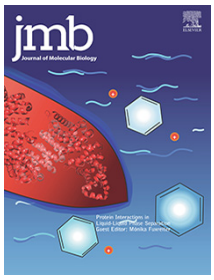
Review Article



Nuclear-Import Receptors Counter Deleterious Phase Transitions in Neurodegenerative Disease

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Abstract

Nuclear-import receptors (NIRs) engage nuclear-localization signals (NLSs) of polypeptides in the cytoplasm and transport these cargo across the size-selective barrier of the nuclear-pore complex into the nucleoplasm. Beyond this canonical role in nuclear transport, NIRs operate in the cytoplasm to chaperone and disaggregate NLS-bearing clients. Indeed, NIRs can inhibit and reverse functional and deleterious phase transitions of their cargo, including several prominent neurodegenerative disease-linked RNA-binding proteins (RBPs) with prion-like domains (PrLDs), such as TDP-43, FUS, EWSR1, TAF15, hnRNPA1, and hnRNPA2. Importantly, elevated NIR expression can mitigate degenerative phenotypes connected to aberrant cytoplasmic aggregation of RBPs with PrLDs. Here, we review recent discoveries that NIRs can also antagonize aberrant interactions and toxicity of arginine-rich, dipeptide-repeat proteins that are associated with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) caused by G_4C_2 hexanucleotide repeat expansions in the first intron of *C9ORF72*. We also highlight recent findings that multiple NIR family members can prevent and reverse liquid–liquid phase separation of specific clients bearing RGG motifs in an NLS-independent manner. Finally, we discuss strategies to enhance NIR activity or expression, which could have therapeutic utility for several neurodegenerative disorders, including ALS, FTD, multisystem proteinopathy, limbic-predominant age-related TDP-43 encephalopathy, tauopathies, and related diseases.

Introduction

A distinctive feature of several fatal and presently incurable neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), multisystem proteinopathy (MSP), and limbic-predominant age-related TDP-43 encephalopathy (LATE), is the depletion of specific RNA-binding proteins (RBPs) with prion-like domains (PrLDs) from the nucleus and their accumulation in cytoplasmic aggregates in degenerating neurons.[1–8] For example, TDP-43 exhibits this pathological phenotype in ~97% of ALS cases and ~50% of FTD cases, whereas another RBP with a PrLD, FUS, displays nuclear depletion and cytoplasmic aggregation in ~1% of ALS cases and ~9% of FTD cases.[1,8] It is suggested that the loss of nuclear function of these RBPs coupled to a gain of toxic function due to cytoplasmic accumulation and aggregation may syner-

gize to elicit neurodegeneration.[1,6,8–10] We have suggested that agents that reverse the cytoplasmic mislocalization and aggregation of these RBPs *and* restore their nuclear localization and function could be powerful therapeutics.[6,11–18] Remarkably, nuclear-import receptors (NIRs) have emerged as agents capable of eliciting such a therapeutic effect.[16,19]

NIRs are members of the karyopherin family of proteins that bind tightly to nuclear-localization signals (NLSs) of polypeptide cargo in the cytoplasm.[20–22] NIRs are subdivided into a small family of karyopherin-α (Kapα) proteins, which engage classical NLSs and subsequently bind to a member of the larger karyopherin-β (Kapβ) family that enables nuclear import.[22] However, Kapβs can directly recognize non-classical NLSs (e.g. proline tyrosine [PY]-NLSs) independent of Kapαs.[22] Kapβs are flexible, superhelical proteins that typically comprise ~20 consecutive HEAT (Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1) repeats, a type of protein tandem repeat structural motif composed of two alpha helices linked by a short loop (Figure 1(A)).[22] Once bound to the NLS, NIRs can transport their cargo across the nuclear-pore complex (NPC) and into the nucleoplasm.[22]

The NPC is an intricate structure[23] which operates as a size-selective barrier to prevent macromolecules with a molecular weight greater than ~30 kDa (or a Stokes radius greater than ~3 nm) from passively diffusing in and out of the nucleus.[24] This barrier is established by FG-rich nucleoporins, which form a phase-separated state inside the NPC channel.[25–28] NIRs can penetrate rapidly through this phase and transport cargo into the nucleus.[29] Once inside the nucleoplasm, the small GTPase Ran in its GTP-bound state binds to the incoming NIR, dissociating the NIR-cargo complex.[22] Cargo is thus released into the nucleus where a high concentration of RNA keeps the incoming RBP soluble so it can perform its regular function, and the NIR is recycled for further rounds of nuclear transport.[22,30–32] By contrast, in the cytoplasm Ran is found in the GDP-bound state, which has a low affinity for NIRs, permitting NIR-cargo interactions.[22]

Beyond this classical function in nuclear transport, NIRs are now understood to operate in the cytoplasm to chaperone and disaggregate NLS-bearing clients.[19,33–43] In this context, NIRs engage cognate NLSs to inhibit and reverse physiological and deleterious phase transitions of their cargo (Figure 1(B)), which include several prominent neurodegenerative disease-linked RBPs with PrLDs, including wild-type and disease-linked mutant forms of TDP-43, FUS, EWSR1, TAF15, hnRNPA1, and hnRNPA2.[19,33–44] For example, Karyopherin-β2 (Kapβ2; also known as Transportin 1) can prevent and reverse fibrillization of wild-type

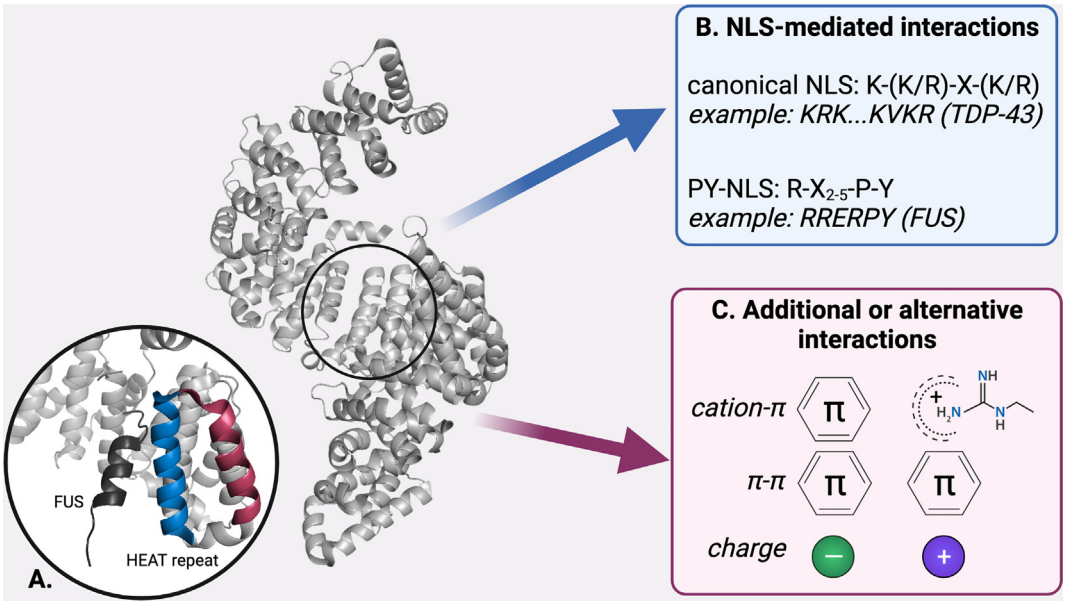

Figure 1. Specific and general interactions enable NIRs to chaperone cargo. (A) NIRs like Kapβ2 (PDB: 4FDD) share a similar structure, with roughly 20 paired helical HEAT repeats coiled into an alpha solenoid structure. HEAT repeats comprise an outer helix (raspberry) and an inner, cargo-facing helix (blue; FUS-PY-NLS cargo, black). NIRs interact with cargo via two non-exclusive mechanisms. (B) Many NIRs will specifically interact with cargo bearing an NLS. For example, Kapβ2 will interact with cargo bearing a PY-NLS, like FUS and hnRNPA1. (C) NIRs can also bind to cargo through additional or alternative interactions, including cation-π, π-π, and electrostatic interactions. Such interactions can occur with cargo that bears an NLS, and with cargo that has no known NLS sequence, such as the arginine-rich DPRs produced in c9ALS/FTD. This figure was made with BioRender.

FUS, EWSR1, TAF15, hnRNPA1, hnRNPA2, and several disease-linked variants.[19] Kapβ2 also prevents and reverses FUS liquid–liquid phase separation (LLPS).[19,33,34,36] Moreover, Importin α (Impα and Kapβ1 (also known as importin β) cooperate to prevent and reverse TDP-43 condensation and fibrillization.[19,37] Importantly, elevated NIR expression can mitigate degenerative phenotypes connected with aberrant aggregation of RBPs with PrLDs in model systems.[19,34,45] Indeed, NIRs can disaggregate cytoplasmic inclusions formed by RBPs with PrLDs and return these proteins to the nucleus, thereby restoring their native function.[19] In this way, NIRs may simultaneously eliminate: (1) any gain of toxic function due to cytoplasmic mislocalization and aggregation of the RBP; and (2) any loss of nuclear RBP function, two facets of disease that likely conspire to drive neurodegeneration.[19] Thus, NIRs join a growing class of ATP-independent protein disaggregases.[14,46–49] These exciting advances have been reviewed in detail elsewhere.[16–18,20,50–52]

In this review, we focus on recent developments concerning how NIRs can antagonize aberrant interactions and toxicity of dipeptide-repeat proteins (DPRs) that are produced via repeat-associated non-AUG (RAN) translation[53] of the G_4C_2 hexanucleotide repeat expansion (HRE) in *C9ORF72* that cause ALS and FTD.[37,54] We also highlight recent findings that multiple NIR family members can prevent and reverse the LLPS of specific cargo bearing RGG motifs.[35] In ALS/FTD and related degenerative disorders, NIRs can be mutated,[55] expressed at lower levels,[52,56] sequestered in stress granules (SGs)[57] and aggregated structures,[52,58–60] or fail to effectively recognize post-translationally modified cargo[61] or disease-linked mutant NLSs.[19,62–68] Moreover, NIRs are critical for neuronal maintenance and function, and mutations in NIRs are associated with human developmental delays, neurologic deficits, and dysmorphic features.[69] Thus, we close the review by discussing strategies to enhance NIR activity or expression, which could have therapeutic utility for several presently untreatable disorders.[16]

NIRs as Safeguards against Toxic DPRs

A large G_4C_2 HRE in the first intron of the *C9ORF72* gene is the most common genetic cause of ALS and FTD (termed c9ALS/FTD).[10,70–73] Patients with c9ALS/FTD can have hundreds to thousands of G_4C_2 repeats in the first intron of *C9ORF72*, whereas healthy individuals typically harbor ~2–23 repeats.[10,70–74] In c9ALS/FTD, the G_4C_2 HRE is bidirectionally transcribed into toxic repeat RNAs, which are RAN-translated to yield five different DPRs: poly(GA), poly(GP), poly(PR), poly(GR), and poly(PA).[71,75–77] In c9ALS/FTD models, arginine-rich DPRs (R-DPRs) are particularly toxic to neurons due to their positive charge and wide range of interacting partners.[78–81] More specifically, poly(GR) and poly(PR) can directly interact with the PrLD-containing RBP TDP-43, altering its phase-separation behavior and accelerating its aggregation both *in vitro* and in cells.[37,82] In fact, poly(GR) and poly(PR) are notorious for their ability to disrupt the LLPS of multiple RBPs through interactions with low-complexity domains (LCDs), and their ability to disturb the dynamics of several membraneless organelles, including SGs, nucleoli, nuclear speckles, Cajal bodies, and heterochromatin.[71,80,81]

Given the high affinity of NIRs to arginine- or lysine-rich NLSs, it was postulated that NIRs might also target R-DPRs.[37] Several NIRs were identified as modulators of R-DPR toxicity in c9ALS/FTD models, suggesting a mechanistic link between NIRs and R-DPRs.[54,83–85] More recent studies found that R-DPRs directly interact with multiple NIRs, including Impα, Kapβ1, and Kapβ2, causing an interruption in nucleocytoplasmic trafficking.[37,86] Specifically, high concentrations of R-DPRs promote the insolubility of NIRs, disrupting their ability to bind and import their NLS-containing cargo.[37,86] As such, this mechanism provides an explanation for why TDP-43 nuclear import deteriorates in c9ALS/FTD.[37]

With the direct link between DPRs and NIRs now revealed, can we steer the chaperoning power of NIRs to combat R-DPR-associated toxicity? R-DPRs undergo RNA-stimulated phase separation,[87] which likely leads to R-DPR accumulation in aggregated structures in c9ALS/FTD.[76,77] Importantly, Kapβ1 and Kapβ2 suppress RNA-stimulated poly(GR) condensation, whereas Impα3 is ineffective.[37] Interestingly, while R-DPRs in molar excess can directly interact with NIRs and impair TDP-43 nuclear import, equimolar or elevated levels of Kapβ1 or Kapβ2 can shield R-DPRs, thereby suppressing their pathological interactions with TDP-43.[37] Thus, increasing the concentration of NIRs prevents R-DPR phase separation, prevents R-DPRs from engaging in deleterious interactions, and also restores the nuclear localization of TDP-43 as it becomes available to interact with its own NIRs.[37] These findings suggest that the reported reduction in the endogenous concentrations of NIRs associated with neurodegenerative disease may contribute to the pathogenesis of c9ALS/FTD, and that interventions aiming to elevate NIR levels are a promising therapeutic strategy to combat R-DPR toxicity in c9ALS/FTD.[52]

Besides R-DPRs, another c9ALS/FTD-linked DPR, poly(GA), as well as chimeric DPR species such as GA:GP can form cytoplasmic inclusions that may inhibit nuclear import of TDP-43.[88,89] Indeed, in hippocampal neurons, poly(GA) expression results in robust TDP-43 cytoplasmic mislocalization.[88] However, overexpression of Impα3 or Impα4, which may be involved in nuclear import of TDP-43,[56,90] can likely restore TDP-43 to the

nucleus.[88] Other repeat-expansion disorders can also produce DPRs, such as spinocerebellar ataxia 36 (SCA36), which presents with poly(PR) and poly (GP).[89,91] Thus, NIRs may also be promising therapeutics to mitigate DPR toxicity in SCA36.

Multiple NIRs Exhibit Chaperone and Disaggregation Activity

The family of Kapβ proteins is large, containing over a dozen subfamilies.[92] These proteins vary in their structure, directionality of transport (nuclear-export factors such as Crm1 are also members of the Kapβ family), and cargo repertoire.[92] And, whereas some cargo proteins show a clear preference for a single NIR, others can be ferried by multiple karyopherins, either individually or in concert with one another.[93–99] In addition to trafficking cargo proteins in and out of the nucleus, Kapβ family members like Kapβ2 can also act to prevent and reverse the self-assembly and aggregation of proteins, including those implicated in neurodegenerative disease.[16] However, it had been an unexplored question as to whether this disaggregation activity was a feature of karyopherin proteins in general, or an exclusive capability of only some. Focusing on the disease-associated RBP FUS, recent work from Baade et al. now establishes that multiple Kapβ family members can act as potent chaperones both *in vitro* and in cells.[35]

To uncover the network of Kapβ proteins that FUS interacts with, Baade et al. performed pull-down assays using cell lysates or purified proteins and found that, in addition to Kapβ2, FUS also binds to Kapβ1, transportin-3, importin-7, importin-13, and exportin-4.[35] Like Kapβ2, transportin-3, and importin-7 (either on its own or as a heterodimer with Kapβ1) interact stably with FUS, forming complexes that were sensitive to the addition of Ran-GTP.[35] Although these complexes were stable, of the NIRs tested, Kapβ2 demonstrated the strongest binding to FUS.[35] It is well established that Kapβ2 binds to FUS via its PY-NLS,[62] but it was unclear if these other NIRs were interacting with FUS in the same way. Thus, Baade and colleagues assessed the binding of each NIR to truncated constructs of FUS.[35] They found that instead of interacting with the PY-NLS, Kapβ1, transportin-3, importin-7, and Kapβ1/importin-7 interact with FUS via its arginine- and glycine-rich RGG domains (Figure 1(C)).[35] Interestingly, when the arginine residues of the RGG domains were mutated to lysine, the binding of Kapβ1, transportin-3, importin-7, and Kapβ1/importin-7 was impaired, underscoring the specific importance of arginine in mediating the interaction between Kapβ proteins and their cargo.[35,36,40,100]

Not only do multiple Kapβ proteins bind to FUS, but they can also chaperone its material state.[35] In the absence of any NIR, purified FUS protein will undergo LLPS.[19,33,37,101] Addition of equimolar levels of Kapβ1, transportin-3, importin 7, or Kapβ1/importin-7 each prevented and reversed FUS LLPS, indicating that these NIRs both bind to and chaperone FUS.[35] Baade et al. also observed this chaperoning activity in cells, where addition of any of the NIRs tested was able to suppress the association of exogenous MBP-FUS with stress granules.[35] The work from Baade and colleagues illustrates that FUS self-assembly can be modulated by multiple Kapβ proteins and suggests that the mechanism by which this chaperone activity occurs may be a universal feature of Kapβ family members.[35] Although Kapβ1, transportin-3, importin 7, or Kapβ1/importin-7 can prevent and reverse FUS LLPS,[35] it remains unclear whether Kapβ1, transportin-3, importin 7, or importin β/7 can reverse the formation of FUS fibrils like Kapβ2.[19] Kapβ2 interacts more avidly with FUS than the other NIRs, which may confer stronger FUS chaperone and nuclear-import activity.[35] Further studies into the many Kapβ family members and their respective abilities to chaperone disease-related cargo proteins will therefore be an intriguing area for future research.

Of particular interest is Transportin-3. Intriguingly, mutations in transportin-3 have been connected to congenital limb-girdle myopathy,[55,102–107] indicating that transportin-3 is critical for human health. Transportin-3 binds to multiple proteins with RGG-motifs, including the cold-inducible RNA-binding protein which is involved in responding to cell stress.[100] Transportin-3 has also been shown to chaperone another disease-related cargo, the arginine-rich nuclear-speckle protein SRRM2.[108,109] In tauopathies, including FTD and Alzheimer's disease, tau disrupts nuclear speckles and sequesters SRRM2 in cytoplasmic inclusions, which reduces SRRM2 splicing activity.[110,111] Thus, upregulation of transportin-3 might enable extraction of SRRM2 from cytoplasmic tau aggregates and restore SRRM2 to the nucleus, which may mitigate degenerative phenotypes in tauopathies.[112]

NIRs also affect phase transitions in physiological contexts. For example, Impα/Kapβ1 regulate the material state of Targeting Protein for XKlp2 (TPX2), a spindle-assembly factor whose activity must be tightly regulated for proper cell cycle progression.[44,113] TPX2 condensation promotes its activity, whereas Impα and Kapβ1, either alone or combined, inhibit TPX2 condensation through a combination of specific binding and general low-affinity interactions.[44,113]

Interestingly, there are NIR family members that bind to cargo with no defined consensus NLS. For example, Transportin-3 and importin 13 can each bind dozens of cargo proteins with no annotated NLS.[114] Surprisingly, despite their close similarity, Transportin-3 and importin 13 have little overlap in which cargo they recognize, and for each NIR, the cargo vary in terms of sequence and structure.[114] Hence, there is yet much to uncover with respect to potential NIR:cargo interactions.

Leveraging NIRs as Therapeutic Agents

As newly appreciated dissolvases,[18] Kapβ family members represent an exciting target to pursue in developing drugs for diseases related to the adoption of aberrant material states by various proteins.[16] There already exist several compounds that affect Kapβs, however these mainly work to prevent activity by occupying the cargo-binding surface of their target.[115] As such, there is an opening to develop compounds that can stimulate Kapβ activity (Figure 2A).

What would such compounds look like? One approach could be to target disassembly of Ran-GTP:Kapβ complexes in the nucleus to promote Kapβ turnover (Figure 2(A, i)).[116] Increasing the efficiency with which free Kapβ proteins are made available would increase the relative levels of unbound Kapβ, thereby potentially increasing their apparent activity.

There is also the opportunity to discover novel small molecules to enhance Kapβ activity directly (Figure 2(A, ii)). To this end, employing a high-throughput screening approach to identify compounds that stimulate Kapβ activity would be an exciting avenue to pursue. Such a screen could be done *in vitro* using purified proteins. For example, it will be of great interest to screen for drug-like compounds that enhance the ability of Kapβ2 to prevent or reverse FUS fibrillization, or Impα/Kapβ1 to prevent and reverse TDP-43 fibrillization. Likewise, screening campaigns might also be considered in cell-based models to find drug-like compounds that enhance the ability of NIRs to restore nuclear localization of TDP-43 or FUS in response to stress or disease conditions.

Several structures of Kapβ2 bound to disease-linked cargo, including hnRNPA1, FUS, and ALS-linked FUS variants are available.[40,62,117] These structures could facilitate the design of small mole-

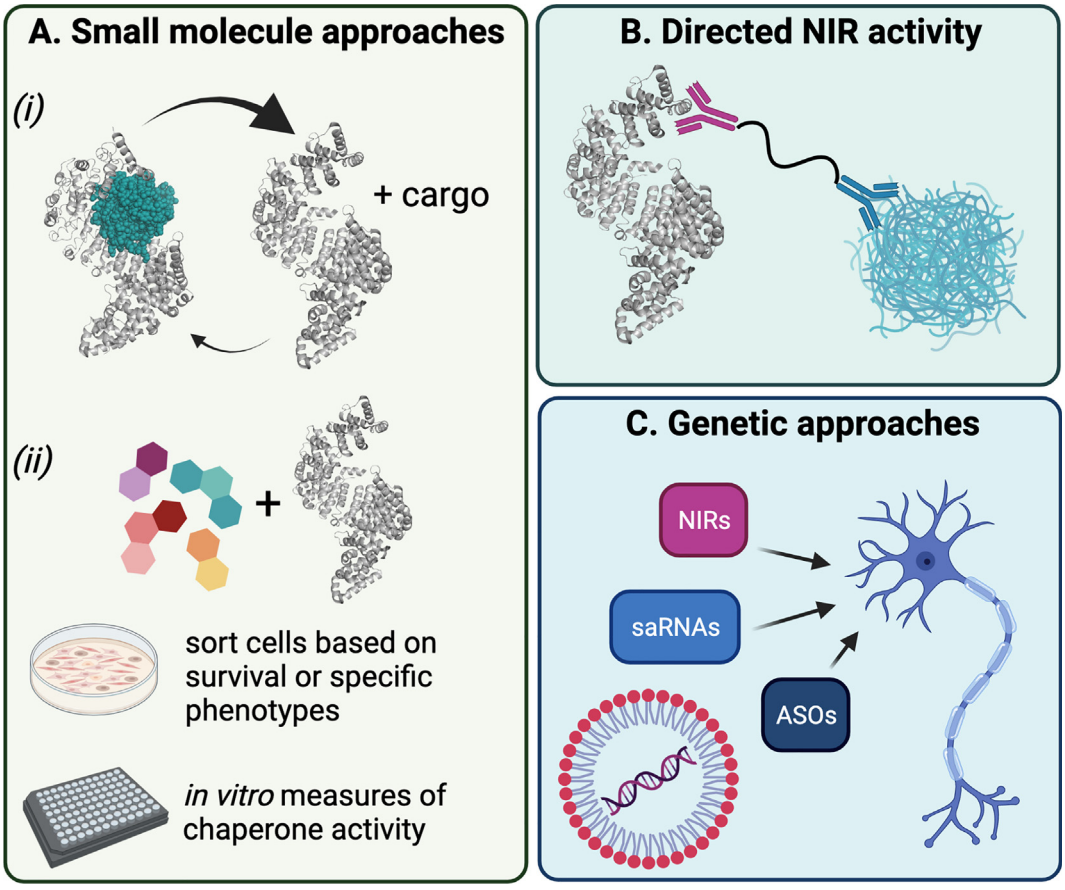

Figure 2. Therapeutic strategies to enhance NIR activity. (A) One potential means by which NIR activity could be enhanced is through the use of small-molecule drugs. For example, a molecule that promotes the release of Ran-GTP (shown in teal) from NIRs in the nucleus would increase the pool of free NIRs available for binding to cargo (i). Alternatively, small molecule libraries or engineered compounds could be used to perform *in vivo* or *in vitro* screens for chaperone activity (ii). (B) Another approach could use a PROTAC-like molecule to direct NIRs to specified targets. (C) Finally, NIR activity could be augmented using genetic approaches to deliver saRNAs or ASOs to elevate NIR expression, or to deliver sequences via AAVs or lipid-containing nanoparticles to express NIRs directly. This figure was made with BioRender.

cules that increase the affinity of Kapβ2 for these specific cargo. This approach may be particularly important for disease-linked hnRNPA1 and FUS variants with mutations in the PY-NLS that weaken the interaction with Kapβ2.[62,67,68] Ideally, compounds could be uncovered that restore the affinity of Kapβ2 for disease-linked cargo to similar levels observed with wild-type cargo. Although structures of Impα/Kapβ1 bound to the TDP-43 NLS are not yet available, other structures of Impα family members bound to cargo have been solved,[118–123] and these could also inform drug design. Here, it will be important to ensure that small-molecules do not make NIR-cargo interactions so tight that they cannot be dissociated by Ran-GTP, as release of cargo is essential for restoring nuclear activity and NIR recycling.[16]

Small-molecule drugs that increase the expression of specific NIRs or even globally upregulate NIRs may also be an interesting therapeutic strategy. How NIR expression is regulated in response to nuclear-transport stress remains poorly understood, and further studies in this area are likely to be informative. For example, it would be important to uncover compounds that globally upregulate nuclear-transport pathways akin to compounds that induce the heat-shock response to upregulate a battery of molecular chaperones.[124–130] Such compounds could provide a critical boost to nuclear transport to combat neurodegeneration.

Another strategy to boost NIR chaperone activity could resemble recent work with proteolysis-targeting chimeras (PROTACs).[131] PROTACs are small molecules that comprise a moiety that targets an E3-ubiquitin ligase tethered to a recruiting compound that binds to a protein of interest.[131] Thus, PROTACs enable specific ubiquitination and subsequent degradation of a target protein, and have been successfully used in a wide range of applications.[131] However, in ALS/FTD, the disease-relevant proteins TDP-43 and FUS serve vital roles in biology, including in RNA metabolism, axonal transport, and responding to DNA damage and other stressors.[6,8,34,132–136] A PROTAC-type model that disaggregates but does not degrade its target protein is therefore a potential adaptation well suited to Kapβ activity. Here, instead of an E3 ligase, Kapβ proteins could be utilized to target phase-separated and aggregated proteins, including those with a mutated NLS or no NLS at all (Figure 2(B)). In promoting a locally high concentration of NIRs, this strategy would liberate the target protein from deleterious assemblies, allowing it to resume its normal activities.

In addition to a compound-based treatment, genetic approaches are also becoming an increasingly tractable strategy for preventing or reversing disease states (Figure 2(C)). For example, delivery of specific NIRs to neurons could be achieved using adeno-associated viruses (AAVs).[137,138] After a single administration of AAV, neurons can be successfully transduced, enabling stable expression of a therapeutic gene of interest.[139] The safety of this approach has been established in numerous clinical trials for neurodegenerative diseases[140–143] and AAVs delivering specific genes are now FDA-approved drugs.[144,145] Nonetheless, caution is still needed.[146,147]

Additional approaches for gene delivery might also be considered, including lipid-containing nanoparticle-mediated delivery of chemically-modified mRNAs to afflicted neurons akin to the technology that has produced highly effective mRNA vaccines (Figure 2(C)).[15,148,149] Antisense oligonucleotides (ASOs) have also been used to increase the expression of certain proteins by facilitating specific splicing events for productive gene expression (Figure 2(C)).[150,151] Alternatively, small-activating RNAs (saRNAs) can be used to activate the expression of target genes with the RNA-induced transcriptional activation complex (Figure 2(C)).[152,153] These approaches could be used to supplement production of NIRs, which undergo age- and disease-related changes in expression levels.[52,56,154]

Perspectives

It is now clear that NIRs perform a range of beneficial chaperone and dissolvase activities that could, if channeled appropriately, provide profound therapeutic effects for several presently fatal neurodegenerative disorders, including ALS, FTD, MSP, LATE, and tauopathies.[16,112] However, several challenges lie ahead. These include ensuring that elevating or enhancing NIR activity does not result in unanticipated off-target effects or undesirable effects such as disturbing the nuclear:cytoplasmic ratio of various cargo in a manner that is detrimental to cell viability. Likewise, some cytoplasmic condensates formed by RBPs with PrLDs, such as TDP-43 myogranules[155] or RNA-transport granules[34,135,156,157], serve beneficial functions, which ideally would not be perturbed by elevated NIR activity. Indeed, it will be important to disperse pathological condensates and simultaneously preserve beneficial condensates. Despite these challenges, the ability of NIRs to reverse the cytoplasmic mislocalization and aggregation of specific cargo *and* restore their nuclear localization and function could enable the development of powerful therapeutics, which warrants intense investigation.

CRediT authorship contribution statement

Hana M. Odeh: Visualization, Writing – original draft, Writing – review & editing. **Charlotte M. Fare:** Visualization, Writing – original draft, Writing

– review & editing. **James Shorter:** Visualization, Writing – original draft, Writing – review & editing.

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Declaration of Competing Interest

H.M.O. and C.M.F. declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. J.S. is a consultant for Dewpoint Therapeutics, Maze Therapeutics, Vivid Sciences, Korro Bio, ADRx, and RBNC Therapeutics.

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