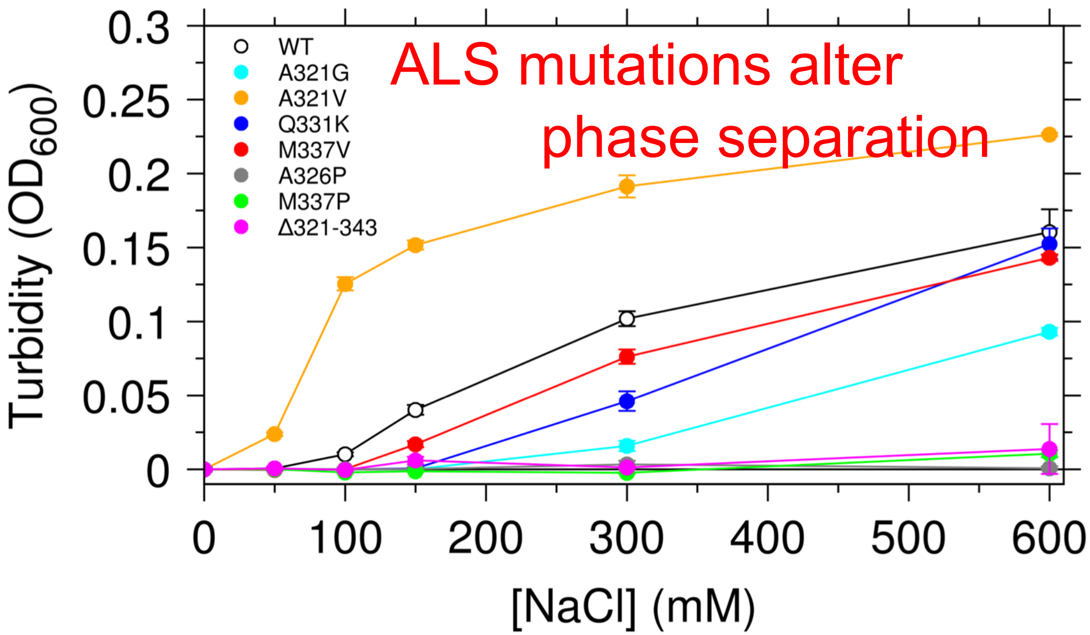

Requires helical region…

ALS A321V: prone to *liquid to aggregate conversion*

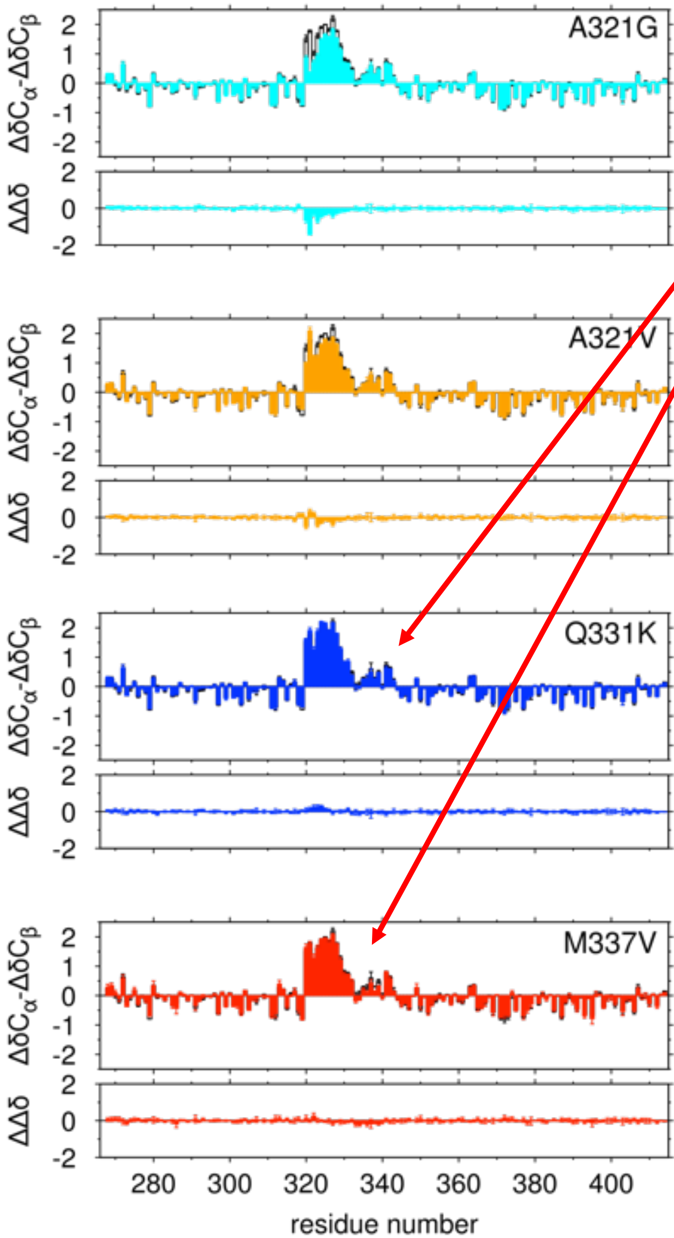



Helical region is cooperatively formed

ALS mutations alter phase separation



# TDP-43 helical structure

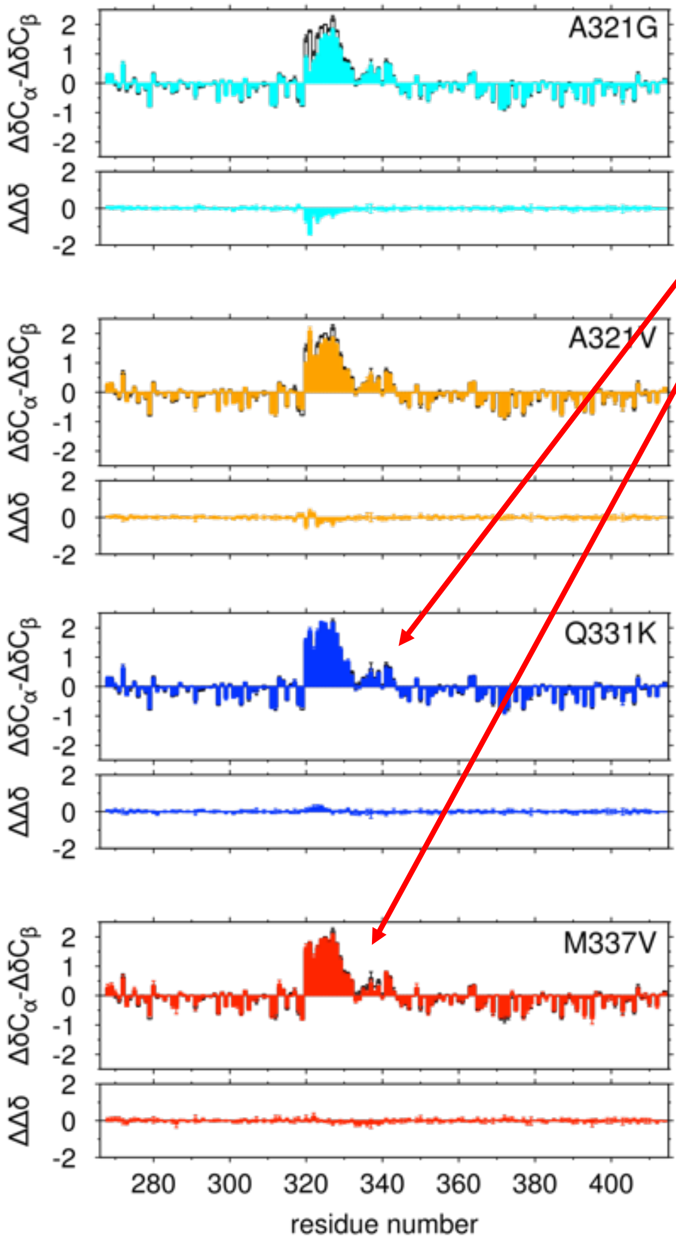



Disruption of helical structure of monomer?

*No change for Q331K or M337V*

*can't explain change in phase separation*

# TDP-43 helix-helix assembly

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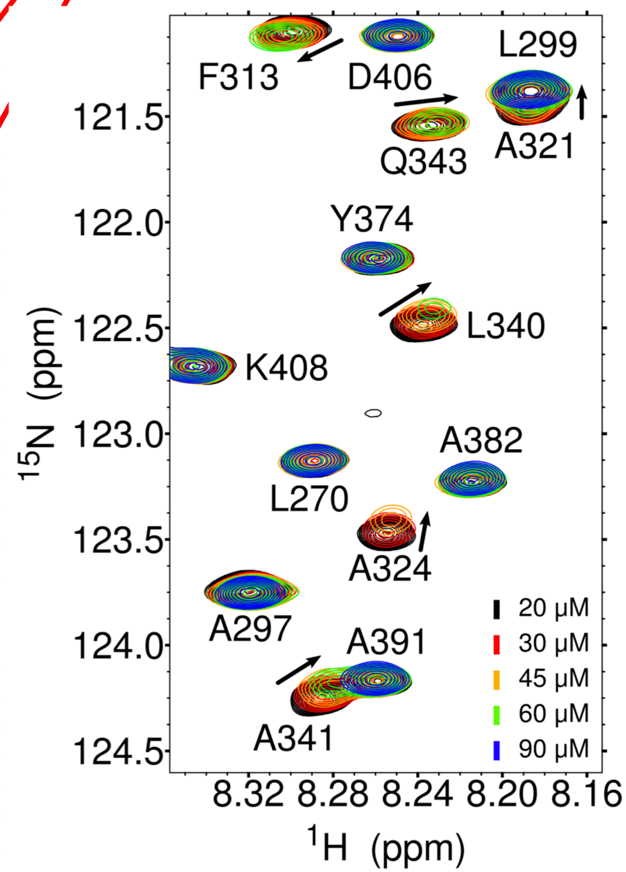
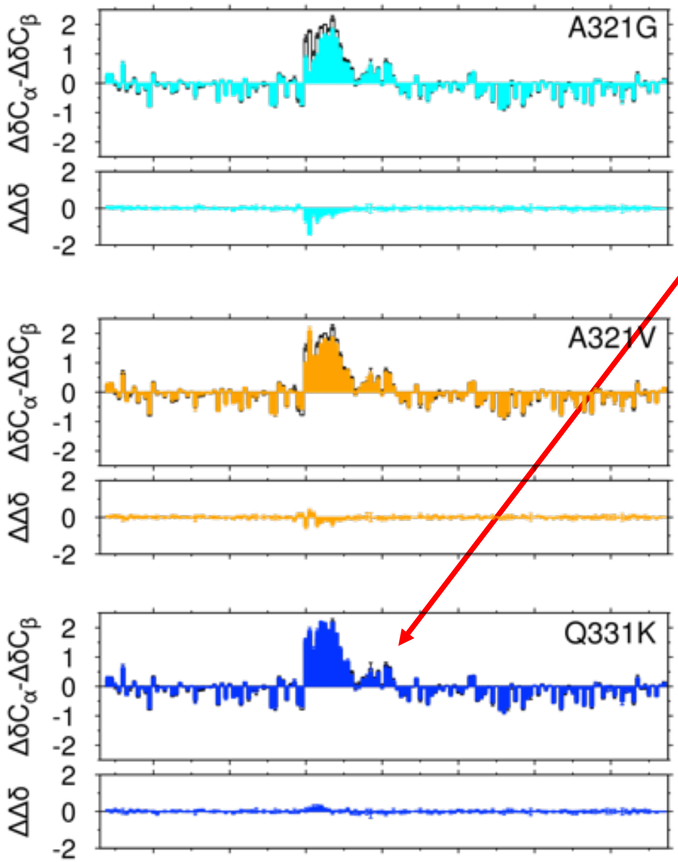



But we noticed:

Wild type TDP-43 self interacts at low salt (without phase separation)

# TDP-43 helix-helix assembly




Disruption of helical structure of monomer?

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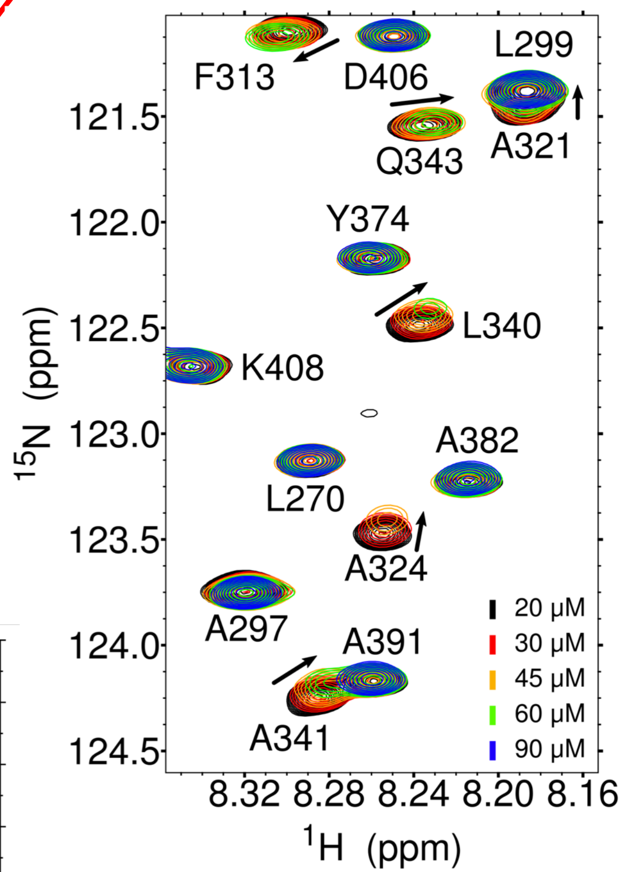


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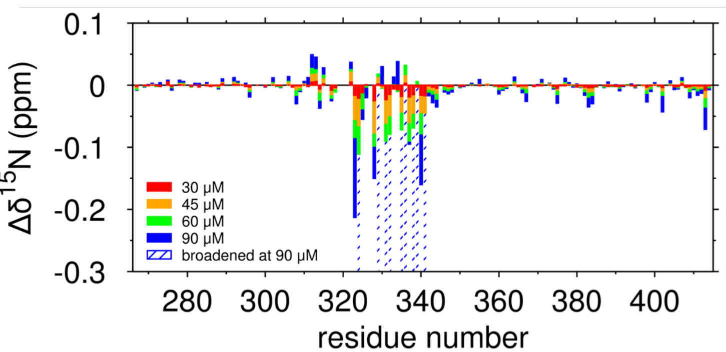
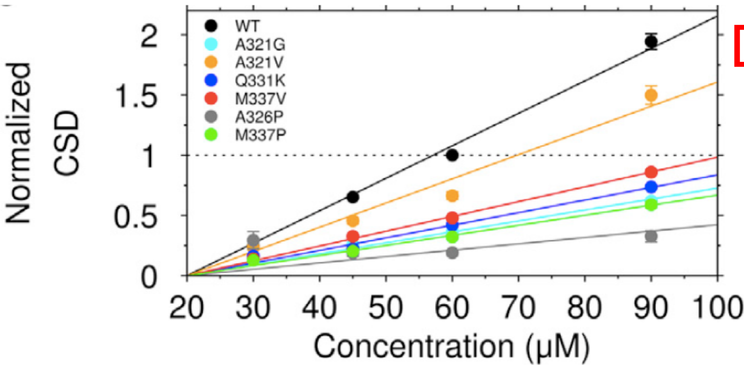



Self interaction at the 321-340 region

# TDP-43 helix-helix assembly

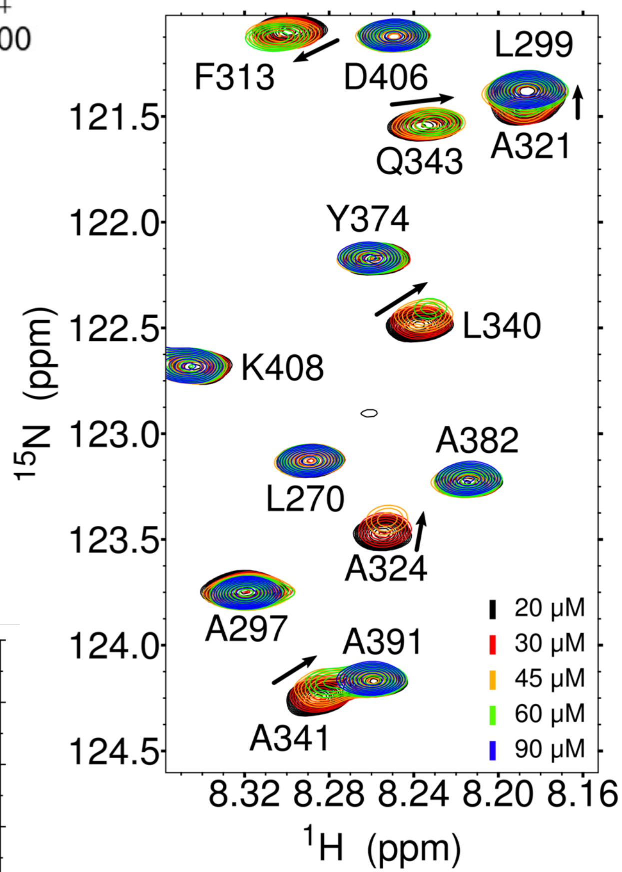



Disruption of helical structure of monomer?

*No change for Q331K or M337V, can't explain change in phase separation*

**ALS mutations disrupt helix-helix interactions**

- even those that don't disrupt monomer structure
- Structured, helix-helix contacts → assembly

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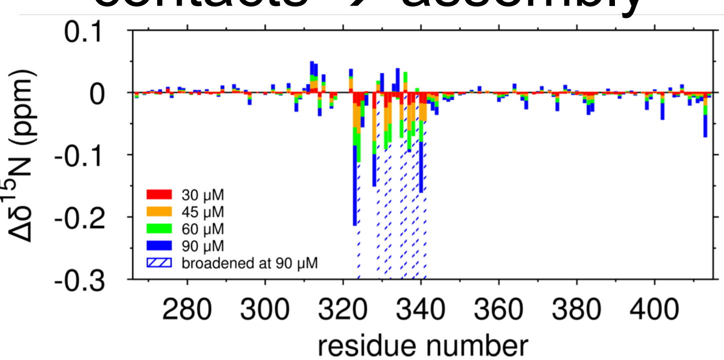
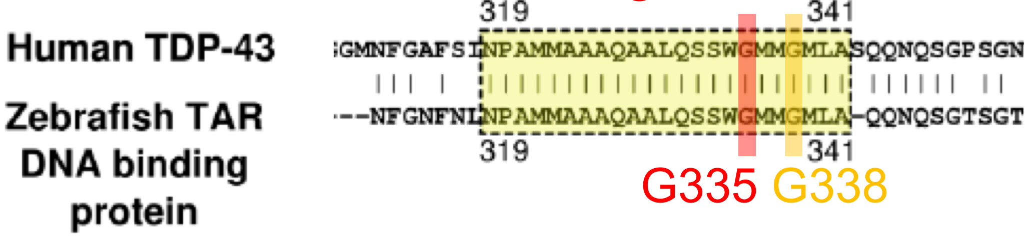



Self interaction at the 321-340 region

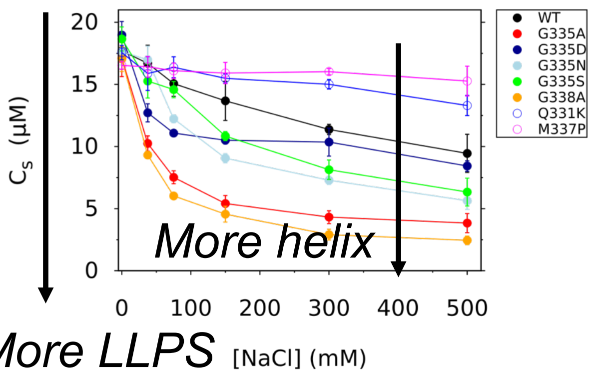
# TDP-43 helix-helix assembly

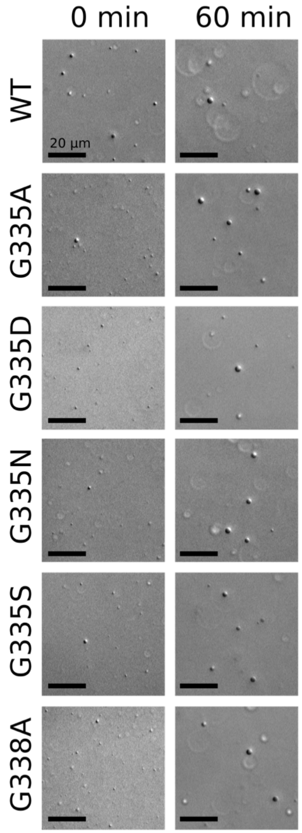
## G335 and G338 discourage helical assembly?



# TDP-43 helix-helix assembly

## G335 and G338 discourage helical assembly



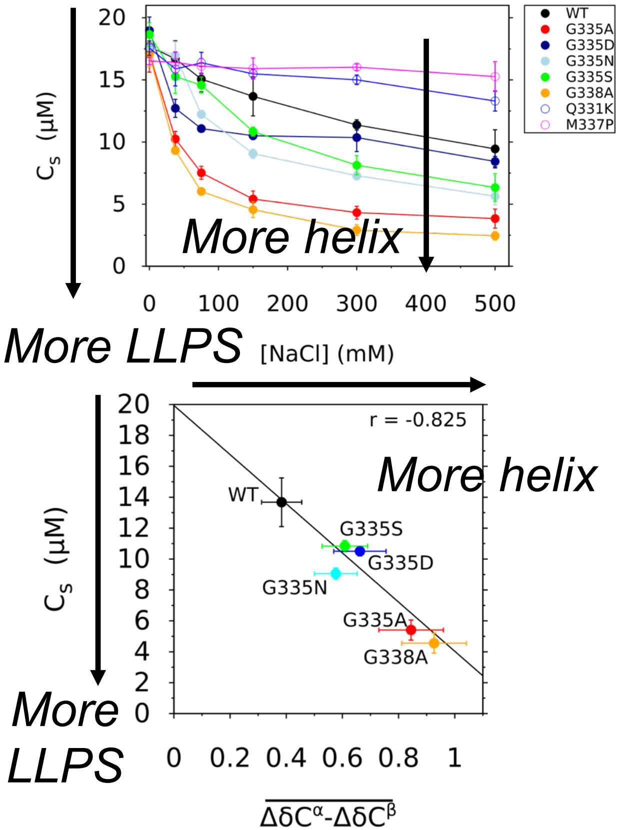



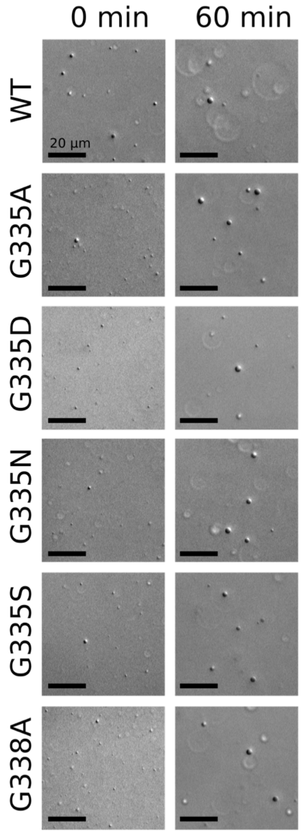

G335A, G338A, and other variants enhance helix-helix interaction and LLPS *in vitro*

Conicella and Dignon et al., *in prep*

# TDP-43 helix-helix assembly

## G335 and G338 discourage helical assembly

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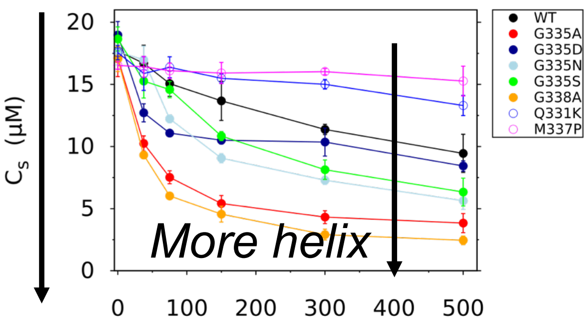
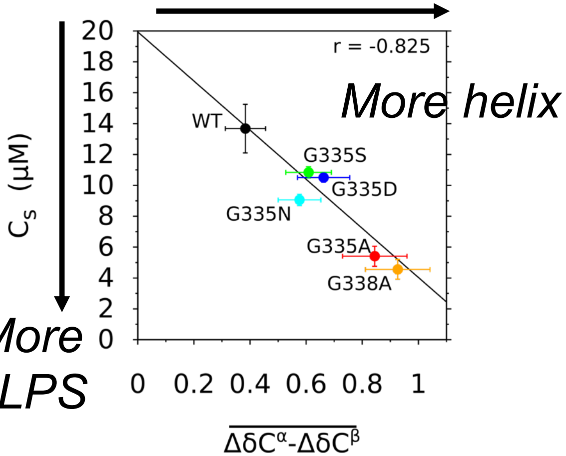
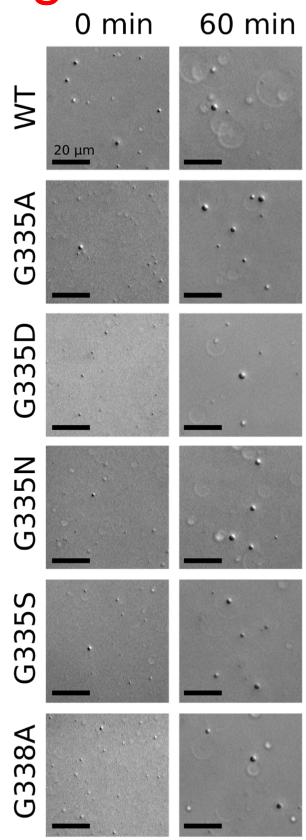
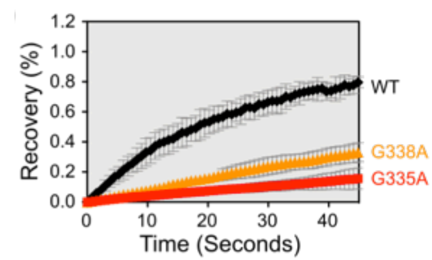
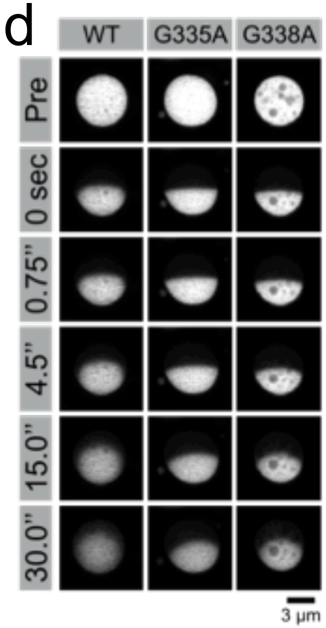



G335A, G338A, and other variants decrease fluidity of TDP-43 reporter droplets in cell



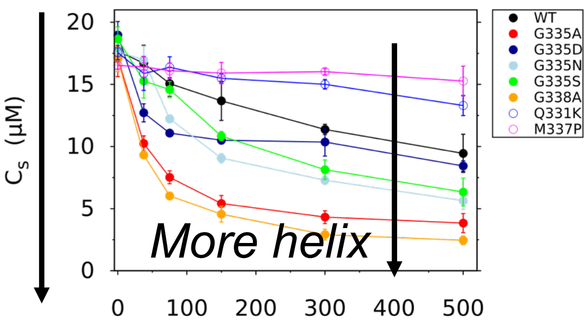

G335A, G338A, and other variants enhance helix-helix interaction and LLPS *in vitro*

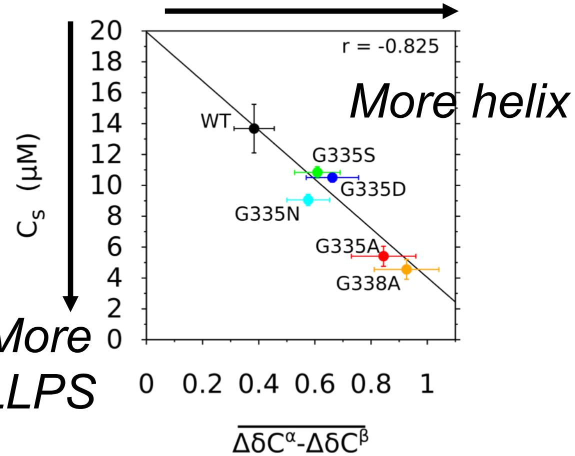
Conicella and Dignon et al., *in prep*

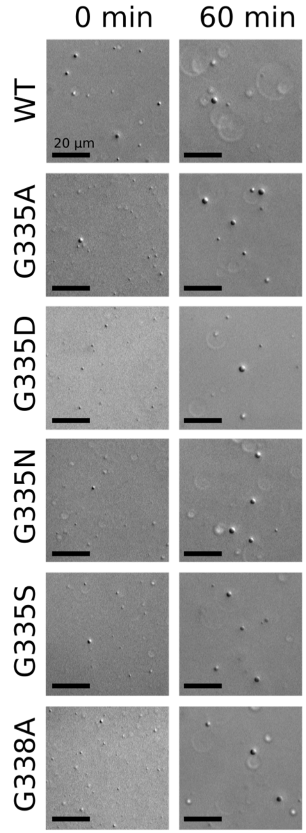
**Broder Schmidt (Stanford)**

# TDP-43 helix-helix assembly

## G335 and G338 discourage helical assembly

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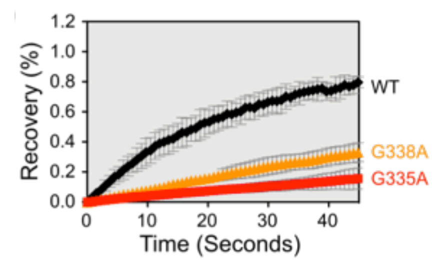
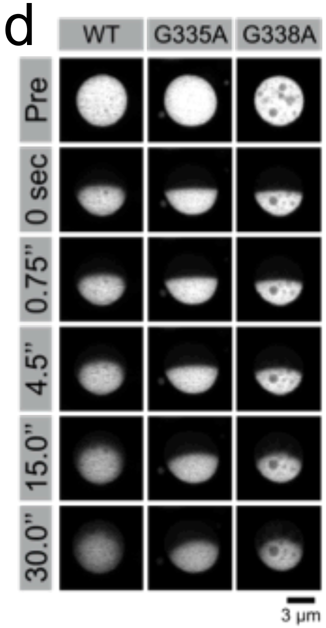
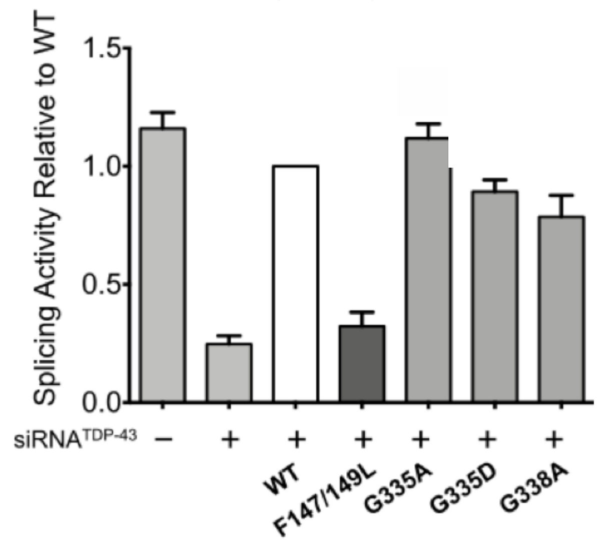


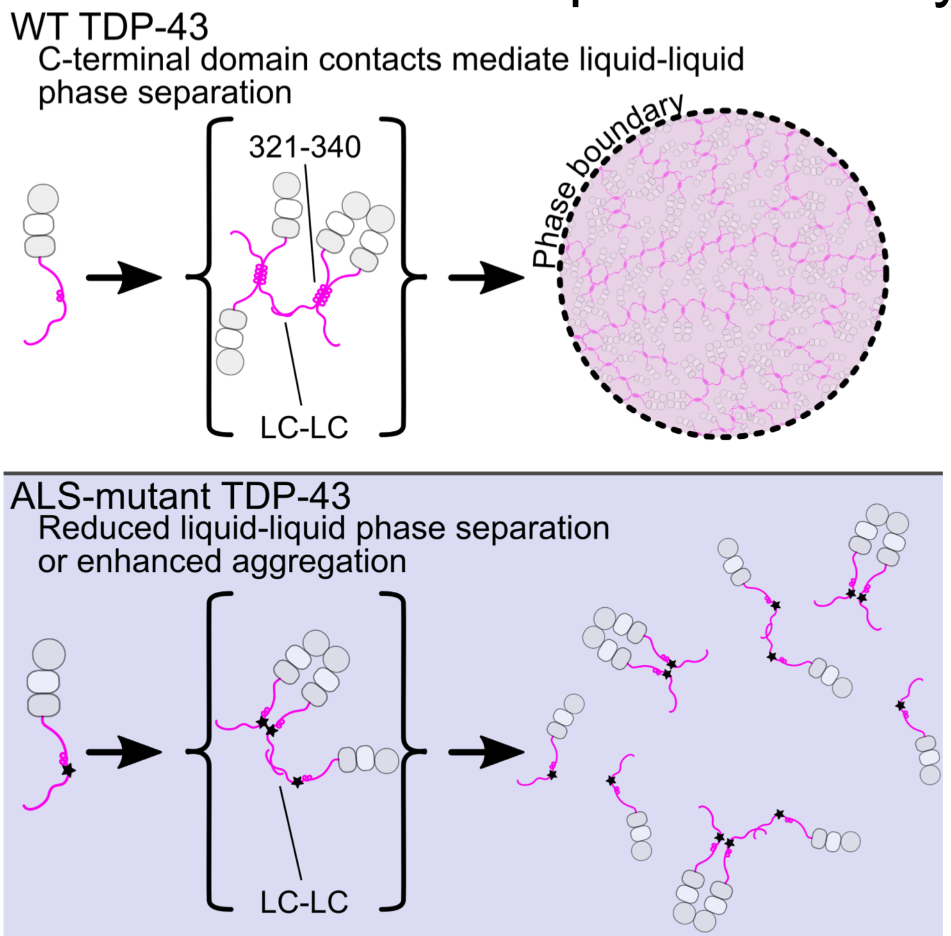

G335A enhances splicing activity of TDP-43

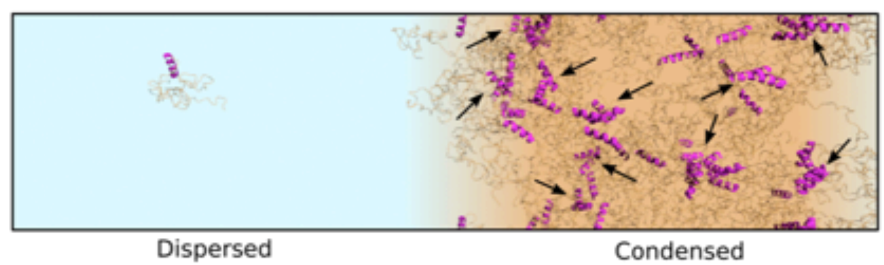
**(Yuna Ayala, SLU)**

# Seeing inside granules

## TDP-43: helical regions

- boost disordered region LLPS
- extend/enhance upon assembly



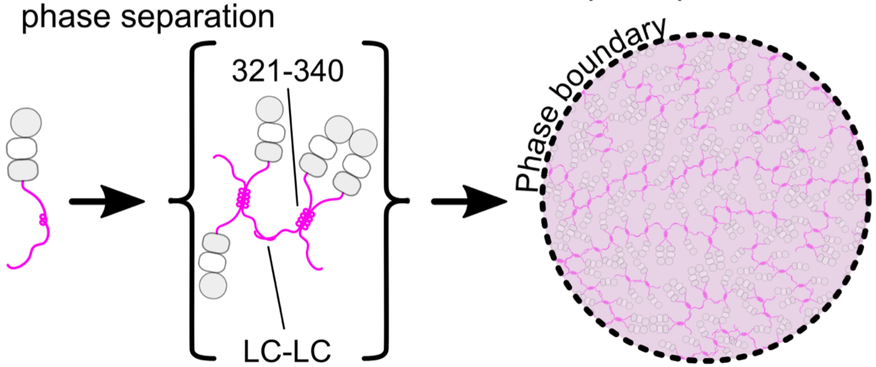




Conicella AE, Zerze GM, Mittlal J, Fawzi NL, *Structure.* (2016)

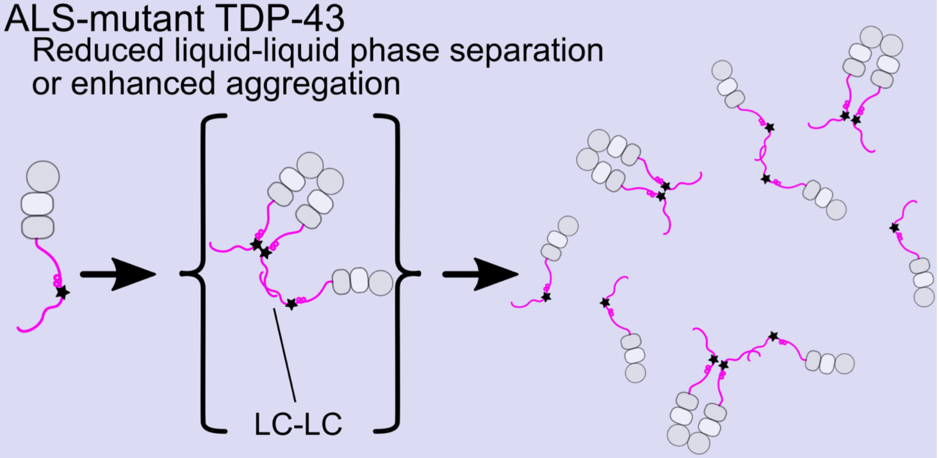
# Seeing inside granules

**TDP-43:** helical regions
- boost disordered region LLPS
- extend/enhance upon assembly

WT TDP-43
C-terminal domain contacts mediate liquid-liquid phase separation

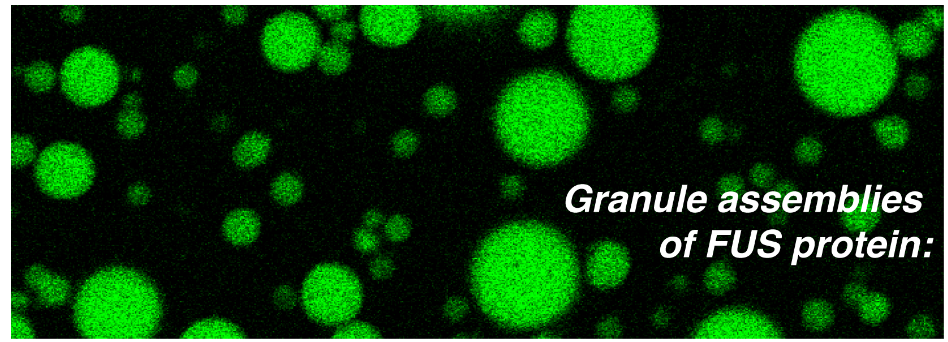


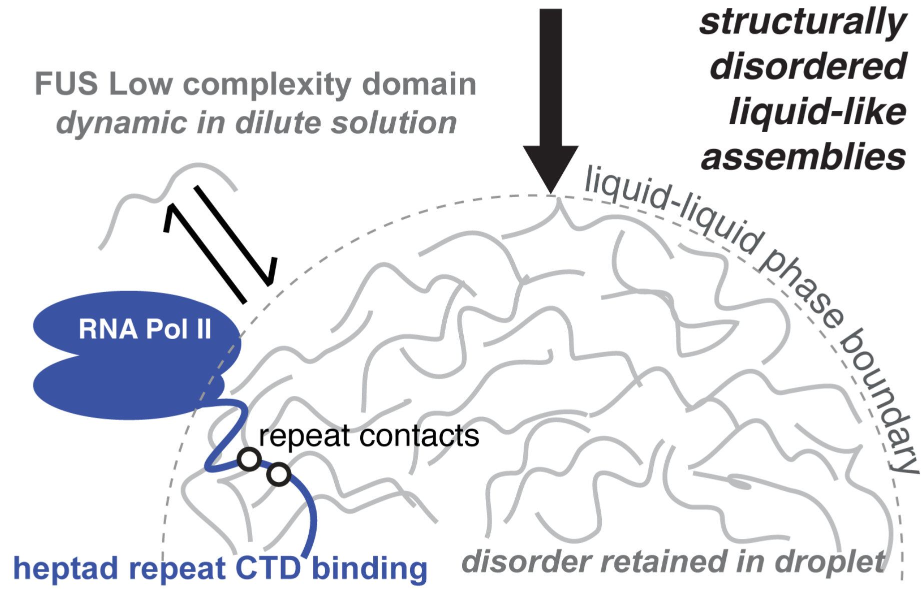

ALS-mutant TDP-43
Reduced liquid-liquid phase separation or enhanced aggregation



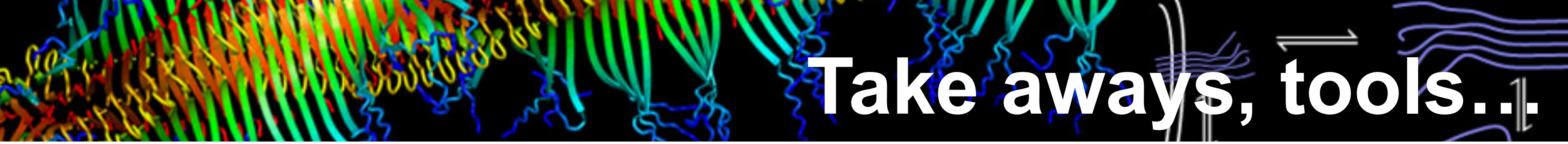

Conicella AE, Zerze GM, Mittlal J, Fawzi NL, *Structure.* (2016)

**FUS:** disordered structure predominates even inside phase separated environment




Burke KA, Janke AM, Rhine CL, Fawzi NL, *Molecular Cell.* (2015)



# Take aways, tools…

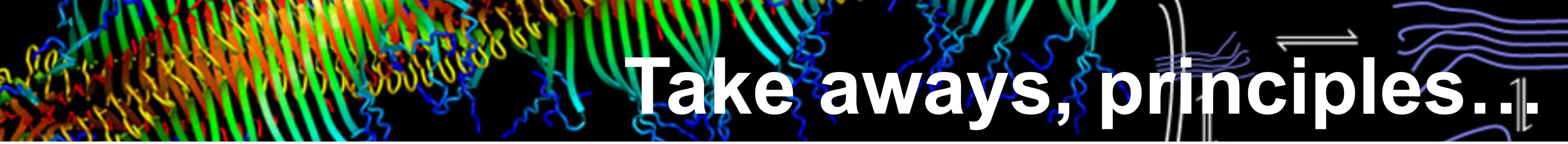
- **NMR "sees" disorder domains at atomic scale**
  - Atomic details of interactions, structure, effect of mutations and post-translational modifications

- **Combining with microscopy to see micron-scale behavior**

- **Simulations predictive insight into structure**

  - *with Jeetain Mittal, Lehigh University*

- **CARS microscopy probes structure in droplets**
  - *with Sapun Parekh, MPI Polymer / UT Austin*



# Take aways, principles…

- **Low complexity domains show predominant disorder**
  - Before and after liquid-liquid phase separation (LLPS)
  - Observed for proteins: FUS, TDP-43, and hnRNP A2

- **Structure can contribute to LLPS**
  - short helix-helix interaction embedded in TDP-43
  - Linear chain formation of TDP-43 N-terminal domain
  - But the primary contacts of LC domains are dynamic, distributed and degenerate

- **Disease mutations and post-translational modifications alter LLPS, aggregation, toxicity in cell**
  - Potential regulatory role and therapeutic opportunity

- **Titration of RNA (and salt) stimulates, then disfavors, select protein LLPS**

# Acknowledgements


**Fawzi group:**

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Anna Bock
Scott Watters
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BROWN

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SKELETAL HEALTH AND REPAIR

NSF

HFSP
HUMAN FRONTIER SCIENCE PROGRAM
FUNDING FRONTIER RESEARCH INTO COMPLEX BIOLOGICAL SYSTEMS
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ALS ASSOCIATION

RHODE ISLAND FOUNDATION

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*Basic Discoveries for Better Health*

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Rhode Island INBRE

***Collaborators:***

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Sapun Parekh - *MPI Polymer/UT Austin*
Jeetain Mittal and Greg Dignon - *Lehigh*
Frank Shewmaker - *USUHS*
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Tanja Mittag and Erik Martin - *St Jude*
Yuna Ayala - *St Louis University*
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Tony Hyman and Richard Wheeler - *MPI Dresden*