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

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Looking for Postdoc!

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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.
Take aways, tools...

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Amino acid #n

Amino acid #n+1
• NMR “sees” disorder domains at atomic scale
  • Atomic details of interactions, structure, effect of mutations and post-translational modifications

![Diagram of amino acids and NMR frequency analysis]

- All the nitrogen nuclei are distinct (low notes = low frequency)
- All the hydrogen nuclei are distinct (high notes = high frequency)

Amino acid #n
Amino acid #n+1
Take aways, tools...

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

1. Frequency of $^1$H nucleus
2. Frequency of $^{15}$N nucleus attached to H
3. All the nitrogen nuclei are distinct
4. All the hydrogen nuclei are distinct

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

Bell figures from: *Pocket Guide to Biomolecular NMR*, by Michaeleen Doucleff
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**
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
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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Mutations in NLS and Low complexity (LC aka SYGQ-rich) regions are associated with amyotrophic lateral sclerosis, leading to protein aggregation.

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


**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
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?
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?
“Prion-like” Low complexity (LC) domains: unusual sequences

Rich in amino acids:
- serine (S)
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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)

Adapted from: Schmidt and Görlich, *Elife* 2015
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 150 mM NaCl pH 5.5 @ 25 °C
FUS LC is unstructured

FUS LC structural work by Kathleen Burke


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

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

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

Phosphomimetics disrupt phase separation when RNA present or with high salt

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
What is the structure of FUS LC inside a “droplet”?

1 mM FUS LC -- unstable

Cloudy, phase separated

FUS LC phase separation
What is the structure of FUS LC inside a “droplet”?

1 mM FUS LC -- unstable

Cloudy, phase separated

Low temp

More spin

<100 μM

Phase separated Dispersed

Two phases

~7 mM
Atomic details of FUS LC droplets

FUS LC primarily disordered in liquid phase separated state
- Phase-separated state overlays with dispersed

~7 mM FUS LC at 25 °C derived from an original sample:
1 mM FUS LC in 50 mM MES/BisTris 150 mM 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 an 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 (>10 mM) dispersed (0.05 mM)

100x signal in phase-separated vs control

~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.

Cool + Slow centrifugation in NMR tube

Phase separated FUS LC

FUS data from Anastasia Murthy

Dilute phase

Concentrated phase
Using NMR spectroscopy to see within the phase separated state

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

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.

Dilute phase

Concentrated phase

Drastically slowed FUS LC diffusion in LLPS

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<th>Diffusion coefficient ($\mu m^2/s$)</th>
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<td>Buffer     440.0 ± 40.9</td>
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<tr>
<td>Condensed FUS LC n.a.</td>
</tr>
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<td>Buffer    69.4 ± 7.47</td>
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Gradient Ratio
Using NMR spectroscopy to see within the phase separated state

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

Even diffusion of buffer slows down.

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

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.

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Drastically slowed FUS LC diffusion in LLPS

Push experiment to limit to see slow FUS LC diffusion
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.

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

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

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

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

More phase separation

More glutamines
Glutamine residues contribute to FUS LC LLPS

Glutamine substitutions modulate FUS LC LLPS

More phase separation

More glutamines

Protein Remaining in Supernatant (μM)

[NaCl] mM

8M urea 0 150 300 600 1000

SSYQGQPQSGSYSQPSYGQGGQQQSYGQQQSYNPPQGYGQQNQYNS

Hydrogen Bonds

Backbone
Side chain

Residue #
Hydrophobic interactions contribute to FUS LC LLPS

**FUS LC LLPS induced by kosmotropic salts**

More phase separation

![Graph showing protein remaining in supernatant vs salt concentration](image-url)
Hydrophobic interactions contribute to FUS LC LLPS

FUS LC LLPS induced by kosmotropic salts

More phase separation

Hydrophobic contacts mediated by glutamine and tyrosine
Hydrophobic interactions contribute to FUS LC LLPS

FUS LC LLPS induced by kosmotropic salts

Hydrophobic contacts mediated by glutamine and tyrosine

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
What insights to the function of TDP-43 can be obtained from its structure?


Ling et al., Neuron, 2013.

Multimerization
What is role of NTD

RNA-binding

Mediates aggregation in ~90% of ALS
Site of 50 out of 52 known ALS mutations
What insights to the function of TDP-43 can be obtained from its structure?

TDP-43 CTD contains **helical** region

Human TDP-43

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Zebrafish TAR DNA binding protein

**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

Helical region is cooperatively formed

ALS mutations alter phase separation

Requires helical region...
TDP-43 CTD contains helical region

Helical region is cooperatively formed

ALS mutations alter phase separation

Requires helical region...

ALS A321V: prone to liquid to aggregate conversion
TDP-43 helical structure

Disruption of helical structure of monomer?
No change for Q331K or M337V
can’t explain change in phase separation
Disruption of helical structure of monomer?

No change for Q331K or M337V can’t explain change in phase separation

But we noticed:

Wild type TDP-43 self interacts at low salt (without phase separation)
Disruption of helical structure of monomer?

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

But we noticed:

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

Self interaction at the 321-340 region
ALS mutations disrupt helix-helix interactions
- even those that don’t disrupt monomer structure
- Structured, helix-helix contacts → assembly

Disruption of helical structure of monomer?
No change for Q331K or M337V, can’t explain change in phase separation

But we noticed:
Wild type TDP-43 self interacts at low salt (without phase separation)

Self interaction at the 321-340 region
G335 and G338 discourage helical assembly?
G335 and G338 discourage helical assembly

G335A, G338A, and other variants enhance helix-helix interaction and LLPS \textit{in vitro}

Conicella and Dignon et al., \textit{in prep}
G335 and G338 discourage helical assembly

More LLPS

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

Conicella and Dignon et al., in prep
G335 and G338 discourage helical assembly

**More helix**

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

**More LLPS**

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

Conicella and Dignon et al., *in prep*
G335 and G338 discourage helical assembly.

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

G335A enhances splicing activity of TDP-43 (Yuna Ayala, SLU).

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

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

FUS: disordered structure predominates even inside phase separated environment

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

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- 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
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- NSF
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