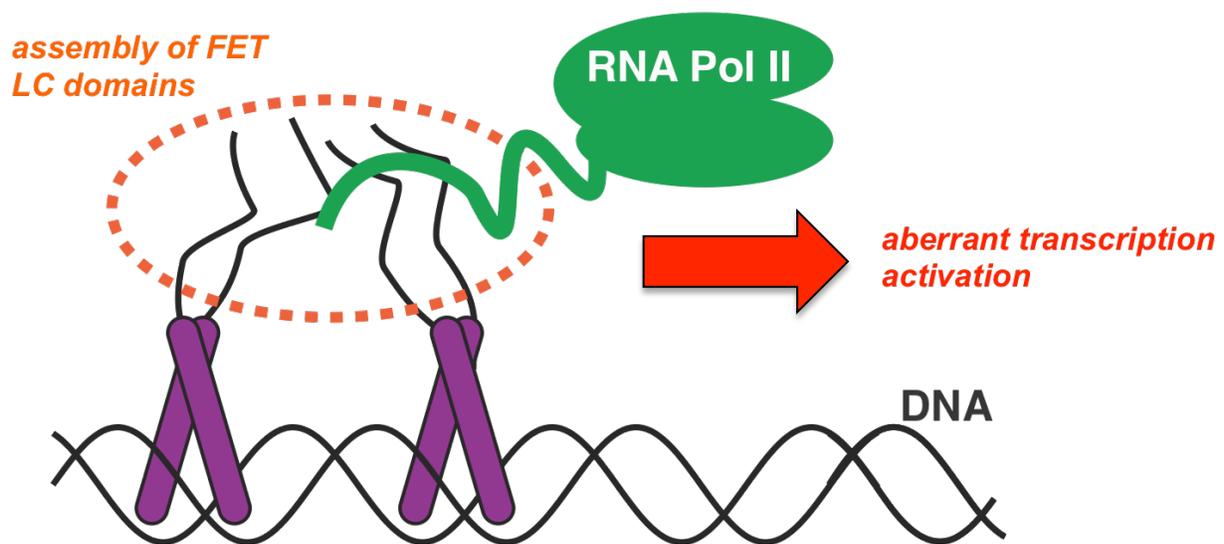


Structure and dynamics of RNA polymerase II C-terminal domain in complex with cancer-linked FET protein assemblies



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STATEMENT OF CONTRIBUTIONS

- The title page graphic was adapted from Dr. Fawzi. Unless otherwise noted, all other figures are my own and were generated from data I personally collected.
- NMR experiments: Dr. Fawzi, Dr. Burke, Alexander Conicella, and Dr. Clarkson set up and helped me run all relaxation and triple resonance experiments. I independently ran most HSQC experiments.
- Bacterial plasmids: Rute Silva and Dr. Burke cloned MBP-FUS FL. CTD27-52 was synthesized by DNA2.0, and I cloned CTD27-37, CTD38-52, and CTD43-52. mCherry-FUS LC and mCherry-TAF15 LC were gifts from the McKnight lab.
- Dr. Burke contributed to assignments of CTD27-52.

GLOSSARY

- **β -turns:** Common protein structural motif that is most often defined as a sequence of four amino acid residues containing a hydrogen bond between the carbonyl of the first residue and the amide of the fourth residue
- **CARM1:** Coactivator-associated arginine methyltransferase 1; enzyme that has been shown to methylate RNA polymerase II CTD's single arginine residue (R1810)
- **CON spectrum:** ^{13}C - ^{15}N NMR spectrum that correlates resonances of carbonyl carbons with resonances of amide nitrogens; provides a fingerprint of a protein much like an HSQC
- **CTD:** RNA polymerase II carboxy-terminal domain
- **CTD degenerate repeats:** In the mammalian RNA polymerase II CTD, heptad repeats 27-52 deviate from the YSPTSPS consensus sequence
- **EWS:** Ewing Sarcoma Breakpoint Region 1, an RNA-binding protein in the FET family
- **FET:** Family of proteins important in RNA biogenesis (FUS, EWS, TAF15)
- **FUS:** Fused in Sarcoma, an RNA-binding protein in the FET family
- **HSQC spectrum:** ^1H - ^{15}N heteronuclear single quantum coherence NMR spectrum; correlates amide proton resonances with amide nitrogen resonances and provides a 'fingerprint' of a protein
- **Intrinsically disordered proteins:** Proteins without an ordered or fixed three-dimensional structure (includes random coils, pre-molten globules, and partially unstructured multi-domain proteins); disorder is not due to denaturation or unfolding
- **Left-handed polyproline II helix (PPII):** Type of protein secondary structure in which adjacent amino acid residues adopt backbone dihedral angles near $\phi = -75^\circ$, $\psi = 150^\circ$
- **Low-complexity (LC) domain:** Protein domain with a simple amino acid sequence; FUS, EWS, and TAF15 each have a LC domain that is thought to act as a transcriptional activator
- **mRNA:** Messenger ribonucleic acid that is transcribed from deoxyribonucleic acid (DNA) by RNA Polymerase II; the ribosome translates mRNA into proteins
- **NOE:** Nuclear Overhauser effect; NMR measurement that can be used to study protein structure and interactions and relies on the transfer of energy through dipole-dipole couplings
- **PIC:** Pre-initiation complex; complex of RNA polymerase II and general transcription factors that assembles at the promoters of genes before transcription begins in eukaryotes and archaea
- **R_1 :** Rate of longitudinal relaxation; reciprocal of T_1 , the time it takes for the z component of nuclear spin magnetization to recover its thermal equilibrium value
- **R_2 :** Rate of transverse relaxation; reciprocal of T_2 , the time it takes for nuclear spin magnetization to decohere
- **ΔR_2 :** Difference in rate of transverse relaxation; if ^{15}N ΔR_2 measurements are calculated for a monomer in the presence and absence of larger, NMR-invisible fibrils, ^{15}N ΔR_2 can be interpreted as a lower bound for the first order on-rate for the monomer binding the fibrils
- **TAF15:** TATA Box Binding Protein (TBP)-Associated Factor 15, an RNA-binding protein in the FET family
- **TEV protease:** Tobacco Etch Virus protease that specifically cleaves at EXXYXQ(G/S) motifs; we use TEV protease to cleave MBP and histidine tags from our proteins
- **Triple resonance experiments:** CBCA(CO)NH, HNCACB, HNCO, HN(CA)CO, HNCA, HNN; these $^1\text{H}_\text{N}$ -detected NMR experiments correlate $C\alpha$ and $C\beta$ chemical shifts with NH groups by transferring magnetization between amide protons, amide nitrogens, and carbon atoms, and they are used to assign the backbone of a protein

ABSTRACT

Translocations of FET protein (**F**US, **E**WS, **T**AF15) low complexity (LC) domains onto transcription factor DNA-binding domains are known to cause cancer (Arvand and Denny, 2001; Guipaud et al., 2006; Lessnick and Ladanyi, 2012). Because FET LC domains are believed to be potent transcriptional activators, FET LC fusions are thought to cause aberrant transcription of genes related to cell growth and survival (Kwon et al., 2013). But what makes FET LC domains such potent transcriptional activators? Various *in vitro* models have suggested that higher order assemblies of FET LC domains recruit RNA Polymerase II CTD to promoters, leading to formation of the pre-initiation complex (Kwon et al., 2013; Schwartz et al., 2013; Burke et al., 2015). While others have shown that TAF15 LC fibrils recruit the degenerate half of RNA polymerase II carboxy-terminal domain (CTD), structural details of the complex formed between self-assembled FET proteins and the CTD remain unknown.

Here we detail the first nuclear magnetic resonance (NMR) study of the intact degenerate repeat half of the CTD (CTD27-52). We report ^1H , ^{15}N backbone resonance assignments as well as key structural and dynamic parameters of CTD27-52, which verify that the unphosphorylated degenerate half of the CTD exists in an entirely disordered conformation. We then characterize the dynamics of the RNA polymerase II CTD27-52 in complex with TAF15 LC fibrils. In the presence of TAF15 LC fibrils, backbone resonances within the first 7 heptads of the degenerate half of RNA polymerase II CTD exhibit heightened transverse relaxation, consistent with regions mediating direct binding of TAF15 LC fibrils. Our findings help characterize the mechanism by which higher order assemblies of FET LC domains recruit RNA polymerase II CTD, which is critical for understanding the role of FET translocations in cancer. Separately, and perhaps more significantly, our backbone resonance assignments of unphosphorylated CTD27-52 facilitate future investigations of residue-specific interactions between the CTD and numerous transcription initiation factors.

INTRODUCTION

Discovery of the RNA Polymerase II CTD. RNA polymerase II is the principle enzyme mediating gene expression in eukaryotic cells. Its most significant role is catalysis of transcription of pre-mRNA from DNA. RNA polymerase II's long, flexible carboxy-terminal domain (CTD) was first discovered in 1985 (Corden et al., 1985; Allison et al., 1985). Although essential for life (Nonet et al., 1987), the CTD is often not required for transcription initiation and mRNA synthesis *in vitro* (Payne et al., 1989; Zehring et al. 1990; Buratowski et al. 1990; Kang et al., 1993; Akoulitchev et al., 1995), suggesting it is not the 'catalytic essence' of RNA polymerase II (Corden et al., 2013).

The CTD distinguishes RNA Polymerase II from its enzymatic relatives, RNA Polymerase I and RNA Polymerase III, which catalyze transcription of pre-rRNA in the nucleolus and pre-tRNA in the nucleoplasm, respectively (Corden et al., 2013). The CTD is thought to help coordinate interactions between RNA Polymerase II and various proteins critical for transcription and co-transcriptional activities. Besides recruiting RNA polymerase II to the pre-initiation complex through interaction with the Mediator and general transcription factors (GTFs) bound to gene promoters, the CTD may help: 1) recruit pre-mRNA 5'-end capping enzymes; 2) facilitate efficient splicing alongside transcription; 3) coordinate proper cleavage and polyadenylation of pre-mRNA 3'-ends; 4) initiate transport of mRNA to the cytoplasm; and 5) contribute to DNA damage repair (Corden et al., 2013).

Cracking the CTD Code. The mystique of the CTD has prevailed for more than three decades, largely because no consensus has been reached on the CTD's structure or the unique roles of its numerous post-translational modifications. In fact, the potential complexity of the CTD code has increased exponentially since its discovery, as newly significant posttranslational

modifications continue to emerge. Models of the CTD code rely on the assumption that the CTD's pattern of posttranslational modifications coordinates specific steps of the transcription cycle (Jasnovidova et al., 2013). Phosphorylation of residues Tyr1, Ser2, Thr4, Ser5, and Ser7 within each of 52 imperfect $Y_1S_2P_3T_4S_5P_6S_7$ heptad repeats is the most widely studied modification. Briefly, researchers believe that unphosphorylated CTD interacts with Mediator and recruits RNA polymerase II to the pre-initiation complex (PIC); phosphorylation of Ser5 then helps release CTD from the PIC as well as recruit 5'-end capping enzymes. Next, RNA polymerase II enters the elongation phase of transcription, and pSer5 levels decrease while pSer2, pThr4, and pTyr1 levels rise, facilitating recruitment of splicing factors and chromatin-modifying enzymes. Lastly, pThr4 levels decrease near the 3'-end of the gene while pSer2 levels peak, allowing the CTD to associate with cleavage and polyadenylation machinery. Following termination of transcription, the CTD is rapidly dephosphorylated (Corden et al., 2013).

Besides phosphorylation of the CTD, many other post-translational modifications have been observed. Glycosylation of Ser5 and Ser7 within each heptad may prevent aberrant phosphorylation during formation of PICs (Ranuncolo et al., 2012). Methylation of the CTD's single arginine residue by CARM1 inhibits expression of small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) (Sims et al., 2011), and CTD with methylated lysine residues is highly enriched at promoters of active genes (Dias et al., 2015). Since methylation and glycosylation introduce steric blocks and preclude formation of hydrogen-bonds and salt bridges, it is plausible that such modifications occlude high-specificity binders of the CTD during the earliest stages of transcription. In this way, they may prevent induced folding of the CTD and preserve its random coil structure, discussed in more detail below.

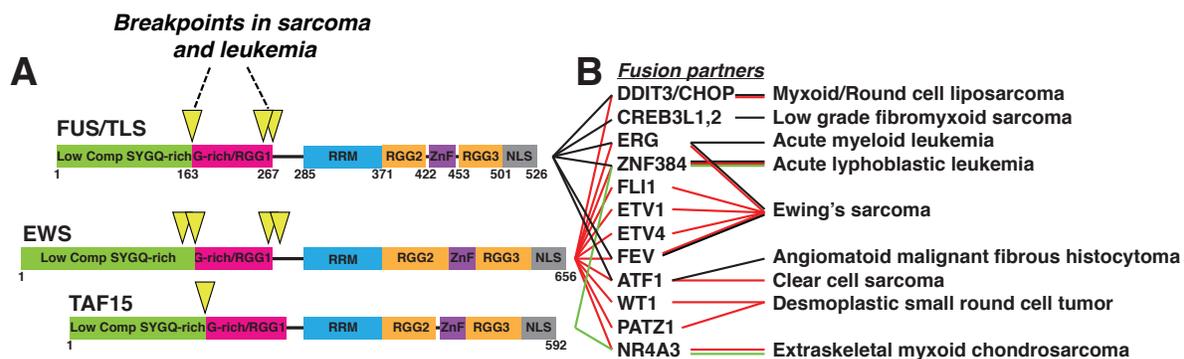
Solving the CTD's Structure. The structure of the CTD continues to stump researchers because its Y₁S₂P₃T₄S₅P₆S₇ consensus sequence introduces complications in the interpretation of circular dichroism (CD) spectra and nuclear magnetic resonance (NMR) data. Furthermore, the disorder and flexibility of the CTD precludes visualization by either X-ray crystallography or cryo-electron microscopy. Hence, even though the cryo-electron microscopy structure of the RNA polymerase II elongation complex was recently solved at 3.4 angstrom resolution, the CTD was invisible within the structure (Bernecky et al., 2016). Theoretical studies have proposed a compact yet flexible conformation based on energy minimization of a polyproline helix (Matsushima et al., 1990), while early NMR studies have proposed a unique β -spiral conformation based on nuclear Overhauser effects (NOEs) that suggest the CTD forms β -turns at its two SPXX (SPTS, SPSY) motifs (Cagas et al., 1995). By generating CD spectra of the same eight-consensus-repeat fragment that Cagas et al. studied with NMR spectroscopy, Bienkiewicz et al. contested Cagas et al.'s proposed β -spiral conformation. Since the CD spectra of the synthetic CTD peptide did not exhibit "classical" structural components, they used convex constraint analysis (CCA) to conclude that at equilibrium, CTD monomers are primarily unordered with a small fraction adopting a left-handed polyproline II helix (PPII) conformation. Interestingly, the same unordered + PPII conformation dominated CD spectra of full-length murine CTD. Bienkiewicz et al. argue that Cagas et al.'s use of NOEs biased their observation of β -turns, because transient β -turn populations could lead to distinctive NOEs even if the peptide is primarily disordered. Thus, they concluded that under physiological conditions, neither β -turns nor PPII conformations were heavily populated in either CTD fragments or full-length CTD. However, they caution that different regions of the CTD may adopt distinct conformations, a phenomenon unobservable by low-resolution CD studies (Bienkiewicz et al., 2000).

Complexes of CTD and Binding Partners. Crystal structures of the CTD in complex with binding partners show that interactions between the CTD and specific transcription factors, phosphatases, and mRNA capping enzymes often couple CTD binding and CTD folding (Jasnovidova et al., 2013). Because interactions requiring a transition from disorder to order are generally of low affinity but high specificity, these domains' interactions with the CTD are most likely transient. Consequently, the CTD has been compared to a fly casting apparatus that localizes RNA polymerase II to transcription factories by increasing local concentrations of interacting proteins. This theory may help explain why RNA polymerase I doesn't have a CTD, since it is already trapped within the nucleolus (Corden et al., 2013).

Role of CTD in Transcription Initiation. The dominance of the CTD's folded state among well-studied interactions does not imply it is more physiologically relevant than the CTD's unfolded state. This dominance merely reflects the challenges inherent in studying intrinsically disordered proteins. New reports show that a random coil conformation of the CTD is avidly recruited by purportedly amyloid-like fibrils of both full-length FUS protein and FET protein low complexity (LC) domains *in vitro* (Schwartz et al., 2013; Kwon et al., 2013). These studies have hypothesized that higher order assemblies of FET LC domains recruit the CTD to promoters, leading to formation of the pre-initiation complex. The pathological significance of this interaction is discussed in more detail below. Because the structural disorder of the CTD may facilitate interaction with other intrinsically disordered proteins during transcription initiation, it is important to note that the CTD is predominantly unphosphorylated during recruitment to promoters (Corden et al., 2013). Given that phosphorylation extends the CTD, making it a more suitable platform for enzymes and other factors that bind with high-specificity (Laybourn et al., 1989; Zhang et al., 1991), it is plausible that a compact, random coil

conformation heightens the CTD's affinity for transcription activation factors while simultaneously occluding binding partners that induce a more structured conformation.

Role of FET Proteins in Cancer. The FET family of proteins consists of fused in sarcoma (FUS), Ewing sarcoma breakpoint region 1 (EWS), and TATA Box Binding Protein (TBP)-Associated Factor 15 (TAF15). FUS, EWS, and TAF15 are abundant heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins that bind both RNA and DNA and affect multiple steps of RNA biogenesis (Schwartz et al., 2015). FET proteins share a similar domain structure (**Figure 1A**), which includes a low complexity (LC), putatively disordered SYQG-rich N-terminal domain known to promote FET protein self-assembly as well as a folded RNA Recognition Motif (RRM) and zinc-finger domain believed to mediate RNA binding.



¹**Figure 1. Translocations of FET protein low complexity (LC) domains on DNA-binding domains cause cancer.** (A) FET family proteins (FUS, EWS, TAF15) share a similar domain structure. Their low-complexity (LC) SYGQ-rich N-terminal domains are putatively disordered and seed FET self-assembly. Their RNA recognition motifs (RRMs) and zinc fingers (ZnF) are folded and bind RNA. (B) Translocations of FET LC domains onto DNA-binding domains of transcription factors are linked to numerous types of cancer. Yellow triangles mark examples of breakpoints in sarcoma and leukemia.

¹ Figure 1 was adapted from a presentation given by N.L. Fawzi. at Columbia University in January of 2016.

Previous studies have shown that polymerization of FET LC domains into amyloid-like fibrils correlates with not only recruitment of the CTD but also transcription activation. Given that fusions of FET LC domains to transcription factor DNA-binding domains are known oncoproteins, it is plausible that interaction between FET assemblies and the CTD directly leads to aberrant transcription of growth factor and cell survival genes implicated in various cancers (Kwon et al., 2013). Interestingly, Kwon et al. observed that FET LC assemblies more readily bind the CTD's degenerate repeat half than its tandem consensus repeat half, further supporting a unique role for the CTD's degenerate repeats in transcription activation. Surprisingly, they found that TAF15 LC assemblies bind the CTD with greater affinity than FUS LC assemblies (Kwon et al., 2013). They hypothesized that charge attraction between aspartic acids in the TAF15 LC domain (absent from FUS LC) and lysines in degenerate CTD repeats mediates the heightened affinity (Kwon et al., 2013). However, the heightened affinity may also be explained by lesser polymerization of FUS LC assemblies compared to TAF15 LC assemblies. Other studies of full-length FUS protein report that its RNA-binding domain is critical for polymerization (Schwartz et al., 2013). Schwartz et al. found that fibrillar FUS assemblies, seeded by RNA, readily recruit the CTD *in vitro*. Since RNA does not seed FUS LC assembly (Burke et al., 2015), it is plausible that different interactions mediate polymerization of FUS LC and native FUS. Moreover, FUS may require both its LC and RNA-binding domains to form higher order assemblies comparable to that of TAF15 LC.

The prevalence of oncogenic FET LC fusions (**Figure 1B**) suggests that FET LC domains are particularly potent transcriptional activation domains. The combined work of the Cech and McKnight labs indicates that polymerization of FET LC domains may explain their noteworthy transcriptional activation capabilities. Even so, structural details of the complex

formed between self-assembled FET LC fibrils and the CTD are largely unknown. Thus, our characterization of the structure and dynamics of the degenerate half of RNA polymerase II CTD in complex with TAF15 LC fibrils seeks to answer two questions posed by Kwon et al.: 1) Why do TAF15 LC fibrils bind the CTD with greater affinity than FUS LC fibrils?; and 2) Why do TAF15 LC fibrils bind degenerate CTD repeats more avidly than consensus repeats?

MATERIALS AND METHODS

Bacterial Plasmids. My primary DNA construct, CTD27-52, is a codon-optimized form of the degenerate repeat half of the CTD of DNA-directed RNA polymerase II subunit RPB1 (residues 1773-1970) incorporating a TEV cleavable N-terminal leader sequence and hexahistidine tag (Peti and Page, 2007) and synthesized by DNA2.0. All other CTD DNA constructs were generated from CTD27-52 by either PCR site-directed mutagenesis or restriction enzyme subcloning. For instance, mutant CTD26 T66A refers to CTD27-52 with an alanine substituted for a threonine at position 66 in CTD27-52 (which matches to residue 1835 of DNA-directed RNA polymerase II subunit RPB1). Table 1 of Appendix A contains my full library of CTD constructs. mCherry-TAF15 LC and mCherry-FUS LC constructs were provided by the McKnight lab.

Protein Expression. RNA polymerase II CTD/MBP-FUS full-length: Expression plasmids were transformed into BL21 Star (DE3) cells (Life Technologies) and grown overnight in starter cultures. Uniformly ^{15}N -labeled protein (or $^{15}\text{N}/^{13}\text{C}$ -labeled or unlabeled protein) was expressed in either M9 media with ^{15}N ammonium chloride and ^{13}C glucose as the sole nitrogen and carbon sources, respectively, or LB. One liter cultures were inoculated with 50 mL starter cultures and grown at 37 °C, 200 rpm to an OD600 between 0.6-0.8. Cultures were then induced

with 0.5 mM IPTG and harvested after growing 4 hours at 37°C, 200 rpm. Bacteria were pelleted and stored at -80 °C until purification.

mCherry-FUS/TAF15 LC: Expression plasmids were transformed into BL21 Star (DE3) cells (Life Technologies) and grown overnight in starter cultures. One liter LB cultures were inoculated with 50 mL starter cultures and grown at 37 °C, 200 rpm to an OD600 between 0.6-0.8. Cultures were then induced with 0.5 mM IPTG and harvested after growing overnight at 16°C, 200 rpm. Bacteria were pelleted and stored at -80 °C until purification.

Protein Purification. RNA polymerase II CTD: Cell pellets were resuspended in 30 mL of pH 7.4 20 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole and lysed in an Emulsiflex C3. Cell lysate was cleared by centrifugation (20,000 rpm for 1 hour at 4 °C). Protein remained soluble in the supernatant following centrifugation. The supernatant was then filtered with a 0.22 µm syringe filter and loaded onto a 5 mL HisTrap HP column. Protein was eluted with a gradient of 10 to 300 mM imidazole in pH 7.4 20 mM sodium phosphate. Fractions containing protein (as determined by protein gel) were pooled and dialyzed overnight at room temperature into pH 7.4 20 mM sodium phosphate 10 mM imidazole. TEV protease was added to samples (at a ratio of roughly 2 mL of .3 mg/mL TEV protease per 30 mL of ~40 µM protein sample) at the start of dialysis in order to cleave the protein's hexahistidine tag. The storage buffer of TEV protease was pH 7.5 50 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, 50% glycerol, and 0.1% Triton-X-100. Following dialysis and TEV cleavage, samples were again filtered with a 0.2 µm syringe filter and loaded onto a 5 mL HisTrap HP column. Cleaved protein was retrieved from the flow-through. Purity was confirmed to be >99% by protein gel, ratio of absorbance at 280 nm to 260 nm, and two-dimensional NMR. Protein was then concentrated to between .5 to 1 mM using centrifugal filtration with a 10 kDa cutoff (Amicon, Millipore) and

buffer exchanged at 4 °C into pH 7 20 mM Tris-HCl, 200 mM sodium chloride, 20 mM BME, 0.5 mM EDTA. Samples were then flash frozen and stored at -80 °C.

mCherry-FUS/TAF15 LC (Protocol adapted from Kwon et al., 2013): Cell pellets were resuspended in 30 mL of pH 7.5 20 mM Tris-HCl, 500 mM sodium chloride, 10 mM imidazole, 5 mM DTT and lysed in an Emulsiflex C3. Cell lysate was cleared by centrifugation (20,000 rpm for 1 hour at 4 °C). Protein remained soluble in the supernatant following centrifugation. The supernatant was then filtered with a 0.22 µm syringe filter and loaded onto a 5 mL HisTrap HP column. Protein was eluted with a gradient of 10 to 500 mM imidazole in pH 7.5 20 mM Tris-HCl. Fractions containing protein (as determined by protein gel) were pooled and dialyzed overnight at room temperature into pH 7.5 20 mM Tris-HCl, 200 mM sodium chloride, 0.5 mM EDTA, and 20 mM BME.

MBP-FUS full-length: For use in turbidity experiments, MBP-FUS cell pellets were resuspended in 20 mM sodium phosphate, 10 mM imidazole, and 300 mM sodium chloride pH 7.4 buffer and lysed in an Emulsiflex C3 and the cell lysate was cleared by centrifugation (20,000 g for 60 minutes). The lysate was then loaded onto a HisTrap HP 5 ml column and protein was eluted with a gradient of 10 to 300 mM imidazole. Fractions containing the most pure MBP-FUS as determined by protein gel were pooled and loaded onto a HiLoad™ 26/600 Superdex™ 200 prep grade size exclusion chromatography column and washed with 20 mM sodium phosphate 300 mM sodium chloride pH 7.4 buffer. Fractions containing solely MBP-FUS were again determined by protein gel and then concentrated to roughly 10 mg/mL using centrifugal filtration with a 10 kDa cutoff (Amicon®, Millipore®) and stored at 4 °C. Purity was confirmed to be >99% by protein gel and ratio of absorbance at 280 nm to 260 nm.

Hydrogel Formation. (Protocol adapted from Kwon et al., 2013). Following dialysis, mCherry-FET LC protein was concentrated to roughly 65 mg/mL at room temperature using centrifugal filtration with a 10 kDa cutoff (Amicon, Millipore). 100 μ L aliquots were incubated at 4 $^{\circ}$ C for 2 days.

Transmission Electron Microscopy. Aliquots for microscopy studies were taken from 100 μ L aliquots of \sim 65 mg/mL mCherry-TAF15 LC hydrogels that were first diluted with 100 μ L of pH 7 20 mM Tris-HCl, 200 mM sodium chloride, 20 mM BME, 0.5 mM EDTA. Aliquots were taken before and after sonication. 4 μ L of solution was spotted onto ultrathin carbon film on holey carbon support grids (product code 01824, Ted Pella, Reading, CA), washed three times with deionized water, stained with 5 μ L of 3% uranyl acetate (Electron Microscopy Sciences, Hatfield, PA) for approximately 1 minute, blotted, and air-dried. Sample grids were imaged with a Philips 410 transmission electron microscope (Protocol adapted from Conicella et al., 2014).

Preparation of NMR Samples. CTD27-52: For the purposes of HSQC spectra comparison, uniformly 15 N-labeled CTD samples were diluted to 50 μ M in pH 7 20 mM Tris-HCl, 200 mM sodium chloride, 20 mM BME, 0.5 mM EDTA and a 90% H₂O/10% D₂O mixture and kept at 4 $^{\circ}$ C. For use in assignment experiments, uniformly 13 C/ 15 N-labeled CTD27-52 samples were diluted to either 523 μ M (for CBCA(CO)NH, HNCACB, HNCO, HN(CA)CO, and HNCA experiments) or 700 μ M (for the HNN experiment) in the same buffer and temperature conditions listed above. For measurement of backbone motions of CTD27-52, 15 N-labeled CTD27-52 samples were diluted to 250 μ M in pH 7 20 mM Tris-HCl, 200 mM sodium chloride, 20 mM BME, 0.5 mM EDTA and a 90% H₂O/10% D₂O mixture and kept at 4 $^{\circ}$ C. Extinction coefficients calculated by ProtParam (Wilkins et al., 1999) were used to estimate sample concentrations.

CTD27-52+mCherry-TAF15 LC fibrils: For binding experiments, 100 μ L aliquots of ~65 mg/mL mCherry-TAF15 LC hydrogels were first diluted with 100 μ L of pH 7 20 mM Tris-HCl, 200 mM sodium chloride, 20 mM BME, 0.5 mM EDTA. I then sonicated the diluted hydrogel sample for 5 10-second cycles at 12% power, with 50-second breaks in between. NMR samples consisted of 250 μ M CTD27-52+8mg/mL of sonicated mCherry-TAF15 LC fibril diluted in pH 7 20 mM Tris-HCl, 200 mM sodium chloride, 20 mM BME, 0.5 mM EDTA and a 90% H₂O/10% D₂O mixture and kept at 4 °C.

Solution NMR Experiments. All NMR experiments were recorded at 4 °C using a Bruker Avance III HD NMR spectrometer operating at either 850 or 500 MHz ¹H frequency equipped with a Bruker TCI z-axis gradient cryogenic probe. Experimental sweep widths and acquisition times (i.e. resolution) and the number of transients were optimized for the necessary resolution, experiment time, and signal-to-noise for each experiment type but kept constant for the same experiment conducted with different peptide samples (i.e., CTD27-52, CTD27-43, CTD38-52, CTD26 A66T, etc.) (Protocol adapted from Burke et al., 2015).

Triple resonance assignment experiments (CBCA(CO)NH, HNCACB, HNCO, HN(CA)CO, a high resolution HNCA, and HNN) were performed on samples of uniformly ¹³C/¹⁵N-labeled CTD27-52. Sweep widths were 10 ppm in ¹H, 20 ppm in ¹⁵N, 6.5 ppm in ¹³C', 56 ppm in ¹³C for C α /C β experiments and 22 ppm for the HNCA. Experiments comprised 2048*, 42-50*, 60*, 128*, and 25* complex data pairs in the direct ¹H, indirect ¹⁵N, indirect ¹³C α /C β , indirect ¹³C α , and indirect ¹³CO dimensions, respectively, and were processed with NMRPipe using default linear prediction parameters for either constant time or real time indirect dimensions and assigned in CARA (Keller, 2005). For the purposes of HSQC spectra comparison among 50 μ M CTD samples, data were apodized with a cosine squared bell function

in the ^1H dimension and a cosine bell function in the ^{15}N dimensions. For the purposes of peak-picking in CARA, an HSQC of 523 μM CTD27-52 was measured at 850 MHz ^1H frequency with 4096 direct ^1H points and 512 indirect ^{15}N points and sweep widths of 13 ppm and 20 ppm, centered at 4.9 ppm and 116.8 ppm, respectively. This high resolution HSQC was processed by first apodizing data with 5 Hz exponential line broadening for the ^1H and ^{15}N dimensions before applying the same cosine bell function in both the ^1H and ^{15}N dimensions (Protocol adapted from Burke et al., 2015).

Motions of the backbone of CTD27-52 were probed using ^{15}N R_1 , temperature-compensated ^{15}N R_2 , and heteronuclear NOE experiments using standard pulse sequences (hsqct1etf3gpsi3d, hsqct2etf3gpsitc3d, hsqcnoef3gpsi, respectively, from Topspin 3.2, Bruker). Each interleaved experiment comprised 128* \times 2048* complex data pairs in the indirect ^{15}N and direct ^1H dimensions, respectively, with sweep widths of 15 ppm and 10.5 ppm, centered at 118.783 ppm and 4.973 ppm. ^{15}N R_2 experiments had a Carr-Purcell-Meiboom-Gill (CPMG) field of 556 Hz and an interscan delay of 2.5 s. The seven interleaved CPMG R_2 relaxation times (i.e., loop lengths) were 16.3 ms, 260.8 ms, 179.3 ms, 32.6 ms, 114.1 ms, 81.5 ms, and 163 ms at 850 MHz and 16.3 ms, 260.8, 32.6, 130.4, 97.8, 179.3, and 65.2 ms at 500 MHz. Data were processed with NMRPipe (Delaglio et al., 1995) and apodized with a cosine squared bell function in the ^1H dimension and a cosine bell function in the ^{15}N dimension. Best-fit R_2 relaxation rates were calculated using least squares optimization of $^1\text{H}/^{15}\text{N}$ peak intensities to a single exponential function (Protocol adapted from Burke et al., 2015).

Turbidity Assays. To assess the effect of increasing protein concentration on the turbidity of FUS, MBP-FUS protein was diluted into 20 mM Tris-HCl 150 mM sodium chloride pH 7.4 buffer and 2.5 μL of TEV protease (0.3 mg/mL, final concentration in sample is 0.015

mg/mL) or its storage buffer (50 mM Tris pH 7.5, 1 mM EDTA, 5 mM DTT, 50% glycerol, 0.1% Triton-X-100) was added for a final volume of 50 μ L in 96 well clear plates (Costar®). To assess the effect of RNA on FUS turbidity, yeast RNA was first desalted into 20 mM Tris-HCl 150 mM sodium chloride pH 7.4 buffer using Thermo Scientific™ Zeba™ Spin Desalting Columns. Desalted RNA (0.25 mg/mL) was then added to 0.5 mg/mL MBP-FUS in the prep described above. To assess the effect of salt on FUS turbidity, MBP-FUS protein was diluted into 20 mM Tris-HCl pH 7.4 buffer with various sodium chloride concentrations. Upon the addition of TEV protease or its storage buffer, turbidity measurements were recorded every five minutes using a SpectraMax® M5 Microplate Reader for a total of 6 readings in a 30 minute period. Experiments were conducted in triplicate and then averaged (Protocol adapted from Burke et al., 2015).

RESULTS

¹H, ¹⁵N Backbone Resonance Assignments of RNA Polymerase II CTD27-52. The carboxy-terminal domain (CTD) of the largest subunit of human RNA polymerase II is composed of 52 tandem heptad repeats modeled after the consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$. Unlike heptad repeats proximal to the catalytic core of RNA polymerase II, heptad repeats in the distal half of the CTD deviate significantly from the consensus sequence, with the majority of heptads containing at least one residue substitution. While the yeast CTD contains only 26 heptad repeats with few degeneracies, mammalian degeneracies have been conserved throughout 175 million years of evolution (Corden et al., 2013) and increase the CTD's potential for posttranslational modifications. While inconclusive, previous structural studies suggest that the CTD exists in equilibrium between a dominant unordered conformation and less populous β -turn

and PPII helix conformations (Matsushima et al., 1990; Cagas et al., 1990; Bienkiewicz et al., 2000). However, these studies have relied on synthetic peptides composed of solely consensus repeats to model the structure of the full CTD. Therefore, the effect of degeneracies on the structure of the CTD has not been studied with high resolution.

We analyzed the intact degenerate repeat half of the CTD (CTD27-52) using solution NMR spectroscopy in order to measure secondary structure population on a residue-by-residue level. Since NMR chemical shifts are sensitive reporters of the overall structure of a protein, we first assigned the backbone amide resonances of the HSQC to residues of CTD27-52. CTD27-52's highly repetitive and proline-rich sequence (**Figure 2A**) made these assignments particularly challenging. Since proline residues lack an amide proton, they are not visible in an HSQC spectrum and prevent resonance transfer in traditional triple resonance experiments, thus impeding sequential assignments. We briefly pursued using carbon detect experiments to assign CTD27-52, but due to significant overlap of peaks in the ^{13}C , ^{15}N CON spectrum (as well as the prohibitive expense of carbon labeling multiple samples), this route was abandoned. We also ran (H)N(COCO)NH experiments (Yoshimura et al., 2015) in an attempt to obtain $i+2$ nitrogen shifts by using CO mixing to transfer resonance through proline residues ($\text{CO}_i \leftrightarrow \text{CO}_{i+1}(\text{proline}) \leftrightarrow \text{CO}_{i+2}$), but unfortunately the concentration of our sample did not provide adequate signal-to-noise. Since CTD27-52 aggregates at concentrations above 1 mM, we abandoned this route as well.

Ultimately, we used standard $^1\text{H}_\text{N}$ detected triple resonance experiments (HNCO, HN(CA)CO, CBCA(CO)NH, HNCACB, a high resolution HNCA, and HNN) in combination with HSQCs of 19 CTD variants to assign all non-overlapped backbone resonances of CTD27-52 (**Figure 2B**). See the appendix for a catalog of CTD variants (**Table S1**). Sixteen CTD27-52

mutants were critical for completing our assignments because single residue mutations produce local perturbations in chemical structure that are observed as chemical shift perturbations in the HSQC spectrum of the protein. For example, mutating residue Y77 to an alanine (i.e., mutant CTD27-52 Y77A), produced an HSQC spectrum wherein residues near Y77 were noticeably shifted from their wild-type positions (**Figure S1**). We chose to mutate CTD27-52 residues to alanines because the backbone resonance of an alanine appears downfield and was thus unlikely to overlap with residues we were trying to assign. We also created three truncated CTD peptides, CTD27-37, CTD38-52, and CTD43-52, which helped us verify our assignments.

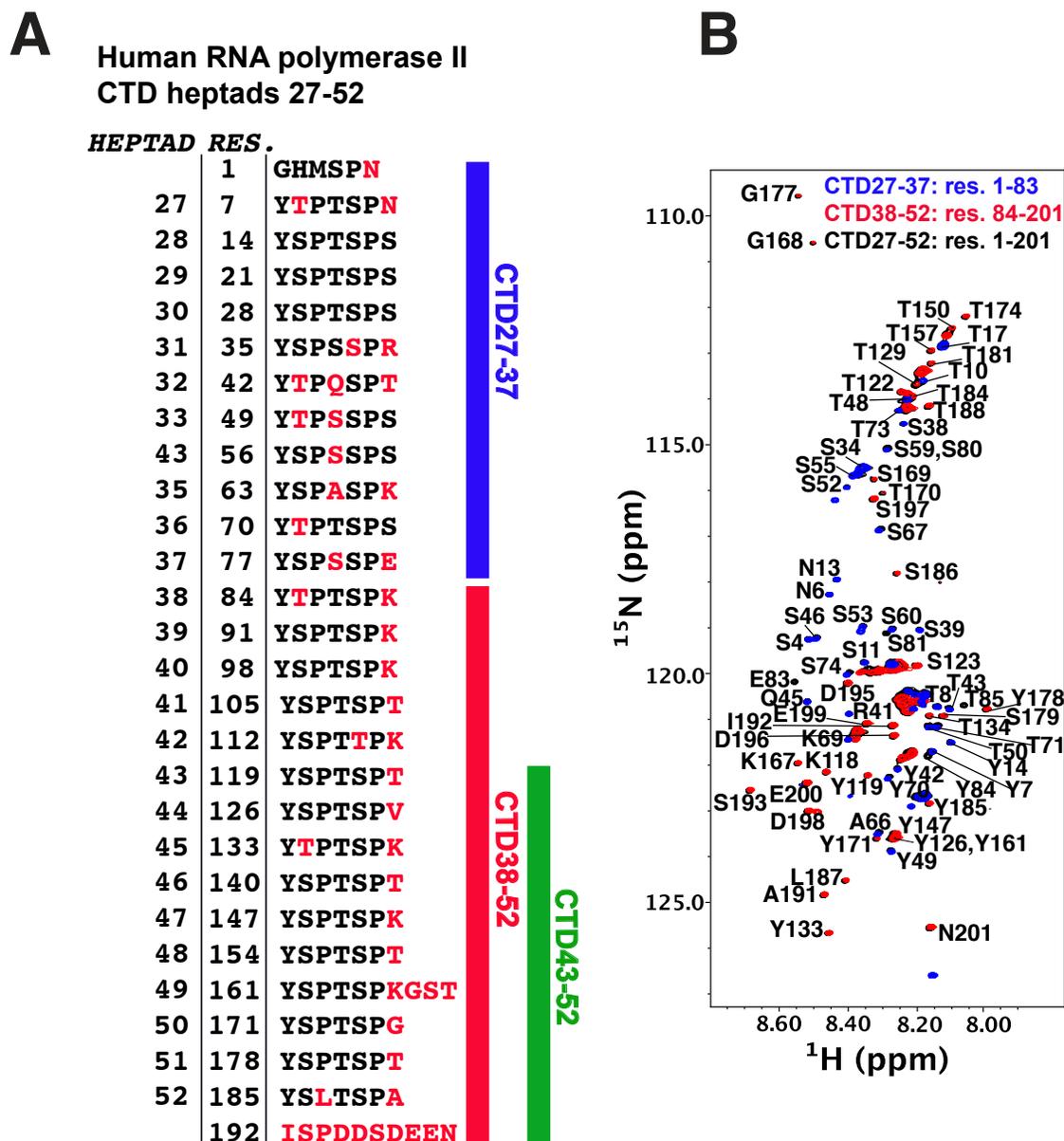


Figure 2. The NMR spectrum (^1H - ^{15}N heteronuclear single quantum coherence, HSQC) of CTD27-52 is remarkably well-resolved given its highly repetitive, proline-rich sequence. (A) Sequence of human RNA polymerase II CTD heptads 27-52. Degeneracies from the consensus YSPTSPS repeat are shown in red and truncated CTD peptides are labeled on the right. Note that residues 1-3 are left over after TEV cleavage of the protein's hexahistidine tag (i.e., res. 1-3 are not part of the native sequence). (B) HSQC overlay of CTD27-52 (black), CTD27-37 (blue), and CTD38-52 (red). Assignments of all non-overlapped backbone resonances are shown in black. Assignments in overlapped regions are omitted for clarity.

RNA Polymerase II CTD27-52 is structurally disordered. The narrow chemical shift dispersion (centered around ~ 8.3 ppm) observed in the HSQC of CTD27-37 is indicative of a disordered protein. Additionally, measurements of protein backbone motions provide further evidence for the uniform disorder of CTD27-52. NMR relaxation experiments (^{15}N R_2 , ^{15}N R_1) and heteronuclear nuclear Overhauser (hetNOE) effects are sensitive to motions on the picosecond to nanosecond timescale, and irregularities in these measurements within a protein reflect slower rotational diffusion of structured regions. Since ^{15}N R_2 , ^{15}N R_1 , and hetNOE values are uniform across CTD27-52 (**Figure 3A**), it is most likely uniformly disordered.

Interestingly, CTD27-52's ^{15}N R_2 values are field-dependent - relaxation of backbone resonances is uniformly higher at a field strength of 850 MHz compared to 500 MHz (**Figure 3B**). Even so, since ^{15}N R_2 measurements at 850 MHz and 500 MHz are highly correlated with no obvious outliers, this data further suggests that CTD27-52 does not sample significant populations of structured conformation on the microsecond to millisecond timescale.

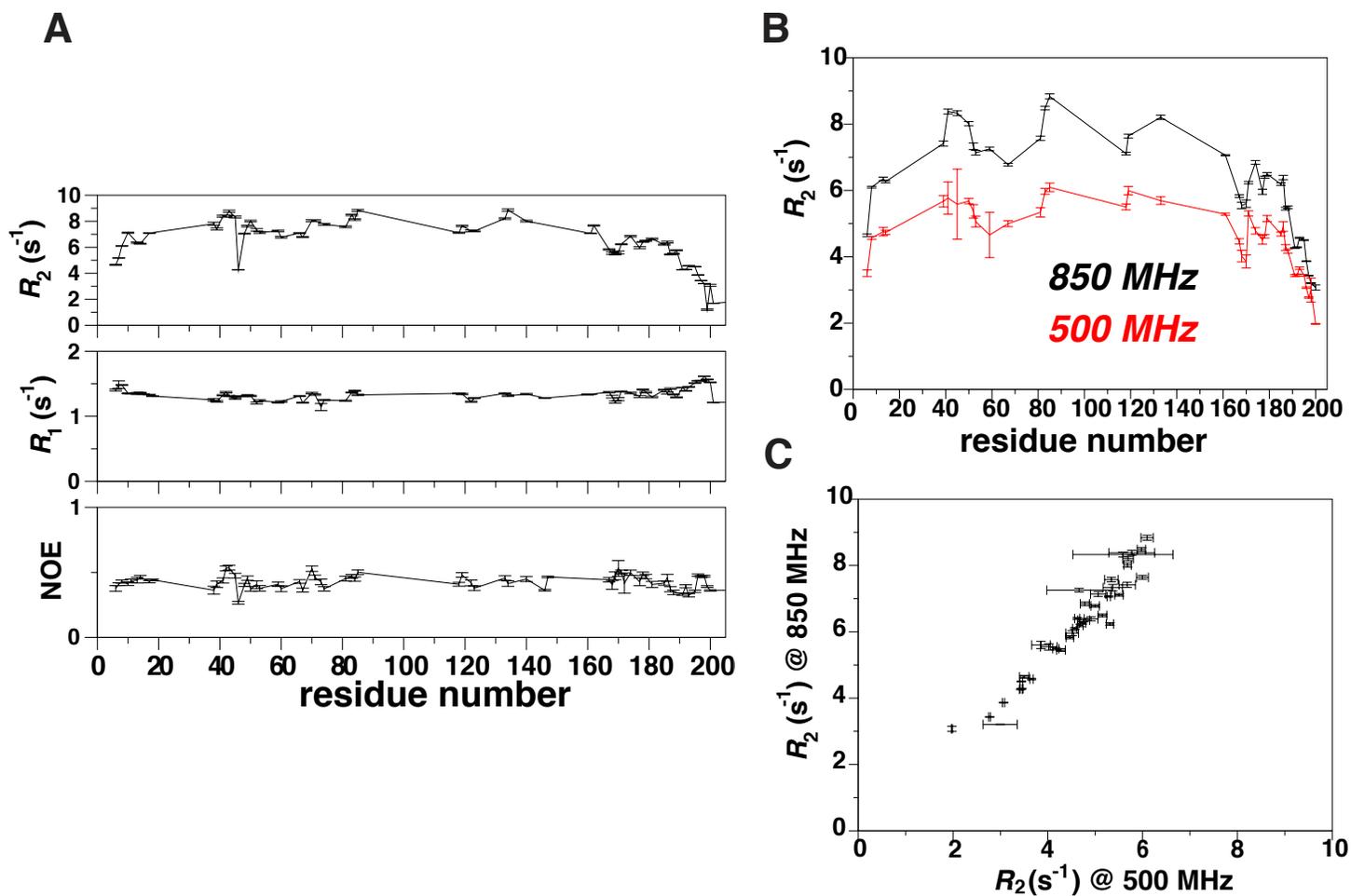


Figure 3. RNA Polymerase II CTD27-52 is structurally disordered. (A) ^{15}N R_2 , ^{15}N R_1 , and hetNOE values (top, middle, and bottom panels, respectively) for dispersed CTD27-37 protein are consistent with disorder across the entire domain. (B) Transverse relaxation of CTD27-52 (^{15}N R_2) is field dependent: ^{15}N R_2 measurements at 850 MHz are uniformly higher than ^{15}N R_2 measurements at 500 MHz. (C) Correlation of ^{15}N R_2 at 850 MHz versus ^{15}N R_2 at 500 MHz. The absence of outliers further suggests CTD27-52 is entirely disordered.

Sonicated mCherry-TAF15 LC hydrogel fibrils bind RNA polymerase II CTD27-52 in a residue-specific manner. The McKnight lab was the first to develop a protocol for 4°C polymerization of mCherry-tagged FET LC domains into hydrogels of purportedly amyloid-like fibrils (Han et al., 2012; Kato et al., 2012). While using fluorescence microscopy to monitor FET LC hydrogel trapping of the CTD, they discovered that polymerization of FET LC domains correlates with recruitment of the CTD (Kwon et al., 2013).

In light of these exciting findings, the Fawzi lab obtained the McKnight lab's mCherry-tagged FET LC constructs. Using NMR spectroscopy, we sought out to test whether mCherry-TAF15 LC fibrils recruit CTD27-52 in a residue-specific manner. Incubating high concentrations (roughly 65 mg/ml) of mCherry-TAF15 LC monomer at 4°C for approximately 48 hours produced a hydrogel as expected (**Figure 4A**). We then sonicated the mCherry-tagged TAF15 LC hydrogel in order to make NMR samples of CTD27-52+8mg/mL of polymerized mCherry-TAF15 LC. We carefully recorded the concentration of mCherry-TAF15 LC at the start of incubation (i.e., before hydrogel formation) and thus estimated the final concentration of mCherry-TAF15 LC in our NMR samples based on the volume of sonicated hydrogel added. Using transmission electron microscopy, we verified that mCherry-TAF15 LC fibrils were not destroyed by sonication (**Figure 4B**).

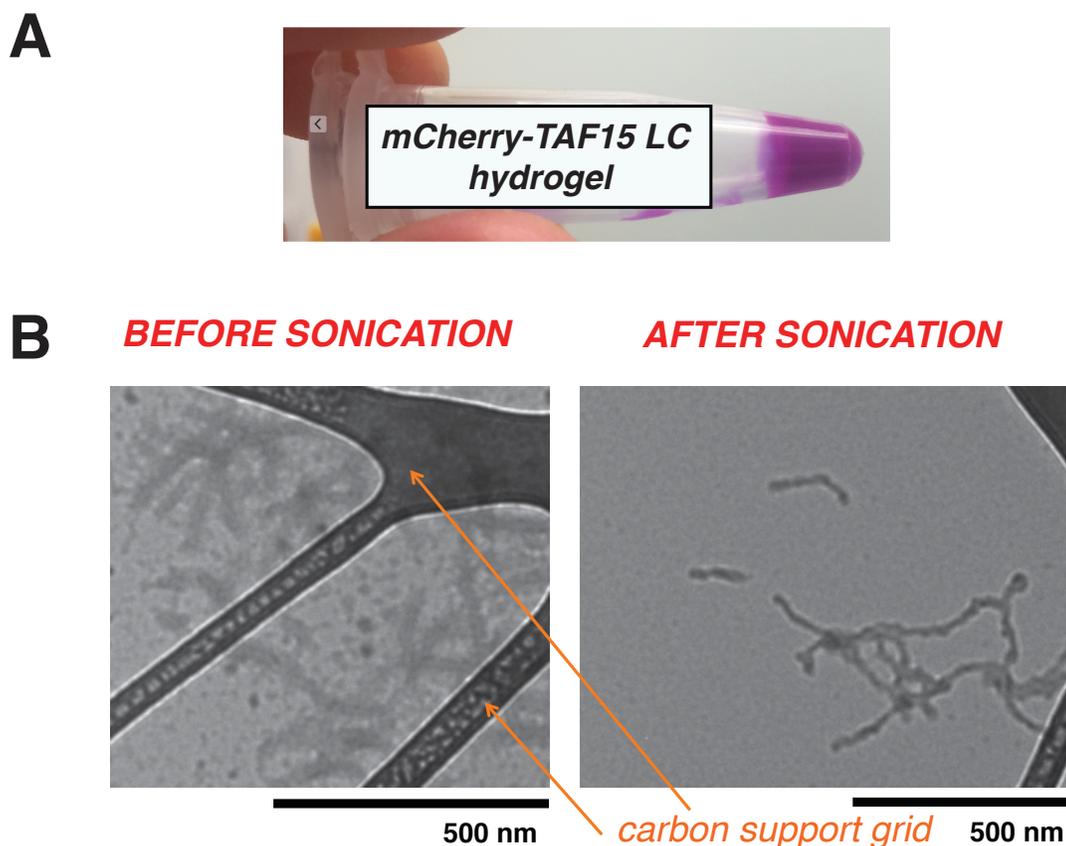


Figure 4. mCherry-TAF15 LC forms a hydrogel of polymeric fibers that are not destroyed by sonication. (A) 48-hour incubation at 4°C causes mCherry-TAF15 LC monomer to form a hydrogel, recapitulating McKnight lab results. (B) mCherry-TAF15 LC hydrogel fibrils are not destroyed by sonication. Hydrogel samples pre- and post-sonication are visualized by transmission electron microscopy. Polymeric fibers are roughly 500 nm in length before sonication.

Adding sonicated mCherry-TAF15 LC fibrils to CTD27-52 did not produce any chemical shift differences in the HSQC spectrum of CTD27-52. However, we observed large differences in the R_2 of CTD27-52 resonances in the presence and absence of sonicated mCherry-TAF15 LC fibrils. We then measured the difference in transverse relaxation rate (ΔR_2) of CTD27-52 in the presence and absence of mCherry-TAF15 LC fibrils in order to characterize the fibril-bound state of CTD27-52. When a monomeric species (i.e., CTD27-52) is bound to a much larger species (i.e., mCherry-TAF15 LC fibrils), R_2 is exceptionally fast, relaxing (i.e. decaying/destroying) transverse magnetization much faster in the bound state than in the unbound state. Hence, lifetime line broadening, the decay in transverse magnetization due to

visiting the bound state, gives rise to a measurable increase in R_2 , ΔR_2 . If R_2 in the bound state were infinitely fast, the value of ΔR_2 would be equal to the first order “apparent” kinetic on-rate, $k_{\text{on}}^{\text{app}}$. Therefore ΔR_2 can be interpreted as a lower bound for the first order on-rate for a monomeric species binding a much larger, NMR invisible 'dark' state such as a hydrogel fibril. In this way, measurements of ^{15}N ΔR_2 reflect the rate at which a monomer reversibly binds a much larger species (Fawzi et al., 2011; Fawzi et al., 2012; Fawzi et al., 2014).

We report that ΔR_2 values are maximal for heptads 27-33 of CTD27-52. Our data suggest that the N-terminal half of CTD27-52 more frequently mediates CTD binding of TAF15 fibrils than the C-terminal half of CTD27-52. Backbone amide resonances for residues Asn6, Tyr7, Thr8, Asn13, Tyr14, Arg41, Tyr42, Thr43, and Gln45 in CTD27-52 consistently have the largest ΔR_2 values. Furthermore, the first 6-7 heptads of CTD27-52 show heightened ΔR_2 values relative to the rest of CTD27-52 independent of magnetic field strength (**Figure 5A**). Because these ΔR_2 measurements reflect the rate at which CTD27-52 residues reversibly bind to mCherry-TAF15 LC hydrogel fibers, they suggest that residues near the N-terminus of CTD27-52 more readily interact with mCherry-TAF15 LC fibrils.

While we do observe a magnetic field dependence in ΔR_2 of CTD27-52, the dependency is linear with no significant deviations (**Figure 5B**), so there is no evidence of any intermediate

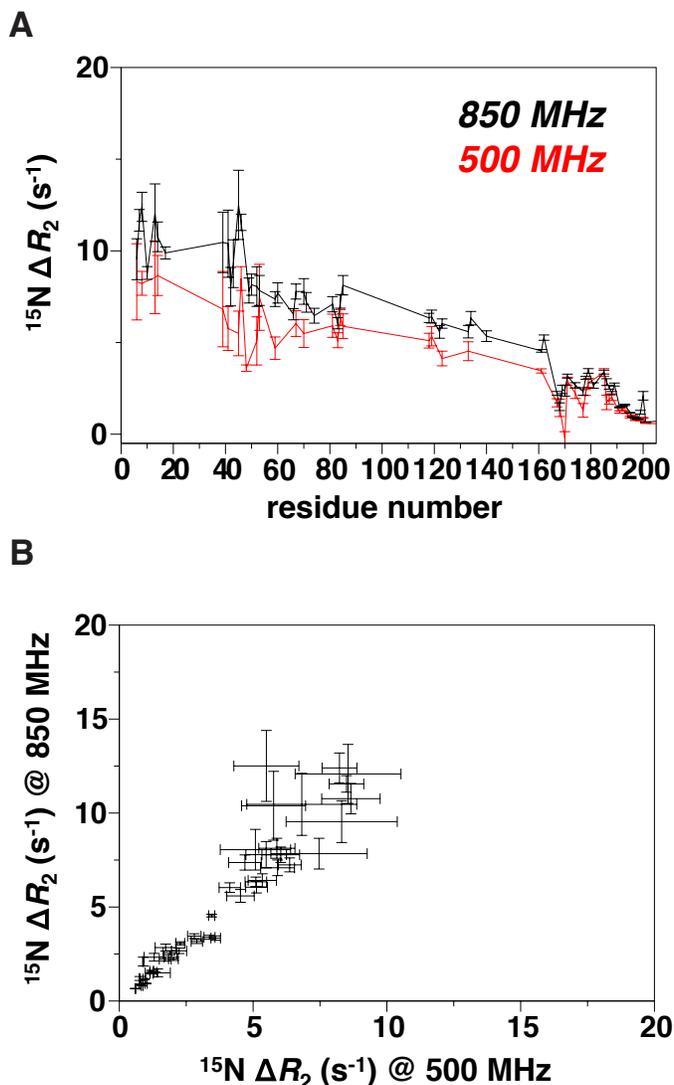


Figure 5. CTD heptads 27-33 (residues 1- 50 of CTD27-52) mediate recruitment by mCherry-TAF15 LC fibrils.

(A) $^{15}\text{N } \Delta R_2$ reflects the rate at which CTD27-37 residues reversibly bind mCherry-TAF15 LC fibrils. Independent of magnetic field strength, the first 6-7 heptads of CTD27-52 show heightened ΔR_2 values relative to the rest of CTD27-52. (B) Linear field dependency of $^{15}\text{N } \Delta R_2$.

chemical exchange (i.e., interaction of residues) within the CTD27-52 monomer itself. The field dependence of ΔR_2 allows us to draw a few additional conclusions about the dynamics of CTD27-52 in complex with mCherry-TAF15 LC fibrils - namely, the lower values of ΔR_2 at lower magnetic field tell us that R_2 of CTD27-52 in its bound state must be on the order of the dissociation rate (k_{off}) of the complex. The rationale for this conclusion is R_2 typically has a linear field dependence for large molecules. R_2 of CTD27-52 in its bound state is on the order of or slower than k_{off} , because if R_2 were significantly faster, there would be complete lifetime line broadening at both fields. Field dependence of ΔR_2 indicates that, at least at the lower

magnetic field, the R_2 rate is not much faster than k_{off} . Also, since we know that maximal ΔR_2 values provide a lower bound for the association rate (k_{on}) of CTD27-52 in complex with mCherry-TAF15 LC fibrils, we can conclude that CTD27-52 monomers visit the NMR-invisible mCherry-TAF15 LC fibril state with an on-rate of more than 11 s^{-1} .

For our first set of NMR experiments, we calculated ΔR_2 by subtracting R_2 values of CTD27-52 in buffer alone from R_2 values of CTD27-52 in the presence of sonicated mCherry-TAF15 LC fibrils. As a result, this set of experiments failed to test whether monomeric mCherry-TAF15 LC or other contaminants within our sonicated mCherry-TAF15 LC hydrogel samples caused our observed residue-specific differences in ΔR_2 of CTD27-52. In order to account for this possibility, we devised a new set of experiments. By centrifuging a freshly sonicated mCherry-TAF15 LC hydrogel sample, we were able to pellet all polymerized fibrils (**Figure 6A**). We then carefully removed the supernatant. The clear color of the pelleted sample's supernatant suggests that our sonicated samples contain negligible amounts of free mCherry-tagged monomer. Thus, almost all mCherry-TAF15 LC must partition into the hydrogel species. We then compared the R_2 values of CTD27-52+supernatant to R_2 values of CTD27-52+sonicated mCherry-TAF15 LC fibrils (**Figure 6B**). Since our ΔR_2 calculations from this set of experiments are almost identical to that of our first set of experiments, it is clear that polymeric fibrils of mCherry-TAF15 LC are responsible for the residue-specific differences in ΔR_2 of CTD27-52.

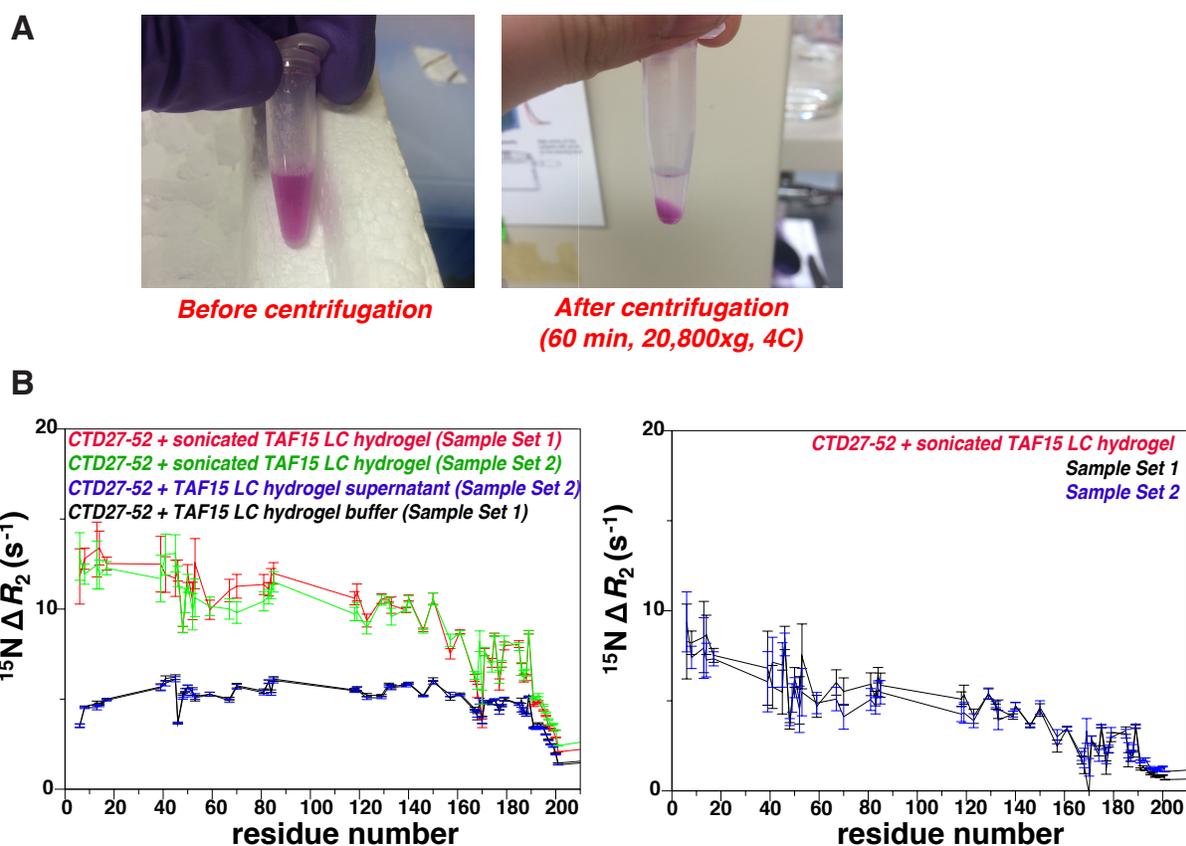


Figure 6. mCherry-TAF15 LC fibrils (rather than monomeric mCherry-TAF15 LC or contaminants) are responsible for residue-specific recruitment of CTD27-52 (A) Centrifugation (60 min, 20,800xg, 4°C) of a freshly sonicated mCherry-TAF15 LC hydrogel sample pellets all polymerized fibrils. Supernatant is entirely clear (i.e., free of mCherry-TAF15 LC monomer). (B) Comparison of different sets of solution NMR data verifies reproducibility of data. R_2 of CTD27-52+supernatant of mCherry-TAF15 LC fibrils is indistinguishable from R_2 of CTD27-52+buffer control. Soluble contaminants and/or monomeric mCherry-TAF15 LC are not responsible for residue-specific increases in $^{15}\text{N} \Delta R_2$ of CTD27-52.

Separately, the heightened ΔR_2 of backbone amide resonances corresponding to residues Asn6, Asn13, and Gln45 led us to wonder whether asparagine and glutamine side chains help mediate recruitment of CTD27-52 by mCherry-TAF15 LC fibrils. Since amide resonances derived from the side chains of asparagine and glutamine residues are visible in the HSQC spectrum, we quantified their $^1\text{H}/^{15}\text{N}$ chemical shift propensities (CSPs) in the presence of mCherry-TAF15 LC fibrils (**Figure 7**). CSPs of CTD27-52 amide backbone resonances are approximately zero throughout the protein, and the amide resonances of asparagine and

glutamine side chains exhibit this same trend. We also quantified the intensity ratios of Asn6, Asn13, and Gln45 side chain resonances in the presence and absence of mCherry-TAF15 LC fibrils. The low intensity ratios of Asn6, Asn13, and Gln45 side chain resonances are approximately equivalent to the low intensity ratios of their corresponding backbone resonances. New studies must be devised to more rigorously test whether asparagine and glutamine side chains are critical mediators of the interaction between CTD27-52 and mCherry-TAF15 LC fibrils.

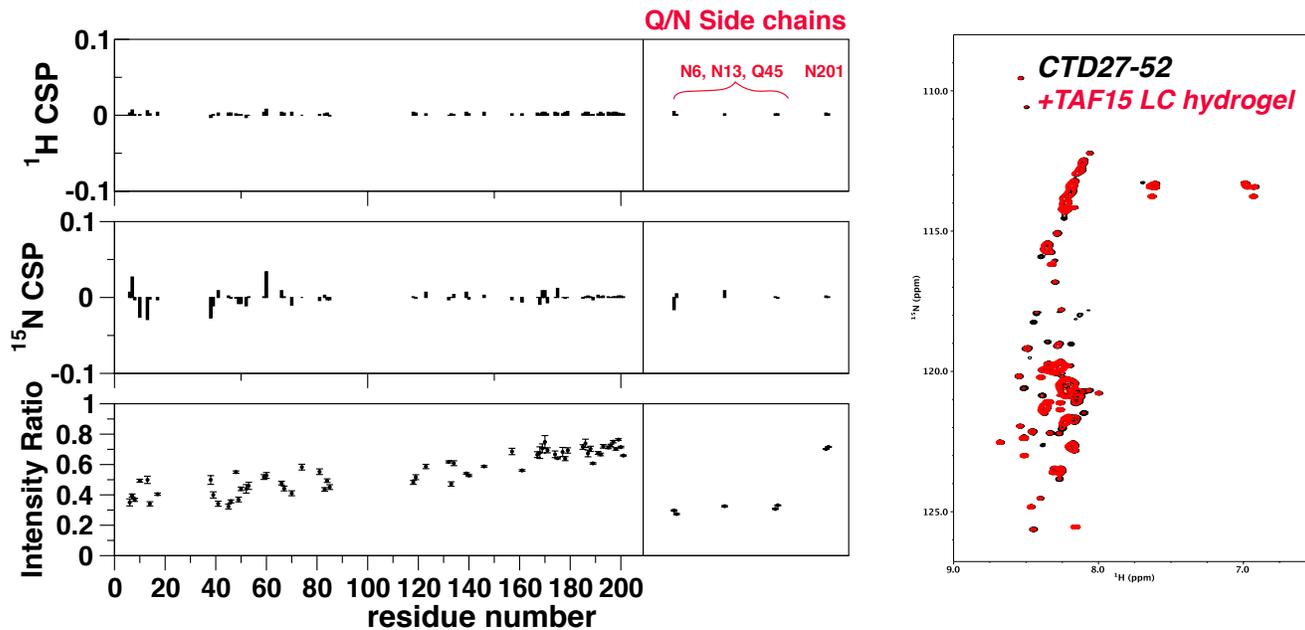


Figure 7. Quantification of chemical shift perturbations and loss of intensity in the HSQC of CTD27-52 in the presence of mCherry-TAF15 LC fibrils. (Left panels) $^1\text{H}/^{15}\text{N}$ chemical shift propensities (CSPs) of CTD27-52 in the presence of mCherry-TAF15 LC fibrils. CSPs of CTD27-52 amide backbone resonances are approximately zero throughout the protein, and the amide resonances of asparagine and glutamine side chains exhibit this same trend. Low intensity ratios of Asn6, Asn13, and Gln45 side chain resonances are approximately equivalent to the low intensity ratios of their corresponding backbone resonances. (Right panel) Overlay of HSQC spectra of CTD27-52 in the presence and absence of mCherry-TAF15 LC fibrils. Significant loss of intensity is observed for residues interacting with mCherry-TAF15 LC fibrils.

Given our above characterization of RNA polymerase II CTD27-52 in complex with mCherry-TAF15 LC fibrils, we now return to the two questions posed by Kwon et al.: 1) Why do TAF15 LC fibrils bind the CTD with greater affinity than FUS LC fibrils?; and 2) Why do TAF15 LC fibrils bind degenerate CTD repeats more avidly than consensus repeats? At this point, it is still unclear whether lesser polymerization of FUS LC can fully explain why TAF15 LC fibrils more avidly bind the CTD. Curiously, sonicated samples of mCherry-FUS LC hydrogel produced no difference in transverse relaxation of CTD27-52 (*our unpublished data*). Conversely, our ΔR_2 measurements clearly show that CTD27-52 binds TAF15 LC fibrils in a residue-specific manner. It is possible that these residue-specific interactions are unique to the CTD27-52:TAF15 LC fibril complex. Nonetheless, it is also possible that FUS LC fibrils are simply smaller and/or less stable than TAF15 LC fibrils. Our observations of mCherry-FUS LC hydrogels hint that FUS LC hydrogels may be less stable than TAF15 LC hydrogels, because despite having approximately equivalent concentrations at the start of 4°C incubation, FUS LC hydrogels more readily solubilized, even before sonication.

Native FUS self-assembles to form a liquid phase-separated state. Our recently published work on interactions mediating self-assembly of native FUS offers additional insight into this dilemma. The Fawzi lab has extensively characterized interactions mediating phase separation of FUS LC domain into liquid droplets. We report that much like mCherry-FUS hydrogels, FUS LC droplets are stabilized by low temperatures and high salt (Burke et al., 2015). Using turbidity assays, I tested the effect of protein concentration, salt and RNA on self-assembly of native protein. I found that native protein self-assembles to form a liquid phase-separated state at concentrations as low as 1 μM (**Figure 8, top panel**), whereas FUS LC requires a concentration of at least 50 μM to phase separate under similar buffer and temperature

conditions. While increasing salt concentration aids phase separation of FUS LC, a 3-fold increase in sodium chloride concentration had little effect on phase separation of native FUS (**Figure 8, bottom left panel**). Additionally, low mass ratios of RNA:full-length FUS promote self-assembly of the native protein (**Figure 8, bottom right panel**), whereas RNA does not affect self-assembly of FUS LC domain (*our unpublished data*).

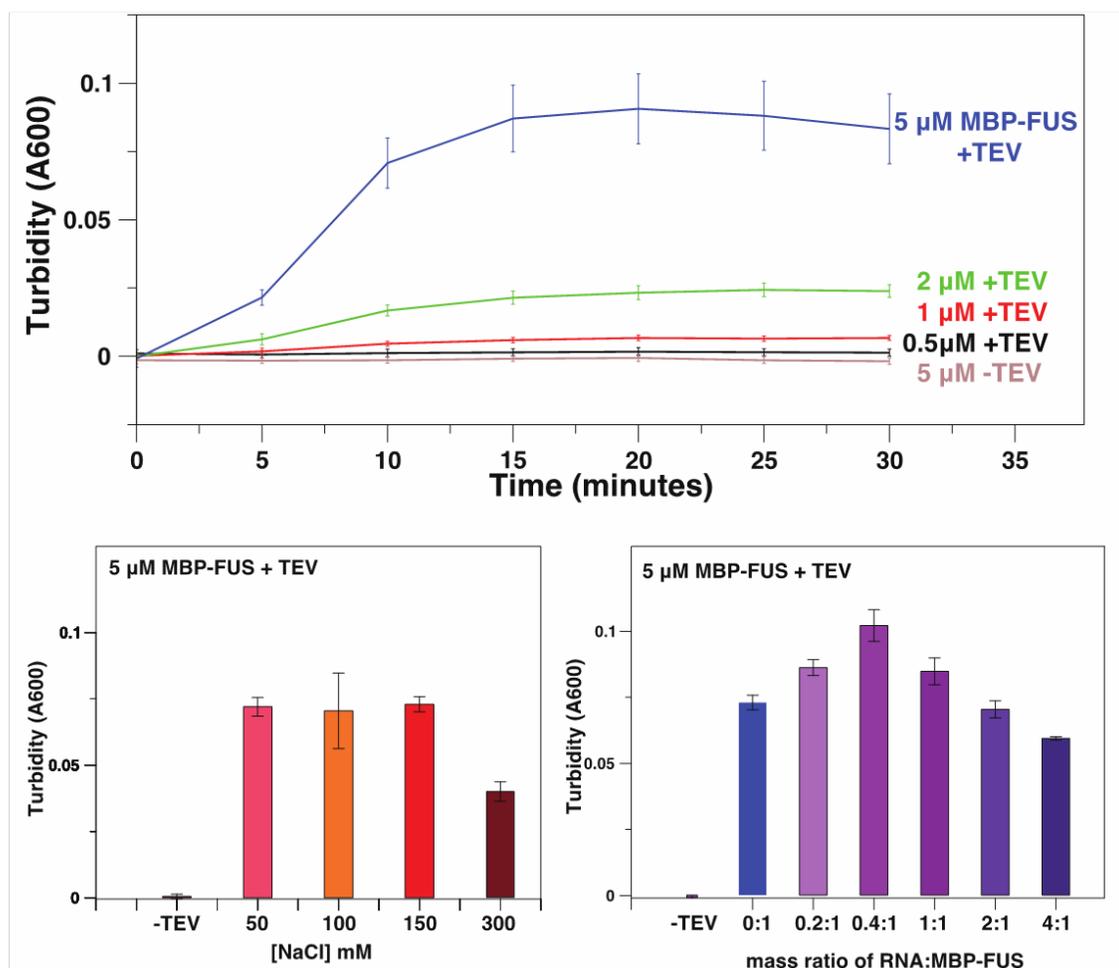


Figure 8. Interactions mediating self-assembly of native FUS are distinct from that of FUS LC (Top) FUS forms a liquid phase-separated state at concentrations as low as 1 μM after an N-terminal maltose binding protein (MBP) fusion is cleaved with TEV protease. (*Bottom left*) Low RNA:MBP-FUS ratios by mass enhance FUS droplet formation. (*Bottom right*): Changing ionic strength by increasing NaCl concentration does not affect droplet formation at $[\text{NaCl}] < 300$ mM, suggesting that interactions between FUS molecules are not held together primarily by electrostatic interactions. Bar chart turbidity values were measured 15 minutes after the addition of TEV protease.

In summary, my work on interactions mediating droplet formation of native FUS complements Schwartz et al.'s findings on fibrillization of native FUS, since they showed that RNA seeds fibrillization and consequently claimed that FUS's RNA-binding domains promote polymerization. My work also indicates that different interactions mediate self-assembly of FUS LC versus native FUS. As a result, fibrils of mCherry-FUS LC may be quite different from fibrils of native FUS. Future studies on interactions mediating phase separation of TAF15 LC will be necessary for understanding whether TAF15 LC polymerizes more readily than FUS LC.

In regards to the second question posed by Kwon et al., degeneracies within CTD heptad repeats 27-33 may provide important clues for why TAF15 LC fibrils preferentially bind the degenerate half of the CTD. Given that amide backbone resonances for residues Asn6, Asn13, and Gln45 consistently show heightened transverse relaxation in the presence of TAF15 LC fibrils, it is plausible that TAF15 LC fibrils actively recruit the side chains of the CTD's degenerate asparagine and glutamine residues.

DISCUSSION

Our investigation of the structure of the intact degenerate repeat half of the CTD of human RNA polymerase II represents the first NMR study of the CTD's non-consensus repeats. Our report of CTD27-52's disordered structure, verified by the narrow chemical shift dispersion of its HSQC spectrum as well as uniform relaxation rates and heteronuclear Overhauser effects across the entire protein, is not surprising. Bienkiewicz et al. predicted that full-length CTD exists in a predominantly disordered conformation almost twenty years ago. However, their prediction was made based on low-resolution CD studies.

Previous NMR studies of the CTD have focused solely on the CTD's consensus repeats. Since peptides containing more than one consensus repeat produce NMR spectra with almost entirely overlapped resonances (Cagas et al., 1995), researchers have never attempted to assign the resonances of a multiple-repeat CTD peptide. Fortuitously, the degenerate repeat half of the CTD produces a remarkably well-resolved and disperse HSQC spectrum at a field strength of 850 MHz, allowing me to assign 103 of CTD27-52's 148 non-proline residues. 67 of these 103 assignments correspond to non-overlapped, well-resolved peaks. Our truncated CTD27-52 proteins produce even better resolved HSQC spectra - for instance, we have assigned 50 of CTD43-52's 59 non-proline residues, and 38 out of these 50 assignments correspond to non-overlapped, well-resolved peaks.

Our assignments of the backbone resonances of CTD27-52 provide a structural fingerprint of the intact, unphosphorylated degenerate repeat half of the RNA polymerase II CTD. These assignments are particularly useful for two reasons: 1) Recent work has shown that the majority of CTD heptads are not phosphorylated *in vivo* (Suh et al., 2016; Schuller et al., 2016), making characterization of the CTD's unphosphorylated state even more relevant to

understanding the transcription cycle; and 2) Post-translational modifications associated with the earliest steps of transcription localize to the degenerate repeat half of the CTD, making CTD27-52 pertinent to understanding transcription initiation. Up until very recently, phosphorylation studies of the CTD have been exclusively performed using CTD-phospho-specific antibodies. Antibody detection does not allow high-resolution analysis of the CTD's phosphorylation pattern because individual heptads within the CTD cannot be distinguished; therefore, it is impossible to determine how many heptads are phosphorylated and at what sites, not to mention the problematic effects of antibody cross-reactivity and variable affinity (Corden et al., 2016). Using genetic and mass spectrometric approaches in an effort to overcome the limitations of antibody detection, Schuller et al. and Suh et al. report that the vast majority of the CTD's phosphorylation sites are unphosphorylated. Their work underscores the physiological relevance of the CTD's unphosphorylated heptads, and our assignments of the unphosphorylated degenerate repeat half of the CTD perfectly complement high-resolution genomic and mass spectrometric approaches. This is because our assignments facilitate residue-specific analysis of the effects of post-translational modifications on CTD27-52's interactions with RNA-processing and transcription factors. For example, phosphomimetic mutations can easily be introduced into our CTD27-37 construct. By measuring differences in transverse relaxation of individual backbone resonances of mutated CTD27-37 proteins in the presence and absence of target proteins, the effect of phosphorylation on binding of individual residues can be investigated. In this way, our assignments of CTD27-37 promote residue-specific investigations of the CTD code.

Our structural characterization of CTD27-52 in complex with TAF15 LC fibrils is the first residue-specific investigation of the CTD's role in transcription initiation. In the presence of TAF15 LC fibrils, backbone resonances corresponding to residues in the N-terminal half of

CTD27-52 exhibit increased transverse relaxation relative to residues in the C-terminal half. Since differences in transverse relaxation can be interpreted as the rate at which CTD27-52 monomer reversibly binds to TAF15 LC fibrils, our data suggests that degenerate heptads 27-33 more rapidly bind TAF15 LC fibrils. Since the CTD's eight lysine residues are distributed among heptads 35-49, our data does not support Kwon et al.'s hypothesis that lysine residues in the degenerate half of the RNA polymerase II CTD mediate interactions with TAF15 LC fibrils. Unfortunately, backbone resonances for the CTD's eight lysines are almost all overlapped in the HSQC of CTD27-52, which complicates our interpretation of transverse relaxation rates. However, bulk R_2 analysis of overlapped lysine resonances parallels the general trend in ΔR_2 - that is, the closer a residue is to the acidic tail of CTD27-52, the less it interacts with TAF15 LC fibrils.

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APPENDIX A

Heptad Truncations	Mutants	
CTD27-37 (residues 1778-1852)	CTD27-52 T10A	CTD27-52 P117A
CTD38-52 (residues 1853-1970)	CTD27-52 P16d	CTD27-52 Y126A
CTD43-52 (residues 1887-1970)	CTD27-52 P33A	CTD27-52 P142A
	CTD27-52 S39A	CTD27-52 T150A
	CTD27-52 S53A	CTD27-52 Y154A
	CTD27-52 A66T	CTD27-52 T157A
	CTD27-52 Y77A	CTD27-52 T164A
	CTD27-52 S88A	CTD27-52 T174A
	CTD27-52 S99A	CTD27-52 P176A
	CTD27-52 S109A	

Table S1. Library of CTD bacterial plasmids used to assign all non-overlapped resonances in the HSQC spectrum of CTD27-52.

Human RNA polymerase II
CTD heptads 27-52

HEPTAD	RES	SEQUENCE
	1	GHMSPN
27	7	Y T PTSPN
28	14	YSPTSPS
29	21	YSPTSPS
30	28	YSPTSPS
31	35	YSPSSPR
32	42	Y T PQSPT
33	49	Y T PSSPS
43	56	YSPSSPS
35	63	YSPASPK
36	70	Y T PTSPS
37	77	Y S PS S PE
38	84	Y T PTSPK
39	91	YSPTSPK
40	98	YSPTSPK
41	105	YSPTSP T
42	112	YSPT T PK
43	119	YSPTSP T
44	126	YSPTSP V
45	133	Y T PTSPK
46	140	YSPTSP T
47	147	YSPTSPK
48	154	YSPTSP T
49	161	YSPTSP K GST
50	171	YSPTSP G
51	178	YSPTSP T
52	185	Y S LTSPA
192		ISPDDSDEEN

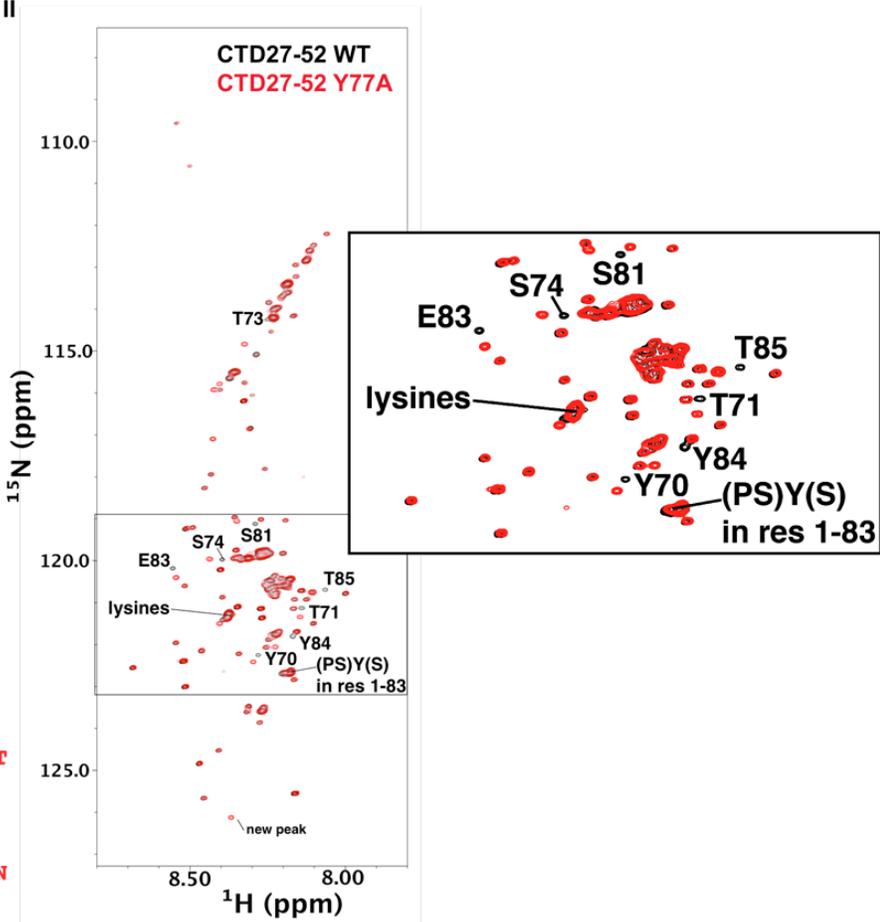


Figure S1. Example of how CTD27-52 mutants aid in assignment of the HSQC spectrum. By overlaying the HSQC spectra of wild-type CTD27-52 (black) and CTD27-52 Y77A (red), chemical shift perturbation of residues near Y77A can be observed. The mutated residue is highlighted in the sequence, and perturbed chemical shifts of nearby residues are labeled in black (see magnified inset). The new backbone resonance for A77 appears downfield.