Granulins modulate liquid–liquid phase separation and aggregation of the prion-like C-terminal domain of the neurodegeneration-associated protein TDP-43

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This article contains Figs. 1 and 2.

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The abbreviations used are: FTLD, frontotemporal lobar degeneration; TDP, TAR DNA-binding protein; PUS, fused in sarcoma; AD, Alzheimer disease; LLPS, liquid–liquid phase separation; PGRN, progranulin; GRN, granulin; CTD, C-terminal domain; DIC, differential interference contrast; ThT, thioflavin T; ThS, thioflavin S; FRAP, fluorescence recovery after photobleaching; Aβ, β-amyloid; LD, liquid droplet; AU, absorbance unit.

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TAR DNA-binding protein 43 (TDP-43) has emerged as a key player in many neurodegenerative pathologies, including frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Hallmarks of both FTLD and ALS are the toxic cytoplasmic inclusions of the prion-like C-terminal fragments of TDP-43 CTD (TDP-43 C-terminal domain), formed upon proteolytic cleavage of full-length TDP-43 in the nucleus and subsequent transport to the cytoplasm. Both full-length TDP-43 and its CTD are also known to form stress granules by coacervating with RNA in the cytoplasm during stress and may be involved in these pathologies. Furthermore, mutations in the PGRN gene, leading to haploinsufficiency and diminished function of progranulin (PGRN) protein, are strongly linked to FTLD and ALS. Recent reports have indicated that proteolytic processing of PGRN to smaller protein modules called granulins (GRNs) contributes to FTLD and ALS progression, with specific GRNs exacerbating TDP-43–induced cytotoxicity. Here we investigated the interactions between the proteolytic products of both TDP-43 and PGRN. Based on structural disorder and charge distributions, we hypothesized that GRN-3 and GRN-5 could interact with the TDP-43 CTD. We show that, under both reducing and oxidizing conditions, GRN-3 and GRN-5 interact with and differentially modulate TDP-43 CTD aggregation and/or liquid–liquid phase separation in vitro. GRN-3 promoted insoluble aggregates of the TDP-43 CTD while GRN-5 mediated liquid–liquid phase separation. These results constitute the first observation of an interaction between GRNs and TDP-43, suggesting a mechanism by which attenuated PGRN function could lead to familial FTLD or ALS.

Frontotemporal lobar degeneration (FTLD) is a neurodegenerative disorder characterized by progressive changes in behavior, speech, and personality among elderly patients (1, 2). These changes are brought up on by gradual atrophy of the frontal and anterior temporal lobes of the brain (3–6). Based on histopathology, heritable FTLD can be classified into the following subtypes: the FTLD-TDP form, which is characterized by the presence of cytoplasmic inclusions of TAR DNA binding protein 43 (TDP-43) and constitutes about half of all cases of FTLD (7); FTLD-Tau, in which cytoplasmic inclusions of hyperphosphorylated tau are observed; and FTLD-FUS, which is associated with inclusions of an RNA-binding protein called fused in sarcoma (FUS) (8–12). The FTLD-TDP pathology is further subclassified based on genetic and clinical presentation (13). Autosomal dominant mutations to the progranulin gene (PGRN) are associated with type A, whereas the hexanucleotide repeat expansions in C9orf72 are present in type B cases (13). Inclusions of TDP-43 are also observed in patients with ALS (14–16). In addition to mutations in TDP-43, those in several genes have been linked to ALS, including FUS, superoxide dismutase 1 (SOD1), and hexanucleotide repeat expansions in C9orf72. Because TDP-43–based neuropathology is present in ~50% of ALS cases and ~97% of ALS cases (17), the protein represents a molecular link connecting these neurodegenerative diseases as a clinicopathological spectrum of the proteinopathies (15, 18–20). It has also come to light that TDP-43 may contribute to the pathogenesis of Alzheimer disease (AD) via both β-amyloid (Aβ)–dependent and independent pathways (21).

Furthermore, TDP-43 abnormalities have also been associated with traumatic brain injury (chronic traumatic encephalopathy) in both preclinical and clinical studies (7) and observed in cognitively impaired persons of advanced age with hippocampal sclerosis, Huntington’s disease, and some other maladies (22). Because of the widespread prevalence of TDP-43 in neurodegenerative pathogenesis, all abnormalities involving the
protein are classified into the category of TDP-43 proteinopathies (22, 23).

TDP-43 is a member of the heterogeneous ribonucleoprotein family involved in transcriptional regulation and mRNA splicing in neurons (24). The protein consists of an N-terminal domain and two RNA recognition motifs followed by a low-complexity, glycine-rich C-terminal domain (25). The protein is known to dimerize through the N-terminal domain, whereas specific phosphomimic mutants within this domain disrupt its aggregation, liquid–liquid phase separation (LLPS), and RNA splicing activity (26, 27). Although mainly localized in the nucleus, in the diseased state, a fraction of the full-length TDP-43 is proteolytically cleaved, and the thus generated 25- and 35-kDa (C25 and C35) C-terminal fragments are translocated into the cytoplasm. These fragments form toxic insoluble inclusions observed in ALS and FTLD-TDP patients (19, 28, 29). Under stress, both full-length and TDP-43 C-terminal fragments are also known to bind RNA and translocate to the cytosol, where they undergo LLPS to form the membraneless organelles called stress granules (29). LLPS has been increasingly observed in many cellular processes and in modulating many cellular functions (30–39). The precise mechanism of stress granule dynamics in TDP-43 pathophysiology remains unclear, but many proteins are known to interact with and partition into the phase-separated droplets of TDP-43 (40, 41). In FTLD and ALS, the aberrant LLPS of TDP-43 has emerged as a key mechanism for aggregation of the protein (40, 42, 43).

Progranulin (PGRN) is a 63.5-kDa secreted protein expressed in many cells, including neurons and microglia (44–46), with pleiotropic roles in both physiological and pathological processes (47–51). The protein consists of seven and a half cysteine-rich modules called granulins (GRNs) (Fig. 1a) (47, 49, 52–54), which are generated by proteolytic processing of PGRN by many proteases. PGRN and GRNs have been observed to coexist in vivo with opposing inflammatory functions (55). In FTLD and ALS, the aberrant LLPS of TDP-43 has emerged as a key mechanism for aggregation of the protein (40, 42, 43).

Figure 1. Sequence, disorder, and charge distributions of the proteins used in this study. a, the sequence of the TDP-43 CTD (267–414, 14.5 kDa), which constitutes the major part of the toxic 25-kDa TDP-43 C-terminal fragment with a hexahistidine tag on the N terminus and sequences of GRN-3 and GRN-5 with putative disulfide bonds. A conservative mutation of Tyr to Trp (asterisk) was made to GRN-3, which is a conserved residue in all GRNs. Both GRNs were used under fully reduced (free thiols) and oxidized conditions. b–d, computational evaluation of the per-residue intrinsic disorder predispositions of the TDP-43 CTD (b), GRN-3 (c), and GRN-5 (d). Here, the outputs of PONDR® VLXT, PONDR® FIT, PONDR® VL3, PONDR® VSL2, IUPred_S, and IUPred_L were averaged to generate mean disorder profiles of different computational data query proteins. e–g, diagrams of linear net charge per residue (NCPR) for the TDP-43 CTD (e), GRN-3 (f), and GRN-5 (g), generated by the CIDER computational platform.
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somal storage disease (56). The production of GRNs in the haploinsufficient but not the null state in FTLD suggests that they are key players in the disease phenotypes. This also indicates that the extenuation of PGRN function could arise from its increased proteolytic processing to generate GRNs (55). Indeed, GRNs have been shown to interact with TDP-43 and exacerbate its levels and toxicity in Caenorhabditis elegans, establishing that PGRN cleavage to GRNs could represent an important part of the disease process in FTLD (57). Despite growing evidence of the involvement of GRNs, their precise mechanisms in FTLD and related pathologies remain unclear. In this report, we sought to understand the interactions between GRNs and the TDP-43 CTD, both of which are proteolytic products of their precursors, under reducing and oxidizing conditions in vitro. Our premise for investigations under reducing and oxidizing conditions stems from our previous observation that GRN-3 is active in both reduced and oxidized forms (58, 59). Furthermore, GRNs’ presence in both extra- and intracellular space led us to hypothesize that they may play a role under redox fluctuations. We here specifically investigated whether GRN-3 and GRN-5 are able to modulate LLPS or formation of insoluble inclusions of the TDP-43 CTD, which provides insights into the potential role of GRNs in FTLD and ALS pathologies.

Results

Structural disorder and charge distribution among GRNs and the TDP-43 CTD suggest potential interactions

The 14.5-kDa construct of TDP-43 that constitutes residues 267–414 in the C-terminal domain (TDP-43 CTD) is a major part of the C25 proteolysis fragment that forms aberrant inclusions within the cytosol in FTLD and ALS patients (Fig. 1a) (16). Fig. 1b shows that the TDP-43 CTD is largely disordered, and previous study indicated that this protein contains a sequence of low complexity that is involved in LLPS (60). On the other hand, GRNs (1–7) are small, ~6-kDa proteins with the conserved sequence X2–3CX5–6CX5CCX5CCX6 CCXDXXXHCCPX5CX5–6CX (Fig. 1a). The 12 conserved cysteine residues form six putative intramolecular disulfide bonds (61) (Fig. 1a). Fig. 1c and d, show that, despite the fact that these proteins contain high levels of cysteine, which is the strongest order-promoting residue, GRN-3 and GRN-5 are predicted to be characterized by high levels of disorder. This is in line with the results of our previous studies, where we have shown that, under reducing conditions, GRN-3 is fully disordered, whereas under oxidizing conditions, the protein obtains thermal stability via disulfide bonds without significant gain in overall structure (59, 62).

As a first step in understanding the effect of GRNs on TDP-43, here we report the interactions of GRN-3 and GRN-5 with the TDP-43 CTD under both reducing and oxidizing conditions. GRN-5 was expressed and purified using the established method for GRN-3 (59), with some minor modifications (see “Experimental procedures”). As observed previously with GRN-3 (59, 62), we observed that GRN-5, too, under both reducing and oxidizing conditions, shows a random-coil disordered structure (Fig. S1). The intrinsic disorder predisposition of TDP-43 and four other proteins (SOD1; FUS; a coflin-binding protein, C9orf72, and polypeptides generated as a result of its intronic hexanucleotide expansion; and actin-binding profilin-1 (PFN1)), which are considered major drivers of ALS and FTLD pathogenesis, revealed significant levels of disorder among them (63, 64). Here we set out to determine whether disorder and charge distribution among the TDP-43 CTD and GRNs indicate interaction propensities. The primary sequence of GRN-5 is enriched in negatively charged residues and lacks positively charged residues with a net pl of 4.01. On the other hand, the TDP-43 CTD has an appreciable number of positively charged amino acids with a net pl of 9.52 (Fig. 1a). The computed disorder predispositions show a high degree of disorder for the entire sequence of the TDP-43 CTD (values > 0.5; Fig. 1b), whereas GRN-3 and GRN-5 show disordered regions in both N- and C-terminal regions (Fig. 1, c and d). The linear net charge per residue plots for these proteins generated by the CIDER computational platform (65) show that positively charged residues are preferentially concentrated within the 50 N-terminal residues of the TDP-43 CTD (Fig. 1e), whereas negatively charged residues are distributed over the entire sequence of GRN-5 (Fig. 1g). Importantly, ANCHOR analysis (66, 67) of the TDP-43 CTD construct utilized in this study revealed the existence of several disorder-based binding regions (residues 1–40, 66–99, 115–123, and 135–145). These regions represent molecular recognition features, intrinsically disordered segments that undergo folding upon interaction with specific binding partners. Based on the counter-ionic character of the two proteins, we hypothesize that GRN-5 could interact with the TDP-43 CTD and mediate LLPS. In addition, GRN-3 was chosen to test our hypothesis, as this protein is also intrinsically disordered and has a pi of 5.33, but it contains both positive and negatively charged residues (Fig. 1, a and f).

We also checked the amino acid sequence–based predispositions of the TDP-43 CTD, GRN-3, and GRN-5 to undergo LLPS. To this end, the CatGRANULE algorithm was utilized, which predicts LLPS propensity based on the analysis of the phase separation features linked to its primary sequence composition, structural disorder, and nucleic acid binding propensities (68). This analysis indicated that the CatGRANULE scores for the TDP-43 CTD, GRN-3, and GRN-5 were 3.88, −3.06, and −2.83, respectively, indicating that only the TDP-43 CTD has an intrinsic propensity for granule formation associated with liquid demixing under physiological conditions. It has to be borne in mind that the positive CatGRANULE score for the TDP-43 CTD can be attributed to the fact that the algorithm was trained on the TDP-43 sequence. However, the negative CatGRANULE scores for GRNs are independent and decoupled from use of the TDP-43 sequence for algorithm training.

GRN-5, but not GRN-3, initiates LLPS of the TDP-43 CTD

To see whether GRNs mediate TDP-43 CTD phase separation, the two proteins were coincubated at a 2:1 molar ratio, respectively, and the samples were monitored for turbidity increase and droplet formation by differential interference contrast (DIC) microscopy under both oxidizing (GRN-3 or GRN-5) and reducing (rGRN-3 or rGRN-5) conditions (Fig. 2).
At room temperature, 20 μM TDP-43 CTD buffered in 20 mM MES (pH 6.0) without salt in the presence of 40 μM GRN-5 or rGRN-5 instantly formed a turbid solution, similar to the known phase separation shown by the TDP-43 CTD–RNA mixture (Fig. 2a). On the other hand, incubation of the TDP-43 CTD by itself or with 40 μM GRN-3 or rGRN-3 did not show turbidity (Fig. 2a). To define the phase boundaries of LLPS by the TDP-43 CTD under increasing GRN concentrations, 20 μM TDP-43 CTD was incubated with GRN-3, rGRN-3, GRN-5 and rGRN-5 in the absence of salt (Fig. 2b). Both GRN-5 and rGRN-5 induced LLPS at concentrations as low 10 μM, whereas neither GRN-3 nor rGRN-3 induced any LLPS, even at high concentrations (Fig. 2b). Next, the phase boundaries of TDP-43 CTD LLPS as a function of GRN concentration and ionic strength were determined (Fig. 2c–g). TDP-43 CTD control in the absence of GRNs but with increasing NaCl concentrations showed no LLPS below the physiological concentration of 150 mM salt (Fig. 2c). GRN-3 showed LLPS above 100 mM NaCl over a large GRN concentration range (Fig. 2d). rGRN-3 showed LLPS under a relatively narrow range of salt concentrations above 300 mM (Fig. 2e). Both GRN-5 and rGRN-5 induced LLPS over a broad range of GRN and salt concentrations (Fig. 2f and g). Only low GRN concentration and low ionic strength failed to induce LLPS of the TDP-43 CTD suggesting that the LLPS is induced by electrostatic interactions between TDP-43 CTD and GRN-5 or rGRN-5, which are counter-ionic to each other (Fig 1e and g). To visualize LLPS, the samples were observed under a DIC microscope for 2:1 coincubation of GRN:TDP-43 CTD under the same buffer conditions as mentioned above.

The TDP-43 CTD control, along with the mixture containing GRN-3 or rGRN-3, did not show formation of liquid droplets for up to 6 h (Fig. 2h). In contrast, liquid droplets were observed almost instantaneously when the TDP-43 CTD was coincubated with rGRN-5, GRN-5, or RNA (Fig. 2h). The droplets, which initially were numerous and small, grew in size by coalescing with one another over the next 6 h of incubation, displaying fluid-like characteristics. Together, the data suggest GRN-5 and rGRN-5, but not GRN-3 or rGRN-3, are able to significantly initiate/enhance phase separation of the TDP-43 CTD at low salt concentrations.

**GRN-3 and rGRN-5** **differently modulate LLPS and formation of ThT-positive aggregates of the TDP-43 CTD**

In FTLD and ALS pathologies, the TDP-43 CTD is known to form toxic inclusions in the cytosol. However, ambiguity remains regarding the nature of these inclusions with both the presence of thioflavin T (ThT)–positive and ThT-negative aggregates has been reported (69–72). To see how GRNs affected the formation of TDP-43 CTD inclusions, we monitored ThT fluorescence for TDP-43 CTD samples in the presence of increasing concentrations of GRN-3, rGRN-3, GRN-5, or rGRN-5 at 37 °C (Fig. 3). The sample of 20 μM TDP-43 CTD alone in 20 mM MES buffer (pH 6.0) showed a typical sigmoidal increase in fluorescence with a lag time of ~ 8 h (Fig. 3, ●), indicating formation of ThT-positive species. For GRN-3 incubation, increasing its stoichiometry from 0.1- to 4-fold excess to that of the TDP-43 CTD showed an increasing lag time of aggregation (Fig. 3a). The 0.1-fold incubation showed the
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Figure 3. Modulation of TDP-43 CTD aggregation by GRNs. a–d, the aggregation kinetics of 20 μM TDP-43 CTD in 20 mM MES buffer (pH 6.0) at 37°C monitored by ThT fluorescence with varying molar equivalence (2–80 μM) of GRN-3 (a), rGRN-3 (b), GRN-5 (c), and rGRN-5 (d). The inset shows the enlarged areas (boxed with dashed lines), highlighting the lag times during aggregation. The data were fitted to a Boltzmann sigmoidal function. e–h, localization of the aggregate-specific dye ThS, observed by confocal fluorescence microscopy of the 1:2 reaction samples of TDP-43 CTD:GRN from a–d immediately (0 h) and after 36 h of incubation. i, images of the TDP-43 CTD control from the reaction in a. Scale bars = 20 μm. Bf, bright-field view.

smallest lag time with 12 h, whereas 4-fold excess GRN-3 showed the highest lag time of ~22 h. Similarly, under fully reducing conditions, 0.1- to 4-fold excess of rGRN-3 also inhibited TDP-43 CTD aggregation, but to a much lesser degree than GRN-3, with lag times ranging between 8 and 15 h (Fig. 3b). Similarly, incubation of GRN-5 at increasing concentrations (0.1- to 4-fold excess) with the TDP-43 CTD showed overall inhibition of TDP-43 CTD fibrillation by increasing the lag times (12 to 36 h, respectively) (Fig. 3c). On the other hand, incubation of rGRN-5 with the TDP-43 CTD showed no discernable lag time but an instantaneous linear increase in ThT fluorescence for all stoichiometric incubations (Fig. 3d, insets). These data suggest that GRN-3 and GRN-5 interact differently with TDP-43 CTD to modulate the latter’s aggregation. Furthermore, instantaneous LLPS observed in the presence of GRN-5 and rGRN-5 (Fig. 2) raises the possibility of the ThT dye partitioning into the droplets formed, resulting in higher ThT fluorescence. To investigate this, the reactions were visualized by fluorescence microscopy using another amyloid binding dye called thioflavin S (ThS). Both ThT and ThS are known to detect amyloid aggregates (73, 74). A 10 μM buffered solution of ThS was added to samples containing 20 μM TDP-43 CTD alone (Fig. 3i) and in the presence of 40 μM GRN: GRN-3 (Fig. 3e), rGRN-3 (Fig. 3f), GRN-5 (Fig. 3g), and rGRN-5 (Fig. 3h). Instantaneously upon addition, TDP-43 CTD alone and with GRN-3 or rGRN-3 showed no aggregate formation, but a dispersed blue fluorescent haze was observed (Fig. 3, e, f, and i, 0 h). It has to be noted that use of a 420–480 nm band pass emission filter generates a blue color for ThS. The same reactions after 36 h showed more concentrated areas of ThS that coincided with the presence of inclusions (Fig. 3, e, f, and i, 36 h). GRN-5 and rGRN-5, on the other hand, showed the presence of ThS within the droplets even at 0 h (Fig. 3, g and h, 0 h), which continued up to 36 h (Fig. 3, g and h, 36 h). These data suggest that ThS (or ThT) could partition into droplets. The enhanced fluorescent signals could emerge because of partitioning, and/or a small amount of ThT-positive species could be present within the droplets.

GRNs colocalize with the TDP-43 CTD droplets or aggregates

To probe whether the droplets are complex coacervates of the TDP-43 CTD and GRNs or formed without GRNs partitioning into the separated phase, fluorescence microscopy was used to observe the colocalization of the two proteins. The TDP-43 CTD and GRNs, labeled with HiLyte 647 and HiLyte 405, respectively, were incubated separately under oxidizing and reducing conditions and monitored for 36 h (Fig. 4). As expected, the TDP-43 CTD by itself did not form phase-separated droplets immediately after incubation (Fig. 4a, 0 h), and over the next 36 h, sparse deposits of aggregated protein were observed (Fig. 4a, 18 h and 36 h). Coincubation of GRN-3 or rGRN-3 with the TDP-43 CTD showed no LLPS immediately after incubation (Fig. 4, b and c, 0 h), parallel to the observation by DIC and turbidity (Fig. 2). Even after 36 h, no phase-separated liquid droplets but insoluble fibrillar deposits were observed (Fig. 4, b and c, 18 h and 36 h) that bound to amyloid dyes as shown above (Fig. 3). Moreover, the GRNs were observed to be localized in the vicinity of TDP-43 CTD deposits as well as within them (Fig. 4, b and c, 36 h). On the other hand, coincubation of both GRN-5 and rGRN-5 with the TDP-43 CTD resulted in emergence of well-defined liquid droplets immediately after incubation (Fig. 4, d and e, 0 h). The number of droplets increased during the next 36 h of incubation, with some becoming more distorted spherical droplets (Fig. 4, d and e, 18 h and 36 h). Importantly, both GRNs (Fig. 4, d and e, blue) colocalized within droplets formed by the TDP-43 CTD (Fig. 4, d and e, red). To further glean insights into the physical nature of the droplets or insoluble inclusions formed, fluorescence recovery after photobleaching (FRAP) was conducted (Fig. 4, f–j). After 36 h of incubation, the TDP-43 CTD control showed no recovery after bleaching, indicative of the insoluble solid
nature of the aggregates (Fig. 4). Similar behavior was observed with coinubcation of the TDP-43 CTD with GRN-3 or rGRN-3 (Fig. 4, g and h), suggesting that GRN-3 under both reduced and oxidized states promoted insoluble fibrils of the TDP-43 CTD, as seen in the microscopy images. However, GRN-5 in both redox states (GRN-5 and rGRN-5) showed exponential fluorescence recovery immediately after incubation, indicating the fluid property of the droplet (Fig. 4, i and j, 0 h). After 36 h, the extent of recovery was slightly mitigated indicating possible “gelation” of the droplet (Fig. 4, i and j, 36 h). Together, the data further confirm that both GRN-5 and rGRN-5 induce LLPS with the TDP-43 CTD by coacervation. On the other hand, GRN-3 does not induce LLPS but forms insoluble deposits of aggregated forms of the TDP-43 CTD, indicating the differential specificity for the two GRNs in interacting with the TDP-43 CTD. In other words, both GRNs could initiate different biophysical processes; the presence of GRN-3 or rGRN-3 led to insoluble aggregates, and those of GRN-5 or rGRN-5 resulted in droplet formation that could maturate into gelated drops or aggregates over time.

To further confirm and quantify the partitioning and colocalization of the TDP-43 CTD and GRNs, the relative amounts of the proteins within the droplets as well as the deposits were quantified by MALDI-TOF MS. Using a known amount of...
This observation is similar to the recently observed ability of GRN-3 and rGRN-3 to form fibrils of β-amyloid(1–42) (Aβ42) peptide involved in AD, where minimal GRN was observed in the pellet (58). On the other hand, the ratio of GRN-to-TDP-43 CTD obtained from the sedimented droplets of TDP-43 CTD and GRN-5 or rGRN-5 coincubation did not show a linear increase with increasing concentrations of GRN, as observed with GRN-3 (Fig. 5, h and i). For coincubation of GRN-5 and rGRN-5, the GRN-to-TDP-43 CTD ratio remained at 0.75 and 1.1, respectively, over the entire range of molar ratios. It is known that the phenomenon of coacervation depends on a defined valency of interactions among the molecules that remain fixed within the dense phase (75–78). One could speculate that the network of weak interactions necessary to stabilize the TDP-43 CTD droplets could be achieved by accommodating equimolar proportions of GRN-5 or rGRN-5 within the droplets.

GRNs modulate liquid droplets formed by the TDP-43 CTD and RNA

One of the main aspects of TDP-43 pathobiology is the coacervation with RNA molecules that form membraneless organelles in the form of stress granules (79–81). To see whether GRN-3 and GRN-5 are able to interact with and modulate liquid droplets (LDs) of the TDP-43 CTD and RNA, torula yeast RNA extract was coincubated with the TDP-43 CTD in the presence or absence of GRNs. As expected, control LDs containing a mixture of 20 μM TDP-43 CTD and 40 μg/ml RNA showed high turbidity (Fig. 6a). Solutions containing a mixture of LDs and 40 μM of GRN-5 showed an increase in turbidity compared with the control (Fig. 6a). The sample containing 40 μM rGRN-5 and LDs did not show appreciable change in turbidity. Similarly, solutions containing a mixture of LDs and GRN-3 or rGRN-3 showed a slight decrease or no change in turbidity levels compared with the control (Fig. 6a). To see how GRNs affect the formation of ThT-positive inclusions of LDs, the reactions were monitored by ThT fluorescence (Fig. 6, b and c). As expected, coincubation of RNA with the TDP-43 CTD did not show ThT increase as they formed LDs (Fig. 6, b and c) compared with TDP-43 CTD alone (Fig. 6, b and c). Inclusion of rGRN-3 in the RNA and TDP-43 CTD mixture significantly increased the rate of ThT-positive aggregates of TDP-43 CTD (Fig. 6b) but slightly delayed aggregation compared with TDP-43 CTD alone or with rGRN-3 (Fig. 6b). Similar augmentation of aggregation was observed with coincubation of GRN-3 and the TDP-43 CTD–RNA mixture (Fig. 6b), although the increase in the rate of aggregation was less pronounced with GRN-3 compared with rGRN-3, as observed previously in Fig. 3. Coincubation of rGRN-5 with the TDP-43 CTD–RNA mixture “rescued” aggregation of the TDP-43 CTD, with a linear increase in ThT fluorescence observed within hours of incubation (Fig. 6c). However, augmentation of ThT-positive aggregates was also pronounced with coincubation of GRN-5 (Fig. 6c). These data indicate that GRNs are able to modify LDs to promote aggregates of the TDP-43 CTD.

To further understand the mechanism of GRNs’ influence on LDs, coincubation of fluorescently tagged samples was monitored by DIC microscopy. As expected, TDP-43 CTD–RNA showed LD formation immediately after incubation that

![Figure 5. Quantitation of sedimented droplets and pellets from coincubation of the TDP-43 CTD and GRNs.](image-url)
remained for 36 h (Fig. 6d). Coincubation of GRN-3 or GRN-3 with the mixture of TDP-43 CTD and RNA showed neither a marked change in droplet shape or size nor GRN colocalization of GRNs within the droplet (Fig. 6, e and f). After 36 h, a marginal increase in LD size was observed, with GRN colocalized within the droplets (Fig. 6, e and f). However, the samples showed distortions in the morphology of LDs, accompanied by an overall reduction in size (Fig. 6, e and f). Addition of GRN-5 to the TDP-43 CTD–RNA mixture also showed immediate formation of droplets, with GRN colocalized within the droplets, which fused and grew bigger in size during the next 36 h, with both the TDP-43 CTD and GRN-5 colocalizing within the LDs (Fig. 6g). Addition of rGRN-5 to the TDP-43 CTD–RNA mixture led to droplet formation, which grew in number but not in size during the same time (Fig. 6h). One can also conclude that the increase in ThT fluorescence (Fig. 6c) can be explained by...
the fact that ThS (and likely ThT) is able to partition into droplets and fluoresces as observed (Fig. 3). rGRN-5 also colocalized with the TDP-43 CTD within the LDs.

To probe the internal dynamics of the LD droplets, FRAP was monitored temporally on the labeled TDP-43 CTD in the presence of RNA and GRNs. The fluorescence recovery rates for control LDs formed by the TDP-43 CTD and RNA showed nearly identical rates at 0 and 36 h, indicating preservation of internal mobility and dynamism over the incubation period (Fig. 6i). LDs formed in the presence of either GRN-3 or rGRN-3 showed a marked level of decrease in the internal mobility of the droplets during the 36 h of incubation (Fig. 6, j and k). Similarly, LDs formed in the presence of both redox forms of GRN-5 displayed decreased fluorescence recovery after 36 h, indicating inhibition of internal mobility, which could indicate maturation of the droplets into gels, a process commonly known as gelation (Fig. 6i, GRN-5, and Fig. 6 m, rGRN-5). Similar to the observation of GRN-5 and the TDP-43 CTD, where gelated droplets eventually transitioned to solid aggregates (Fig. S2), it is possible that these gelated LDs also eventually form insoluble aggregates of the TDP-43 CTD and RNA. The cellular ramifications of this on stress response by stress granules remains to be seen.

Discussion

The link between PGRN and familial FTLD/ALS is well-established, with the implication of autosomal dominant mutations in PGRN that lead to the protein’s haploinsufficiency. The loss of PGRN function in these pathologies is also speculated to arise at a posttranscriptional level with increased PGRN proteolysis (82). GRN immunopositivities have indeed been observed in a region-specific manner in both AD and FTLD human brains (83). Furthermore, the production of GRNs in haploinsufficient but not null-state familial FTLD suggests that they may be key players in the disease phenotypes. Support for this thought comes from the observation that GRNs interact with TDP-43 and exacerbate the latter’s levels and toxicity in C. elegans (57). Moreover, the recent discovery of PGRNs/GRNs being associated with lysosomal dysfunction (84) also posits the question whether GRNs could be involved in the autophagic fate of TDP-43 inclusions under redox stress. The results presented here attempts to answer some of the key questions regarding GRNs and the TDP-43 CTD, such as how GRNs modulate the dynamics between LD and fibril formation of the TDP-43 CTD and whether LDs facilitate the formation of insoluble TDP-43 CTD inclusions.

The findings of this study showcase how GRN-3 and GRN-5 modulate the dynamics of the TDP-43 CTD in forming insoluble aggregates or LDs under both oxidizing and reducing conditions. It is clear from the data that GRN-3 and GRN-5 affect TDP-43 CTD aggregation via disparate mechanisms (Fig. 7); although GRN-3 induces formation of insoluble inclusions, GRN-5 coacervates with the TDP-43 CTD to undergo LLPS under both reduced and oxidized forms, an interaction likely to be driven by the counterionic electrostatic characteristics of the TDP-43 CTD and the negatively charged GRN-5 (85–87). Furthermore, GRN-3 and GRN-5 also modify LDs formed by coacervation of RNA and the TDP-43 CTD. Specifically, GRN-3 in both redox states accelerates the formation of ThT-positive,
insoluble aggregates of the TDP-43 CTD. This observation supports the one by Salazar and co-workers (57), who observed exacerbation of TDP-43 toxicity in C. elegans by GRN-3 and GRN-7. GRN-5, on the other hand, seems to remodel LDs by inducing gelation within the 36-h time period, which changes to fibril formation after an extended time period (Fig. S2). At this point, the cellular ramifications of gelation of LDs by GRN-5 are unclear, but our ongoing investigations will glean insights into this aspect in the future. Nevertheless, our results provide the first in vitro evidence of an interaction between GRN and the TDP-43 CTD.

The increasing relevance of GRNs in FTLD and associated neurdenerative disorders imparts significance to the results presented in this study. Despite its significance, how haploinsufficiency of PGRN results in diminished TDP-43 inclusions in FTLD and ALS patients remains unknown. Common mutations associated with PGRN haploinsufficiency have been linked to mutant mRNA degradation (88), which several groups have identified to lead directly to reduced circulating levels of PGRN (89–91). A few have also argued that increased proteolysis of PGRN is the reason for haploinsufficiency of PGRN results in TDP-43 inclusions in FTLD and ALS (88), which several groups have identified to lead directly to reduced circulating levels of PGRN (89–91). A few have also argued that increased proteolysis of PGRN is the reason for haploinsufficiency, which leads to an increase in GRN levels (57). A recent study established that more effective proteolytic processing of cathepsin D, an aspartyl protease known to cleave PGRN, was estimated spectrophotometrically using molar extinction coefficients of 6250 M⁻¹ cm⁻¹ for GRN-3 and 7740 M⁻¹ cm⁻¹ for GRN-5 at 280 nm. The number of free cysteines was calculated from Elman’s assay and by iodoacetamide labeling, as reported previously (62). rGRN-3 and rGRN-5 were generated by addition of 2 mM tris(2-carboxyethyl)phosphine to the HPLC-fractionated protein for 2 h at room temperature and were used as the reduced form. ¹⁵N-labeled GRN-3 and GRN-5 were generated by growing the cells in M9 minimal medium enriched with ¹⁵NH₄Cl.

**TDP-43 CTD**—The plasmid for TDP-43 CTD expression was a gift from Dr. Nicolas Fawzi at Brown University (Addgene plasmid 98669, RRID: Addgene 98669). The protein was expressed as a fusion construct with a His tag (His₆) at the N-terminus, followed by a tobacco etch virus cleavage site. The plasmid was expressed in E. coli BL21 Star™ (DE3) cells (Life Technologies). Transformed cells were grown at 37°C in Luria-Bertani medium supplemented with 100 µg/ml kanamycin. Overexpression of the protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM at an A₆₀₀ of 0.5–0.7 AU. After overnight induction at room temperature (~25°C), cells were harvested by centrifugation (15,000 × g, 4°C) and used immediately or stored at −20°C. The cells were resuspended in lysis buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 5 mM imidazole, and 6 mM urea). 0.5 mM PMSF was also added to the resuspension. Cells were lysed using Misonix XL-2000. The lysate was centrifuged at 20,000 × g at 4°C for 1 h to remove cellular debris. The supernatant was incubated with nickel-nitrilotriacetic acid resin at 4°C for 2 h. The slurry was resuspended in a Kimble Kontes Flex column and washed with two wash buffers (20 mM Tris (pH 8.0), 500 mM NaCl, and 6 mM urea) with first containing 15 mM and a second wash of 30 mM imidazole, and protein was eluted in elution buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 6 mM urea, and 150 mM imidazole). Eluate was buffer-exchanged into storage buffer (20 mM Tris (pH 8.0), 500 mM NaCl, and 2 mM urea) and concentrated using Amicon Ultra-Centrifugal units (Millipore). Concentrated protein was flash-frozen and stored at −80°C. Concentrated aliquots were thawed on ice and desalted into 20 mM MES (pH 6.0) using Zeba desalting spin columns (Thermo) and used for studies.

**Thioflavin-T fluorescence**

The aggregation kinetics of the TDP-43 CTD were monitored using ThT on a BioTek Synergy H1 microplate reader. 20 µM TDP-43 CTD under varying reaction conditions was incubated at 37°C for 36 h in the presence of 10 µM ThT. Samples were excited at 440 nm, and emission was monitored at 480 nm. The ThT kinetics data were fit using the Boltzmann function on Origin 8.5.

**MALDI-TOF MS**

Pellet and droplet characterization of the coincubated protein complexes was performed on a Bruker Daltonics Microflex LT/SH TOF-MS system. Samples from aggregation kinetics reactions were recovered after a period of 36 h. The recovered samples were spun down at 18,000 × g, and the supernatant was decanted. The pellet obtained was then resuspended in an equal volume of reaction buffer (20 mM MES, pH 6.0). The pellet and supernatant fractions were then prepared for MALDI-MS by mixing with 6.5 ng of insulin (external standard). Additionally, the

**Experimental procedures**

**Expression and purification of recombinant proteins**

Granulins (GRN-3 and GRN-5)—Unlabeled GRN-3 was expressed and purified from Escherichia coli SHuffle™ cells (New England Biolabs) as described previously (62), whereas unlabeled GRN-5 and ¹⁵N-labeled GRN-5 were expressed in Origami 2 DE3 (Invitrogen). Briefly, the proteins were expressed as a GRN:trxA fusion construct and purified using immobilized-nickel affinity chromatography. The fusion protein was then cleaved by addition of restriction-grade thrombin (bovine, BioPharm Laboratories) at 1 unit/1 mg of protein to remove both trxA and the His tag. The reaction was incubated at room temperature (~25°C) for 22–24 h. The protein was then fractionated on a semipreparative Jupiter® 5 µm 10 × 250 mm C18 reverse-phase HPLC column (Phenomenex) using a gradient elution of 60%–80% acetonitrile containing 0.1% TFA as described previously (62). The concentration of the proteins was estimated spectrophotometrically using molar extinction coefficients of 6250 M⁻¹ cm⁻¹ for GRN-3 and 7740 M⁻¹ cm⁻¹ for GRN-5 at 280 nm. The number of free cysteines was calculated from Elman’s assay and by iodoacetamide labeling, as reported previously (62). rGRN-3 and rGRN-5 were generated by addition of 2 mM tris(2-carboxyethyl)phosphine to the HPLC-fractionated protein for 2 h at room temperature and were used as the reduced form. ¹⁵N-labeled GRN-3 and GRN-5 were generated by growing the cells in M9 minimal medium enriched with ¹⁵NH₄Cl.

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pellet samples were dissolved with formic acid in a 1:1 ratio to allow disaggregation of fibrils and other insoluble complexes. Both aliquots were then spotted onto a Bruker MSP 96 MicroScout Target with a 1:1 ratio of sample:sinapinic acid matrix in saturated acetonitrile and water. Instrument calibration was performed using Bruker Protein Calibration Standard I (Bruker Daltonics).

DIC

The phase separation was monitored using DIC on a Leica DMIL LED microscope under ×20 or ×40 magnification with a DIC polarizing filter. The reactions were observed on clear-bottom Nunc™ MicroWell™ 96-well microplates (Thermo).

Fluorescence microscopy

For colocalization studies of GRNs and the TDP-43 CTD during their interactions, labeling of the respective proteins with amine-reactive fluorescent dyes was performed, and visualization was done on a Zeiss LSM 510 Meta confocal microscope at ×40 or ×63 magnification under oil immersion with a DIC polarizing filter. Briefly, the TDP-43 CTD was labeled with HiLyte-647 (AnaSpec), and GRN-3 and GRN-5 were labeled with HiLyte-405 (AnaSpec). Free dyes were removed via desalting columns (Zeba desalting spin columns, Thermo) for the labeled TDP-43 CTD and Clarion™ MINI spin columns (Desalt S-25, SorbTech) for labeled GRN-3 and GRN-5. 20 μM TDP-43 CTD was mixed with 40 μM GRN-3 and GRN-5 or 40 μg/ml RNA. A ratio of 1% labeled: 99% unlabeled proteins was used for both the TDP-43 CTD and GRNs. Reactions were performed using the following experimental setup. A glass coverslip was attached onto one end of a two-side open-ended 96-well plate (Greiner Bio-One). Reactions were loaded into a well and covered with an optically clear plate-sealing film (Thermo). Reactions were then monitored at certain time points. Similarly, to observe the localization of ThS, 10 μM ThS (AAT Bioquest) was added to a sample containing 20 μM TDP-43 CTD with 40 μM GRNs. Samples were visualized using the setup described above on a Zeiss LSM 510 Meta confocal microscope with 420–480 band-pass emission filter.

FRAP

FRAP assays were performed on open-ended 96-well plates as described above. 10 μM TDP-43 CTD was mixed with 20 μM GRN-3 and GRN-5 or 20 μg/ml RNA. Reactions were monitored 10 min after mixing to allow deposition of suitable droplets onto the bottom of the well, which were used for bleaching. The TDP-43 CTD labeled with HiLyte-647 (AnaSpec) was used as the probe in the assays. An LSM 510 Meta confocal microscope at ×63 magnification under oil immersion with a DIC polarizing filter and FRAP module was used. The region of interest was bleached with a 633 nm laser line with 100% bleaching intensity for 750 iterations, and imaging was performed at 5% laser intensity for 120 s after bleaching. The numerical aperture was kept at 0.69 AU.

NMR spectroscopy

The heteronuclear multiple quantum coherence NMR spectra for 20 μM 15N-labeled GRN-5 or rGRN-5 resuspended in 20 mM MES (pH 6.0) with 10% D2O was acquired on a Bruker Advance III-HD 850-MHz NMR spectrometer equipped with a Bruker TCI cryoprobe at the high-field NMR facility of the University of Alabama, Birmingham as described previously (62).

Computational analysis of the TDP-43 CTD, GRN-3, and GRN-5

The per-residue intrinsic disorder predispositions of the TDP-43 CTD, GRN-3, and GRN-5 were evaluated by a set of commonly used disorder predictors (PONDR® VLXT (93), PONDR® VSL2 (94), and PONDR® VSL3 (95), available on the PONDR site (http://www.pondr.com);3 PONDR® FIT (96), accessible at the DisProt site (http://original.disprot.org/metapredictor.php); and the IUProdel computational platform, available at the IUProdel site (https://iuprodel2a.elte.hu/) that allow identification of either short or long regions of intrinsic disorder, IUProdel-L, and IUProdel-S (97–100). For each query protein, the outputs of these individual predictors were used to calculate a consensus disorder profile by averaging disorder profiles of individual predictors. The outputs of these tools are represented as real numbers between 1 (ideal prediction of disorder) and 0 (ideal prediction of order). A threshold of 0.5 or more was used to identify disordered residues and regions in query proteins. A protein region was considered flexible when its disorder propensity was in a range from 0.2 to 0.5. Results of this multiparametric computational analysis are presented in the form of mean disorder propensity, calculated by averaging disorder profiles of individual predictors. Use of consensus for evaluation of intrinsic disorder is motivated by empirical observations that this approach usually increases the predictive performance compared with use of a single predictor (96, 101–108). The CIDER computational platform (65) was used to generate linear net charge per residue diagrams for query proteins, whereas the sequence-based propensities of the TDP-43 CTD, GRN-3, and GRN-5 for granule formation associated with liquid demixing under physiological conditions were evaluated by the CatGRANULE algorithm (68), which generates positive and negative scores for proteins capable and not capable of granule formation, respectively. Finally, the presence of disorder-based binding sites in the TDP-43 CTD was evaluated by the ANCHOR algorithm (66, 67).

Author contributions—A. A. B. and V. N. U. data curation; A. A. B. and V. N. U. formal analysis; A. A. B., V. N. U., and V. R. investigation; A. A. B., V. N. U., and V. R. writing-review and editing; V. R. conceptualization; V. R. supervision; V. R. funding acquisition; V. R. writing-original draft; V. R. project administration.

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