Do not curse the darkness of the spinal cord, light TDP-43

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Cytoplasmic inclusions containing the transactivation response element (TAR) DNA-binding protein-43 (TDP-43) aggregates are hallmarks of neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (Arai et al., 2006; Neumann et al., 2006). Despite the well-recognized correlation between TDP-43 aggregation and neuronal degeneration, whether this relationship is causal has remained unclear. The recent advent of the optoDroplet technique for controlling protein-protein interaction through light illumination has allowed the generation of droplets containing intrinsically disordered proteins in cells with an unprecedented spatiotemporal precision (Shin et al., 2017). Moreover, the use of this optogenetic approach to explore TDP-43 uncovered the neurotoxicity associated with TDP-43 phase transitions in cultured neurons (Mann et al., 2019; Zhang et al., 2019). Here, we discuss our recent discovery of novel facets of TDP-43, based on the use of an optogenetic TDP-43 variant (opTDP-43) interrogated in zebrafish motor neurons, in which the *in vivo* dynamic nuclear-cytoplasmic relocation and the clustering of TDP-43 can be observed directly due to the transparent zebrafish body (Asakawa et al., 2020). Our results showed that optogenetically clumped opTDP-43 mislocalizes to the cytoplasm and damages motor neurons before the development of large cytoplasmic aggregates, which are similar to those found in the ALS patients. This unexpected finding raises the possibility that the onset of motor neuron dysfunction caused by TDP-43 in ALS occurs much earlier than previously anticipated; therefore, future efforts should be made to identify the cellular environments and insults that facilitate pathological TDP-43 oligomer formation to better understand, and potentially intervene in, the prodromal phase of ALS and other TDP-43 proteinopathies.

ALS is a devastating neurological disease, in which the upper and lower motor neurons progressively degenerate, leading to fatal paralysis due to relentless muscular atrophy. A characteristic feature of degenerating motor neurons in sporadic ALS, which accounts for greater than 90% of total ALS cases, is the deposition of an aggregated form of TDP-43 in the cytoplasm. The remaining 10% cases are associated with monogenic heritability (familial ALS), and approximately 4% of familial ALS cases have been linked to the *TARDBP* gene, which encodes TDP-43, demonstrating a causal role for TDP-43 in the pathogenesis of some forms ALS. At present, however, whether the etiological mechanisms of sporadic ALS, where *TARDBP*/TDP-43 mutation is absent, are shared with the TARDBP-linked familial ALS remains known.

TDP-43 is an evolutionarily conserved heterogeneous nuclear ribonucleoprotein that regulates various aspects of RNA metabolisms including transcription, splicing, and mRNA stability and transport. Under normal physiological conditions, TDP-43 is primarily localized in the nucleus, where it forms homo-oligomers through its N-terminal domain (**Figure 1A** and **B**) (Afroz et al., 2017). On the other hand, pathological TDP-43 aggregation is prominent in the cytoplasm and mediated by the C-terminal intrinsically disordered region containing prion-like glutamine/asparagine-rich and glycine-rich regions, suggesting that the modes of TDP-43 multimerization may influence its subcellular localization, or *vice versa*. In addition, cytoplasmic degradation of TDP-43 protein by the ubiquitin-proteasome system and autophagy also contributes to the nuclear enrichment of TDP-43. Although a full picture of TDP-43 regulation in normalcy and disease has not yet been determined, the complexity and robustness of TDP-43 regulation can be readily implied by the convergence of the nuclear import signal and the poly-ubiquitination site within the N-terminus homo-oligomerization domain (Khosravi et al., 2020).

Among these multilayered TDP-43 regulations, multimerization has been particularly challenging to study in isolation. Consequently, how TDP-43 multimerization contributes to TDP-43 toxicity is not entirely understood. Cytoplasmic TDP-43 aggregation has frequently been observed in cellular and animal models upon overexpression of wild-type or mutant TDP-43 and often accompanied by toxicity. However, whether the observed toxicity stems from multimerization and/or overexpression is difficult to determine. To circumvent this difficulty, the optoDroplet approach, in which protein-protein interactions are light-activatable via the oligomerization of the Arabidopsis Cryptochrome-2/CRY2 tag (Shin et al., 2017), has been successfully applied to examine the TDP-43 phase transitions (Mann et al., 2019; Zhang et al., 2019). Light-induced optoTDP-43 phase transitions have been shown to cause toxicity in cultured neurons (Mann et al., 2019). In the following paragraphs, we describe efforts to extend these pioneering disease-in-a-dish approaches to disease-in-a-fish systems to develop an ALS model animal (Asakawa et al., 2020).

ALS is characterized by the selective vulnerability of motor neuron among other cell types in the central nervous system. Therefore, toxicity associated with multimerized TDP-43 in ALS, if any, likely strikes at inherent characteristics of motor neurons. To explore multimerization-dependent TDP-43 toxicity in the context of motor neurons, we developed an optogenetic TDP-43 system in zebrafish. This tiny minnow possesses a near-transparent body during the juvenile stage, which allows external light to efficiently reach various cell types in the body, including the spinal motor neurons. We first developed an opTDP-43, in which the mRFP1-tag is fused to the N-terminus and the CRY2olig-tag is fused to the C-terminus of TDP-43 (**Figure 1A**), based on the expectation that the CRY2olig tag would efficiently promote inter-molecular interaction between adjacent opTDP-43 molecules via the C-terminal intrinsically disordered regions, thereby phase separation. opTDP-43 was functional as TDP-43 under dark conditions as it was able to rescue the blood circulation defect of TDP-43 knockout fish and to display typical nuclear localization. Our *in vivo* characterization of opTDP-43, however, was initially disappointing because opTDP-43 did not alter the nuclear-enriched localization in the embryonic epithelial cells or differentiated myofibers in zebrafish, both of which are large sized and easy to access microscopically, after a couple of hours of illumination with a confocal blue laser, whereas the control mRFP1-CRY2olig proteinrequired less than 10 minutes to form aggregates. Before abandoning this project, we noticed that opTDP-43 gradually develops cytoplasmic foci in the early differentiating muscle cells of the embryos, following a couple of hours of light illumination, suggesting that the cytoplasmic TDP-43 mislocalization may represent a cell-type-dependent phenomenon. The much longer illumination duration needed for opTDP-43 to form cytoplasmic foci than for mRFP1-CRY2olig implies an inhibitory effect of the TDP-43 module on CRY2olig-dependent ultimerization in opTDP-43, potentially through an antagonizing effect of N-terminus-dependent homo-oligomerization (Afroz et al., 2017). To examine the response of opTDP-43 to the light illumination in motor neuron, we targeted opTDP-43 expression to the spinal motor neurons by using an mnr2b-Gal4 driver. Surprisingly, unlike in the other cell types examined, opTDP-43 gradually mislocalized to the cytoplasm and dispersed throughout the cell in motor neurons, without forming distinct foci in the cytoplasm after a couple of hours of light illumination. Nuclear opTDP-43 localization was restored after the illumination ceased. This transient opTDP-43 mislocalization is most likely independent of endogenous TDP-43, as non-optogenetic EGFP-tagged TDP-43 did not mislolcalize to the cytoplasm during light activation, despite the observed opTDP-43 mislocalization.

Does this transient cytoplasmic opTDP-43 mislocalization do any harm to the motor neurons? To address this question, we employed a prdm14-Gal4 driver line for the primary motor neuron CaP, which is present as a single unique cell in each spinal hemi-segment and has a defined innervation territory in the ventral myotome. We found that axon outgrowth of CaP expressing opTDP-43 was diminished when the fish was illuminated with a blue light for three hours just before the CaP begins to arborize its axon. Moreover, the axon shrinkage accompanied by the loss of neuromuscular synapses was observed when the blue light was shined against aroborized axons during the later stages. These observations demonstrated that the opTDP-43 multimerization by transient light illumination causes neurotoxicity leading to myofiber denervation, providing evidence that multimerized TDP-43 exerts toxicity prior to developing into large cytoplasmic aggregates.

Although the mechanisms underlying the low aggregation propensity of opTDP-43 in the motor neurons remained unknown, we attempted to reproduce opTDP-43 aggregation by further extending the illumination duration. By designing an arena in which fish can freely swim above a blue light-emitting diode light panel, we demonstrated that opTDP-43 eventually developed into distinct cytoplasmic foci, after 24 hours of illumination. Fluorescence recovery after photobleaching experiments revealed that these opTDP-43 foci had very low protein exchange rates, demonstrating that these opTDP-43 foci represented immobile protein assemblies (aggregates). Intriguingly, non-optogenetic EGFP-TDP-43 also became incorporated into the opTDP-43 aggregates after several days of illumination, suggesting that opTDP-43 aggregates eventually gain the seeding capacity for TDP-43 aggregation. These opTDP-43 aggregates were partially and differentially recognized by the antibodies against phospho-S409/S410 and some components of conventional stress granules, demonstrating that opTDP-43 aggregates are

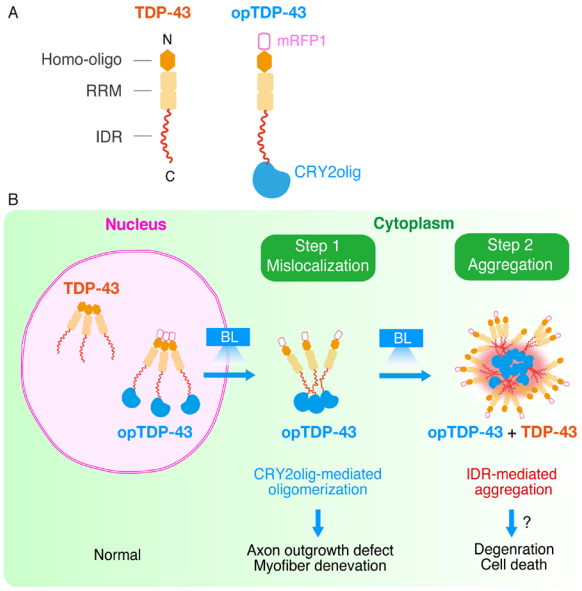

Figure 1 | **Optogenetic phase transition of TDP-43 in the motor neuron.**
(A) Structures of TDP-43 and opTDP-43. (B) Cytoplasmic TDP-43 in different forms may cause toxicity via distinct mechanisms. CRY2olig-driven opTDP-43 oligomerization promotes pathological changes in motor neurons, such as axon retraction, which is associated with myofiber denervation, prior to the accumulation of distinct cytoplasmic aggregates. Whether CRY2olig-driven opTDP-43 aggregates are toxic to motor neurons and whether CRY2olig-driven aggregates eventually deplete endogenous nuclear TDP-43 pools remain unknown. BL: Blue light; IDR: intrinsically disordered region; RRM: RNA-binging domain; TDP-43: cytoplasmic inclusions containing the transactivation response element DNA-binding protein-43.

heterogeneous protein assemblies with ALS pathologies. Although whether opTDP-43 aggregates resulted in toxic effects remained to be proved, fish depositing the aggregated opTDP-43 carrying a familial ALS mutation (A315T) in the motor neurons displayed motor decline after prolonged illumination albeit at a low frequency.

In summary, these observations provide evidence for a hypothesis that the formation of cytoplasmic TDP-43 aggregates can be divided into different phases and that cytoplasmic TDP-43 in different forms may cause toxicity via distinct mechanisms (**Figure 1B**). What mechanisms mediate acute and long-term toxicity following opTDP-43 multimerization? Given the wide range of RNAs bound by TDP-43, acute toxicity may derive from the dysregulation of the target RNAs, potentially leading to profound proteostatic stresses in the nucleus, cytosol or cytoplasmic organelles such as mitochondria. Alternatively, a pathological form of the TDP-43 oligomer itself may exert toxicity, although the identity of such an oligomer and the cellular environments and insults that facilitate its assembly remain elusive. In a long term, the seeding capacity of opTDP-43 might eventually deplete the nuclear TDP-43 pool, leading to a TDP-43 loss-of-function defect.

Perhaps, the most surprising observation is that the oligomerization of opTDP-43 alone shifts the opTDP-43 equilibrium from the nucleus to the cytoplasm in the motor neurons. To solve this puzzle, it is important to determine how the oligomerization of opTDP-43 alters its interaction with nucleocytoplasmictransport, post-translational midification and protein degradation systems in the context of motor neuron leading to a net increase in cytoplasmic TDP-43 pool. In addition to these cell biological regulations of TDP-43, the recent biophysical observation that heterotypic molecular interactions within a biomolecular condensate give rise to vectorial flux, such as the exclusion of matured ribosomal subunits from the nucleolus (Riback et al., 2020), raises a possibility that optogenetically-induced alterations of opTDP-43:protein and opTDP-43:RNA interaction networks in the nucleus might result in an exclusion of opTDP-43 oligomers and their efflux. In the cytoplasm, phase transitions of RNA-binding proteins are influenced by a concentration of RNAs available for interaction as shown in the G3BP-mediated stress granule assembly (Guillen-Boixet et al., 2020; Sanders et al., 2020; Yang et al., 2020), and RNA-binding antagonizes its neurotoxic phase transitions of TDP-43 (Mann et al., 2019). Therefore, the cytoplasm with a relatively low RNA concentration may serve as an environment in which TDP-43 is prone to undergo toxic phase transitions once its concentration rises. In the future efforts, both biology and biophysics are essential for understanding the mechanisms of the cytoplasmic shift of opTDP-43 equilibrium, which may be relevant for understanding how the normally nuclear-enriched TDP-43 increases its cytoplasmic pool in a prodromal stage of ALS.

The optical inaccessibility of the spinal cord has been and will continue to be a major obstacle to understanding the dynamic nature of TDP-43 in the spinal motor neurons during both healthy and diseased states. We envisage that optogenetic TDP-43 should serve as a candle to overcome the darkness of the spinal cord.

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