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1 Phase separation and molecular ordering of the prion-like do- 2 main of the thermosensory protein EARLY FLOWERING 3

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17

18 **Abstract:** Liquid-liquid phase separation (LLPS) is an important mechanism enabling the dynamic
19 compartmentalisation of macromolecules, including complex polymers such as proteins and nu-
20 cleic acids, and occurs as a function of the physicochemical environment. In the model plant, *Ar-*
21 *abidopsis thaliana*, LLPS by the protein EARLY FLOWERING3 (ELF3) occurs in a temperature
22 sensitive manner and controls thermoresponsive growth. ELF3 contains a largely unstructured
23 prion-like domain (PrLD) that acts as a driver of LLPS in vivo and in vitro. The PrLD contains a
24 poly-glutamine (polyQ) tract, whose length varies across natural Arabidopsis accessions. Here, we
25 use a combination of biochemical, biophysical and structural techniques to investigate the dilute
26 and condensed phases of the ELF3 PrLD with varying polyQ lengths. We demonstrate that the
27 dilute phase of the ELF3 PrLD forms a monodisperse higher order oligomer that does not depend
28 on the presence of the polyQ sequence. This species undergoes LLPS in a pH and temperature-
29 sensitive manner and the polyQ region of the protein tunes the initial stages of phase separation.
30 The liquid phase rapidly undergoes aging and forms a hydrogel as shown by fluorescence and
31 atomic force microscopies. Furthermore, we demonstrate that the hydrogel assumes a semi-ordered
32 structure as determined by small angle X-ray scattering, electron microscopy and X-ray diffrac-
33 tion. These experiments demonstrate a rich structural landscape for a PrLD protein and provide a
34 framework to describe the structural and biophysical properties of biomolecular condensates.

35

36 **Keywords** *Liquid-liquid phase separation, hydrogel, small angle X-ray scattering, atomic force microscopy, fluores-*
37 *cence microscopy, EARLY FLOWERING 3, thermosensing, Arabidopsis thaliana*

38 Introduction

39 Compartmentalisation into biomolecular condensates, or membraneless organelles, helps
40 to regulate the biochemistry of the cell by dynamically concentrating and sequestering different
41 components including proteins such as transcription factors, RNA-binding proteins and co-factors
42 and nucleic acids¹⁻⁹. Proteins with intrinsically disordered regions (IDRs) and low complexity
43 prion-like domains (PrLD) often act as drivers of LLPS, separating into a highly concentrated
44 protein-rich phase and a dilute phase under specific conditions¹⁰⁻¹². While simple polymers have

45 been successfully studied experimentally and modelled using theoretical methods such as course-
46 grained (CG) simulation and atomistic models, quantifying and predicting the behaviour of PrLD
47 proteins as a function of physicochemical variables is challenging due to the complexity in the
48 amino acid sequence of proteins^{13–19}. The interactions that contribute to the metastable condensed
49 phase include many transient, short-range interactions including pi-pi, cation-pi, dipole, electro-
50 static and hydrophobic interactions, all of which may be present in a given polypeptide. These
51 weak and low-specificity contacts are often present in disordered proteins and will occur intramo-
52 lecularly in the dilute phase and both intra- and intermolecularly in the condensed phase. The for-
53 mation and dynamics of phase separation mediated by PrLD proteins is often highly sensitive to
54 pH, ionic strength and temperature and will vary as a function of the properties of the amino acids
55 (i.e. polar, charged, hydrophobic, aromatic) in the PrLD sequence^{20–23}. While this dynamic re-
56 sponse of PrLD proteins is technically challenging to study, it may play a critical physiological
57 function, allowing PrLD proteins to act as sensors of the *in vivo* cellular environment and to alter
58 physiological responses accordingly. For example, the poly(A)-binding protein (Pab1) in yeast and
59 the recently characterised circadian clock protein EARLY FLOWERING3 (ELF3) in Arabidopsis
60 have been shown to act as direct *in vivo* temperature sensors and to alter developmental re-
61 sponses^{6,24}. Thus, an understanding of the molecular basis of environmental sensing by PrLD pro-
62 teins is a pre-requisite to engineering these properties for the creation of tailored responses to
63 stresses such as temperature changes in the cell.

64 ELF3 displays a well-characterised ability to undergo LLPS *in vitro* and *in vivo* and exists
65 with natural sequence variation within the PrLD associated with specific phenotypes, suggesting
66 a physiological role for LLPS^{6,26}. ELF3 is a largely disordered protein with a C-terminal PrLD.
67 The PrLD contains a poly-glutamine repeat (polyQ) that exhibits different lengths from 7 to 29
68 glutamines across 181 natural Arabidopsis accessions^{25,26}. Previous experiments in Arabidopsis
69 plants grown at 17 °C, 22 °C and 27 °C demonstrated that the length of the polyQ has a mild but
70 statistically significant effect on hypocotyl elongation, a commonly used measure of ther-
71 moresponsive growth⁶. While ELF3 PrLD with 7 glutamines (Q7), from the lab strain Columbia-
72 0, has been shown to phase separate *in vitro* in a temperature-dependent manner, the effects of
73 varying polyQ length on the dynamics of phase separation is not known⁶. In order to better under-
74 stand the biophysical basis of ELF3 condensation, we verified that ELF3 Q0, Q7 and Q20 could
75 form puncta *in vivo* and performed biochemical, structural and biomechanical studies of the cor-
76 responding PrLD regions, demonstrating that LLPS of ELF3 PrLD with varying polyQ lengths is
77 sensitive to temperature and pH. We further show that the dilute phase, unlike a canonical intrin-
78 sically disordered protein, exists as a monodisperse higher order oligomer and that upon liquid-
79 liquid phase separation forms a new species with distinct microenvironments and different biome-
80 chanical properties. This species is able to further undergo aging into an ordered hydrogel. ELF3
81 PrLD exhibits a distinct structural landscape, with the dynamics and biomechanical properties of
82 the condensed liquid and gel phases modulated by the length of the polyQ region.

83

84 **Results**

85

86 *In vivo* puncta formation of ELF3 Q0, Q7 and Q20

87 Based on our previous studies of the full-length and PrLD of ELF3, the protein undergoes
88 LLPS *in vivo* and *in vitro* with the PrLD required and sufficient for phase separation⁶. To verify
89 formation and dynamics of condensate formation *in vivo*, the sequence of the *ELF3* gene encoding

90 polyQ lengths of 0 (deletion mutant), 7 (from accession Columbia-0) and 20 (from accession
91 Sandåkra-2 (San-2)) glutamines tagged with mVenus was expressed under an inducible promoter
92 in tobacco leaf epidermal cells. The formation of puncta was observed for all three constructs
93 (Figure 1). Fluorescence recovery after photobleaching (FRAP) experiments were performed to
94 investigate the dynamics of the proteins in the puncta. The puncta for all constructs exhibited partial
95 recovery after photobleaching, with fluorescence intensities after photobleaching ranging from
96 about 50% for Q0 and Q7 (Figure 1A and B) to 30% for Q20 (Figure 1c) of the prebleach intensity.
97 This indicates that the puncta are comprised of a mixture of mobile and immobile species, as has
98 been observed for many other systems²⁷. In order to more robustly characterize the dynamics and
99 structure of the condensate, detailed in vitro studies of the PrLD region of the protein required for
100 phase separation were performed.

101

102 *In vitro characterization of the ELF3 PrLD*

103 Using optical imaging, turbidity assays (A_{440}) and phase diagrams, we examined the effects
104 of temperature, protein concentration and pH on LLPS of ELF3 PrLD with polyQ lengths of 0, 7
105 and 20 glutamines (Figure 2 and Supplementary Figure S1A). All PrLD constructs underwent
106 LLPS, however temperature and pH had subtle but measurable effects on phase separation for the
107 three constructs studied. Increasing temperature triggered LLPS for Q0, Q7 and Q20 constructs
108 and exhibited reversibility as shown qualitatively (Figure 2A) using temperature steps. Absorbance
109 measurements at A_{440} were used to further examine this behaviour, with Q0 and Q7 behaving sim-
110 ilarly, exhibiting an LLPS transition temperature occurring at 31.2 ± 0.4 °C and 28.1 ± 0.3 °C, re-
111 spectively (Figure 2B). In comparison, the Q20 construct started phase separation at a lower tem-
112 perature, as shown by the non-zero normalised absorbance between 15 °C and 20 °C and a sloping
113 bottom plateau. The A_{440} gradually increased for Q20 as the temperature was raised with a T_m of
114 33.0 ± 1.5 °C (Figure 2B). The T_m values were calculated based on the first temperature ramps, as
115 denoted by the filled blue squares in Figure 2b. The effects of temperature were largely reversible
116 for all polyQ constructs tested, with the PrLD proteins switching between the dilute and condensed
117 phase as monitored by A_{440} (Figure 2A and B). It should be noted that, due to the experimental set-
118 up for the A_{440} measurements, where the highest temperature measured was 40 °C, the parameters
119 extracted from the fitting, in particular for Q20, should be considered as estimations as no plateau
120 was reached and above 40 °C the proteins began to irreversibly precipitate. The observed demixing
121 of the ELF3 PrLD proteins with increasing temperature is indicative of hydrophobic and aromatic
122 interactions driving phase separation due to the favourable enthalpic contributions to the free en-
123 ergy of the system under the temperature regime of interest and positive entropic effects due to
124 counter ion and/or hydration water release, as has been observed for other LLPS systems^{20,28-30}.
125 All constructs underwent phase separation under the conditions tested and expansion of the polyQ
126 repeat from 0 to 20 glutamines had a relatively small effect on the transition temperature onset of
127 phase separation.

128 In addition to temperature variables, pH changes also affected phase separation, with the
129 longer polyQ construct (Q20) forming spherical droplets over a wider range of protein concentra-
130 tions and pH (pH 8.0-9.0) than the Q0 and Q7 constructs, which only underwent LLPS over a
131 narrow pH range of 8.5-9.0 under the same ionic strength buffer conditions (Figure 2C). Fusing a
132 C-terminal GFP to the PrLD constructs resulted in the formation of spherical droplets with the
133 same overall trend observed for the unfused proteins, with the Q20 construct undergoing phase
134 separation at a pH range of 6.5-8.5 versus the Q7 and Q0 constructs that behaved in a similar

135 manner, with phase separation occurring between pH 7-8.5 (Supplementary Figure S1B). Extend-
136 ing the polyQ region results in a broader range of LLPS formation with respect to pH and temper-
137 ature. This may be due to the relatively short polyQ tracts for Q0 and Q7, with longer polar gluta-
138 mine stretches acting to prevent precipitation and keeping the protein in the condensed droplet
139 phase over a wider range of pH and temperatures.

140

141 *Fluorescence recovery after photobleaching in vitro.*

142 While pH and temperature affected LLPS, we questioned whether there were inherent dif-
143 ferences with respect to dynamics or stability of the condensed phase due to varying polyQ length.
144 Fluorescence recovery after photobleaching (FRAP) experiments were performed on Q0, Q7 and
145 Q20 PrLD constructs fused to GFP to investigate this possibility. All proteins formed droplets as
146 the pH of the solution was reduced step-wise (0.2 pH units) from 9.4 to 8.4 and 40 minutes of
147 equilibration after each pH change. These experiments revealed the formation of a low mobility
148 condensate with negligible fluorescence recovery over minutes (Figure 2D). No clear differences
149 were observed for the different samples, with all samples exhibiting formation of gel-like droplets.
150 This suggests that while temperature change results in more reversible liquid-liquid phase separa-
151 tion, at least under fast temperature ramps, pH changes favoured gel formation after initial con-
152 densation.

153

154 *Biomechanical measurements by AFM*

155 To further characterise the condensed phase and determine whether there were differences
156 in the biomechanical properties of the samples, atomic force microscopy (AFM) and AFM-coupled
157 confocal microscopy³¹ experiments were performed (Figure 3 and Supplementary Fig. S2-S3). All
158 samples were applied to a glass coverslip and imaged in liquid for AFM topography and force
159 curve measurements. Lower pH and longer adhesion times resulted in the formation of the gel state
160 whereas higher pH and shorter adhesion times maintained the droplets in a more liquid-like state
161 and droplets were considered a “highly viscous liquid” if they exhibited fusion with other droplets
162 and at least 10% fluorescence recovery over 2 minutes. For these samples, in agreement with mod-
163 els describing nanoscale indentation of liquid interfaces,^{32,33} the stiffness was calculated based on
164 a linear fit to the force vs. distance curves with between 9 and 13 measurements taken from the
165 centre of each droplet to avoid edge artefacts, which are present when the droplet size is compara-
166 ble to the size of the AFM tip height ($\approx 3.5 - 7 \mu\text{m}$). Such a force versus indentation linear regime
167 can directly be related to the liquid-liquid interfacial tension³³. This linear model is in good agree-
168 ment with the data versus parabolic models for isotropic elastic solids. The droplets for all samples
169 exhibited variable mechanical properties, suggesting the presence of harder and softer regions
170 within individual droplets, with measurements ranging from 3 to 9 mN/m (Table 1 and Figure 3).
171 This is likely due to liquid-hydrogel phases coexisting in the droplet and the more hydrogel pre-
172 sent, the greater the deviation from linearity of the force as a function of distance. In contrast to
173 the liquid condensed phase, the gel-state exhibited no fluorescence recovery, no fusion and a non-
174 linear force versus indentation regime that could be fit using a Hertz model (applicable to solid
175 samples) and the Young’s modulus calculated³³ (see comparison between indentation cycles per-
176 formed on liquid-like and hydrogel droplets in Supplementary Figure S2C, were deviation from
177 linearity in the liquid phase curve can also be due to the presence of an out of contact Debye’s
178 screening length region³³). Interestingly, gel transition of the samples only partially affected over-
179 all droplet morphology since all droplets retained a quasi-spherical shape (Figure 4), however

180 indentation cycles showed a non-linear force *vs.* distance regime as opposed to the linear force *vs.*
181 distance pattern observed for the highly viscous liquid droplet samples. In addition, the mechanical
182 response changed for the hydrogel samples. For all hydrogel samples, the stiffness ranged between
183 5-100 kPa, with ELF3 PrLD Q0 showing a consistently lower Young's modulus than the Q7 and
184 Q20 constructs (Table 1). It should be noted that constructs in Figure 4A exhibit higher rigidity
185 than the values reported in Table 1 because they were imaged with a higher AFM loading rate (see
186 Experimental Methods), required to decrease the acquisition time while providing sufficient pixels
187 for high resolution, thus the apparent "stiffer" response at higher loading rate is due to viscoelastic
188 behaviour. Wide-field fluorescence and high-resolution AFM imaging of the samples further re-
189 vealed a heterogeneity within the droplets, suggesting the formation of discrete microenvironments
190 (Figure 4). The microenvironments exhibited a stacked or layered structure, with varying step-like
191 height profiles ranging from ~20 nm to 200 nm (Figure 4D and Supplementary Figure S3). These
192 experiments represent the first examples, to our knowledge, of the use of AFM to determine the
193 biomechanical properties of macromolecular condensates in the liquid phase, allowing us to probe
194 the molecular organisation of molecules within individual droplets.

195

196 *SAXS of dilute and condensed phases*

197 In order to further investigate the transition from the dilute to condensed phase, condensed
198 phase dynamics and the potential structuration and ordering of microenvironments within the con-
199 densate, we performed a series of small angle X-ray scattering (SAXS) experiments (Supplemen-
200 tary Table 1 and Figure 5). Scattering curves were determined for each sample (ELF3 PrLD Q0,
201 Q7 and Q20) in the dilute phase using an online HPLC purification step in order to remove any
202 aggregates (Figure 5A). All proteins eluted as symmetrical peaks after the void volume of the
203 column. In the dilute phase with monodisperse samples, there should be little or no contribution to
204 the scattering from other species such as aggregates or the condensed phase, and the scattering
205 curve will then reflect the form factor, $P(q)$, providing information on the size and shape of the
206 individual molecules in solution. Based on polymer theory, the conformation the molecules adopt
207 in the dilute phase can be predictive of their phase behaviour, providing a technically simple way
208 to gain insight into the condensed phase³⁴. Calculating the radius of gyration (R_g) based on fitting
209 the linear portion of the Guinier region, yields values between 72-76 Å for the three samples (ELF3
210 PrLD Q0, Q7 and Q20) and the maximum dimension, D_{max} , values of 272, 273 and 294 Å, respec-
211 tively. Surprisingly, based on these measurements the estimated molecular weights, even in the
212 dilute phase, of the molecular species corresponds to a multimeric ~28-30-mer assembly (Supple-
213 mentary Table 1). This higher order oligomeric state was further confirmed by size-exclusion chro-
214 matography multiangle laser light scattering (Supplementary Figure S7). As expected, the largest
215 R_g and D_{max} values were calculated for ELF3 PrLD Q20 due to its increased length from expansion
216 of the polyQ region (Table 2). Furthermore, these dilute phase measurements demonstrate that for
217 all PrLDs, the species in solution is relatively globular, as shown in the Kratky plot (Figure 5B).
218 The compact spherical structure of a monodisperse higher order oligomer, in contrast to an ex-
219 tended conformation often observed for intrinsically disordered proteins, coupled with the lack of
220 predicted secondary or tertiary structure, suggests that the presence of weak multivalent interac-
221 tions may be sufficient to exclude solvent and that hydrophobic amino acid sequences lacking
222 predicted secondary structure, partially organise the protein molecules, likely a prerequisite for
223 phase separation by ELF3³⁵. As liquid-liquid phase separation requires multivalent interactions,
224 the dilute phase organisation may recapitulate these types of interactions intramolecularly or as
225 smaller oligomeric units, with proteins predicted to phase separate potentially exhibiting a more

226 compact structure versus an extended conformation from non-phase separating disordered pro-
227 teins³⁵.

228 LLPS of the samples was triggered by a temperature increase and resulted in changes to
229 the scattering curves (Figure 5C and Supplemental Figure S5). In the liquid-liquid separated phase,
230 the SAXS measurements demonstrate an increase in the R_g and a more extended conformation
231 based on the Kratky plot calculations (Table 2 and Figure 5D). Changes in R_g in the condensed
232 state have been previously observed for proteins undergoing LLPS and ascribed to nucleation
233 events³⁵. The LLPS samples are more heterogeneous than the online HPLC purified samples in the
234 dilute phase, as reflected in the upturn of the scattering curve as $I(q)$ approaches 0 (Figure 5C), but
235 exhibit a clear trend in R_g , with an increase in the R_g in the condensed phase, suggesting possible
236 elongation of the polypeptide chains or an increase in the overall size. In addition to these changes
237 in the R_g , a structure factor peak, $S(q)$, formed in the low q region of the scattering curves that
238 increased in height with increasing temperature (Figure 5C and Supplemental Figure S5). This
239 $S(q)$ (structure factor) peak is indicative of loose lamellae within the condensed phase, and a cal-
240 culated spacing (d_{hkl}) of approximately 155, 163 and 167 Å for ELF3 PrLD Q0, Q7 and Q20,
241 respectively, similar to the calculated layer spacing observed in AFM experiments. It should be
242 noted that the layered arrangement is distinct from the characteristic fibril formation observed for
243 amyloids,³⁶ but a less compact lamellar phase characteristically observed for lipid membranes and
244 polymer lamellae^{37,38}. To further investigate the temperature effects to the condensed phase, tem-
245 perature ramps ELF3 PrLD Q7 and Q20 exhibited an increase in the structure factor peak as the
246 temperature was increased, whereas ELF3 PrLD Q0 exhibited little change, suggesting it had
247 passed through the liquid phase and had undergone aging to the more stable hydrogel, losing the
248 capacity to easily transition between the condensed and dilute phases (Supplemental Figure S5).

249

250 *Structural characterization of the gel phase*

251 The ordered species in the condensate were further characterized using stable gel samples,
252 obtained by aging of the condensed liquid phase at pH 7.7-8 for ~1 hr at 4 °C for Q0, Q7 and Q20.
253 These samples no longer exhibited reversibility between dilute and condensed phases with tem-
254 perature or pH change and coalesced into large non-spherical amorphous species. Gel samples
255 were characterised using transmission electron microscopy (TEM) and X-ray diffraction. Negative
256 stain TEM confirmed regions with a stacked structure in the gel phase for all polyQ constructs,
257 with a stack spacing estimated at ~40-50 Å (Figure 6A). This stack spacing is smaller than ob-
258 served for AFM and SAXS experiments and may be due to the longer aging of the sample and/or
259 dehydration effects of sample preparation, however a highly ordered organisation of molecules is
260 observed. The TEM experiments exhibit inhomogeneity in the samples with certain regions exhib-
261 iting well-ordered molecular stacks and other regions showing no clear organisation.

262 X-ray diffraction experiments for the ELF3 PrLD Q20 construct exhibited a weak powder
263 diffraction ring at 4-5 Å (Figure 6b), similar to observations for hydrogel forming FUS constructs,
264 denoting a long-range molecular ordering with a powder diffraction ring arising from the distance
265 between sheets of polypeptide chains³⁹. The ELF3 PrLD Q0 and Q7 constructs showed very dif-
266 fuse scattering in the 4-5 Å range (Supplemental Figure S6), due to either diffraction measurements
267 from an amorphous region exhibiting less molecular stacking or indicating an overall less well-
268 ordered hydrogel for the ELF3 PrLD Q0 and Q7 constructs.

269

270 **Discussion**

271 Proteins that drive LLPS are under active study not only due to their key biological role in
272 cellular compartmentalisation, but also for diverse applications in biomaterials and drug-deliv-
273 ery^{20,40–44}. Characterisation of the dilute and condensed phases and the effects of exogenous vari-
274 ables including temperature and pH is experimentally challenging due to the complexity of the
275 protein polymer and the intra- and intermolecular interactions at play during the nucleation and
276 condensation process. Here, we demonstrate the utility of complementary biophysical and struc-
277 tural techniques to determine the dynamics and structure of different phases of a PrLD protein as
278 a function of pH and temperature. Phase diagrams, A₄₄₀ assays and SAXS measurements demon-
279 strate that the dilute phase is a higher order oligomer and formation of the condensed phase is
280 triggered by pH changes and an increase in temperature. While phase separation of the ELF3 PrLD
281 does not depend on the polyQ regions, variation in polyQ length, which occurs in natural Ara-
282 bidopsis accessions, alters LLPS formation with respect to temperature and pH. Upon aging, an
283 ordered low mobility gel phase quickly forms in vitro and can also be observed in vivo, as puncta
284 do not exhibit full fluorescence recovery. AFM, SAXS, EM and X-ray diffraction of the gel phase
285 are all consistent with a stacked/layered structure in the biomolecular condensate, with extensive
286 ordering occurring in the aged hydrogel. The physiological function of these different phases re-
287 mains an open question requiring further investigation. However, with these detailed structural and
288 biophysical studies the oligomeric state and the demixing properties of the protein can be measured
289 and quantified, providing a foundation for altering these variables and determining their physio-
290 logical function.

291 The varying conformations and dynamics of the ELF3 PrLD polypeptide- from a surpris-
292 ingly large and monodisperse oligomeric species in the dilute phase, to a solvent excluded liquid
293 condensed phase and finally to a highly ordered hydrogel - demonstrate how the intrinsically dis-
294 ordered polypeptide is able to access different regions of structure space as a function of the phys-
295 icochemical environment. The low-affinity and transient interactions of the ELF3 PrLD, consisting
296 of both intra- and intermolecular interactions, may facilitate the priming of the molecules for phase
297 separation and condensation via the formation of a large oligomeric species in the dilute phase^{45,46}.
298 While the ELF3 PrLD has little predicted secondary structure, it is still able to form a defined and
299 homogeneous ~28-30 oligomeric assembly in solution. Based on the SAXS studies, this species is
300 compact and globular, likely serving as an intermediate for phase separation which would occur
301 via the fusion of oligomeric spheres. Whether this state is specific to the ELF3 PrLD or more
302 common to PrLD phase separating proteins is unknown and will require further study.

303 Examination of the primary amino acid sequence of ELF3 PrLD demonstrates a number of
304 proline clusters and aromatic residues that may act as “stickers” for LLPS and/or oligomerisation,
305 although the specific amino acids driving this remain to be determined. In other systems that un-
306 dergo LLPS and fibril or hydrogel formation, various motifs important for steric zippers, such as
307 those in amyloid fibrils, and kinked beta sheets have been identified. Studies of LLPS in the protein
308 FUS, for example, reveal the presence of specific peptide motifs that act as drivers for phase sep-
309 aration and hydrogel formation. These low-complexity, aromatic-rich, kinked segments (LARKs),
310 have been shown to self-assemble into stacked structures and protofilaments, with short LARK
311 hexapeptides able to form kinked beta sheets that are less stable than beta amyloid fibrils but still
312 exhibit long-range ordering^{39,47}. The ELF3 PrLD does not possess canonical LARK peptide se-
313 quences and increasing temperature generally disrupts LARK-type peptide interactions, in contrast
314 to increasing temperature triggering LLPS for ELF3 PrLD, suggesting other mechanisms are crit-
315 ical for the observed self-organizing behavior of the protein that likely depend on specific hydro-
316 phobic interactions⁴².

317 While focused on a specific PrLD, these studies provide a framework for the in vitro char-
318 acterization of condensate-forming biomolecules using complementary biophysical and structural
319 techniques. The in vivo implications and prevalence of long-range molecule ordering of PrLD-
320 containing proteins such as ELF3 are still to be fully explored. This may be a much more general
321 phenomenon for other condensate-forming species. For example, the formation of discrete hetero-
322 geneous assemblies, or pleiomorphic ensembles, within the same droplet has been observed in
323 vivo for scaffold proteins and signalling complexes, such as the PDGF receptor complex and the
324 Wnt signalosome, allowing for the cooperative assembly of different components while retaining
325 some conformational flexibility of the components^{48,49}. Stacked structures in condensates have
326 been observed for other IDR-containing proteins such as FLOE1, a plant water-sensory protein
327 important for seed germination. Substitution of tyrosine and phenylalanine for serine residues in
328 the aspartate-serine rich (DS) domain of FLOE1 resulted in gel formation of the mutant in vivo
329 with ordered structures observed by TEM and 3-D tomography⁵⁰. Phase separation and gelation of
330 FLOE1 was driven by aromatic residues in the glutamine/proline/serine-rich (QPS) domain, in
331 particular tyrosine residues.

332 Taken together, these experiments have allowed a detailed in vitro characterisation of ELF3
333 PrLD condensates using complementary biophysical and structural techniques that have not been
334 previously applied to biological molecules in dilute and condensed phases. The molecular ordering
335 observed in vitro for ELF3 PrLD may be a much more general phenomenon than previously ap-
336 preciated and may help explain the variability in fluorescence recovery after photobleaching ob-
337 served for many membraneless organelles^{5,51,53}. An important and remaining challenge is to deter-
338 mine the physiological role of condensed species with different mobilities and whether long-range
339 order and molecular stacking has a physiological role in ELF3 function. This will require further
340 study including the re-engineering of the PrLD of ELF3 to alter LLPS and an examination of the
341 effects in vivo at the cellular and phenotypic level.

342 **Experimental Methods**

343 **Protein Expression and purification of ELF3PrLD and ELF3PrLD-GFP**

344 ELF3 PrLD (Q7, residues 388-625, AT2G25930, *Arabidopsis thaliana* ecotype Columbia), Q0,
345 Q20, ELF3 PrLD-GFP Q7, Q0 and Q20 were cloned into the expression vector pESPRIT2 using
346 the Aat II and Not I sites as previously described⁶. To generate the GFP constructs the stop codon
347 for ELF3 PrLD Q0, Q7 and Q20 was removed by site-directed mutagenesis using the QuikChange
348 protocol (Agilent) and a GFP-tag was added to the C-terminus as previously described⁶. All pro-
349 teins were overexpressed in *E. coli* BL21-CodonPlus-RIL cells (Agilent). Proteins used for phase
350 diagrams, FRAP, AFM, SAXS, EM and X-ray diffraction experiments were expressed and puri-
351 fied as follows. Briefly, cells were grown in LB media supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin
352 and 35 $\mu\text{g ml}^{-1}$ chloramphenicol at 37 °C and 120 rpm. At $\text{OD}_{600\text{nm}} = 0.8$, the temperature was
353 lowered to 18 °C and protein expression was induced with 1 mM isopropyl-b-D-1-thiogalactopy-
354 ranoside (IPTG). After 16 h, the cells were harvested by centrifugation at 5000 x g and 4 °C for
355 15 min. For all constructs, cells were resuspended in 100 mM Bis-Tris propane pH 9.4, 300 mM
356 NaCl, 20 mM imidazole, 1 mM TCEP (tris(2-carboxyethyl)phosphine (TCEP) and EDTA-free
357 protease inhibitors (ThermoFisher). Cells were lysed by sonication and the cell debris pelleted at
358 50000 x g and 4 °C for 30 min. The supernatant was applied to a 1 ml Ni-NTA column pre-equi-
359 librated in resuspension buffer (lysis buffer without protease inhibitors), washed with resuspension
360 buffer and high salt buffer (100 mM Bis-Tris propane pH 9.4, 1 M NaCl, 20 mM imidazole, 1 mM

361 TCEP). The proteins were eluted in 100 mM Bis-Tris-propane pH 9.4, 300 mM NaCl, 300 mM
362 imidazole and 1 mM TCEP. Protein purity was determined by SDS-PAGE, and the fractions of
363 interest were pooled and dialysed for ~2 h at 4°C in 50 mM Bis-Tris-propane pH 9.4, 500 mM
364 NaCl, 1 mM TCEP. Final protein concentration was 4-8 mg/ml for all samples. Bis-Tris-propane
365 was used due to its wide buffering pH range. For the turbidity assays the Bis-Tris-propane was
366 replaced with CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) buffer, as the proteins were sta-
367 ble and soluble at this pH at protein concentrations. Purification was performed as for above, with
368 the Bis-tris propane buffer replaced by CAPS pH 9.7. Final dialysis was performed against 50 mM
369 CAPS pH 9.7, 200 mM NaCl, 1 mM TCEP and proteins diluted to ~0.4 mg/ml for turbidity assays.

370 **Transient expression in *Nicotiana benthamiana* for FRAP**

371 The coding sequence of *ELF3Q0*, *ELF3Q7* and *ELF3Q20* were cloned into the GreenGate cloning
372 system by digestion and ligation. For mVenus tagged constructs, GreenGate reactions with a β -
373 estradiol inducible promoter were performed as previously described⁵³. The *Agrobacterium tume-*
374 *faciens* strain harbouring the pSOUP helper plasmid was transformed with the above-described
375 plasmids. mVenus tagged fusion proteins were co-expressed with the p19 silencing repressor in
376 epidermal leaf cells of *N. benthamiana*. Induction with β -estradiol was carried out as described⁵⁴.

377 **In vivo FRAP measurements and analysis of ELF3 condensates**

378 FRAP measurements were carried out with a confocal laser scanning microscope (inverted
379 LSM880, ZEISS) equipped with a 40x water objective (C-Apochromat, NA 1.2, ZEISS). mVenus
380 was excited with an argon laser at 514 nm. For FRAP measurements, single condensates in or
381 around the nucleus were chosen. Time series of 65 frames were acquired to observe fluorescence
382 recovery. Acquisition times were approximately 300 ms per frame. Fluorescence recovery was
383 analysed individually for each bleached condensate by drawing narrow ROIs⁵⁵. For normalisation,
384 calculation of mobile fraction and half-life ($\tau_{1/2}$) a plugin written at the Stowers Institute for medical
385 research (<https://research.stowers.org/imagejplugins/index.html>) was used in Fiji. 9-10 individual
386 puncta were photobleached from different cells, and used in each analysis. FRAP curves were
387 generated from all data and mean curves with S.D. are shown. Experiments were performed at
388 room temperature.

389 **Light scattering turbidity assays**

390 The light scattering assay was performed in a Cary 100 UV-vis spectrometer (Agilent Technolo-
391 gies UK Ltd., Stockport, UK) as previously reported⁶. Briefly, the absorbance at 440 nm was mon-
392 itored for samples containing buffer alone (50 mM N-cyclohexyl-3-aminopropanesulfonic acid
393 (CAPS) pH 9.7, 200 mM NaCl, 1 mM TCEP), ELF3 PrLD Q0, Q7 or Q20 (15 μ M, ~0.4 mg/ml)
394 in quartz cuvettes (path length 10 mm) with increasing temperature (10-40 °C; 1 °C min⁻¹), and the
395 spectra were normalized with respect to buffer alone. A transition temperature (T_m) was deter-
396 mined by fitting the spectrum with a 4-parameter sigmoidal equation using GraphPad Prism 9.4.0
397 and the sigmoidal 4PL equation, which is defined as $y=A + (B-A)/(1+(T_m/x))^C$, where y is the
398 normalised turbidity at 440 nm, x is the temperature in °C, A and B are bottom and top plateaus
399 with the same units as y, C is the slope factor or Hill slope and T_m is the midpoint transition
400 temperature. Reported values are from the curves shown in Fig. 2a with the filled blue squares for
401 the first temperature increase from 10-40°C. T_m values were $31.2 \pm 0.4^\circ\text{C}$ (ELF3 PrLD Q0), 28.1
402 $\pm 0.3^\circ\text{C}$ (ELF3 PrLD Q7) and $33.0 \pm 1.5^\circ\text{C}$ (ELF3 PrLD Q20). Due to the experimental set-up,

403 where the highest temperature measured was 40 °C, the parameters extracted from the fitting, in
404 particular for ELF3 PrLD Q20 should be considered as estimations as no plateau was reached and
405 above 40 °C the proteins began to irreversibly precipitate. To monitor reversibility, the turbidity
406 was monitored for an increasing temperature ramp (10 to 40 °C; 1 °C min⁻¹) followed by decreasing
407 the temperature (40 to 10 °C; 1 °C min⁻¹) and this cycle was repeated twice in total for ELF3 PrLD
408 Q0, Q7 and Q20.

409 **Phase diagrams**

410 To generate phase diagrams for ELF3 PrLD Q0, Q7 and Q20 and ELF3 PrLD Q0-, Q7-, Q20 -
411 GFP, the pH of the dialysis buffer (50 mM Bis-Tris propane pH 9.4, 500 mM NaCl and 1 mM
412 TCEP) was gradually decreased by 0.2 pH units and at each pH the solution was allowed to equil-
413 ibrate for at least 40 min. The solution was monitored for increased turbidity due to liquid droplet
414 formation visually and by optical microscopic examination using the Olympus CKX41 bright field
415 microscope with a Photometrics CoolSNAP cf2 camera at 20x magnification. All solutions were
416 at 4 °C and kept in the cold room except for brief visualisation under a microscope at room tem-
417 perature on cooled glass slides (4°C). For each phase diagram, measurements were performed at
418 pH 9.5, 9.0, 8.8, 8.6, 8.4, 8.2, 8.0, 7.8, 7.6, 7.4 and 5 different protein concentrations (0.5, 1.0, 2.0,
419 4.0, 6.0 mg/ml).

420 **Temperature-induced LLPS images**

421 Samples of ELF3 PrLD Q0, Q7 and Q20 at ~ 4 mg/ml in 50 mM Bis-Tris propane, pH 9.2, 500
422 mM NaCl and 1 mM TCEP (Q0 and Q7) or 50 mM Bis-Tris propane, pH 8.8, 500 mM NaCl and
423 1 mM TCEP (Q20), 500 mM NaCl, 1 mM TCEP were visualised at 4°C, 22°C and 4°C using an
424 Olympus CKX41 bright field microscope with a Photometrics CoolSNAP cf2 camera at 20x mag-
425 nification. 50 µl samples were cooled to ~ 4°C and 5 µl of solution was applied to a cooled glass
426 slide and quickly imaged. The samples were then heated to 22°C in a heating block for ~5 min. to
427 induce phase separation. 5µl of solution was applied to a room temperature glass slide and imaged.
428 The tubes were then cooled to ~4°C for 15 min. and imaged as described.

429 **Confocal imaging and fluorescence microscopy in vitro**

430 For droplet visualization and photobleaching experiments of ELF3 PrLD Q0-, Q7- and Q20- GFP
431 proteins, liquid droplet formation was induced by dialysis as described above with GFP labelled
432 ELF3 PrLD protein concentration of ~4 mg/ml and starting dialysis conditions of 50 mM Bis-Tris-
433 propane pH 9.4, 500 mM NaCl with the pH gradually reduced in units of 0.2 until the solution
434 became turbid (~pH 7.8). All dialysis steps were performed in a cold room at 4 °C. Once turbidity
435 was observed, a 10 µl drop of solution was applied to a glass slide and all subsequent measure-
436 ments performed at room temperature. The drop was covered with a cover slip and mounted and
437 visualized using an objective-based total internal reflection fluorescence (TIRF) microscopy in-
438 strument composed of a Nikon Eclipse Ti, an azimuthal iLas2 TIRF illuminator (Roper Scientific),
439 a 60x numerical aperture 1.49 TIRF objective lens followed by a 1.5x magnification lens and an
440 Evolve 512 camera (Photometrics). For photobleaching experiments, droplets were allowed to ad-
441 here to the coverslip prior to photobleaching to minimize droplet movement during the experiment.
442 Acquisition times were approximately 1 s per image for 30 s and then every 10 s for 270 s. Droplet
443 size was ~2-5 µm with bleaching area of ~ 1 µm. Time-lapse images were acquired at 530 nm.

444 **Correlative AFM-fluorescence experiments**

445 *AFM-confocal fluorescence microscopy:*

446 AFM coupled to confocal fluorescence microscope was developed in-house, allowing AFM im-
447 aging and fluorescence recovery after photobleaching experiments on the same droplets (Supple-
448 mentary Fig. S3a)³¹. AFM images were acquired using a Nanowizard 4 (JPK Instruments, Bruker)
449 mounted on a Zeiss inverted optical microscope and equipped with a Tip Assisted Optics (TAO)
450 module and a Vortis-SPM control unit. The AFM cantilever optical beam deflection system makes
451 use of an infrared low-coherence light source (emission centred at 980 nm). A custom-made con-
452 focal microscope was coupled to the AFM using a supercontinuum laser (Rock-PP, Leukos) as
453 laser source at 20 MHz equipped with an oil immersion objective with a 1.4 numerical aperture
454 (Plan-Apochromat 100x, Zeiss). Fluorescence was collected after a pinhole of 100 μm diameter
455 size (P100D, Thorlabs) by an avalanche photodetector (SPCM-AQR-15, PerkinElmer) connected
456 to an SPC-150 (Becker & Hickl) TCSPC card. An ET800sp short pass filter (Chroma) was used
457 to filter out the light source of the AFM optical beam deflection system. The excitation laser power
458 was measured after the objective at the sample level with a S170C microscope slide power sensor
459 and a PM100 energy meter (both purchased from Thorlabs) and was set in all the experiments to
460 1 μW . Confocal images and FRAP were acquired using a 488/10 nm excitation filter and a 525/39
461 nm emission filter. Simultaneous AFM/confocal images were collected with the AFM tip and con-
462 focal spot positions fixed and co-aligned while the sample was scanned using the TAO module.
463 Full alignment was obtained using at first white field illumination. Then the fine-tuned was
464 achieved measuring the increase of tip luminescence when aligned with the confocal spot. Finally,
465 while imaging the sample, any mismatch between topography and confocal image was adjusted
466 by correcting the tip position.

467 *AFM-wide-field fluorescence microscopy:*

468 AFM coupled wide-field fluorescence microscope was developed in-house, allowing AFM imag-
469 ing and epifluorescence or Total Internal Reflection Fluorescence (TIRF) imaging (Supplementary
470 Fig. S3b)^{51,52}. and based on a Nanowizard 4 (JPK Instruments, Bruker) mounted on a Zeiss in-
471 verted optical microscope. The custom-made epifluorescence/TIRF microscope was coupled to
472 the AFM using a LX 488-50 OBIS laser source (Coherent). We used an oil immersion objective
473 with a 1.4 numerical aperture (Plan-Apochromat 100x, Zeiss). Fluorescence was collected with an
474 EmCCD iXon Ultra897 (Andor) camera. The setup makes use of a 1.5x telescope to obtain a final
475 imaging magnification of 150-fold, corresponding to a camera pixel size of 81 nm. An ET800sp
476 short pass filter (Chroma) was used in the emission optical path to filter out the light source of the
477 AFM optical beam deflection system. The excitation laser wavelength was centred at 488nm and
478 the power was measured before the objective with a PM100 energy meter (purchased from
479 Thorlabs) and was optimized in all the experiments in the range of 1-5 μW . We used an acousto-
480 optic tuneable filter (AOTFnc-400.650-TN, AA opto-electronics) to modulate the laser intensity
481 and record fluorescence images using an ET525/50 nm (Chroma) as emission filter.

482 In both correlative AFM - TIRF / confocal, AFM images were acquired in QI mode with a scan
483 size ranging from 10 μm \times 10 μm to 50 μm \times 50 μm and with 256x256 or 128/128 lines/pixels.
484 Quantitative imaging mode (QI) was used to generate a force curve for each recorded pixel. Force
485 curves were employed to evaluate droplets mechanical response. Typical force distance curves
486 were recorded with a loading rate of \approx 10 μm /s, with large indentation cycle lengths ranging from

487 5 μm to 10 μm and maximal peak force of 1 – 1.5 nN. All parameters were optimized to provide
488 image acquisition stability, considering sample low rigidity and having the cantilever immersed in
489 a crowded environment with micrometric large droplets diffusing. Curves were then employed to
490 extract all mechanical parameters for both liquid and gel phase constructs reported in Table 1, as
491 well for the AFM images in Fig. 3 and Fig. 4. Gel phase constructs reported in Fig. 4, less dynamic
492 on the coverslip and in solution, exhibited more stable force vs distance curves and could be im-
493 aged using a tip speed of 200 $\mu\text{m}/\text{s}$, ensuring a faster acquisition time. Because of the micrometric
494 height of the droplet comparable with the size of the AFM tip, only the curves acquired on the top
495 of the droplets were considered, in order to avoid edge artifacts, non-negligible when the pyramidal
496 tip facets are in contact with the droplets and present in the AFM images reported in Fig. 3 and
497 Fig. 4c.

498 From the force curves on droplets in liquid phase, the stiffness k_{eff} was calculated based on a linear
499 fit to the force vs indentation δ (Eq. 1)^{32,33}.

$$500 \quad F = k_{\text{eff}} \delta \quad (1)$$

501 Indentation cycles performed on droplets in gel phase were treated using a Hertz contact model,
502 leading to the evaluation of the Young's modulus E (Eq. 2).

$$503 \quad F = \frac{\frac{4}{3}E}{1-\nu^2} \tan(\theta) \delta^2 \quad (2)$$

504 Where ν is the Poisson's ratio of 0.5, conventionally used for soft incompressible isotropic mate-
505 rials which are elastically deformed, and θ is the pyramidal tip angle. A comparison between in-
506 dentation cycle performed on LLPS liquid phase (red) or gel condensates (blue) is shown in Fig.
507 S4 where equations 1 and 2 have been used to fit the experimental data. The Young's modulus
508 images reported in Fig 4a exhibit a higher E compared to the values reported in table, which is
509 likely due to the higher AFM tip speed (loading rate) employed for the acquisition of the force vs
510 distance curves, suggesting a viscoelastic behaviour for all constructs in the gel phase.

511 Biolever mini (AC40TS, Olympus) and MSNL (Bruker) AFM cantilevers were purchased from
512 Nano Bruker. Biolevers mini have a resonance frequency of ≈ 30 kHz in liquid, nominal stiffness
513 of 0.1 N/m and a 7 μm high tetrahedral tip. MSNL are V-shaped cantilevers with small tip radius
514 (≈ 2 nm) suited for high resolution imaging, and nominal stiffness ranging from 0.01 to 0.6 N/m,
515 depending on the cantilever. Images in Fig. 3 and Fig. 4 in the main manuscript were generated
516 using AC40TS and MSNL AFM cantilevers, respectively. In all AFM experiments, the inverse
517 optical lever sensitivity and lever stiffness of the cantilevers were calibrated using the “contact-
518 free” method of the JPK AFM instruments, making use of a combination of a Sader and thermal
519 methods^{56,57}. For droplets in gel phase, we used the MSNL-E for Q0 and Q7 constructs and the
520 MSNL-F for the Q20 ones, with nominal spring constant of 0.1 N/m and 0.6 N/m respectively,
521 assuming $\theta = 22.5^\circ$ to fit experimental data with equation (2). The fundamental resonance was
522 used with a correction of 0.817 at room temperature and in liquid environment. Circular glass
523 coverslips (25 mm diameter, 165 μm thick) were purchased from Marienfeld. They were cleaned
524 by a first cycle of sonication in 1 M KOH for 15 min, rinsed with deionized water 20 times, and
525 finally subjected to a second sonication cycle in deionized water for 15 min. For measurements in
526 the gel phase, GFP-labelled ELF3 PrLD Q0, Q7 and Q20 samples in buffer A (50 mM Bis-Tris
527 propane, pH 9.4, 500 mM NaCl and 1 mM TCEP) at 4 mg/ml were dialysed stepwise against

528 buffer B at pH 9.0 (100 mM Bis-Tris propane, 300 mM NaCl, 1 mM TCEP) for 45 minutes, fol-
529 lowed by buffer B at pH 8.0 and finally buffer B at pH 7.6 for 15 minutes. The fast pH changes
530 led to formation of spherical droplets with gel-like properties. The final protein concentration used
531 for all measurements was ~2 mg/ml as calculated using A_{280} measurements on a NanoDrop (Ther-
532 moFisher) and adjusted by dilution with buffer B at pH 9.4. A 10 μ L sample of protein was applied
533 to a clean glass cover slip and mounted on the AFM. After incubation for 2 minutes to allow
534 droplets to adhere to the surface, 300 μ L of buffer A was added and finally imaged by correlative
535 AFM-TIRF fluorescence (Fig. 4 in main manuscript). Total sample preparation time for AFM
536 measurements was ~ 15-20 min. including sample slide preparation, cantilever tuning and image
537 acquisition time (11 min. for a 256x256 pixel image). We have noted that droplets become more
538 gel-like over time as they are allowed to adhere to the surface of the microscope slide.

539 From correlative confocal-AFM experiments, the LLPS liquid-like droplet contact angle can be
540 evaluated. For a spherical droplet, it is given by⁵⁸

541
$$\theta = 2 \tan^{-1}\left(\frac{h+r}{a}\right) \quad (3)$$

542 θ can be evaluated using the morphological information obtained from both confocal (parameter
543 a) and AFM images (parameter $h+r$, maximal height of the droplet) as shown in Supplementary
544 Fig. S5. Shadow vertically asymmetry in Fig. S5c can be partially due to the tetrahedral geometry
545 of the probe as well as to the 10° cantilever inclination in respect to the glass coverslip (imposed
546 by the AFM cantilever holder). Horizontal asymmetry can be partially due to the excitation beam
547 not fully perpendicular to the glass coverslip and to a horizontal inclination of the cantilever. Con-
548 tact angles for droplets were found in the range between 120° and 150° suggesting a low wettability
549 associated to quasi-spherical droplet shapes as shown in Fig. 3 in main manuscript.

550 **Transmission electron microscopy experiments**

551 Samples of gel phase Q0, Q7 and Q20 ELF3 PrLD were prepared by stepwise dialysis of proteins
552 at 4 mg/ml in 50 mM Bis-Tris propane, pH 9.4, 500 mM NaCl and 1 mM TCEP. The pH was
553 decreased in units of 0.2, with each dialysis step performed for 40 min at 4°C until pH 7.8. The
554 sample became cloudy and then formed amorphous gel-like deposits, which were stored for at
555 least 1hr at 4°C before image collection. The viscous gel was resuspended by fast pipetting to
556 obtain a suspension of small gel pieces. Around 4 μ l of sample was applied to the mica-carbon
557 interface of a mica sheet with a layer of evaporated carbon film. The carbon film was floated off
558 the mica sheet in ~600 μ l of 2% (wt/vol) sodium silicotungstate. The sample was then transferred
559 onto a 400-mesh copper TEM grid and air-dried. Images were taken on a Thermo Fisher Technai
560 F20 microscope operating at 200 kV. Images were acquired using a Thermo Fisher Ceta camera.

561 **SAXS measurements**

562 SAXS experiments were performed at the European Synchrotron Radiation Facility (ESRF) on the
563 BioSAXS beamline BM29^{59,60} for dilute phase, HPLC experiments and on beamline B21 at Dia-
564 mond Light Source (SM26313-1, SM26314-2) for temperature ramp experiments⁶¹. For dilute
565 phase experiments, an online HPLC system (Shimadzu) was attached directly to the sample inlet
566 valve of the BM29 sample changer. Protein samples at 4 mg/ml (ELF3 PrLD Q0) or ~8 mg/ml
567 (ELF3 PrLD Q7, Q20) were loaded into vials and automatically injected onto the column (Super-
568 ose 6 3.2/300 Increase GE Healthcare) via an integrated syringe system. Buffers were degassed

569 on-line and a flow rate of 0.05 ml/min at room temperature was used for all sample runs. For
570 measurements of the dilute phase for ELF3 PrLD Q0, Q7 and Q20, 50 mM Bis-Tris propane pH
571 9.4, 500 mM NaCl and 1 mM TCEP was used for all samples. Prior to each run the column was
572 equilibrated with at least 3 column volumes of buffer and the baseline monitored. All data from
573 the run were collected using a sample to detector (Pilatus 2M Dectris) distance of 2.81 m corre-
574 sponding to an q range of 0.008-0.45 \AA^{-1} . Due to column separation of the sample, some dilution
575 effects will occur prior to measurement. 1800 frames (2 sec/frame) were collected. Initial data
576 processing was performed automatically using the Dahu pipeline, generating radially integrated,
577 calibrated and normalised 1-D profiles for each frame^{62,63}. All frames were then further processed
578 using Scatter IV⁶⁴, briefly, the frames were dropped into the software and 50 – 100 frames selected
579 for the background buffer. Using the heat plot a selection of similar frames (cyan in colour) were
580 selected and merged. 30 frames corresponding to the highest protein concentration were merged
581 and used for all further data processing and model fitting.

582 For the temperature ramp experiment for ELF3 PrLD Q7, samples were placed into the BioSAXS
583 robot (Arinax, France) with both the sample changer and the vacuum cell both set at 4 °C. 50 μL
584 of sample and matching buffer containing 50 mM CAPS, pH 9.7, 300 mM NaCl, 1mM TCEP were
585 individually loaded and 10 frames of 1 second were taken. Temperature was raised in both the
586 sample changer and the vacuum cell and incubated for 5 minutes before measurement. Tempera-
587 ture series of 4 °C, 12-15 °C, and 22-24 °C were taken. All frames were compared to the initial
588 frame and matching frames were merged for buffer and samples. Scatter IV¹⁰ was then used to
589 subtract buffer from sample and to generate R_g and plots.

590 **X-ray diffraction measurements**

591 X-ray diffraction experiments for Q0, Q7 and Q20 ELF3PrLD were performed on beamline ID23-
592 2 at the ESRF. Briefly, samples were purified as described above and dialysed directly against 50
593 mM BTP, pH 7.8, 250 mM NaCl and 1 mM TCEP at a concentration of ~ 4 mg/ml for 3 h, after
594 which time the protein formed an amorphous gel. The sample were spun down to collect the gel.
595 The gel was directly mounted in a mesh litholoop (Molecular Dimensions). Diffraction was meas-
596 ured at room temperature. Diffuse powder rings were observed at ~ 4 -5 \AA , consistent with van der
597 Waal's distance between interacting molecules.

598 **Multi angle laser light scattering experiments**

599 50 μl of ELF3 PrD Q0, Q7 or Q20 at a concentration of ~ 4 -7mg/ml were loaded onto an S200
600 Increase size-exclusion column (Superdex 200 Increase10/300 GL, GE Healthcare) at a flow rate
601 of 0.5 ml min⁻¹. The column was pre-equilibrated with 50 mM Bis Tris propane at pH 9.4, 1
602 M NaCl, 1 mM TCEP and connected to a Hitachi Elite LaChrom UV detector and LACHrome
603 Pump L-2130, a multi-angle laser light-scattering detector (DAWN HELEOS II, Wyatt Technology
604 Corporation) and a refractive-index detector (Optilab T-rEX, Wyatt Technology Corporation). The
605 data were processed with the ASTRA 6.1.7.17 software (Wyatt Technology Corporation).

606 **Supporting Information**

607 Experimental materials and methods; supplementary figures for SAXS scattering curves and
608 Kratky plots, additional X-ray protein sequences, additional phase diagrams for GFP labelled pro-
609 teins, AFM and microscopy experimental set-up, linear and Hertz model fits for AFM data,

610 additional AFM images, additional diffraction images, table of SAXS data collection statistics,
611 additional references are provided as Supporting Information.

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619 designed experiments, C.Z. designed experiments and analysed data. The manuscript was written
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647 **Abbreviations**

648 LLPS, liquid-liquid phase separation; PrLD, prion-like domain, IDR, intrinsically disordered re-
649 gion; ELF3, EARLY FLOWERING 3; SAXS, small angle X-ray scattering; AFM, atomic force
650 microscopy; EM, electron microscopy

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855

856 **Figure legends**

857 **Figure 1.** Representative fluorescence recovery after photobleaching (FRAP) images of agroin-
858 filtrated *Nicotiana benthamiana* leaf epidermal cells transiently expressing mVenus labelled
859 ELF3 Q0, Q7 or Q20. White boxes indicate photobleached areas. Panels at left are prior to bleach-
860 ing, middle immediately after bleaching and right after recovery. Mean recovery curves for ELF3
861 Q0, Q7 and Q20 are shown at far right and S.D. are shown. All curves are generated from 9-10
862 individual puncta measurements with each FRAP experiment performed on a different cell.

863 **Figure 2.** ELF3 PrLD with varying polyQ lengths undergoes phase separation. **A)** Reversible tem-
864 perature induced phase separation for ELF PrLD Q0, Q7 and Q20 (4 mg/mL) visualised at 4°C
865 (left), warmed to 22°C (center) and cooled to 4°C (right). **B)** Turbidity assays (A440) of untagged
866 ELF3 PrLD Q0, Q7 and Q20 as a function of temperature. Protein concentration was 15µM (~0.4
867 mg/ml) for each construct. Temperature ramps are shown with red arrows indicating increasing or
868 decreasing temperature. **C)** Phase diagrams for untagged ELF3 PrLD Q0, Q7 and Q20 constructs

869 as a function of protein concentration and pH. Open circles are dilute phase, dark blue LLPS and
870 grey precipitate/gel. LLPS conditions are shaded in light grey for clarity. Circles represent meas-
871 urements at a specific pH (9.5, 9.0, 8.8, 8.6, 8.4, 8.2, 8.0, 7.8, 7.6 and 7.4) and a specific protein
872 concentration (0.5, 1.0, 2.0, 4.0, 6.0 mg/ml) **D**) Fluorescence recovery after photobleaching
873 (FRAP) experiments. Red arrows indicate regions that were photobleached and scale bars are
874 shown. Recovery panels displayed are 3 minutes after photobleaching for all samples. Samples
875 exhibited little fluorescence recovery due to formation of an immobile gel phase.

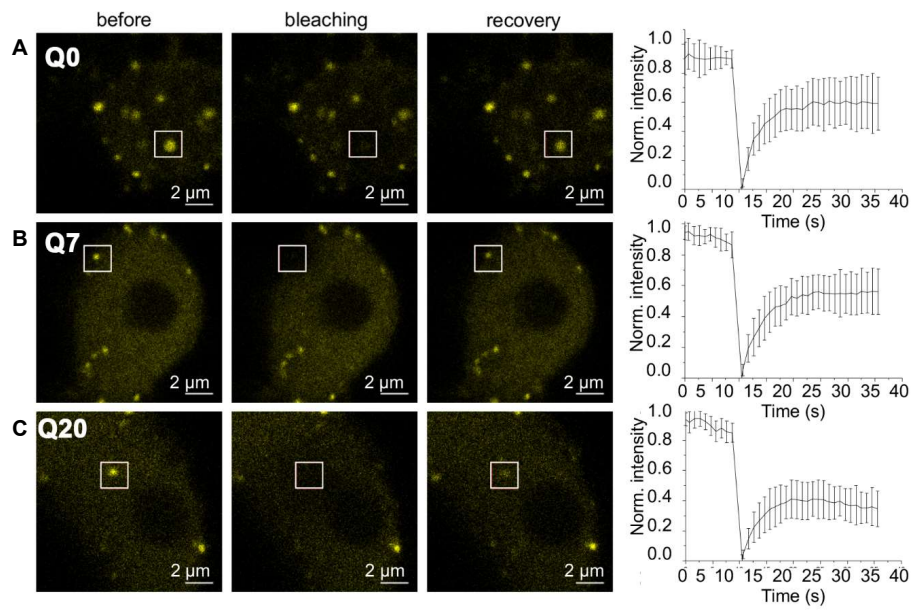
876 **Figure 3.** Coupled AFM and confocal fluorescence microscopy of ELF3 PrLD -GFP constructs.
877 AFM topography mapping is shown at left, fluorescence of the same sample is shown at middle
878 and stiffness measurements are shown at right. Alignment between the AFM and confocal micro-
879 scope was within 1-2 microns for the two microscopes. Scale bar = 5 μm .

880 **Figure 4.** Wide-field total internal reflection fluorescence microscopy and high-resolution AFM
881 of ELF3-GFP PrLD gel droplets. **A**) Young's modulus measurements of the droplets showing in-
882 homogeneous stiffness within individual droplets, scale bar = 1 μm . **B**) Fluorescence images of
883 ELF3 PrLD Q0 (left), Q7 (middle) and Q20 (right), scalebar = 1 μm . Areas of interest are boxed
884 and shown in close-up in (c). **C**) AFM topography of the droplets in a, scalebar = 1 μm . **D**) Height
885 profile measurements across individual droplets (blue lines in c) showing a step pattern between
886 flat layers.

887 **Figure 5.** ELF3 PrLD in the dilute and condensed phase. **A**) SAXS scattering curve for ELF3
888 PrLD Q0 (green), Q7 (pink) and Q20 (dark blue) in the dilute phase measured with online HPLC
889 purification. Inset shows a schematic representation of the dilute and condensed phases as a func-
890 tion of temperature. **B**) Corresponding Kratky plots for the curves shown in (A) The Gaussian
891 shape denotes a globular species in solution. **C**) SAXS scattering curves for ELF3 PrLD Q0
892 (green), Q7 (pink) and Q20 (dark blue) samples in the condensed phase after aging. The structure
893 factor peak due to long-range ordering of molecules within the condensed phase is indicated by an
894 arrow. **D**) Corresponding Kratky plots for condensed phase scattering curves are shown and colour
895 coded as above. The shape of the Kratky plot indicates a more extended and less globular species
896 with the structure factor peak showing more prominently. SAXS scattering curves in (A) and (C)
897 show a vertical offset by a factor of 10 for ELF3 PrLD Q7 and a factor of 100 for Q0 to aid in
898 viewing.

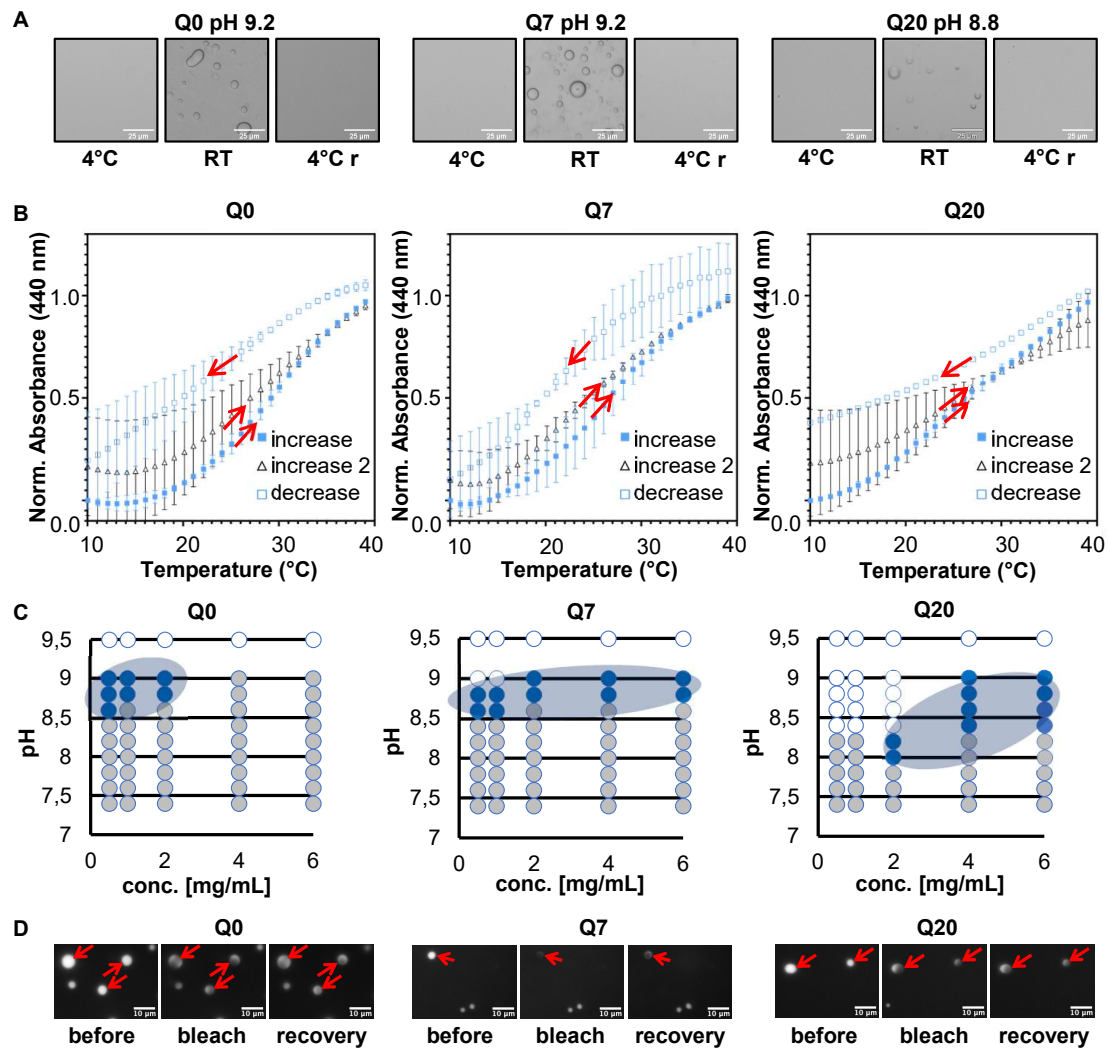
899 **Figure 6.** ELF3 PrLD exhibits stacked molecular organisation in the condensed phase. **A**) Nega-
900 tive stain TEM images of ELF3 PrLD Q0, Q7 and Q20 showing a layered structure in the gel
901 condensed phase. Layered structure can be clearly observed in magnified images of the boxed
902 regions. **B**) X-ray diffraction image for ELF3 PrLD Q20 showing a diffuse powder ring at 4-5 \AA .
903 A close-up is shown with the diffraction ring indicated by the black arrow.
904

Figure 1



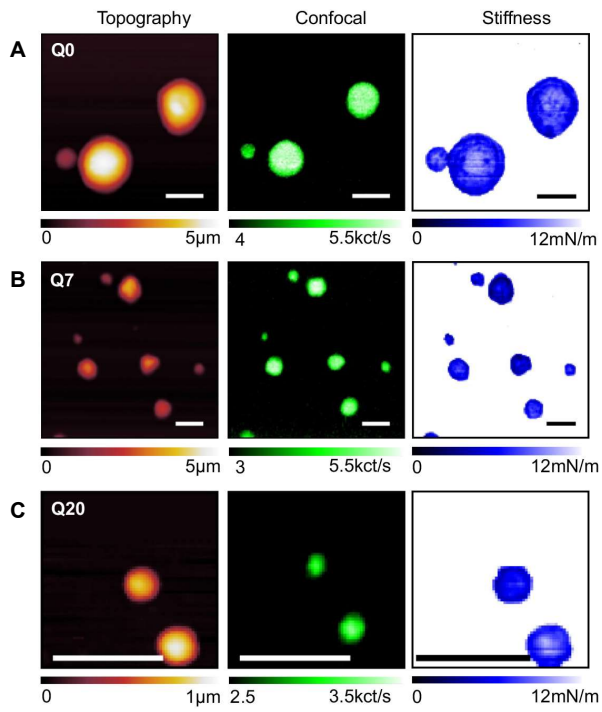
905

Figure 2



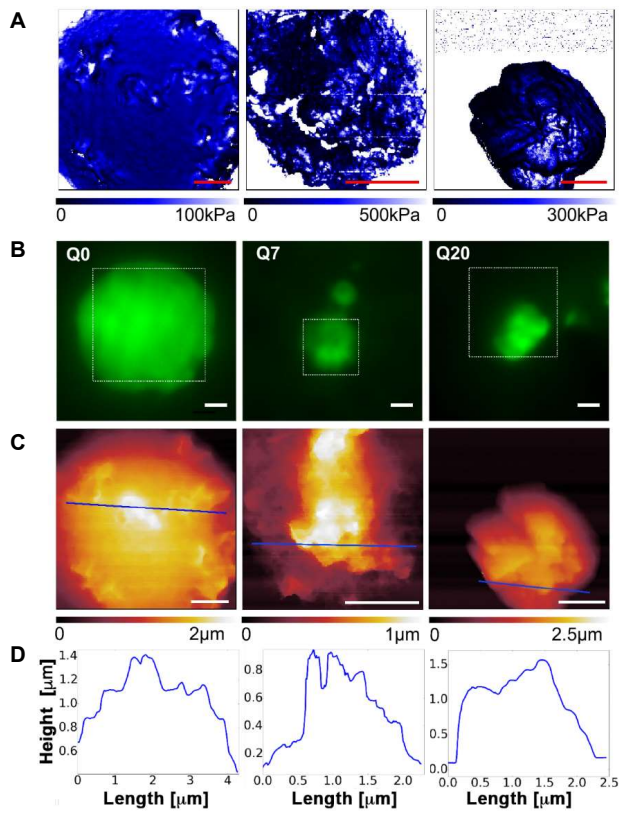
906

Figure 3



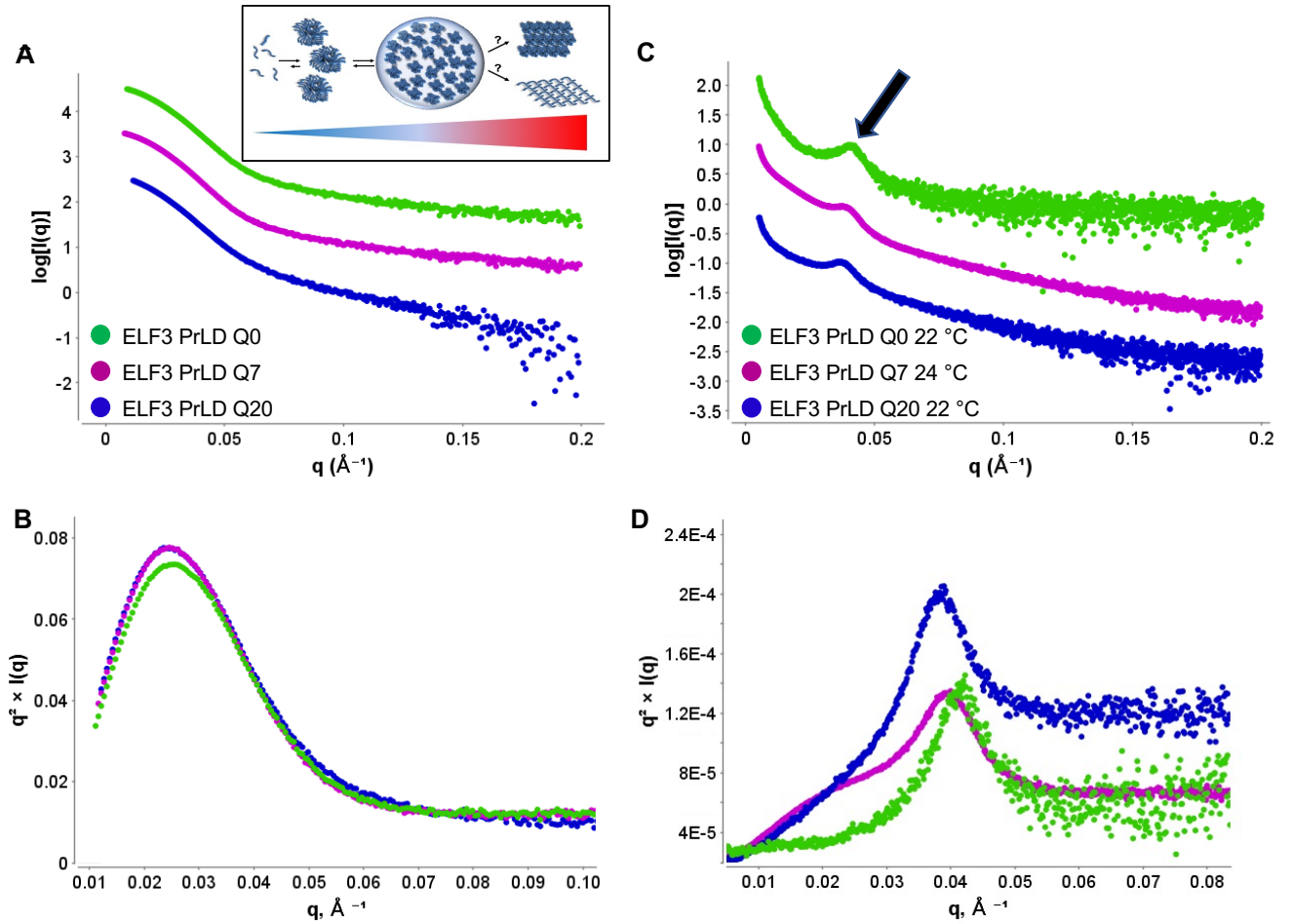
907

Figure 4



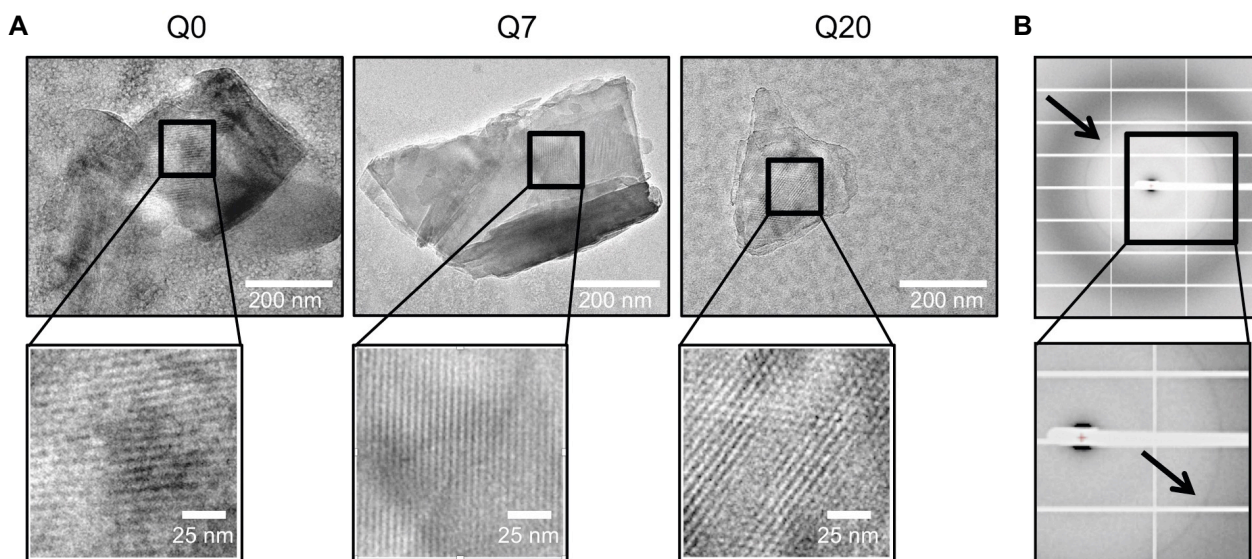
908

Figure 5



909

Figure 6



910