# Chemically oligomerizable TDP-43: a novel chemogenetic tool for studying the pathophysiology of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the progressive loss of both upper and lower motor neurons. Most ALS cases are sporadic, but approximately 5–10% of patients have a familial background. To date, more than 30 familial ALS-causative genes have been identified (Maurel et al., 2018). The clinical manifestation and disease progression of sporadic ALS and familial ALS are similar and often clinically and pathologically indistinguishable, suggesting that they share a common pathophysiology in motor neuronal degeneration. One of the pathological hallmarks of ALS is the mislocalization of a multifunctional nuclear protein, TAR-DNA binding protein 43 (TDP-43). TDP-43 was identified as a primary component of ubiquitin-positive cytosolic inclusion bodies seen in remnant motor neurons in both sporadic and familial ALS (Neumann et al., 2006), and was later recognized as an autosomal dominant familial ALS-causative gene (ALS10) (Sreedharan et al., 2008). Since the cytosolic inclusion of TDP-43 is seen in almost all cases of ALS, regardless of the TDP-43 genotype, TDP-43 is thought to be a central hub molecule, linking both familial and sporadic ALS. Therefore, elucidation of the molecular mechanisms underlying TDP-43-related neurotoxicity would contribute to understanding the pathophysiology of this merciless disease.

TDP-43 is a highly conserved multifunctional protein that plays a pivotal role in a wide variety of critical biological processes, including transcription, splicing, RNA editing, and DNA repair. TDP-43 contains multiple functional domains, such as two RNA recognition motifs, a nuclear localization signal, a nuclear export signal, and a glycine-rich low-complexity domain (LCD) and is mainly located in the nucleus under normal conditions. Most ALS-causative mutations accumulate in the C-terminal LCD, indicating the importance of the LCD in the pathogenesis of ALS (Prasad et

al., 2019). The mislocalization of TDP-43 is supposed to cause not only the loss-of-function of TDP-43 but also the sequestering of essential proteins, resulting in neuronal cell death. The mechanism by which nuclear TDP-43 transforms into cytosolic aggregates remains unknown. As reviewed elsewhere, cleavage of the N-terminus of TDP-43 by various proteases causes loss of the nuclear localization signal and promotes cytosolic aggregation of the C-terminal fragments containing the aggregation-prone LCD (Prasad et al., 2019). However, evidence shows that cytosolic aggregates seen in the spinal cord of ALS patients contain significant amounts of full-length TDP-43, indicating that TDP-43 escapes from the nucleus to the cytosol via unidentified pathways besides protease cleavage. Recently, TDP-43 was reported to undergo liquid-liquid phase separation (LLPS), a biophysical phenomenon that condenses specific molecules as droplets from a homologous solution (Conicella et al., 2016). The C-terminal LCD of TDP-43 facilitates protein-protein interactions and is suggested to promote LLPS. LLPS drastically increases the local concentration of TDP-43, and highly condensed TDP-43 molecules might lead to conformational changes and pathogenic aggregation, followed by seed aggregate propagation. However, due to a lack of methods to manipulate LLPS, it remains undetermined whether LLPS is causal for the mislocalization of TDP-43.

Recent advances in optogenetic tools have enabled researchers to modify the status of the phase of TDP-43 and shed light on the mechanisms underlying aggregation and mislocalization of TDP-43. *Arabidopsis thaliana* cryptochrome-2 (Cry2) is known to self-associate upon blue light exposure, and Shin et al. (2017) utilized Cry2olig, an optimized variant of the Cry2 oligomerizing domain, as a module for inducible intracellular droplets, known as the optoDroplet system. After a short exposure to blue light, proteins with a sticky LCDs form

droplets, whereas proteins without LCDs disperse immediately and do not form droplets; therefore, the optoDroplet system is a useful tool for inducing LLPS and monitoring the tendency of LCD to become oligomerized. Furthermore, the optoDroplet system can work at a subcellular level, and the propagation of phase-separated droplets can be spatiotemporally monitored. Mann et al. (2019) employed the optoDroplet system, fused it to TDP-43, and triggered the oligomerization of TDP-43 in live cells. The oligomerized TDP-43 underwent LLPS, followed by translocation from the nucleus to the cytosol and eventually Cry2olig-TDP-43 formed cytosolic detergent-insoluble aggregates. The substitution of TDP-43 amino acids responsible for RNA binding enhanced aggregate formation, indicating that RNA binding has a negative effect on the phase transition of TDP-43. Consequently, the introduction of an RNA fragment recognizable by TDP-43 ameliorates TDP-43 aggregation and cytotoxicity, increasing the possibility for the development of a novel drug. Asakawa et al. (2020) introduced Cry2olig-TDP-43 into a zebrafish model and examined the effect of TDP-43 oligomerization on neurotoxicity *in vivo*. Once oligomerization was triggered, TDP-43 started to translocate from the nucleus to the cytosol, resulting in neurotoxicity. Importantly, TDP-43 exerted its neurotoxicity before the formation of large cytosolic aggregates, showing that TDP-43-mediated neurotoxicity occurs at an earlier stage than expected. These results also indicate that TDP-43 translocation is promoted by aberrant phase separation of TDP-43, and therefore, mislocalization is caused by the LLPS of TDP-43. The next issue to address is whether oligomerization of TDP-43 indeed causes ALS pathology in a mammalian model. Although the optogenetic Cry2olig system is highly useful for manipulating phase separation *in vitro* and *in vivo*, it still presents several limitations: (1) the selective induction of dimers or oligomers is impossible, (2) blue light is absorbed by hemoglobin, thus the systemic activation of Cry2olig-TDP-43 in a mammalian model is challenging; (3) special equipment is required for the activation of Cry2olig-TDP-43 in a large quantity of cells, and (4) phototoxicity might be problematic if longer irradiation with blue light is necessary.

To overcome these limitations, we

developed a chemically oligomerizable TDP-43 system (Yamanaka et al., 2021). This system adopted the dimer domain of the FKBP-F36V mutant as a clustering module, and homodimerization was induced by a small compound ligand, AP20187. AP20187 has a dumbbell-like shape and can act as a connector between two FKBP dimer domains. The advantages of this system are: (1) a dimer or oligomer can be selectively induced, (2) it is applicable to *in vivo* experiments without resulting in substantial toxicity, and (3) it can be used on a large quantity of cells without special equipment. When one FKBP-dimerizing domain is fused to TDP-43 (D1-TDP-43), the addition of AP20187 triggers dimer formation of TDP-43. When two dimerizing modules are fused to TDP-43 (D2-TDP-43), the addition of AP20187 can induce both dimer and oligomer formation of TDP-43. Surprisingly, the system revealed that the dimerization of TDP-43 via D1 was sufficient to initiate the aggregation of TDP-43, indicating that the intrinsic dimer-prone domain promotes oligomer formation when two TDP-43 molecules exist in close proximity. In particular, the N-terminal domain (NTD) of TDP-43 reportedly induces dimerization of TDP-43, and the LCD of TDP-43 is known to be responsible for the phase separation of TDP-43. In order to identify the domain essential for oligomer formation in D1-TDP-43, we constructed a series of deletion mutants, including ΔNTD and ΔLCD. Deletion analyses indicated that both NTD and LCD are essential for aggregate formation. The difference between the roles of NTD and LCD remains to be determined, but we suppose that dimerization via the NTD may create two TDP-43 molecules in close proximity, and the LCD can entangle these TDP-43 molecules to form aggregates. The accumulated TDP-43 contained some mobile fractions, suggesting that it underwent LLPS. The accumulated TDP-43 eventually translocated from the nucleus to the cytosol, forming cytosolic aggregates. These cytosolic aggregates mimicked the pathological characteristics of ALS inclusion bodies, such as the sequestration of wild-type TDP-43, fused-in-sarcoma (FUS), Sequestosome 1/p62, and stress granule marker TIA1. Oligomerization of TDP-43 also increased the detergent-insoluble fraction and eventually triggered cytotoxicity. These findings strongly indicate that this chemically oligomerizable TDP-43

system is a powerful tool for elucidating the pathophysiology of ALS.

Artificial modulation of LLPS in live cells is now of interest to the field of biology, and as a result, many relevant tools have been engineered. The optoDroplet system, developed by Shin et al. (2017), is an optogenetic tool using the Cry2olig system, and it was the first method to manipulate the LLPS of ALS-causative FUS in live cells. The usefulness of the optoDroplet system in ALS research was also validated in a cell model and a zebrafish model, as previously mentioned. We have demonstrated that the chemogenetic system controls

the phase separation of proteins, similar to the optoDroplet system. As shown in **Figure 1**, these methods are complementary as each method has its own advantages and disadvantages. The optoDroplet and chemogenic methods can be applied to other ALS-causative proteins and neurodegenerative disease-related proteins that undergo LLPS, to unveil their pathophysiology. Aberrant phase separation is now conceptually accepted as one of the common features of aggregate-prone neurodegenerative disease-related proteins, and intervention of their LLPS could contribute to the discovery of a novel drug. In the case of TDP-43,
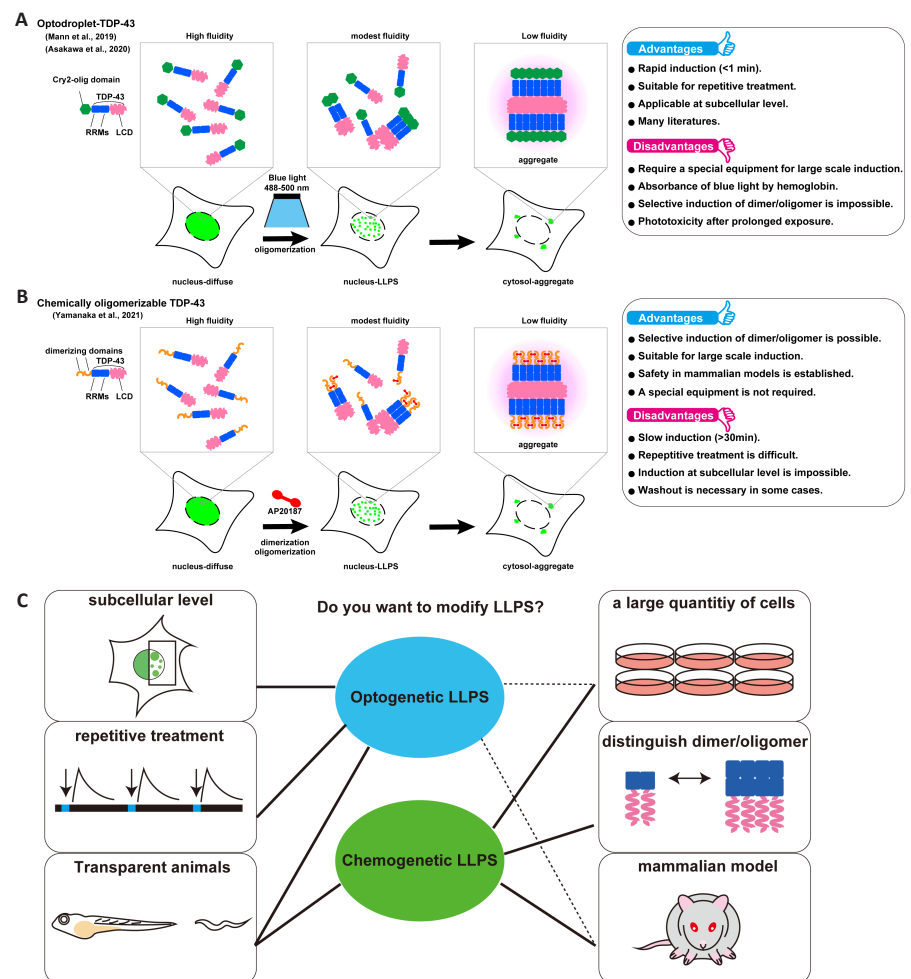


Figure 1 | **Optogenetic and chemogenetic phase separation of TDP-43 followed by cytosolic localization and choosing the tools for manipulating LLPS.**

(A, B) TDP-43 has two RNA recognition motifs (RRMs) and a C-terminal low-complexity domain (LCD). To artificially control its phase status, two different approaches are used. (A) To optogenetically induce phase separation of TDP-43, the Cry2olig domain is fused to TDP-43. Upon blue light exposure, the Cry2olig domain facilitates the oligomerization of TDP-43. (B) To chemically induce homo-oligomerization of TDP-43, multiple FKBP-dimerizing domains are fused to TDP-43 to facilitate oligomerization in the presence of the AP20187 ligand. In the absence of treatment, TDP-43 localizes in the nucleus and is highly mobile. Once oligomerization is triggered, optogenetically or chemogenetically, TDP-43 undergoes phase separation. At this stage, TDP-43 is still mobile but its fluidity is impaired. Eventually, the LCD induces the aggregate formation of TDP-43, and the aggregated TDP-43 mislocalizes to the cytosol and sequesters essential molecules, resulting in cytotoxicity. The advantages and disadvantages of each method are shown. (C) Diagrams for choosing the tools for manipulating LLPS: The optogenetic method and the chemogenetic method are complementary, and we propose here which tool should be more suitable for the listed experiments. FKBP: FK506-binding protein; LCD: low-complexity domain; LLPS: liquid-liquid phase separation; RRM: RNA recognition motif.

the aberrant LLPS can trigger TDP-43 mislocalization, and the intervention of the phase separation/mislocalization process can be a novel therapeutic target for ALS. Babinchak et al. (2020) reported that 4,4'-dianilino-1, 1'-binaphthyl-5,5'-disulfonic acid and its derivatives are potent biphasic modulators of protein LLPS; it can modulate the LLPS of TDP-43 LCD *in vitro*, and modulate the dynamics of stress granules in live cells, without modifying endogenous proteins. Although further study is warranted, this study proposes a new class of compounds with therapeutic potential for ALS. The next area of investigation is whether the attenuation of TDP-43 oligomerization indeed ameliorates the progression of ALS.

In addition to pathophysiological studies, advances in the modulation of LLPS have been applied for the creation of a new type of biochemical machinery in cells. The FKBP dimer domain that we utilized as the oligomerizing module of TDP-43 was adopted by other researchers to manipulate the LLPS of proteins of interest in live cells to control biochemical reactions. For example, Yoshikawa et al. (2021) combined the FKBP dimer domain and an optogenetic tool to create synthetic droplets that recruit and release proteins of interest in live cells. This system can optogenetically and chemogenetically modulate the activity of the protein of interest, and it successfully controls the guanine nucleotide exchange factor, Vav2, to promote cell protrusion (Yoshikawa et al., 2021). Nakamura et al. (2018) developed iPOLYMER (intracellular production of ligand-yielded multivalent enhancers) using tandemly-fused FKBP and its heterotypic interactor, FKBP12-rapamycin-binding domain, and created artificial stress granules in live cells. Although the function of artificial stress granules has not been fully investigated, this study proposes the potential of optogenetic and chemogenetic manipulation of oligomerization-prone proteins in the creation of synthetic membrane-less organelles. Since membrane-less organelles containing stress granules are formed via LLPS, optogenetic and chemogenetic approaches enable the creation of artificial membrane-less organelles whose functions can be designed. These methods will also

contribute to synthetic biology, as well as the study of the origin of life with membrane-less protocells.

In conclusion, the results discussed in this study provide a novel approach to the investigation of ALS pathogenesis using TDP-43. We hope that further investigation, using a combination of optogenetic and chemogenetic tools, will reveal the molecular mechanism underlying the condensation of aggregate-prone proteins via LLPS and subsequent gelation or liquid-solid phase transition, contributing to the development of curative therapy for ALS.

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