Review

Measuring and Interpreting Nuclear Transport in Neurodegenerative Disease—The Example of C9orf72 ALS

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Abstract: Transport from and into the nucleus is essential to all eukaryotic life and occurs through the nuclear pore complex (NPC). There are a multitude of data supporting a role for nuclear transport in neurodegenerative diseases, but actual transport assays in disease models have provided diverse outcomes. In this review, we summarize how nuclear transport works, which transport assays are available, and what matters complicate the interpretation of their results. Taking a specific type of ALS caused by mutations in C9orf72 as an example, we illustrate these complications, and discuss how the current data do not firmly answer whether the kinetics of nucleocytoplasmic transport are altered. Answering this open question has far-reaching implications, because a positive answer would imply that widespread mislocalization of proteins occurs, far beyond the reported mislocalization of transport reporters, and specific proteins such as FUS, or TDP43, and thus presents a challenge for future research.

Keywords: nuclear transport; nuclear pore complex; nuclear transport receptor; C9orf72; amyotrophic lateral sclerosis (ALS)

1. Introduction

1.1. Nucleocytoplasmic Transport

Nucleocytoplasmic transport (NCT) occurs through nuclear pore complexes (NPCs), large protein complexes embedded in the nuclear membrane. NPCs facilitate and regulate the import and export of proteins and RNA between the nucleus and cytoplasm. Small proteins will rapidly diffuse through the NPC, but the passage of larger molecules is hindered by the NPC’s permeability barrier [1,2]. The association of proteins or RNAs with specific importins, exportins, and transportins, grouped together as nuclear transport receptors (NTRs), facilitates the passage of NTR–cargo complexes. Additionally, a pool of importin-β functions as a stable component of the NPC’s permeability barrier [11]. The differential localization of NPC-associated proteins in a single yeast nucleus suggests that NPCs may even have specialized transport functions [12].

In baker’s yeast, there are 19 known NTR [13–16], whereas in human cells, 30 NTRs [17] are identified. The substrate specificity of some, but not all NTRs has been mapped, and jointly they bind a large and diverse group of cargoes, while some redundancies exist [18]. The importin-β superfamily is the largest class of NTRs; they can either directly bind to...
their cargo, or via an adaptor protein, an importin-α isoform [19]. Exportins include CRM1, LOS1, and the MEX67-MTR2 (or human TAP/p15) complex for mRNAs and tRNAs [20], MSN5 for tRNAs, and CAS/CSE1 specifically exports Impα/Kap60 [21]. Of note, while general protein export is mediated only by the major exportin CRM1, many importins cooperate for general protein import. NTRs bind their cargoes through a nuclear localization signal (NLS) or a nuclear export signal (NES) encoded on a cargo molecule.

Figure 1. Nucleocytoplasmic transport can be measured with fluorophore-tagged transport reporters. (A) GFP tagged with an NLS accumulates in the nucleus owing to constant active import, which is faster than the passive leak in the opposite direction. (B) GFP tagged with an NES accumulates in the cytosol owing to constant active export, which overpowers passive influx. (C) GFP tagged with both an NLS and NES sequence accumulates depending on the strongest signal, as it is both actively imported and exported and passively diffuses across the nuclear pore. Passive diffusion (=influx and efflux) is dependent on the concentration gradient of the reporter, the number of NPCs, and their permeability.

Ran, a member of the Ras family of small GTPases, is in its GTP bound state in the nucleus and its GDP bound state in the cytosol. This gradient functions to ensure that the cargoes are accumulated in the nucleus or cytosol by promoting the removal of cargo from importins and the loading of cargo to exportins [22–24]. Whereas importins bind to either the NLS of their cargo or to the RanGTP, exportins bind to both the NES of their cargo and the RanGTP cooperatively. Therefore, cargo release from importins depends on RanGTP binding, while cargo release from exportins needs RanGTP hydrolysis. RanGTP levels in the nucleus are maintained by the guanine nucleotide exchange factor RCC1 or RanGEF. RanGTP is converted to RanGDP in the cytoplasm by the GTPase-activating protein RanGAP. NCT is thus an interplay between the NPCs, with a number of different NTRs recognizing cargo through sorting signals and a gradient of Ran in its GTP or GDP bound state.

1.2. Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurodegenerative disease that affects neurons in the brain and spinal cord. Amyotrophy is the degeneration of spinal motor neurons, which leads to denervation and muscle wasting. Lateral sclerosis refers to the scarring (sclerosis) of the lateral axons, which control voluntary movement [25]. In most cases, the cause for ALS remains unknown, but
5–10% of ALS cases are caused by autosomal dominantly inherited mutations [26], of which almost 40% in Caucasians are in C9orf72 [27]. Specifically, a G4C2 hexanucleotide is repeated up to thousands of times in C9-ALS patients [28,29], and translated via repeat-associated non-AUG (RAN) translation. This non-canonical initiation of translation allows elongation of a repeat sequence in the absence of an AUG starting codon, in multiple reading frames, thus leading to multiple dipeptide repeat proteins (DPRs) [30]. For C9orf72, the sense RNA encodes glycine-alanine (GA), glycine-proline (GP), and glycine-arginine (GR) repeat proteins. The antisense transcript produces proline-arginine (PR), glycine-proline (GP), and proline-alanine (PA) repeat proteins [31,32]. On a molecular level, C9-ALS disease is thus associated with multiple disease-related biomolecules, DNA, RNA, and DPRs. Hence, C9-ALS models introduce the G4C2 repeat DNA, or express or inject a single DPR of a certain length.

1.3. Nucleocytoplasmic Transport and C9orf72 ALS

A growing body of work suggests that changes in components of the nucleocytoplasmic transport machinery are related to neurodegenerative diseases (reviewed in [33–43]), but also specifically ALS. Firstly, the C9orf72 protein was found to interact with both importin-β1 and Ran-GTPase [44]. Moreover, effects on toxicity were reported; it was found that overexpression of RanGAP reduces toxicity of G4C2 repeats [45], downregulation of NTRs enhances G4C2 toxicity [46], and toxicity caused specifically by PR50—50 repeats of PR—is reduced by NTR overexpression [47]. An overview of NCT related genes that modify toxicity can be found in the accompanying review by Vanneste et al [48]. Furthermore, GA50 was reported to bind RanGAP1 in cytoplasmic inclusions [49], and poly-PR and -GR can directly bind to importin-β [50]. Moreover, the nuclear and cytoplasmic distributions of major protein components of NPCs were found to be altered in ALS [51]. Mislocalization or aggregation of NTRs has also been linked to C9-ALS (reviewed by Hutten and Dormann [39]) and mislocalization of NTRs and Nups has been shown in ALS post-mortem tissue and ALS models (reviewed in the accompanying review [48]). PR repeats are reported to directly bind to nuclear pores [52]. Further data linking altered NCT to C9-ALS include the following: nuclear RNA retention [46,52], mislocalization of Pom121 by poly-GA [49], reduced nuclear RCC1 (RanGEF) in C9orf72-carrier induced neurons [47], and increased cytoplasmic localization of neuronal RNA-binding protein Elav [53]. RanGAP was also found to be mislocalized in C9orf72-carrier induced neurons [47], but not in other models of C9orf72-associated neurodegeneration [47,54–56]. Thus, there is evidence from different model systems and experimental methods that nucleocytoplasmic transport components—NTRs, RanGAP, RanGEF, and Nups—are impacted in C9-ALS.

From the plethora of effects on NCT components, one would expect that the kinetics of NCT is impacted in C9-ALS. Establishing if the transport kinetics of NPCs in C9-ALS is altered is important, as a positive answer would suggest a common cause in ALS, i.e., mislocalization of many proteins, including ALS-related FUS and TDP43. A negative answer would suggest the NCT machinery is a robust system, and not causal to the phenotype of C9-ALS. This question has indeed been addressed in seven studies [45,50,52,55,57–59], which are summarized in Table 1; we included only those studies that make use of C9ALS models and mobile reporters. We did not include papers with full length proteins, as their localization may be impacted by other processes, such as retention. Table 1 shows the used reporters (i.e., usually a fluorescent protein with a specific NLS and/or NES sequence), the NTRs associated with the used NLS/NEs (importin-α/β complex, TNPO1 (Kapβ2), and CRM1), the C9-ALS model (i.e., introduction of repeat DNA, or expression or injection of a specific DPR), and the cell model. From a brief look at the joint data, it is not obvious if transport kinetics are altered in C9-ALS, as some experiments report changes, while others do not. To understand what one may conclude from the results from these diverse experimental setups and why there are seemingly inconsistent outcomes, we next discuss commonly used transport assays to then come back to the nuclear transport process in more detail to illustrate the complications in interpreting their outcomes.
Table 1. Transport studies of C9-ALS.

| Reporter (Localization in Wildtype) | NTR | Model | Effect of Indicated C9-ALS DNA Repeats or DPRs on TransportReporter |
|-----------------------------------|-----|-------|-------------------------------------------------------------|
| (A) NLSmCherry-mneon-GFP2x-NESPKI (nuclear) | Impα / β complex CRM1 | (G4C2)30 expression in C9-ALS fibroblasts | Recovery of nuclear fluorescence after FRAP is decreased |
| (B) NLSmCherry-mneon-GFP2x-NESPKI (nuclear) | Impα / β complex CRM1 | (G4C2)30 expression in HeLa Kyoto cells | Nuclear accumulation (N/C) is decreased (GR25) |
| (C) NLSmCherry-mneon-GFP2x-NESPKI (cytoplasmic) | Impα / β complex CRM1 | mCherry-DPR lentiviral expression in HeLa Kyoto cells | Nuclear accumulation (N/C) is decreased (dose-dependent and more so with PR20/GR20 than with PR10/GR10)†† Nuclear accumulation (N/C) is unchanged (PA10/GA10/GR10)‡‡‡ |
| (D) BSA-NLS(Sv40)n (nuclear) | Impα / β complex CRM1 | Peptide injection in U2OS cells | Nuclear intensity is decreased (PR20 dose-dependent) |
| (E) RFP-NLS(Fusion)(nNLS)(nuclear) | Impα / β complex CRM1 | DFR expression in HeLa cells | Cytoplasmic intensity (C/T) is increased (more so with GA149-GFP, than with GA149-GFP-NLS) |

Summary of Experimental Setup and Results of Nuclear Transport Studies in C9-ALS Models

| Reporter (Localization in Wildtype) | NTR | Model | Effect of Indicated C9-ALS DNA Repeats or DPRs on TransportReporter |
|-----------------------------------|-----|-------|-------------------------------------------------------------|
| (A) NLSmCherry-mneon-GFP2x-NESPKI (nuclear) | Impα / β complex CRM1 | (G4C2)30 expression in C9-ALS fibroblasts | Recovery of nuclear fluorescence after FRAP is decreased |
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* K to P mutation in the PKI-NES. † PR175-GFP was lower expressed than the GFF-GR149 and GA149-GFP. †† Reduced protein translation for GR100/PR100 expressing cells. ††† Passive diffusion of GFP and small fluorescent dextrans into the nuclei of permeabilized HeLa cells increased by PR10 and GR10, not by GP10, GA10, or PA10. ‡ Nuclear with dexamethasone. ‡‡ Nuclear accumulation and solubility of control GCR2-GFP2 reporter (lacking NLS) not altered.

References:
- Zeng 2015 [40]
- Shi 2017 [56]
- Liu 2016 [59]
- Vana 2018 [60]
- Hayes 2020 [61]
- Hutten 2020 [62]
1.4. Commonly Used Nuclear Transport Assays

The assays monitoring the kinetics of nuclear import and/or export generally use small inert reporters, such as GFP with a sorting signal, to control and limit reporter retention [60]. A GFP-NES or GFP-NLS reporter will passively pass the NPC down the concentration gradient owing to its small size, but additionally be actively transported owing to the NES/NLS [60]. Thus, its localization at steady state is the result of continuous nuclear import and efflux in the case of GFP-NLS, and import and influx in the case of GFP-NES (see Figure 1). The ratio between the fluorescence levels in the nucleus and cytosol provides the most common transport readout (the N/C ratio). The steady state localization of reporters encoding both an NLS and NES report on the ratio between import and export [61]. Provided that proper controls for the functionality of the NLS and NES are performed, this strategy has the advantage that, with a single cell line/model, one can monitor both import and export.

To measure actual rates of transport and influx/efflux, there are several strategies. Influx rates can be measured by monitoring the passive nuclear entry of larger cargoes, e.g., multimers of GFP, of proteinA, and of maltose-binding protein moieties [1,2]. The passive diffusion through the pores is affected by the overall size and shape of the protein, and its surface properties [62]. For example, surface histidines, cysteines, and arginines will facilitate the movement through the FG-Nup meshwork, and higher surface hydrophobicity increases the NPC passage [62]. Efflux and influx rates can also be measured using the GFP-NLS and GFP-NES reporters by inhibiting active import or export and following the subsequent loss of compartmentalisation. Import and export rates are derived from experiments where the import or export of GFP-NLS or GFP-NES reporters is temporarily disturbed, after which the re-establishment of the equilibrium can be quantified. Jointly, this collection of assays reports on the state steady state ratios between nuclear entry and exit, or yields the individual rates of entry and exit by temporarily altering the steady state.

There are several options to block import or export. CRM1 export can be inhibited by Leptomycin B (LMB) [63], a fungicide that binds CRM1 irreversibly in the NES binding region, thus blocking the binding between CRM1 and its cargo [64,65]. A limitation of the use of LMB may be its off-target effects; it also inhibits the degradation of p53 and upregulates apoptosis in cancer lines [66], limits RNA translation [67], and leads to accumulation of poly(A)$^+$ RNA within the nucleus [68]. Several import inhibitors are available that each block a specific transport route; e.g., importazole [69], ivermectin [70], and free importin-β-binding domain [50] inhibit the importin-α/β route, while the M9M peptide inhibits TNPO1 [71]. Alternatively, 2-deoxyglucose and/or sodium azide deplete cells from ATP, and can thus also be used to disrupt the RanGTP gradient. Depleting RanGTP inhibits major export and import pathways, but the different export routes are more sensitive to RanGTP depletion than the import via importin-α, importin-β, or transportin [72]. Especially in baker’s yeast, inhibition of import has been accomplished by anchor away strategies, such as anchoring of Kap95 to the plasma membrane ATPase using rapamycin inducible dimerization of FRB- and FKBP-domains attached to the NTR and the plasma membrane ATPase [73]. Methods that are performed at steady state and circumvent the use of drugs or poisons are based on fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). In import studies, the nuclear area is bleached, and the recovery of nuclear fluorescence, which relies on the exchange of fluorescent molecules from the cytosol with bleached molecules from the nuclear compartments, is taken as a read-out for import rates [74,75]. As all assays, except those based on FRAP and FLIP, are subject to possible side effects of the interventions inhibiting transport, a combination of interventions is advisable.

Commonly used nucleocytoplasmic transport assays are performed in permeabilized cells, in live cells, or in in vitro assembled oocyte nuclei, and are listed in Table 2. A much used in vitro NCT assay uses digitonin permeabilized cells supplemented with exogenous recombinant transport factors [76]. This assay yields nuclei with functional NPCs.
while allowing to introduce the reporter proteins and NTRs at known concentrations and timepoints. The disadvantages are that permeabilization can relocate or extract soluble proteins [77] including transport factors and ATP [76], and potentially alter transport kinetics. In order to limit method artefacts, permeabilization assays are preferably combined with live-cell imaging [77]. The simplest transport measurements in live cells are those that determine the steady state distribution of GFP-NLS or GFP-NES reporters. Alternatively, as discussed above, the kinetics can be determined either based on the use of poisons to alter the steady state, or based on FRAP or FLIM. Lastly, like permeabilized cells, in vitro assembled oocyte nuclei also provide a system where reporter proteins and NTRs can be introduced at known concentrations and timepoints aiding kinetic measurements. Xenopus egg extracts contain the structural components of nuclei in a disassembled state, but will reconstitute nucleus-like structures around added DNA [78]. These nuclei actively exclude nonnuclear proteins and accumulate nucleoplasmin and proteins with NLSs [79–81], and import can be inhibited with lectin wheat germ agglutin, which binds to glycosylated FG-Nups [82]. Here, a combination of transport models may also provide the best assessment of the question at hand.

Table 2. Benefits and drawbacks of commonly used transport assays.

| Cell permeabilization                                                                 | In vivo N/C ratios; use of inhibitors                                                                 |
|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|
| + Allows addition of specific concentration of cargo/NTR [76,83]                    | + N/C ratio is a robust read-out largely independent of reporter levels [85]                          |
| - Permeabilization can relocate or extract soluble proteins [77] including transport factors/ATP [76]; might deplete NTRs from NPCs; and reduces concentrations of competitive cargo, which increases import rates [84] | - Expression/injection of reporter might be toxic; use of inhibitors to measure rates of nuclear entry and exit might be toxic |
| In vivo FRAP/FLIP                                                                   | In vivo FRAP/FLIP                                                                                     |
| + Direct measurement of kinetics of nuclear entry and exit; area under the curve indicates mobile fraction of the reporter | + Proteins can be added to/immunodepleted from the extract                                               |
| - Expression/injection of reporter might be toxic; photobleaching induces ROS        | - No native cytosolic fraction                                                                      |
| - Allows studying nuclear pore complex assembly, function [86], transport [87,88], and cargo competition [89] | - Allows studying nuclear pore complex assembly, function [86], transport [87,88], and cargo competition [89] |

2. Nuclear Transport Up-Close: Complications When Interpreting Nuclear Transport Data

NCT assays rely on monitoring the localization of proteins to either the nuclear or cytosolic compartment (Figure 1). A change in localization can report a change in import or export efficiency by the joint action of NPCs, NTRs, and Ran. The interpretation of what drives the change in import or export efficiency and the contribution of each specific component (NPCs, NTRs or Ran), however, is often complicated for a number of reasons.

First, cargoes are transported by dedicated transporters, which recognize their cargo via particular NLSs and/or NESs. However, often, a simple “one cargo–one NTR” relation does not exist, as specific NTR-isoforms show preference for certain NLSs, several NTRs can be redundant for one specific NLS, and a protein or protein complex can contain multiple localization sequences (see Table 3 for examples). NLS sequences are, among others, the classical NLS, which has a mono- or bipartite basic amino acid cluster [90]; the
PY-NLS; and a RG-rich NLS [91]. Some importins have their own preferred sequences, such as yeast Kap121, which recognizes a non-conventional lycine-rich sequence [92]. There is not yet a full overview in which sequences are recognized by the importins, but attempts are ongoing to discover more NLSs [93]. The full complement of interactions between NTRs and cargoes in a specific cellular setting has not been established to date, hence a change in localization of one cargo cannot easily be connected to the activity of a specific NTR or NTR isoform.

Second, import rates depend on the availability of the NTRs [84,94,95]. The formation of a complex of NTR and cargo in the crowded cytosol is rate-limiting compared with the fast translocation through the NPC, which is reported to occur on the timescale of milliseconds for commonly used reporters [96]. Therefore, altering the concentration of NTRs impacts transport rates. For example, Kap121 and Kap123 recognize the same NLSs, and when expressed at similar concentrations, import rates are indistinguishable [84]. However, natively, the abundance of Kap123 is higher than that of Kap121, thus making Kap123-mediated import faster [84]. Import rates eventually saturate at high NTR levels, which must be owing to saturation of the NPCs, the RanGTP transport complex dissociation, or both [84]. The expression or availability of NTRs can change under stress conditions. For example, the importin Hikeshi, which uses ATP to import Hsp70 to limit shock-induced damage, is specifically upregulated under heat stress [16]. Moreover, conditions of oxidative stress and heat shock lead to rapid accumulation of Impα in the nucleus [97,98], and arsenite stress accumulates Impα in stress granules [99]. To explain a change in the localization of a reporter mechanistically, one would want to know whether the intervention, e.g., the introduction of DPRs or G4C2 DNA sequences, created a change in the levels of NTRs available for transport.

Table 3. Transport specificity and redundancy.

| I. Transport of Cargo Depends on a Localization Sequence that is Recognized by an NTR |
|---------------------------------|----------------------------------------------------------------------------------|
| NTR                            | NLS/NES                                                                                      |
| Impα/β                         | Sv40/cNLS: PKKKRKV [90]                                                                      |
|                                 | cMyc: PAAKRKVL [100,101]                                                                    |
|                                 | Nucleoplasmin: KRPAATKKAGQAKKKK [102]                                                       |
| TNPO1                          | PY-NLS: R-X_{3,5}-PY motif [103]                                                            |
|                                 | e.g., FUS: RG-rich_{46}RQDRRERPY [104]                                                      |
| Kap104                         | RG-rich motif [91]                                                                           |
| Kap121                         | Lycine-rich sequence [92]                                                                    |
| Attempts to discover more NLSs |                                                                                              |
| CRM1                           | Φ-X_{2,3}-Φ-x_{2,3}-Φ-x-Φ; where Φ is I, M, F, V, and mostly L [105,106]                    |
|                                 | PKI: e.g., MSLNELALKLAGLDI [58]                                                              |
|                                 | HIV-1 Rev: LQLPPLERLT                                                                      |
| II. Redundancy in NTRs for One Type of NLS |                                                                                           |
| NTR1                           | NLSi-Cargo1                                                                                   |
| NTR2                           | NLSi-Cargo1                                                                                   |
|                                      | Different NTRs can recognize the same NLS, and thus transport the same cargo; e.g., Trm1, Trm2A, and Trm2B all bind the PY-NLS, and transport FUS [107]/HuR [108], as well as unique cargoes. |
| NTR1a                          | NLSi-Cargo1                                                                                   |
| NTR1b                          | NLSi-Cargo2                                                                                   |
|                                      | Different isoforms of one NTR may bind the same NLS, but transport other cargoes; e.g., importin-α isoforms bind the classical NLS, but, e.g., STAT1 is bound by α5, but not by α1 [109]. Importin-α3 only recognizes the NLS with appropriate N-terminal flanking residues [110]. |
| III. Redundancy in NTRs for One Type of Cargo |
| NTR1                           | NLSi-Cargo1                                                                                   |
| NTR2                           | NLSii-Cargo1                                                                                   |
|                                      | A group of cargoes, with distinct NLSs, can be recognized by different NTRs; e.g., Kap121 can substitute Kap123 for import of ribosomal proteins [111,112] |
Table 3. Cont.

| IV. Combinations of Localization Sequences |
|-------------------------------------------|
| **NTR1** | NLSi-NLSii-Cargo1 | One cargo may have two different NLSs; e.g., Nxf1 contains a cNLS for importin 4/11/α/β and a PY-NLS for Trn1/Trn2 [113–117]. |
| **NTR2** | NLSi-NLSii-Cargo1 | |
| **NTR1** | NLS-NES-Cargo1 | One cargo may contain an NLS and an NES; cyclin B [118,119] and HIV Rev [105,120] contain an impβ1 NLS and a CRM1 NES. |
| **NTR2** | NLS-NES-Cargo1 | |
| **NTR1** | NES-adaptor Cargo1 | Ribosomal pre60S particles bind to an adaptor protein (NMD3), which contains the NES [121,122]. |
| **NTR2** | | |

Third, import rates also depend on the affinity between NTR and NLS, where over a certain range, a higher affinity will give a higher import rate [84,85,123]. However, a too high binding affinity prevents NTR-cargo complex dissociation and, consequently, inhibits transport [124,125]. Affinities can be temporarily altered by post-translational modifications (PTM), with phosphorylation of NTRs [126,127] and NLSs [128,129], the most studied PTM regulating NCT [130,131]. For example, transcription factors are only required for short periods of time and in small amounts, and sequences close to their NLS are phosphorylated to increase the affinity for importins [128,129] or cause retention in nucleus or cytoplasm [132]. However, methylation [133,134], ubiquitination [135–137], acetylation [138], and sumoylation [139] have also been shown to influence NTR binding. NTR binding can also be altered by physicochemical parameters, as is the case for ROS-induced formation of disulfide bonds between TRN1 and FOXO [140], which is reversed in the more reduced environment of the nucleus [141]. Proteolytic events can unmask NLS and NES sequences [142–144], whereas binding to another protein [145–147] or mRNA [148], or protein multimerization [149,150], can bury sorting sequences. To explain a change in the localization of a reporter mechanistically, one would want to know whether the affinity of the NTR for the NLS is altered. This is often not trivial to determine as the affinities determined with purified protein can be different from those determined in cytoplasmic extract [151] or those observed when multiple cargoes are presented simultaneously [152].

A fourth, and major, complication to interpret what drives the change in import or export efficiency is related to the fact that nuclear import of a specific heterologously expressed transport reporter (Figure 1) is always in competition with the transport of native cargoes. Because most NTRs have more than one specific cargo to transport, an increase in any of the cargoes of that NTR leads to lower N/C ratios of all its cargoes, including the reporter used to study transport [75,89]. Additionally, more cargo for one NTR can limit RanGTP availability to all NTRs, and slow down the recycling of importins and export. These aspects are easily overlooked when interpreting NCT data, but can lead to the false conclusion that the import kinetics by one specific NTR is reduced in a disease model compared with a wild type situation, while all that is changing is the concentration of competing cargo.

Lastly, it should be taken into account that changes in the localization of a reporter can be the result of changes to the NPCs themselves, both in number and in functionality. Recently, a disease-related reduced nuclear abundance of key Nups was shown in C9ALS and sporadic ALS iPSNs [153]. Theoretical considerations predict that fewer NPCs will support a higher accumulation of a GFP-NLS reporter in the nucleus [154], as the number of NPCs is likely limiting for efflux, but not for import [84,89]. Additionally, the deletion of one nucleoporin immediately translates to reduced export [155–161], but because the rate limiting step for import is receptor binding [95,154,162], cells still maintain high import rates even when FG-domains in the NPCs are deleted [5,163]. Specific NTRs preferentially bind specific FG-Nups, hence the presence or absence of certain FG-Nups may affect only specific import routes [6,164,165]. PTM has been reported to change the structure of pores. For example, in yeast, phosphorylation of Nup53 releases its binding partner Nup170, thus exposing binding sites for Kap121 on Nup170, limiting Kap121 import directly [166], a process that may also underlie reduced transport by TRN-1 and Kapβ [127]. Knowing the number and functionality of individual NPCs is a major challenge, and together with
the previously mentioned complications of mapping the full complement of NTRS and cognate cargoes in a cell, these aspects provide the largest challenge in interpreting what underlies a change in the localization of a NCT reporter.

To summarize, in order to assess if the transport of proteins is altered in a disease model, well controlled steady state, or dynamic measurements of the location of mobile, non-toxic reporter proteins suffice. Preferably, multiple NLS- or NES-containing reporters representing different transport pathways are used. To understand what drives a possible change in the transport of these reporters, several additional, and rather complex, questions have to be addressed (summarized in Figure 2). In case of the outermost layers, we enter into what is presently still largely uncharted scientific territory.

All considerations above apply when comparing data obtained using different model systems. Nucleoporin expression changes between different cell types, tissues, and disease-states [167–171], as well as during development and ageing [172,173]. Moreover, the abundance of the NTRs differs between cell types. For example, Kapα levels differ between leukaemic cell lines [151] and between testis, ovary, spleen, heart, kidney, brain, or muscle cells [152,174–177]. Further, whereas TRN2A is expressed in HeLa cells, it is undetectable in HEK293T cells [108]. Kapα2 [178–182] and CRM1 [182–189] have been shown to be upregulated in different types of cancers, and NTR expression is regulated during development [190,191] and differentiation [192,193]. Thus, there is cell type variability of NPCs and cognate nuclear transport factors, which complicates the comparison of results from different studies.

For the studies related to C9-ALS, a further complication in the comparison of results is related to the fact that the DPRs impact cell physiology variably in the used biological systems as they localize differently and are toxic to different extents. In Table 4, we...
summarize the localization of different DPRs in different model systems and highlight whether they convey growth defects (indicated in different colours). For example, poly-PR is generally the most toxic DPR and PA is often non-toxic; this is the case in iPSC neurons [194], *S. cerevisiae* [47], rat neurons [194], mouse neurons, and HEK293 cells [195]. Yet, in zebrafish embryos, PR is less toxic than GR and equally toxic to PA [196]. PR is rarely found in patient material, usually attributed to its high toxicity, but if it is detected, it is found in aggregates in the cytoplasm [197,198]. This localization is not observed in most model systems where PR is nuclear or nucleolar [194,195,199–202]. The reports on poly-GA are diverse, reporting non-toxic [46,47,53,194–196,199,200,203,204] as well as toxic [49,200,205,206] effects. Interestingly, it seems that the toxicity of DRPs increases when cells are further differentiated into neurons [207]. Thus, even independent of the discussed cell type specific differences in the nuclear pores and cognate transport factors, the effect of DPRs on transport in the different C9-ALS models may be variable owing to differences in localization and toxicity.

3. Is Nuclear Transport Altered in C9-ALS?

The seven papers on NCT in C9-ALS [45,50,52,55,57–59] jointly report on 21 experiments assessing the localization of transport reporters in different C9-ALS models (Table 1). Clearly, despite obtaining solid and significant data, it is still difficult to conclude whether the data unambiguously establish that actual effects on NCT kinetics are observed when introducing C9-ALS-related peptides or DNA. How can six papers convincingly show alterations in the localization of transport reporters [45,50,52,55,57,59], whereas the seventh extensively shows no effects can be observed [58]?

Cell type-specific characteristics. When discussing several complications in the interpretation of NCT data, we mentioned cell type-specific transport characteristics, such as distinct NLS–NTR affinities or NTR abundances. This complication is clear when comparing the outcomes of the experiments in B and D (Table 1, for all further references in this section, see Table 1); the same reporter that is excluded from the nucleus in wild type motor neurons localizes to the nucleus in wild type *Drosophila* salivary glands. The same is observed comparing wild type HeLa Kyoto cells (L) and neuronal cells (N), where the reporter localizes differently in both cell types. These differences depending on cell type are also observed when specific DPRs are introduced. Namely, PR<sub>20</sub> peptide injection reduces importin-α/β import in U2OS cells (F), but not in HeLa Kyoto cells (J). Similarly, poly-GA impacts the transport of an NLS<sub>Sv40</sub>-mNeonGreen<sub>2x</sub>-NES<sub>PKI</sub> reporter in neuronal cells (N), but not in HeLa Kyoto cells (L). There may even be subpopulations within a cell type that behave differently. For example, when a subpopulation of cells with stress granules is selected, transport alterations are observed for (L), whereas this was not seen in the whole population [58]. This may be linked to the fact that arginine-rich DPRs can undergo liquid–liquid phase separation, and induce phase separation of a large set of proteins involved in RNA and stress granule metabolism [208]. Jointly, the cell type-specific localization of a reporter in wild type cells illustrates that cell type-specific differences in, among others, NPCs and cognate transport factors impact the transport assays (Table 1, compare L–N, B–D). In addition, the introduction of C9-ALS models adds to the variability between cell types (Table 1, compare F–J, N–L), possibly through the mentioned differences in localization and toxicity (Table 4).

Sorting signals. When discussing the different transport assays available, their possible technical complications were mentioned. A potential issue when using reporters with multiple localization signals relates to the fact that GFP has an intrinsic tendency to accumulate in the nuclear compartment [62,209], irrespective of the presence of an NLS. Nuclear localization of a GFP-based reporter may thus not be driven by import, but rather by influx. For example, the data suggest that the Sv40NLS is not very effective in motor neurons, as it does not promote nuclear accumulation when the NES is removed (E). Without controls, we do not know whether the nuclear accumulation of a reporter is due to import or influx, and the impact of DPRs thus might be on influx rather than active
transport. Determining the effect is complicated, as Hayes et al. show that passive diffusion of GFP and small fluorescent dextrans into the nucleus, and thus influx of molecules is increased by PR$_{10}$ and GR$_{10}$ [50]. Validating the functionality of the sorting signals in reporters with both NLS and NES sequences is thus important to prevent changes in influx being misinterpreted as changes in import.

Transport assays. Changes in transport induced by the introduction of C9-ALS models are quantified in different ways, reporting either the nuclear fluorescence or the cytosolic fluorescence (A–G) or a ratio relative to the total (H–N) or cytosolic (O–P) fluorescence. Measuring the steady state fluorescence in the nucleus relative to the cytosol or total fluorescence is less sensitive to changes in expression level than other readouts such as nuclear (or cytosol) fluorescence alone. This may have impacted the interpretation in this case as poly-PR (and poly-GR) can limit translation [58,204,210]. Hence, it is possible that the expression of the reporter is reduced in the C9-ALS-models (B,C), which caused a lower nuclear fluorescence, rather than an effect on import.

Mobile reporters are the basis of all transport assays, and a quantification of the mobile fraction in FRAP experiments can be used to confirm that they are indeed mobile. However, it is the rate of recovery after bleaching in the nuclear or cytosolic compartment that is a measure for the kinetics of transport. The experiment in (A), for example, measures the change in mobile fraction, rather than actual transport kinetics.

Regarding the choice of transport assays (Table 2) and reporter, we mentioned that it is preferable to assess different transport reporters when studying NCT, as different NTRs each represent unique import pathways with unique parameters—affinities for FG-Nups, expression levels, and competing cargoes—defining their import kinetics. This is illustrated in (H,I) where the localization of a TNPO1 cargo is insensitive to GA$_{149}$, while that of an importin-α/β is sensitive. Moreover, each transport assay has advantages and disadvantages (Table 2), and even though cell permeabilization assays (G) and injections (F,G,J,K) are used frequently in the field, it would be helpful to combine permeabilized studies with live-cell imaging.

Jointly, in the case of these C9-ALS studies, several technical limitations may have impacted the interpretation of the data summarized in Table 1. Hence, at present, it is not firmly established whether the introduction of disease-related biomolecules, G4C2-DNA, RNA, and DPRs, impacts the transport kinetics by NPCs.

Thus, even though convincing data have been obtained confirming a relation to the NCT machinery in C9-ALS in general, it is difficult to conclude from the current transport data if and how C9-ALS affects the actual kinetics of NCT. For future studies, the choice of model systems is critical, both from the perspective of the diverse behaviour of C9-ALS related DPRs (Table 4) and the diversity in the characteristics of NCT in different cells types. Moreover, to facilitate the interpretation of transport assays mechanistically, one would ideally quantify all nucleocytoplasmic transport components (NTR abundance, NPC number and functionality, and the integrity of the Ran gradient), study different NTRs, and combine different transport assays. These in-depth studies are required to answer whether NCT is changed in C9-ALS, and potentially many proteins are mislocalized, leading to cellular alterations causing the disease, or, alternatively, that the NCT machinery is a robust system, and widespread mislocalization of nuclear proteins should not be expected to occur or be causal to the phenotype of C9-ALS. The challenges described are not unique to the case of C9-ALS, and answering the question of how NCT is impacted in neurodegenerative diseases represents a major opportunity for scientists from the nuclear transport and neuroscience communities.
| Cell Type       | PR                          | FA                          | GP                                      | GR                          | GA                          |
|-----------------|-----------------------------|-----------------------------|-----------------------------------------|-----------------------------|-----------------------------|
| Patients        |                             |                             |                                         |                             |                             |
| Frontal cortex  | Cytoplasmic aggregation     | Cytoplasmic aggregation     | Cytoplasmic, paranuclear aggregation    | Cytoplasmic aggregation     | Cytoplasmic, paranuclear     |
|                 | [197,198]                   | [198]                       | [197]                                   | [197]                       | inclusions [205]            |
|                 | (Rare)                      | (Rare)                      | (Rare)                                  | (Rare)                      | (Rare)                      |
|                 | Sparse: Cytoplasmic         | Sparse: Cytoplasmic         | Numerous: Cytoplasmic, cytoplasmic     | Moderate: Cytoplasmic       | Numerous: Cytoplasmic,      |
|                 | inclusions, cytoplasmic     | inclusions, cytoplasmic     | granular, or intranuclear inclusions   | inclusions or intranuclear  | cytoplasmic granular, or    |
|                 | [211]                       | [211]                       | inclusions [211]                        | inclusions [211]            | intranuclear inclusions     |
|                 | (Rare)                      | (Rare)                      | (Rare)                                  | (Rare)                      | (Rare)                      |
| Patients spinal | Nucleolar and extra-nuclear | Nucleolar and extra-nuclear | Nucleolar aggregation, cytoplasmic     | Nucleolar                   | Nucleolar                   |
| chord           | inclusions [196]            | inclusions [196]            | diffuse [210]                           | diffuse, plus nuclear       | diffuse, plus nuclear       |
| iPSC neurons    | Nuclear [196]               | Nuclear [196]               | Diffuse cytoplasmic, plus nuclear       | Nucleolar                   | Nucleolar                   |
| Neurona cells   | Cytoplasmic and nucleus     | Cytoplasmic and nucleus     | Nucleolar aggregation, cytoplasmic      | Cytoplasmic aggregation     | Cell(ol)ary                  |
|                 | [215]                       | [215]                       | diffuse [215]                           | [196,207]                   | aggregation                |
| Rat neurons     | [195,207]                   | [195,207]                   | [196,207]                               | [196,207]                   | [195,207]                   |
| Mouse neurons   | Cytoplasmic diffuse plus    | Cytoplasmic diffuse         | Nucleolar                              | Nucleolar                   | [195,207]                   |
|                 | inclusions [194,213]        | Cytoplasmic diffuse         | diffuse [202]                          | diffuse, preferentially     | diffuse, preferentially     |
|                 | (Rare)                      | (Rare)                      | (Rare)                                  | nuclear                   | nuclear                     |
| NCS34 cells     | Nuclear aggregation         | Cytoplasmic aggregation     | Cytoplasmic diffuse, plus nuclear       | Cytoplasmic, occasionally   | Nucleolar                   |
| (mouse motor    | [196,207]                   | [194,207]                   | diffuse, plus nuclear                   | nuclear aggregation        | diffuse, plus nuclear       |
| neurons)        |                             | Cytoplasmic diffuse         | diffuse cytoplasmic, plus nuclear       | Cytoplasmic diffuse         | diffuse, plus nuclear       |
|                 |                             | Cell(ol)ary aggregation     | cytoplasmic, occasionally nuclear       | Cytoplasmic diffuse         | diffuse, plus nuclear       |
|                 |                             | aggregation                | [196,207]                               | [196,207]                   | [196,207]                   |
| Differeniated   |                             |                             |                                         |                             |                             |
| SH-SY5Y cells   | Nucleolar                   | Nucleolar                   | Nucleolar                               | Nucleolar                   | Nucleolar                   |
| (neuroblastum)  |                             |                             | diffuse, preferentially nuclear         | diffuse, preferentially     | diffuse, preferentially     |
| HER20 cells     |                             |                             | Cell(ol)ary aggregation                 | diffuse, preferentially     | nuclear                     |
| (cancer cells)  |                             |                             | aggregation                             | nuclear                     | diffuse, plus nuclear       |
|                 |                             |                             | Nucleolar                               | Nucleolar                   | diffuse, plus nuclear       |
| Hela cells      | Nucleolar                   | Nucleolar                   | Nucleolar                               | Nucleolar                   | Diffuse cytoplasmic, plus  |
| (cancer cells)  |                             |                             | diffuse, preferentially nuclear         | diffuse, preferentially     | nuclear                     |
| K562 cells      |                             |                             | Cell(ol)ary aggregation                 |                          | Diffuse cytoplasmic, plus   |
| (cancer cells)  |                             |                             | aggregation                             | Cell(ol)ary aggregation     | nuclear                     |
| U2OS cells      |                             |                             | Cell(ol)ary aggregation                 |                          | Cell(ol)ary aggregation     |
| (cancer cells)  |                             |                             | aggregation                             | Cell(ol)ary aggregation     | aggregation                |
| CHO cells       |                             |                             | Cell(ol)ary aggregation                 |                          | Cell(ol)ary aggregation     |
| (Chinese hamster |                             |                             | aggregation                             | Cell(ol)ary aggregation     | aggregation                |
| kidney cells)   |                             |                             | aggregation                             | Cell(ol)ary aggregation     | aggregation                |
| COS cells       |                             |                             | Cell(ol)ary aggregation                 |                          | Cell(ol)ary aggregation     |
| (fibroblast-like |                             |                             | aggregation                             | Cell(ol)ary aggregation     | aggregation                |
| monkey kidney   |                             |                             | aggregation                             | Cell(ol)ary aggregation     | aggregation                |
| cells)          |                             |                             | aggregation                             | Cell(ol)ary aggregation     | aggregation                |
| C. elegans (mus |                             |                             | Cell(ol)ary aggregation                 | Cell(ol)ary aggregation     | aggregation                |
| cell)           |                             |                             | aggregation                             | aggregation                 | aggregation                |
| D. melanogaster |                             |                             | Cell(ol)ary aggregation                 |                          | Cell(ol)ary aggregation     |
| S. cerevisiae   |                             |                             | Cell(ol)ary aggregation                 |                          | Cell(ol)ary aggregation     |
| Zebra fish      |                             |                             | Cell(ol)ary aggregation                 |                          | Cell(ol)ary aggregation     |

Colour coded toxicity from red meaning highly toxic, to green denoting non-toxic, as specified in each paper (not comparatively between model organisms). Reference to toxicity is in the right lower corner.
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Abbreviations

ALS amyotrophic lateral sclerosis
C9-ALS C9orf72-related ALS
DPRs dipeptide repeat proteins
FG phenylalanine glycine repeat
FLIP fluorescence loss in photobleaching
FRAP fluorescence recovery after photobleaching
FUS fused in sarcoma
Ga, GP, GR, PA, PR glycine-alanine, glycine-proline, glycine-arginine, proline-alanine, proline-arginine repeats
LMB leptomycin B
NES nuclear export sequence
NLS nuclear localization sequence
NP/NMP1 nucleophosmin
NPC nuclear pore complex
NTR nuclear transport receptor
Nups nucleoporins
PKI cAMP-dependent protein kinase inhibitor
PTM post-translational modification
RAN translation repeat-associated non-AUG translation
Ran Ras-related nuclear protein
RanGAP Ran GTPase activating protein
RanGEF Ran guanine nucleotide exchange factor
RBP RNA binding protein
ROS reactive oxygen species
SV40 simian virus 40
Rev human immunodeficiency virus type 1 Rev protein
TDP-43 TAR-DNA binding protein 43
TNPO1 Kap β2
TRN1 transportin 1

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