Dissertation der Graduate School of Systemic Neurosciences der Ludwig-Maximilians-Universität München

Identification of the nuclear export pathways of TDP-43 and FUS

Ph.D. (Doctor of Philosophy) Thesis

HELENA EDERLE

Born in Kisel, Russia

21st December 2017

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Dedicated to

ANGELA and JOHANNES WINKLER

ABBREVIATION INDEX

ALS	Amyotrophic lateral sclerosis
C-Terminus	Carboxy-terminus
C9orf72	Chromosome 9 open reading frame 72
CRM1	Chromosome region maintenance 1
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
hnRNP	Heterogenous nuclear ribonucleoprotein
hnRNP-C	Heterogenous nuclear ribonucleoprotein C1/C2
lncRNA	Long non-coding RNA
mRNA	Messenger RNA
mRNP	Messenger ribonucleoprotein
miRNA	MicroRNA
N-Terminus	Amino-Terminus
NES	Nuclear export signal
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NUP	Nulceoporin
PY-NLS	Proline – tyroline nuclear localization signal
RBP	RNA-binding protein
RGG	Arginine (A) – glycine (G) – glycine (G)
RNP	Ribonucleoprotein
RRM	RNA-recognition motif
TDP-43	TAR DNA-binding protein of 43 kDa
UTR	Untranslated region
XPO1	Exportin-1

* List of most common abbreviations used in the manuscript

INDEX

Identification of the nuclear export pathways of TDP-43 and FUS

ABSTRACT

TDP-43 (TAR DNA-binding protein of 43 kDa) and FUS (Fused in sarcoma) are nuclear DNA/RNA-binding proteins that play a key role in ALS (Amyotrophic lateral sclerosis) and FTD (Frontotemporal dementia). In ALS and FTD patients both proteins accumulate in insoluble cytoplasmic inclusions, and it has been shown that defects in nuclear import critically contribute to this pathology. As intranuclear inclusions of TDP-43 and FUS are occasionally found in ALS and FTD patients, it can be speculated that defects in nuclear export of TDP-43 and FUS might also contribute to the pathogenesis of ALS and FTD. However, the mechanisms of nuclear export of TDP-43 and FUS are currently unknown. This thesis was aimed to identify the nuclear export routes of TDP-43 and FUS, in order to address whether the involved factors or signals are altered in ALS and FTD patients.

I could show that both proteins undergo rapid nucleocytoplasmic shuttling independent of predicted putative nuclear export signals (NESs) and the export receptor Exportin-1/CRM1. I could furthermore show that both proteins exit the nucleus independent of RNA-binding and mRNA export factors, excluding export along with bound mRNAs as possible exit route. Moreover, mutation or deletion of various functional domains of TDP-43 and FUS as well as a siRNA-mediated knockdown of another putative export receptor, Exportin-5, did not inhibit nuclear export of both proteins. However, I found that substantial enlargement of TDP-43 and FUS prevented their nuclear egress, suggesting that both proteins leave the nucleus by passive diffusion. Furthermore, nuclear egress of TDP-43 by passive diffusion was enhanced by transcriptional inhibition or mutations that abrogate RNA-binding of TDP-43.

I. INTRODUCTION

1. Amyotrophic lateral sclerosis and frontotemporal dementia: TDP-43 and FUS in neurodegenerative diseases

The neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are characterized as rare diseases with a low prevalence (1-9/100 000). Amyotrophic lateral sclerosis, *inter alia*, known as Lou Gehrig´s or Charcot´s disease, is a neuromuscular disease that causes a progressive death of motor neurons in the cortex, brainstem and spinal cord. This degeneration of motor neurons leads to fatal paralysis and patients eventually die of respiratory failure within 1-3 years after disease onset (Rothstein 2009).

Frontotemporal dementia is the most common type of dementia in the presenile age group (<65 years) that is caused by neuronal death in the frontal and temporal cortical lobes. The clinical symptoms of FTD include a spectrum that ranges from lingual, behavioral to locomotor dysfunctions. Patients suffering from frontotemporal dementia die 7 – 10 years after disease onset (Rademakers, Neumann et al. 2012). ALS and FTD are fatal diseases with currently no available cure.

In 2006, TAR DNA-binding protein of 43 kDa (TDP-43) was identified as the main component of insoluble protein inclusions found in ALS and FTD cases (Neumann, Sampathu et al. 2006). A few years later, protein inclusions containing FUS (Fused in sarcoma) were characterized in a subset of ALS and FTD patients (Neumann, Rademakers et al. 2009). These findings proposed defective RNA metabolism as a common pathomechanism underlying both diseases that could explain why they show an overlap in their clinical pathology (Lillo, Savage et al. 2012). Meanwhile, point mutations in *TARDBP* and *FUS*, the genes encoding for TDP-43 and FUS, were shown to cause dominantly inherited types of ALS and occasionally FTD, suggesting a causal link between TDP-43 and FUS pathology and disease pathogenesis (Lagier-Tourenne, Polymenidou et al. 2010).

2. Nuclear import defects in amyotrophic lateral sclerosis and frontotemporal dementia

In healthy neurons, TDP-43 and FUS are predominantly localized in the nucleus. In addition, small amounts of TDP-43 and FUS can be found in the cytoplasm. However, in glial and neuronal cells of ALS and FTD patients TDP-43 and FUS aggregate in cytoplasmic and occasionally intranuclear protein inclusions (see Figure 1).

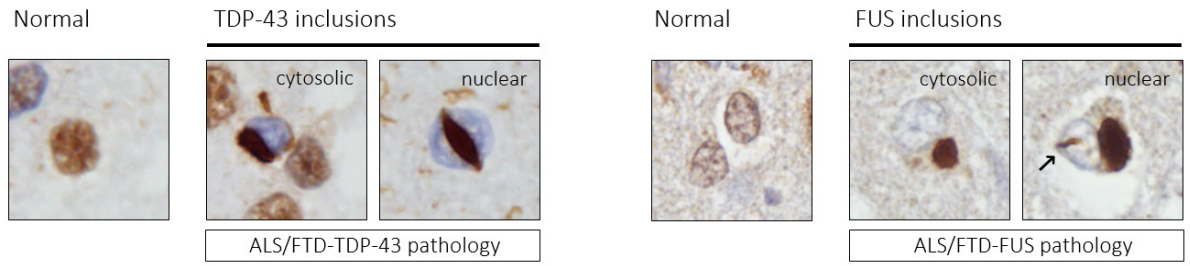


Figure 1. Inclusions of TDP-43 and FUS in ALS and FTD post-mortem brain sections (modified from Ederle and Dormann 2017). TDP-43 and FUS predominantly localize in the nucleus (normal, left panels). In ALS and FTD-affected brains, TDP-43 and FUS aggregate in cytoplasmic and nuclear inclusions (pathology, right panels).

In case of FUS, it was shown that defective nuclear import is responsible for the cytoplasmic mislocalization and neurodegeneration. First, it was demonstrated that mutations in the non-classical proline tyrosine-nuclear localization signal (PY-NLS, see Figure 3) at the C-terminus of FUS disrupts binding to the nuclear import receptor Transportin and thus disturbs proper nuclear import of FUS (Dormann, Rodde et al. 2010). Importantly, Dormann and colleagues determined a correlation between the grade of FUS mislocalization and the clinical phenotype; whereat they could show that patients carrying point mutations that result in severe import defects, such as P525L, suffer from an early disease onset and a rapid disease progression, suggesting that nuclear import defects critically contribute to disease pathogenesis. Mouse models harboring mutations or deletions of the FUS NLS develop age-dependent motor neuron degeneration, finally providing evidence that defective nuclear import of FUS causes neurodegeneration (Sharma, Lyashchenko et al. 2016, Scekic-Zahirovic, Oussini et al. 2017). To explain how mislocalized FUS in the cytoplasm may start to aggregate, “a two hit model of FUS pathology”, later on revised as a “multiple hit model”, was proposed (Dormann and Haass 2011). This model postulates that mislocalization of FUS, driven by import defects, is not sufficient to result in persistent cytoplasmic protein inclusions as seen in ALS and FTD patients. In addition, a second or even a third hit (*e.g.* cellular stress or genetic defects) is required in order to drive FUS in cytoplasmic stress granules that presumably function as precursor of pathological protein inclusions (see Figure 2).

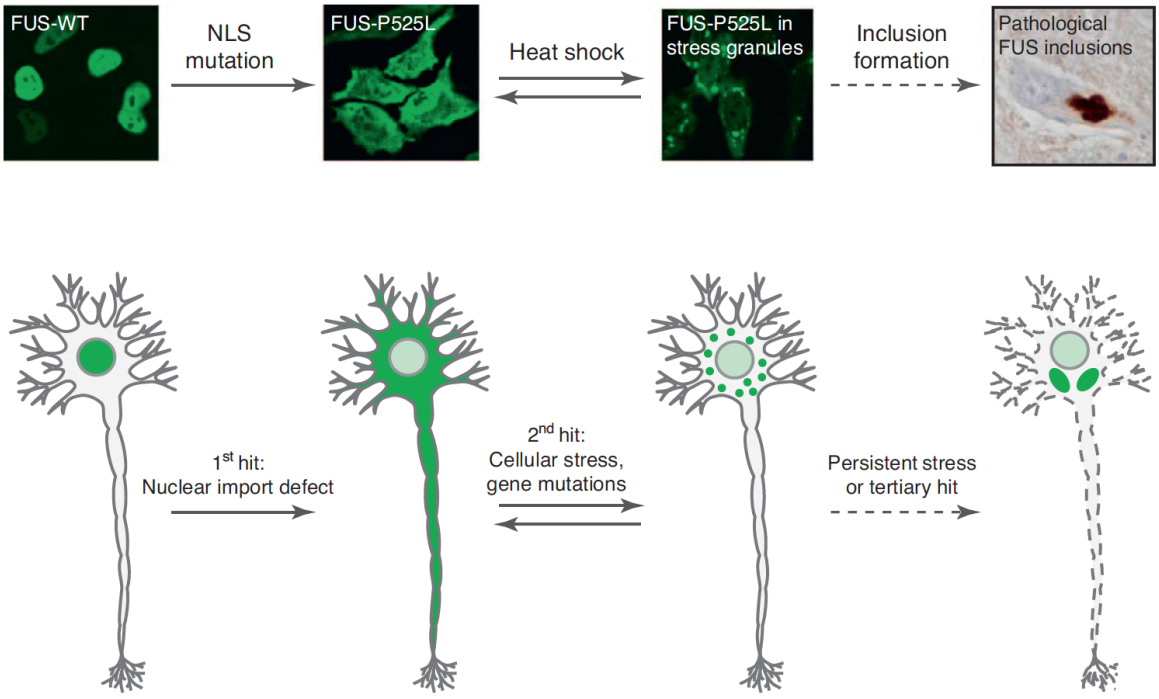


Figure 2. Multiple hit theory as a potential explanation for cytoplasmic FUS inclusions (modified from Dormann and Haass 2011). Normally, FUS is localized within the nucleus (WT). Mutations within the proline-tyrosine nuclear localization signal (PY-NLS, *e.g.* P525L) cause a nuclear import defect that leads to a cytoplasmic redistribution of FUS (first hit). Cellular stressors, such as heat shock, recruit the mislocalized protein into stress granules (second hit) that might represent the preliminary stage of pathological FUS inclusions observed in ALS and FTD patient brains. Permanent FUS inclusions could be caused by unknown persistent stressors that prevent the dissolution of stress granules and thus result in final pathological stage (third hit).

The pathomechanism of TDP-43 mutations remains controversial. Most TDP-43 mutations cluster in the low complexity domain at the C-terminus. Although some mutations were reported to cause a toxic cytoplasmic mislocalization of TDP-43 (Barmada, Skibinski et al. 2010), other studies could not verify that TDP-43 mutations cause a nuclear import defect and pathological redistribution of the protein (Bentmann, Neumann et al. 2012). Nishimura and colleagues identified reduced levels of the export receptor Exportin-2/CAS in the brains of FTD patients with TDP-43 inclusions and demonstrated that reduced CAS protein levels impair nuclear import of TDP-43 in cells (Nishimura, Zupunski et al. 2010). This suggests that nuclear import defects may also contribute to TDP-43 pathology, although more thorough studies are needed to address potential nuclear import defects in TDP-43 proteinopathies.

3. TDP-43 and FUS: of functions and malfunctions

3.1. Functional domains of TDP-43 and FUS

TDP-43 and FUS are ubiquitously expressed DNA/RNA-binding proteins that belong to the hnRNP (Heterogenous nuclear ribonucleoprotein) protein family and have various functions in both the nucleus and the cytoplasm. Both proteins share common functional and structural domains like a RNA recognition motif (RRM) and a low complexity domain that contains a repetitive accumulation of certain amino acids. TDP-43 is characterized by two RNA recognition motifs (RRM1 and RRM2) and a N-terminal classical bi-partite nuclear localization signal (cNLS); while the glycine (Gly)-rich low complexity domain of TDP-43 is located at the C-terminus (see TDP-43 in Figure 3). The low complexity domain of FUS, *inter alia*, is characterized by the accumulation of N-terminal glutamines (Gln), followed by the first arginine-glycine-glycine (RGG1) repeat domain. The remaining two RGG domains (RGG2 and RGG3) are spaced by a RNA recognition motif and a zink finger (ZnF). The proline-tyrosine nuclear localization signal (PY-NLS) of FUS is located at the C-terminus (see FUS in Figure 3). Both proteins contain predicted, but functionally not tested, nuclear export signals (NES) that reside within the respective RRM domains.

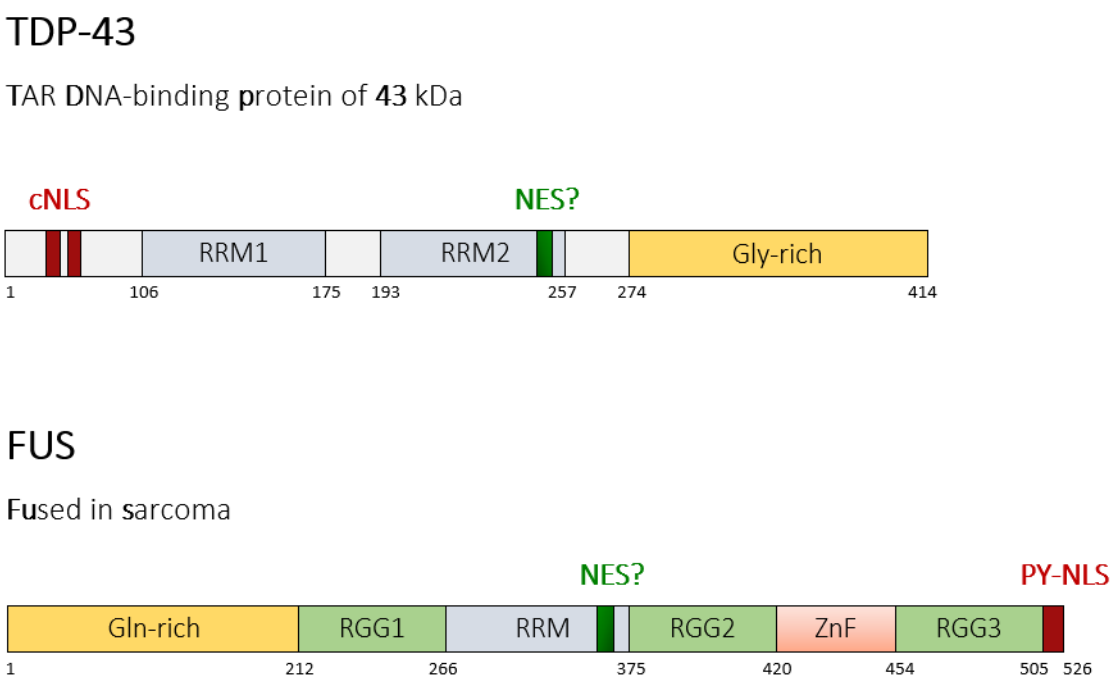


Figure 3. Schematic diagram of functional domains of TDP-43 and FUS. TDP-43 and FUS display commonalities in their domain structure: both proteins contain a RRM (blue) and a low complexity domain (yellow) as a well as predicted nuclear export signal (NES, dark green), located within the respective RRM domain. Furthermore, FUS contains three RGG domains (light green) and a ZnF motif (orange). While TDP-43 is characterized by a classical bi-partite NLS in the N-terminal domain, FUS has a C-terminal PY-NLS (red). Arabic numerics represent amino acids.

3.2. From pre-mRNA to microRNA: functions of TDP-43 and FUS in the nucleus

3.2.1. Nuclear functions of TDP-43

Nuclear functions of TDP-43 and FUS are versatile, ranging from transcriptional regulation and pre-mRNA splicing to processing of non-coding RNAs.

TDP-43 interacts with thousands of RNAs in rodent and human brains, shown by genome-wide *in vivo* studies (Polymenidou, Lagier-Tourenne et al. 2011, Tollervey, Curk et al. 2011). Initially, TDP-43 was found to transcriptionally repress the transactivation response (TAR) element of the human immunodeficiency virus type 1 (HIV-1), thereby regulating its transcription (Ou, Wu et al. 1995). Later on, exonic and intronic RNA targets of TDP-43 involved in various functions were identified, like Dyrk1a RNA that is associated with the development of the nervous system (Sephton, Cenik et al. 2011). Observation of perichromatin fibrils, sites of enhanced nuclear transcription, revealed elevated TDP-43 levels in rat neurons providing additional examples for a role of TDP-43 in transcription (Casafont, Bengoechea et al. 2009). Interestingly, TDP-43 seems to regulate its own transcription by a negative feedback loop whereat it binds to a 3´UTR (untranslated region) region of its own mRNA hypothetically promoting RNA instability (Ayala, De Conti et al. 2011).

Knockdown studies not only provided insights into the role of TDP-43 in transcription but also in splicing regulation: The reduction of TDP-43 caused changes within more than 950 splicing events in rodent brains (Polymenidou, Lagier-Tourenne et al. 2011). TDP-43 was shown to regulate splicing in a variety of different ways: *e.g*. exon skipping of exon 9 of human CFTR (Cystic fibrosis transmembrane conductance regulator) (Buratti, Dork et al. 2001) or splicing abrogation of intronic and exonic enhancers of Apolipoprotein A-II gene in TDP-43 depleted human hepatocarcinoma cells (Mercado, Ayala et al. 2005). An increased incorporation of exons, on the other hand, like in the case of the human survival of motor neuron 2 (SMN2) pre-mRNA upon TDP-43 overexpression, was also reported (Bose, Wang et al. 2008). TDP-43 functions in pre-mRNA splicing are still growing as the number of spliced genes affected by the loss of TDP-43 is constantly increasing (*e.g.* POLDIP 3, SORT1, MADD, STAG2) (Shiga, Ishihara et al. 2012, De Conti, Akinyi et al. 2015, Mohagheghi, Prudencio et al. 2016). Interestingly, TDP-43 was observed to repress splicing of cryptic exons. The lack of this repressive TDP-43 function led to the incorporation of the cryptic exons into mRNAs in murine stem cells, resulting in disrupted translation and finally in nonsense-mediated decay. The same study of Ling and colleagues found that repression of cryptic exons was inhibited in ALS/FTD cases, hypothesizing that this observed loss of function contributes to TDP-43 proteinopathy (Ling, Pletnikova et al. 2015).

In addition to the role of TDP-43 in transcription and pre-mRNA splicing, the association of TDP-43 and other RNA types, *e.g.* micro and long non-coding RNA was examined. MicroRNA (miRNA) and long non-coding RNA (lncRNA) belong to the class of non-coding RNAs; with around 20 nucleotides in

length, microRNAs represent the short version of this RNA type. A RNA transcript length of more than 200 nucleotides in turn characterizes long non-coding RNAs. Long non-coding RNAs do not encode for proteins, even though their implicated functions range from autophagy, protein synthesis and trafficking to proteostasis (Grammatikakis, Panda et al. 2014, Roberts, Morris et al. 2014). MicroRNAs have been extensively studied in the context of the Drosha complex, the key complex processing miRNA with the help of Drosha and Dicer proteins and TDP-43 interacts with both (Gregory, Yan et al. 2004). TDP-43 was shown to stabilize Drosha protein during *in vitro* neuronal differentiation (Di Carlo, Grossi et al. 2013) or to upregulate Dicer mRNA and protein levels when reduced in human neuronal cell models (Colombrita, Onesto et al. 2015). Like in the case of TDP-43 splice targets, the list of miRNAs influenced by TDP-43 depletion is constantly growing, adding let-7b, miR-663 or miR-558-3p as miRNA targets of TDP-43 (Buratti, De Conti et al. 2010, Kawahara and Mieda-Sato 2012). In addition, several examples point towards an interaction of TDP-43 with long non-coding RNAs: Transcript expression of NEAT1 (Nuclear paraspeckle assembly transcript 1) and MALAT1 (Metastasis associated lung adenocarcinoma transcript 1) was shown to be significantly elevated in FTD patient brains with TDP-43-positive protein inclusions (Tollervey, Curk et al. 2011). Moreover, the two isoforms NEAT1 and 2 interact with both TDP-43 and FUS. In spinal motor neurons from sporadic ALS patients, TDP-43 and FUS were found to be enhanced at paraspeckles and bound to NEAT1 and 2, providing a link between TDP-43 and FUS and lncRNAs in disease pathogenesis (Nishimoto, Nakagawa et al. 2013).

3.2.2. Nuclear functions of FUS

FUS, originally found as a chimeric fusion gene in liposarcoma tumors, has various functions it the RNA metabolism, just like TDP-43 (Crozat, Aman et al. 1993). It associates with RNA polymerase II (RNAP II) and key transcriptional factors, *e.g.* TFIID (Transcription factor IID) (Bertolotti, Lutz et al. 1996). In order to regulate RNAP II phosphorylation during transcription, FUS directly interacts with RNAPII, providing examples for a substantial role in transcriptional regulation (Schwartz, Ebmeier et al. 2012). Moreover, there is evidence that FUS binds to transcriptionally active chromatin in the nucleus after its self-assembly. Interestingly, ALS and FTD-associated mutations seem to abrogate the self-assembly of FUS as well as the binding to chromatin, pointing to a potential disease-relevant pathomechanism (Yang, Gal et al. 2014).

Like TDP-43, FUS features different functions in splicing regulation and miRNA biogenesis. FUS knockdown alters pre-mRNA splicing of more than 350 RNA transcripts unraveled by the same knockdown approach that studied splicing changes upon TDP-43 depletion (Lagier-Tourenne, Polymenidou et al. 2012). Rogelj and colleagues even defined that FUS binds along the whole length of

pre-mRNAs, preferably in a “saw-tooth pattern“ at the 5´ end of long introns (Rogelj, Easton et al. 2012). Moreover, FUS seems to be a mandatory co-transcriptional splicing factor for RNAP II and U1 snRNP (U1 small nuclear ribonucleoprotein) (Yu and Reed 2015). Depicted as part of the spliceosome, FUS was demonstrated to interact with key components such as small nuclear RNPs (Ribonucleoproteins), Sm proteins and the SMN protein (Yamazaki, Chen et al. 2012, Gerbino, Carri et al. 2013). The SMN-FUS interaction has also disease-relevant implications: ALS-associated FUS mutations (*e.g.* R495X) reduce Gem bodies, defined as nuclear structures that contain SMN complexes that are able to interact with Cajal bodies, in mouse and patient tissues. Other ALS-linked FUS mutations (*e.g.* R514G, R521G), examined in the same study, modify alternative splicing or influence short and long isoforms (Sun, Ling et al. 2015).

Morlando and colleagues who studied the interaction between FUS and the Drosha complex unraveled FUS association with microRNAs. They found that FUS facilitates the recruitment of Drosha to chromatin by binding to nascent pri-miRNAs. Furthermore, FUS depletion was shown to downregulate miRNAs, like miR-132 or miR-192 (Morlando, Dini Modigliani et al. 2012). Work on the 3´UTR of FUS revealed a regulatory feedback loop that maintains FUS levels by the upregulation of miR-141 and miR-200a that in turn bind to the 3´UTR of FUS and downregulate it. ALS-associated FUS mutations disturb this autoregulatory control circuit (Dini Modigliani, Morlando et al. 2014). To date, examples for FUS interaction with lncRNAs are mainly based on work of the above-mentioned lncRNA NEAT1 that is also associated with TDP-43 (see p. 13-14, “Nuclear functions of TDP-43”) (Nishimoto, Nakagawa et al. 2013).

3.3. mRNA stability, trafficking and translation: functions of TDP-43 and FUS in the cytoplasm

3.3.1. Cytoplasmic functions of TDP-43

TDP-43 and FUS are mainly localized in the nucleus; however, small amounts of both proteins can be found the cytoplasm indicating functions of the two proteins in both compartments (Zinszner, Sok et al. 1997, Ayala, Zago et al. 2008).

Human low molecular weight neurofilament (hNFL) and Histone deacetylase 6 (HDAC6) are two examples of mRNAs stabilized by TDP-43. TDP-43 was shown to directly bind to the 3´UTRs of hNFL and HDAC6 mRNA *in vitro* and *in vivo*, thereby stabilizing it (Strong, Volkening et al. 2007, Fiesel, Voigt et al. 2010). Furthermore, transcripts of G3BP (Ras GTPase-activating protein-binding protein), TIA-1 (Cytotoxic Granule Associated RNA Binding Protein), Tbc1d1 (TBC1 Domain Family Member 1) and Add2 (β-adductin) mRNAs are hypothesized to be stabilized by TDP-43, even though the underlying mechanism remains elusive as no direct interactions of TDP-43 and the transcripts was demonstrated (McDonald, Aulas et al. 2011, Stallings, Puttaparthi et al. 2013, Costessi, Porro et al. 2014). However, TDP-43 was also seen to bind to the 3´UTRs of Vegfa (Vascular endothelial growth factor a) and Grn (Progranulin) mRNA transcripts and to destabilize them, providing evidence for a direct role of TDP-43 in mRNA stability (Colombrita, Onesto et al. 2012).

Active transport of TDP-43 along axons and dendrites as well as TDP-43´s influence on the localization of interacting partners has been demonstrated in several studies, providing evidence for its regulatory role in mRNA localization and transport. The first hint that TDP-43 might be involved in mRNA trafficking came from a study that found TDP-43-positive RNA granules co-localizing with Staufen 1 and FMRP (Fragile X mental retardation protein), two proteins associated with mRNA transport and local neuronal translation (Wang, Wu et al. 2008). Later on, these proposed functions could be verified by additional studies. TDP-43 was shown to localize and even to be actively transported in axons of primary cultured motor neurons. Moreover, TDP-43 was found to co-localize with transport-regulating RBPs (RNA-binding proteins), such as IMP1 (IGF2 mRNA-binding protein 1), HuD/ELAV4 and SMN (Fallini, Bassell et al. 2012). Work by Narayanan and colleagues supported these findings by their studies on TDP-43 at presynaptic membranes of axonal terminals and, hence, proposed an involvement of TDP-43 in mRNA transport into processes (Narayanan, Mangelsdorf et al. 2013). Finally, it was shown that TDP-43 is part of cytoplasmic mRNP (Messenger ribonucleoprotein) transport granules bi-directionally moving RNA along microtubules and that ALS-associated TDP-43 mutation disrupt this transport (Alami, Smith et al. 2014).

The role of TDP-43 in mRNA translation partially overlaps with its functions in mRNA trafficking. Wang and colleagues hypothesized that TDP-43 has multiple regulatory functions in translational control. They found TDP-43 not only co-localizing with proteins involved in mRNA transport and translation in

hippocampal neurons, but also to repress translation *in vitro*. Moreover, they observed the co-localization of TDP-43 with P-body associated proteins GW182 and eIF4E (Eukaryotic translation initiation factor 4E) in dendrites. In characterizing P-bodies as "cytosolic structures that are involved in RNA induced silencing complex-mediated translation repression and mRNA degradation/storage (Parker and Sheth 2007)" (see Wang et al. TDP-43, the signature protein of FTLD-U, is a neuronal activity-responsive factor. J Neurochem 2008, p. 802), they assumed that the association of TDP-43 with P-bodies could point towards functions in local translation in neuronal dendrites (Wang, Wu et al. 2008). This hypothesis was picked up a few years later in a proteomic study showing the interaction of TDP-43 with protein classes involved in both RNA splicing and translation (Freibaum, Chitta et al. 2010). Furthermore, TDP-43 was reported to associate with ribosomes in order to temporally arrest translation when short-term stress occurred (Higashi, Kabuta et al. 2013). The translational control of Futsch/MAP1B by TDP-43 is an actual example for TDP-43 role in translational regulation (Coyne, Siddegowda et al. 2014).

3.3.2. Cytoplasmic functions of FUS

Colombrita and colleagues unraveled the interaction of FUS with the 3′UTRs of Vps54 (Vacuolar protein sorting 54), Nvl (Nuclear VCP-like) and Taf15 (TBP-associated factor 15 RNA-polymerase II) mRNAs. However, this study could not demonstrate a stabilizing role of FUS on these proteins (Colombrita, Onesto et al. 2012). A few years later, FUS was reported to stabilize mRNA of GluA1, a subunit of AMPA receptors, by binding in close proximity to the 3′UTR of its mRNA. The investigated interaction seemed to control the poly (A) tail length and thereby stabilize the GluA1 mRNA (Udagawa, Fujioka et al. 2015).

Like for TDP-43, FUS role in mRNA trafficking came from the observation that FUS clustered in RNP particles at dendritic spines of hippocampal neurons, presumably fulfilling regulatory functions (Belly, Moreau-Gachelin et al. 2005). Two additional studies investigated FUS translocation to dendritic spines. One showed enhanced FUS clusters at dendritic spines upon mGluR5 (Metabotropic glutamate receptor 5) stimulation, arguing that FUS facilitates mRNA transport and translation within dendrites upon neuronal stimulation. The other study looked at FUS-containing RNP granules that interact with the actin-stabilizing protein Nd1-L in dendrites, thus providing further evidence for a role of FUS in mRNA localization and transport (Fujii, Okabe et al. 2005, Fujii and Takumi 2005).

So far, the role of FUS in mRNA trafficking and local translation is not well documented. Yasuda and colleagues found an association between FUS and tumor-suppressor protein adenomatous coli (APC)-RNPs, whereby FUS seemed crucial for an efficient translation of APC associated mRNA transcripts at cell protrusions. Interestingly, the study found that cytoplasmic FUS granules, caused by ALS-linked

mutations (*e.g.* P525L), recruited APC-RNPs and translated mRNA transcripts like Kank2 (Kn motif and ankyrin repeat domains 2) or Pkp4 (Plakophilin4) present in APC-RNPs. Contrary to the postulated view that FUS granules resemble properties of stress granules that repress translation (Yasuda, Zhang et al. 2013).

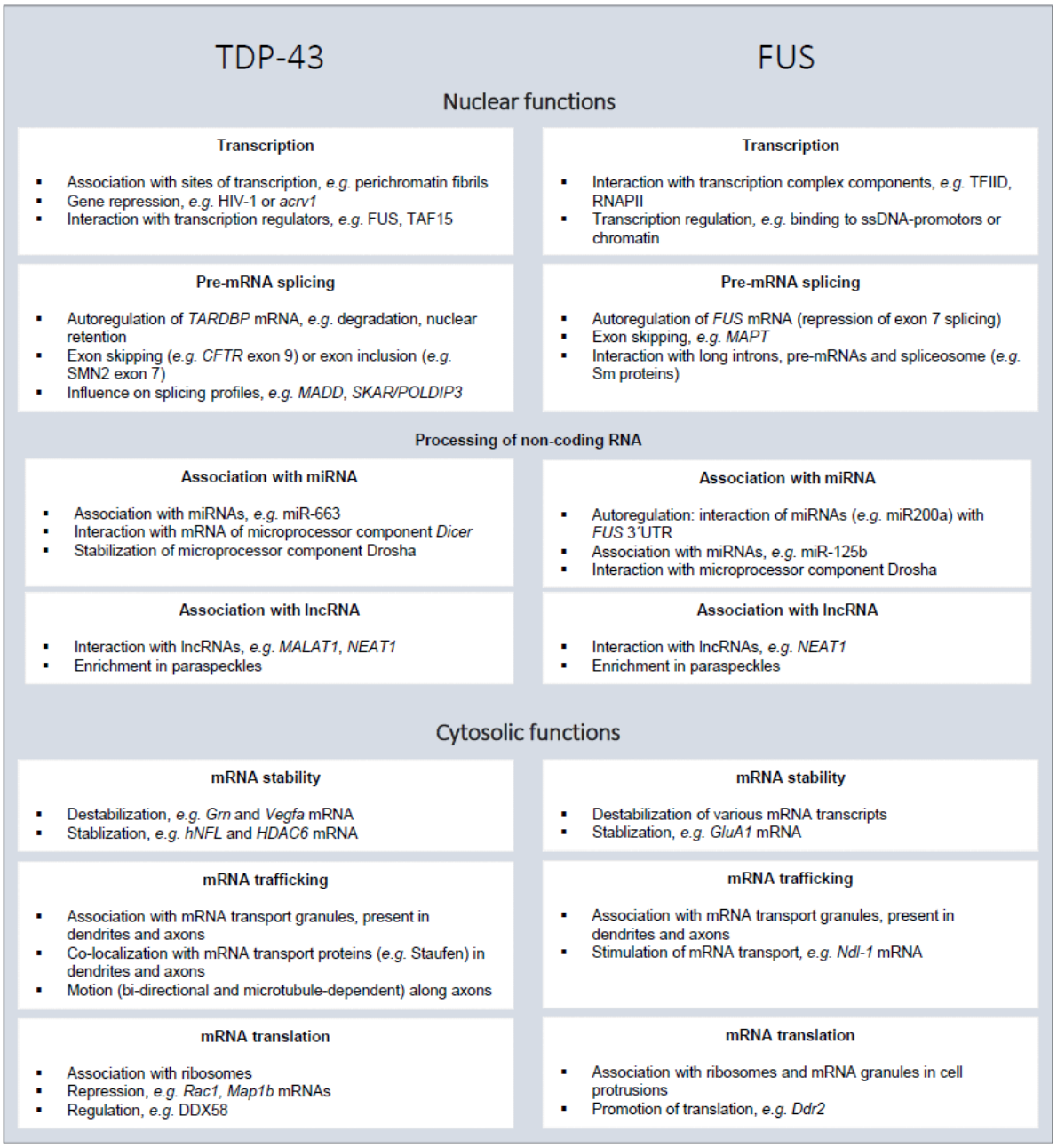


Figure 4. Summarized nuclear and cytoplasmic functions of TDP-43 and FUS (modified from Ederle and Dormann 2017). Exemplary assembly of functions that TDP-43 and FUS fulfill within subcellular compartments (listed interactions of TDP-43 and FUS do not necessarily prove function).

4. Nuclear export of TDP-43 and FUS: of evidence and ideas

4.1. Evidence for nuclear export of TDP-43 and FUS

The continuous shuttling of FUS between the nucleus and cytoplasm was already reported by Zinszner and colleagues in 1997 (Zinszner, Sok et al. 1997). They implemented the interspecies heterokaryon assay to demonstrate shuttling of FUS between the two cell compartments. In addition, they substantiated their results on nucleocytoplasmic shuttling of FUS by injecting an anti-FUS antibody into the cytoplasm. Post-injection, FUS redistributed from the nucleus to the cytoplasm while *de novo* synthesis of proteins was inhibited, thus arguing that cytoplasmic appearance of FUS could only occur if the protein undergoes repeated rounds of shuttling between the nucleus and the cytoplasm.

First evidence for nuclear export of TDP-43 from the nucleus to the cytoplasm was provided by work of Ayala and colleagues in 2008 (Ayala, Zago et al. 2008). Like Zinszner, they implemented the interspecies heterokaryon assay to demonstrate nucleocytoplasmic shuttling of TDP-43 within the cell. Moreover, the group observed retention of TDP-43 in the cytoplasm when transcription was inhibited, a common feature of shuttling proteins like hnRNPs, without addressing the underlying mechanism.

The notion that TDP-43 and FUS can leave the nucleus supports the hypothesis that both proteins fulfill crucial functions not only in the nucleus, where they are mainly found at steady state, but may also play important roles in the cytoplasm.

4.2. Potential export routes: how TDP-43 and FUS could exit the nucleus

4.2.1. Receptor- or mRNA-driven export

Theoretically, nuclear export of TDP-43 and FUS could either be mediated by export receptors, such as Exportin-1/CRM1 or other exportins, or alongside with mature mRNA that exits the nucleus in the form of mRNPs (Messenger ribonucleoproteins) via the mRNA export pathway. Either way, the proteins exit the nucleus through nuclear pore complexes (NPCs), which span the nuclear membrane and are build up by macromolecules called nucleoporins or Nups. The primary function of nuclear pore complexes is to enable active or passive transport between the nucleus and the cytoplasm. Importins and exportins represent such transport receptors that belong to the karyopherin-β (KPNB) receptor family. Exportins or export receptors for example, recognize nuclear export signals (NESs) of their cargo protein and thus directly interact with the cargo. The best-characterized NES is the leucine-rich NES that interacts with the export receptor Exportin-1, also known as CRM1 (Chromosome region maintenance 1), and the Ran-GTP machinery (Dong, Biswas et al. 2009, Monecke, Guttler et al. 2009). Alternatively, export receptors can recognize adaptor proteins and thus indirectly recognize cargos. Nuclear export mediated by export receptors is an energy-requiring process, whereat Ras-like GTPase

Ran controls the association and dissociation between receptor and cargo. Ran exists in two nucleotide-bound states: Ran-GTP is the predominant form present in the nucleus, while Ran-GDP is mainly found in the cytoplasm. Export receptors recruit their cargo in the presence of the high Ran-GTP concentration in the nucleus and dissociate upon hydrolysis of Ran-GTP to Ran-GDP in the cytoplasm (Floch, Palancade et al. 2014, Cautain, Hill et al. 2015).

In contrast, mRNA-mediated export is independent of the Ran GTPase and for most mRNAs promoted by the Nxf1/Nxt1 heterodimer. mRNAs assembled into mRNPs are directed to the Nxf1/Nxt1 heterodimer with the help of the TREX (Transcription-export) complex composed of UAP56, Aly/REF and CIP29 (Bjork and Wieslander 2017). After the association of the mRNPs with the Nxf1/Nxt1 heterodimer, the complex exits the nucleus through the nuclear pore complexes. A subset of mRNAs (*e.g.* ribosomal and small nuclear RNAs) is exported independent of Nxf1/Nxt1 and utilizes NES-containing adaptor proteins in order to exit the nucleus with the Exportin-1/CRM1 receptor (Hutten and Kehlenbach 2007, Carmody and Wente 2009).

4.2.2. Export of TDP-43: receptor-mediated or alongside with mRNA?

Winton and colleagues published a study addressing the role of TDP-43 NLS and NES mutants mimicking pathological phenotypes of ALS and FTD in cultured cells. With regard to nuclear export of TDP-43, they suggested an Exportin-1/CRM1-mediated export of TDP-43 dependent on a potential leucine-rich NES located in the second RNA-recognition domain (RRM2) of TDP-43. In order to address the functionality of this NES, they created two NES mutants by introducing point mutations to the NES-sequence and observed their localization in cells. They showed that the one TDP-43 NES-mutant localized in the nucleus in form of intra-nuclear punctae, which they called nuclear inclusions. Furthermore, they observed a nuclear reorganization of endogenous TDP-43 into punctate inclusions when cells were treated with leptomycin B, an Exportin-1/CRM1 inhibitor (Wolff, Sanglier et al. 1997). As Winton and colleagues did not use *bona fide* nuclear export assays, such as the interspecies heterokaryon assay, to further analyze the generated NES-mutant, the actual proof for a NES-dependent export of TDP-43 mediated by Exportin-1/CRM1 is still missing (Winton, Igaz et al. 2008).

A few years later, Miguel and colleagues generated transgenic *Drosophila melanogaster* models expressing TDP-43 NES-mutants based on Winton´s experimental design and analyzed them *in vivo*. Similar to Winton´s *in vitro* data, the NES-mutants of TDP-43 showed a nuclear localization *in vivo*, co-localizing with nuclear markers. The analysis of the toxicity caused by the NES-mutants revealed neither an effect on the eye morphology of flies, nor on the larval lethality when expression was targeted to muscle cells. Larval lethality was only observed when the NES-mutants were expressed in neurons, even though the lethality was less severe than in flies expressing wild-type TDP-43 or NLS-

mutant TDP-43. Furthermore, expression of TDP-43 NES-mutants reduced the lifetime of adult flies, but notably less than the TDP-43 NLS-mutants (Miguel, Frebourg et al. 2011).

Taking together, so far published work on nuclear export pathways of TDP-43 could neither demonstrate nor disprove functionality of the potential NES or a role of Exportin-1/CRM1 in nuclear export of TDP-43. Thus, mRNA-mediated export of TDP-43 represents a likely route to exit the nucleus, even though the underlying export mechanisms remain elusive.

4.2.3. Export of FUS: alongside with mRNA or a single receptor?

Like for TDP-43, results on potential export pathways of FUS are controversial. First, Sugiura and colleagues studied the biological functions of DDX39, a RNA helicase of the DEAD box RNA helicase family and in their search for potential interactors of DDX39, they identified the proteins Aly and CIP29 and found FUS strongly interacting with CIP29. Since Aly and CIP29 are co-factors involved in mRNA export, Sugiura suggested a multiprotein complex of DDX39, Aly, CIP29 and FUS with potential functions in mRNA export (Sugiura, Sakurai et al. 2007).

Contrary evidence came from work by Kino and colleagues who suggested a NES-based and Exportin-1/CRM1-dependent export of FUS. They analyzed the localization of individual functional FUS domains and found a slight shift of the EGFP-tagged RRM domain to the cytoplasm, in comparison to EGFP alone. Using bioinformatical prediction tools, they predicted a leucine-rich NES within the RRM of FUS, suggesting that the NES might be responsible for the observed redistribution of the EGFP-RRM construct. Moreover, treatment of EGFP-RRM with leptomycin B reduced the cytoplasmic redistribution, implying that Exportin-1/CRM1 might be the involved export receptor. Interestingly, a RGG1-RRM construct did not display an enhanced cytoplasmic redistribution (Kino, Washizu et al. 2011). Subsequently, exome sequencing in a large pedigree with an autosomal dominant form of familial ET (Essential tremor) proposed a rare mutation in the nuclear exporting signal region (NES; p.Q290X) of Fused in sarcoma gene (Merner, Girard et al. 2012). Following up on this finding, Lorenzo-Betancor and colleagues performed exon-sequencing analysis of potential NESs of FUS, TDP-43, TAF15, EWSR1, hnRNPA1 and hnRNPA2B1 in order to look for new mutations associated with Essential tremor. They only identified a rare R471C substitution in EWSR1 in a single subject with familial ET, hence the pathogenicity of this substitution remains equivocal, Moreover, it is unclear whether the putative NESs predicted in this study are actually functional (Lorenzo-Betancor, Ogaki et al. 2014). To my knowledge, there is no further work elaborating on the nuclear export pathways of FUS, leaving it up to further work on this topic.

4.2.4. An alternative possibility: passive diffusion as an export route for TDP-43 and FUS

Passive diffusion is often defined as a process that enables metabolites at a size range of 40-60 kDa to freely pass the nuclear pore. It has long been thought that only molecules below this threshold are able to passively diffuse through nuclear pore complexes (Gorlich 1998). More recently, multiple studies have challenged this view: Seibel and colleagues for example noted that GFP and EGFP multimers (tetramers and hexamers, respectively) exceeded the proposed size limit for passively diffusing proteins in various mammalian cell lines (Seibel, Eljouni et al. 2007). Wang and Brattain also observed the diffusion of GFP chimeric proteins with a size up to 110 kDa in different cell lines, proposing that proteins larger than 60 kDa are able to passively diffuse through nuclear pores (Wang and Brattain 2007). More recently, two studies disproved the common hypothesis that passive diffusion only occurs for proteins below 40-60 kDa (Popken, Ghavami et al. 2015, Timney, Raveh et al. 2016). Instead, they demonstrated that passive diffusion simply becomes slower/less efficient, arguing for a "soft barrier" for passive diffusion. Taking this work into account, it is also possible that TDP-43 and FUS, with a molecular weight of 43 and 53 kDa, respectively, simply leave the nucleus by passive diffusion.

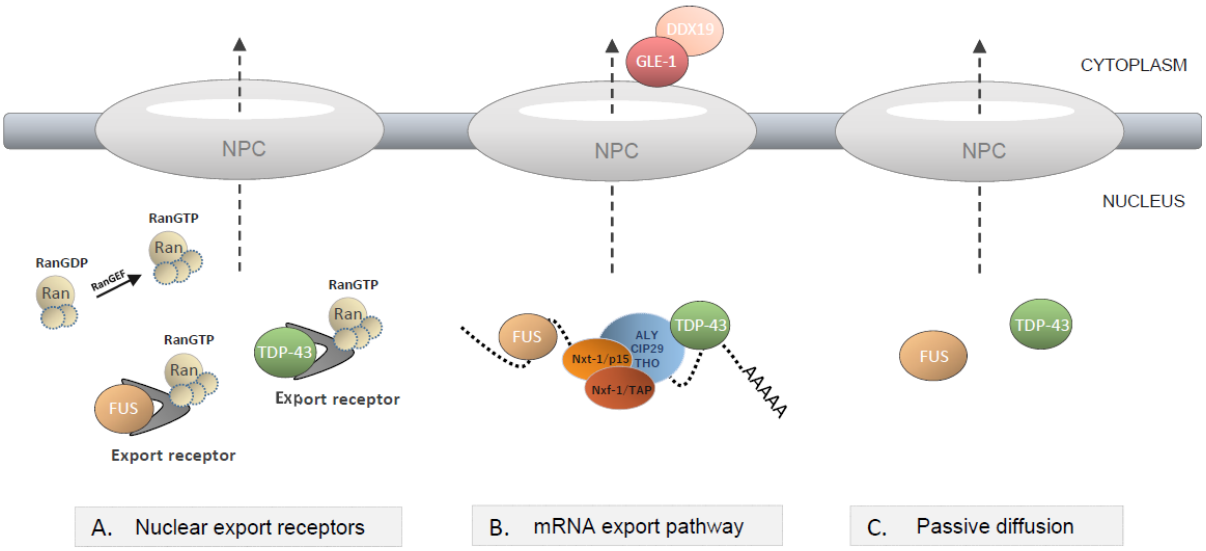


Figure 5. TDP-43 and FUS could leave the nucleus via three potential export routes (modified from Ederle and Dormann 2017). A. Forming a complex with an export receptor like Exportin-1/CRM1 and RanGTP, TDP-43 and FUS could exit the nucleus via an active, receptor-mediated pathway. RanGEF (Guanine nucleotide exchange factor) ensures energy source by the conversion of RanGDP to RanGFP. B. Export together with mRNA represents the second potential export pathway for TDP-43 and FUS. TDP-43 and FUS could either interact with mRNAs associated with the mRNA export complex, or with proteins of the mRNA export complex, such as the Nxf-1/Nxt-1 heterodimer or the TREX complex component Aly/REF. At the cytoplasmic side of NPCs, DDX19 and GLE1 impart the directionality of the mRNA export. C. Passive diffusion is the third option for TDP-43 and FUS to

leave the nucleus; with a molecular weight of 43 kDa (TDP-43) and 53 kDa (FUS), they appear small enough to passively diffuse through the nuclear pore complex (NPC).

5. Ph.D. project goals

Nuclear import defects, especially for FUS, are widely accepted as a culprit for ALS/FTD-associated cytoplasmic inclusions (see p. 10-11 for further information). Whether defects in nuclear export of TDP-43 or FUS also contribute to ALS/FTD pathogenesis and may be responsible for nuclear aggregation of TDP-43 or FUS or cause defects in mRNA processing, is still unknown. The presence of occasional intranuclear TDP-43 and FUS inclusions of ALS and FTD patients indicate that besides nuclear import, nuclear export might be altered in ALS and FTD pathogenesis. Recent genetic screens of C9orf72-associated toxicity link the nucleocytoplasmic transport machinery with C9orf72-ALS/FTD, supporting the hypothesis that nucleocytoplasmic transport might represent a potential pathomechanism in these neurodegenerative diseases.

Therefore, the main objective of my Ph.D. thesis was to identify the unknown nuclear export pathways of TDP-43 and FUS. Specifically, my aims were to find potential signals/sequences in TDP-43 or FUS required for nuclear export, and to identify factors responsible for nuclear export of TDP-43 and FUS (*e.g.* Exportin-1/CRM1 or other nuclear export receptors). Knowing the signals and factors that mediate nuclear export of TDP-43 and FUS will be crucial for testing the hypothesis that nuclear export is altered in ALS/FTD patients. A second objective of my PhD thesis, addressed as a side project, was to identify additional nuclear transport factors contributing to nuclear localization of TDP-43 and FUS. Importin α/β and Transportin are well-known import receptors for TDP-43 and FUS, respectively. However, my preliminary results suggest that additional transport factors may play a role in nuclear localization and possibly in nuclear import of TDP-43 and FUS

II. RESULTS

DECLARATION OF COPYRIGHT AND CONTRIBUTIONS

This section of the Ph.D. thesis was prepared in parallel to the manuscript "Nuclear egress of TDP-43 and FUS is a diffusion-limited process", submitted to Scientific Reports (in revision, but not yet accepted). Therefore, structure and text of this Ph.D. thesis and the manuscript will partially overlap in this regard.

Figure 8C/D/F Figure 9D Figure 10C	Original polyclonal HeLa cells stably expressing mCherry-tagged TDP-43-83AAA and FUS-P525L NLS-mutants generated by Eva Funk (former PhD student of Dorothee Dormann).
Figure 8E Figure 9C Figure 10B	Immunoblotting conduced and analyzed by Claudia Abou-Ajram (technician of research group of Dorothee Dormann).
Figure 9A	Flag-TDP-43-WT and RNA-binding-deficient mutants F2L/ΔRRM1/F4L kindly provided by Emanuele Buratti and Francisco Baralle.
Figure 11A	TDP-43-ΔC-V5 generated by Dorothee Dormann.
Figure 11C	HA-FUS-ΔQ and HA-FUS-ΔQ/RGG1, GFP-FUS-ΔQ/RGG1/RRM, GFP-FUS-ΔRGG2, GFP-FUS-ΔZnF and GFP-FUS-ΔRGG3 generated Ramona Rodde (former technician of research group of Dorothee Dormann).
Figure 13 B/C	pEYFP-NLS-FRB-TDP-43 and pEYFP-NLS-FRB-FUS generated by Christina Funk and Susanne M. Bailer; NEX-TRAP assay conduced and analyzed by Christina Funk and Susanne M. Bailer.
Figure 14B/C/D/E	Cell fractionations and raw data analyzes for quantifications conduced and analyzed by Claudia Abou-Ajram (technician of research group of Dorothee Dormann).
Figure 16B	Original polyclonal HeLa cells stably expressing wild-type mCherry-tagged TDP-43 and FUS generated by Eva Funk (former PhD student of Dorothee Dormann).

Table 1. Summary of contributions to the results-section of the main project

1. Analysis of nuclear export of TDP-43 and FUS in the interspecies heterokaryon assay

The interspecies heterokaryon assay is based on the fusion of cells from two different species. The fusion results in a hybrid cell that contains two nuclei of different species in order to detect nucleocytoplasmic shuttling of proteins that predominantly localize in the nucleus at steady state. When the protein-of-interest undergoes nucleocytoplasmic shuttling, it will be exported from one nucleus and re-imported into the other and shuttling can be measured by *e.g.* using antibodies against the protein-of-interest. In order to prevent import of newly synthesized proteins, the interspecies heterokaryon assay is performed in the presence of protein synthesis inhibitors (Gama-Carvalho and Carmo-Foncesa. 2006). I applied the interspecies heterokaryon assay as a *bona fide* nuclear export assay to assess export of TDP-43 and FUS (see schematic diagram in Figure 6A).

I used HeLa cells stably expressing V5-tagged TDP-43 and HA-tagged FUS fused with mouse embryonic fibroblasts (MEFs) to analyze nuclear export of TDP-43 and FUS in interspecies heterokaryons at

different time points. This demonstrated that both proteins undergo nuclear export with slightly different efficiencies. While TDP-43 can already be detected in the mouse nucleus 30min after fusion and is readily seen in the mouse nucleus of heterokaryons at 2h, FUS signals in the mouse nucleus are weaker 1h post-fusion and take about 5h to reach high levels in mouse nuclei (see Figure 6B). The non-shuttling heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP-C) served as a control to demonstrate efficient translational inhibition by cycloheximide (CHX), as *de novo* synthesis of hnRNP-C in the cytoplasm due to insufficient translational inhibition would lead to the detection of the non-shuttling control protein in the murine nucleus.

Furthermore, I compared the reversible translational inhibitor cycloheximide with the alternative inhibitors emetine (irreversible) (Grollman 1968) and harringtonine (HR, reversible) (Tscherne and Pestka 1975). Cycloheximide inhibits the translocation of ribosomes and thus blocks elongation (Schneider-Poetsch, Ju et al. 2010). Harringtonine immobilizes ribosomes immediately after translation initiation (Ingolia, Brar et al. 2012) and emetine permanently inhibits translation by the irreversible blocking of ribosome movement along the mRNA strand (Pestka 1971).

All tested translational inhibitors displayed sufficient translational inhibition, as the non-shuttling control protein hnRNP-C was not detected in the MEF nuclei 2h (TDP-43) or 3h (FUS) post-fusion, providing evidence that novel protein synthesis in the cytoplasm was successfully suppressed (see Figure 6C). In contrast to hnRNP-C, TDP-43-V5 and HA-FUS undergo shuttling and appear in mouse nuclei of heterokaryons under all three treatment conditions.

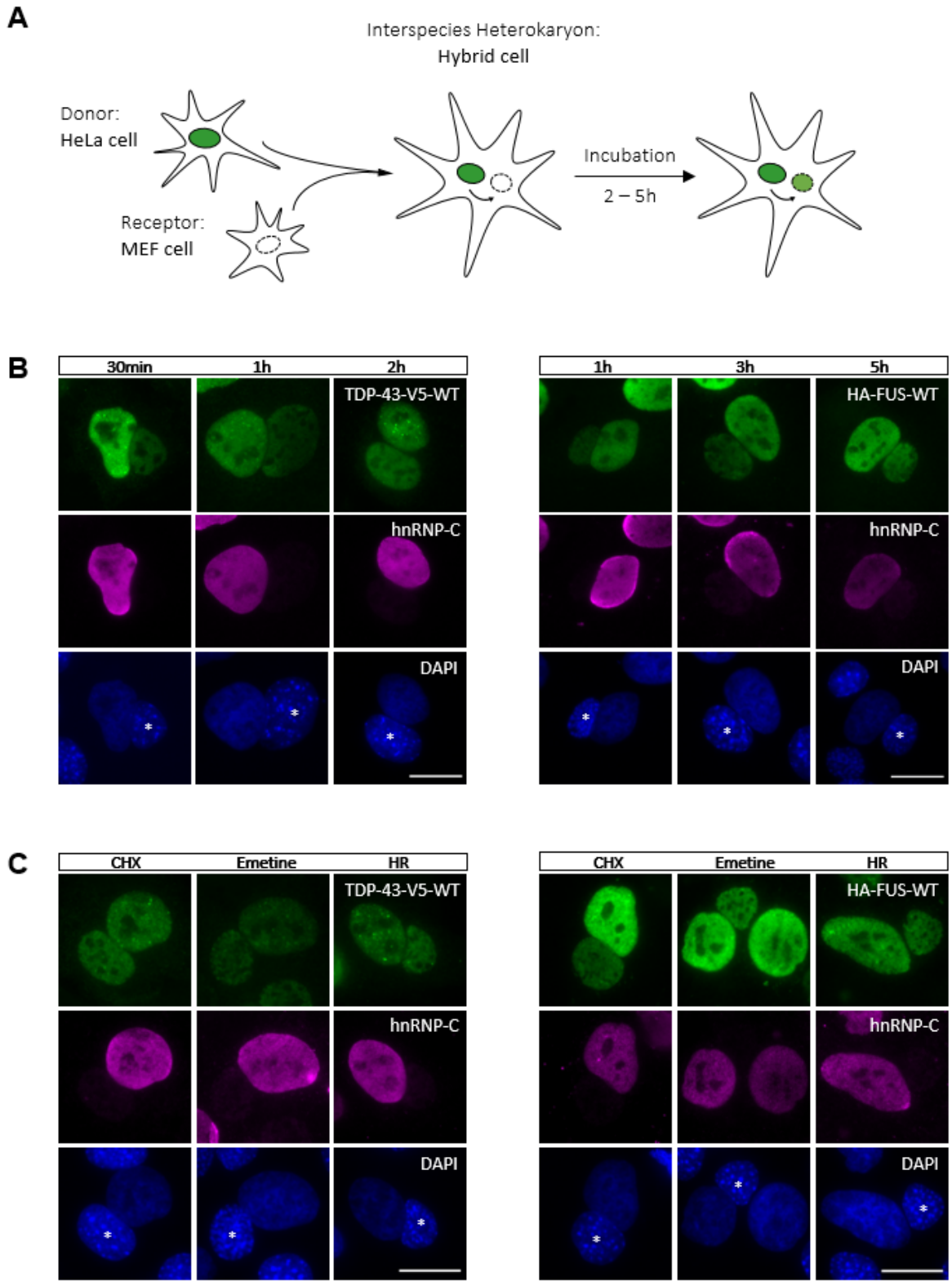


Figure 6. Analyzing nuclear export of TDP-43 and FUS in the interspecies heterokaryon assay

A. Schematic diagram of the interspecies heterokaryon assay. In this assay, fusion of a human HeLa cell expressing the protein-of-interesting (green within "donor" cell) with a mouse embryonic fibroblast (MEF, "receptor") results in a hybrid cell that contains two nuclei from different species ("interspecies heterokaryon"). If the protein-of-interest undergoes nuclear export from the donor, it will appear in the nucleus of the receptor

after an incubation period. To assure detection of nuclear export from the donor to the receptor and not import of newly synthetized protein in the cytoplasm, the heterokaryon assay is performed in the presence of translational inhibitors (*e.g.* cycloheximide) in order to suppress cytoplasmic protein synthesis.

B. Nuclear export of TDP-43 and FUS in the interspecies heterokaryon assay. The fusion of HeLa cells stably expressing V5-tagged TDP-43 or HA-tagged FUS with MEFs produced heterokaryons that were either incubated for 30min, 1h and 2h (TDP-43) or 1h, 3h and 5h (FUS) after fusion. Localization of TDP-43 and FUS (in green; stained with a V5- or HA-specific antibody, respectively) not only in the HeLa but also in the MEF nuclei (see asterisks in DAPI channel) depicts nuclear export of TDP-43 and FUS, while the non-shuttling hnRNP-C control protein remains in the nuclei of HeLa cells (in pink; stained with a human-specific hnRNP-C antibody). Nuclei were visualized using DAPI stain. Scale bars: 20µm.

C. Comparison of translational inhibitors in the interspecies heterokaryon assay. Heterokaryons resulted from the fusion of V5-tagged TDP-43 or HA-tagged HeLa with MEF cells, were either incubated for 2h (TDP-43) or 3h (FUS) after fusion and in the presence of the translational inhibitors cycloheximide (CHX; 75µg/mL), emetine (10µg/mL) or harringtonine (HR; 10µg/mL). Exclusive nuclear hnRNP-C localization indicates an adequate inhibition of *de novo* protein synthesis in the cytoplasm. TDP-43 (green), FUS (green) and hnRNP-C (pink) were visualized by antibody staining with V5 (TDP-43)-, HA (FUS)-, and hnRNP-C-specific antibodies; nuclei were stained with DAPI (blue). Scale bars: 20µm.

2. Predicted Exportin-1/CRM1-dependent nuclear export signals of TDP-43 and FUS are not functional

To address whether TDP-43 and FUS may contain a classical leucine-rich NES recognized by the nuclear export receptor Exportin-1/CRM1, I implemented the bioinformatical NES predictions tools NES finder 0.2 and NetNES 1.1 Server. Using these prediction tools, I determined two putative Exportin-1/CRM1-dependent NESs for each protein that were termed NES-222 (IPKPFRAFAF) and NES-239 (IAQSLCGEDLII) for TDP-43 and NES-289 (VQGLGENVTI) and NES-301 (VADYFKQIGI) for FUS (see Figure 7A).

The predicted NESs within both proteins meet the consensus sequence $\Phi x_{2\text{-}3}\Phi x_{2\text{-}3}\Phi x\Phi$ (Φ are hydrophobic amino acids, spaced by any amino acid, represented by x) recognized by Exportin-1/CRM1 (Kutay and Guttinger 2005). While NES-222 in TDP-43 and NES-301 in FUS are newly predicted NESs, NES-239 in TDP-43 and NES-289 in FUS were already predicted and experimentally addressed by Winton and colleagues (2008) and Kino and colleagues (2010), respectively. However, their functionality was not conclusively confirmed by *bona fide* export assays, like the interspecies heterokaryon assay.

To test the activity of both predicted Exportin-1/CRM1-dependent NESs, I replaced two hydrophobic key amino acids within each putative NESs by alanine (A) (mNES, see Figure 7B) and analyzed these mutants in the interspecies heterokaryon assay. The introduced mutations should abrogate binding to Exportin-1/CRM1 and hence inhibit nuclear export in the interspecies heterokaryon assay, if the identified NESs are truly functional. Since it is possible that both NESs are functional and thus mutation of a single NES does not inhibit Exportin-1/CRM1-dependent export, I additionally constructed double-mutants (double mNES) and tested all constructs in the interspecies heterokaryon assay.

However, none of the introduced mutations inhibited nuclear export of V5-tagged TDP-43 or HA-tagged FUS in the interspecies heterokaryon assay: Both wild-type and mutant proteins readily accumulated in the mouse nucleus (marked by asterisks in DAPI channel) 2h (TDP-43) or 3h (FUS) post-fusion, showing that the identified NESs are not essential for the nuclear export of TDP-43 and FUS and therefore are not functional (see Figure 7C).

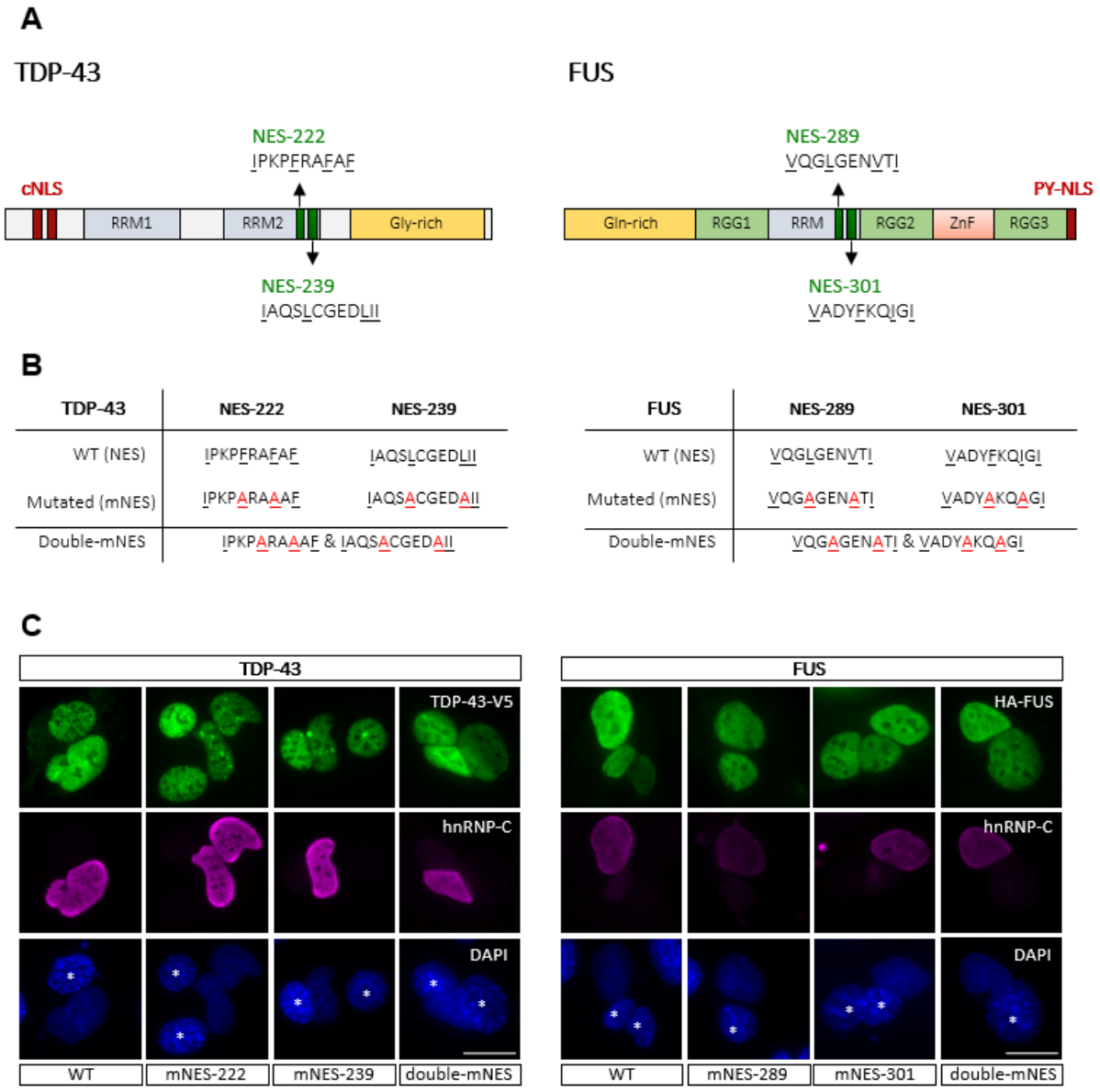


Figure 7. Nuclear export signals (NESs) of TDP-43 and FUS are not functional

A. Graphic scheme of predicted nuclear export signals within TDP-43 and FUS. The putative NESs of both proteins are localized within the respective RRM domain. Hydrophobic key amino acids (underlined) are supposed to mediate binding to Exportin-1/CRM1.

B. Amino acid sequences of Exportin-1/CRM1-dependent NESs of TDP-43 and FUS. Using site-directed mutagenesis, two hydrophobic key amino acids (all hydrophobic amino acids are underlined) within the putative WT-NESs were exchanged for alanine (mNES, underlines in red). Double mutants (double mNES) are characterized by a combination of point mutations within both putative NESs of one protein.

C. NES mutants of TDP-43 and FUS in the interspecies heterokaryon assay. V5-tagged or HA-tagged NES-mutants (in green) undergo nuclear export in the heterokaryon assay as seen by their accumulation in the MEF nuclei (marked by asterisks in DAPI channel); whereas the non-shuttling control hnRNP-C remains within the human nuclei. Visualization of TDP-43 (green), FUS (green) and hnRNP-C (pink) was achieved by V5 (TDP-43)-, HA (FUS)-, and hnRNP-C-specific antibody staining; nuclei were stained with DAPI (blue). Scale bars: 20µm.

3. Nuclear export of TDP-43 and FUS is independent of Exportin-1/CRM1

Mutating the putative NESs of TDP-43 and FUS showed that they are not functional. However, Exportin-1/CRM1 could still mediate nuclear export of TDP-43 and FUS by interacting with alternative NESs that do not match the consensus algorithm and thus were not predicted with the used bioinformatics prediction tools. Alternatively, TDP-43 and FUS could bind interaction partners with a classical Exportin-1/CRM1-dependent NES and thus could be exported via Exportin-1/CMR1 in a piggy-back fashion.

In order to address whether nuclear export of TDP-43 and FUS is mediated by Exportin-1/CRM1, I pharmacologically inhibited Exportin-1/CRM1 with leptomycin B (LMB) in the interspecies heretokaryon assay; leptomycin B is a widely used Exportin-1/CRM1-specific inhibitor (Wolff, Sanglier et al. 1997). However, incubation with this pharmacological inhibitor did not disturb nuclear export of TDP-43 and FUS, as both proteins still accumulated in the mouse nucleus in the interspecies heterokaryon assay (marked by asterisks in DAPI channel in the left panels of Figure 8A). To control for sufficient inhibition by LMB, I treated HeLa cells in parallel with the same inhibitor concentration for 2h and analyzed the subcellular distribution of p62/SQSTM1, a well-characterized Exportin-1/CRM1 substrate (Pankiv, Lamark et al. 2010). This caused a strong shift of the cytoplasmically localized protein to the nucleus, demonstrating efficient inhibition of Exportin-1/CRM1 by LMB (see LMB control in the right panel of Figure 8A).

In addition, I made use of HeLa cells stably expressing mCherry-tagged TDP-43 and FUS NLS-mutants. In these stable cell lines, mCherry-tagged TDP-43 and FUS carry mutations in their NLS that lead to import defects and hence to a partial cytoplasmic redistribution of the proteins (Bentmann, Neumann et al. 2012). If export of TDP-43 and FUS depends on Exportin-1/CRM1, the mCherry-tagged NLS-mutants should shift to the nucleus upon LMB inhibition or upon siRNA-mediated knockdown of Exportin-1/CRM1 (see schematic diagram in Figure 8B). However, neither LMB treatment, nor siRNA-mediated knockdown of Exportin-1/CRM1 showed an effect on the localization of the TDP-43 and FUS NLS-mutants (see Figure 8C, D and E). Furthermore, the combination of siRNA-mediated Exportin-1/CRM1 depletion and LMB (to inhibit residual amounts of Exportin-1, still present in siRNA-treated cells) did not result in a nuclear shift of TDP-43 and FUS NLS-mutants (see Figure 8F).

These results suggest that nuclear export of TDP-43 and FUS is independent of the export receptor Exportin-1/CRM1 and that they exit the nucleus via alternative pathways.

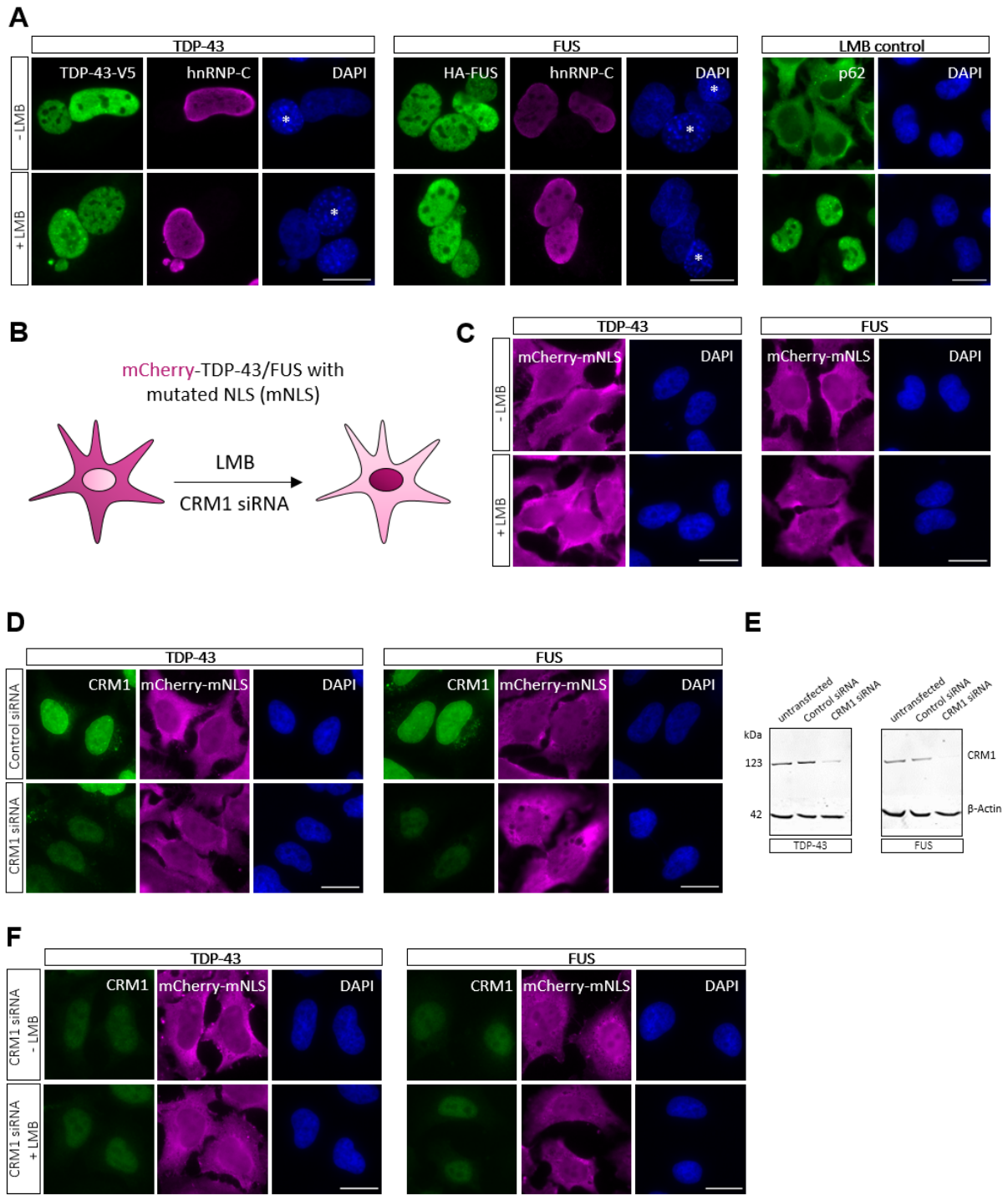


Figure 8. Nuclear export of TDP-43 and FUS does not dependent on Exportin-1/CRM1

A. Leptomycin B (20nM) did not inhibit nuclear export of TDP-43 and FUS. Post-fusion, 2h (TDP-43) or 3h (FUS) incubation with LMB did not disrupt nuclear export in the heterokaryons as TDP-43 and FUS accumulate in the MEF nuclei (marked by asterisks in DAPI channel); whereas LMB treatment (20nM, 2h) was efficient in preventing p62/SQSTM1 (in green) from exiting the nucleus. Scale bars: 20µm.

B. Graphic scheme of experiments with mCherry-tagged TDP-43 and FUS carrying mutations within the NLS (mNLS). The introduced point mutations in TDP-43 (83AAA, replacing amino acid 83-85 with alanine) and FUS (P525L, replacing amino acid 525 with leucine) cause a cytoplasmic protein accumulation due to impaired

nuclear import. Treatment of TDP-43 and FUS-mNLS with LMB or siRNA targeting Exportin-1/CRM1 should lead to a more nuclear distribution, if export of TDP-43 and FUS depends on Exportin-1/CRM1.
C. Treating NLS-mutants of TDP-43 or FUS with LMB (20nM for 2.5h) does not affect cytoplasmic localization of the mutant proteins. Scale bars: 20µm.
D and E. 72h-incubation of TDP-43 or FUS NLS-mutants with control or Exportin-1/CRM1-targeting siRNA does not cause a nuclear retention of TDP-43 or FUS (D). siRNA-mediated knockdown efficiency was evaluated by Western blotting, analyzing the protein levels with an Exportin-1/CRM1-specific antibody; β-actin served as a loading control (E). Scale bars: 20µm.
F. The combination of LMB and Exportin-1/CRM1-specific siRNA does not lead to a nuclear accumulation of TDP-43 and FUS NLS-mutants. In addition to the 72h-incubation of HeLa cells stably expressing mCherry-TDP-43-, and FUS-mNLS with control or Exportin-1/CRM1 siRNA cell were treated with LMB (20nM for 2.5h). This combined treatment did not alter cytoplasmic distribution of TDP-43 and FUS NLS-mutants. Scale bars: 20µm.
Visualization of TDP-43-, or FUS-mNLS (pink) and Exportin-1/CRM1 (green) in C, D and F was achieved using mCherry-, or Exportin-1/CRM1-specific antibodies, respectively; nuclei were stained with DAPI (blue). Scale bars: 20µm.

4. Nuclear export of TDP-43 and FUS is independent of RNA binding or the mRNA export pathway

As multifunctional RNA-binding proteins, TDP-43 and FUS could alternatively leave the nucleus via the mRNA export route, alongside with bound mRNA targets (for further details, see "Potential export routes: how TDP-43 and FUS could exit the nucleus", p. 19-20). In order to analyze the role of mRNA in mediating nuclear export of TDP-43 and FUS, I utilized RNA-binding deficient mutants of TDP-43 and FUS and addressed the impact of silencing mRNA export factors in the above introduced stable cell lines expressing TDP-43 and FUS NLS-mutants.

Binding of TDP-43 to RNA was shown to depend on the RRM1 domain as well as key phenylalanine residues within both the RRM1 and RRM2 domains. TDP-43 mutants F2L (2 point mutations within the RRM1 domain, exchanging phenylalanine for leucine), ΔRRM1 (complete deletion of the RRM1 domain) and F4L (4 point mutations exchanging phenylalanine for leucine within the RRM1 and RRM2 domains,) were shown to be deficient in RNA-binding (Buratti and Baralle 2001, Ayala, De Conti et al. 2011). I utilized these mutants to analyze whether RNA-binding is required for nuclear export of TDP-43.

In addition to these TDP-43 mutants, I engineered two RNA-binding deficient FUS mutants that carry various mutations in motifs that were shown to mediate RNA-binding of FUS (Nguyen, Mansfield et al. 2011, Daigle, Lanson et al. 2013, Liu, Niu et al. 2013, Ozdilek, Thompson et al. 2017): (1) the RRM/ZnF mutant (mRRM/mZnF) contains a combination of multiple point mutations within the RRM and ZnF domains and (2) the RGG mutant (mRGG) is characterized by the replacement of all arginines (R) for lysines (K) within the three RGG motifs (see Figure 9A).

Testing the RNA-binding deficient TDP-43 and FUS mutants in the interspecies heterokaryon assay demonstrated that all mutants accumulated in the mouse nuclei post-fusion as efficiently as the wild-type proteins (Figure 9B). Thus, RNA-binding does not seem to be required for nucleocytoplasmic shuttling of TDP-43 or FUS, suggesting that both proteins leave the nucleus independent of bound mRNAs.

This finding was substantiated by siRNA-mediated knockdown of Aly/REF (Katahira 2012), a key protein of the mRNA export complex, in stably expressing mCherry-tagged NLS-mutants of TDP-43 and FUS. Incubation of cells with Aly/REF-specific siRNA for 72h reduced Aly/REF proteins levels (see Figure 9C) and retained polyA+ RNA in the nucleus (indicated by arrows in the polyA+ RNA channel in Figure 9D), demonstrating substantial Aly/REF depletion (Katahira, Inoue et al. 2009). However this did not cause a nuclear redistribution of TDP-43 and FUS NLS-mutants (see Figure 9D).

Taking the results together, nuclear export of TDP-43 and FUS appears to take place independently of the mRNA export route along with bound mRNAs.

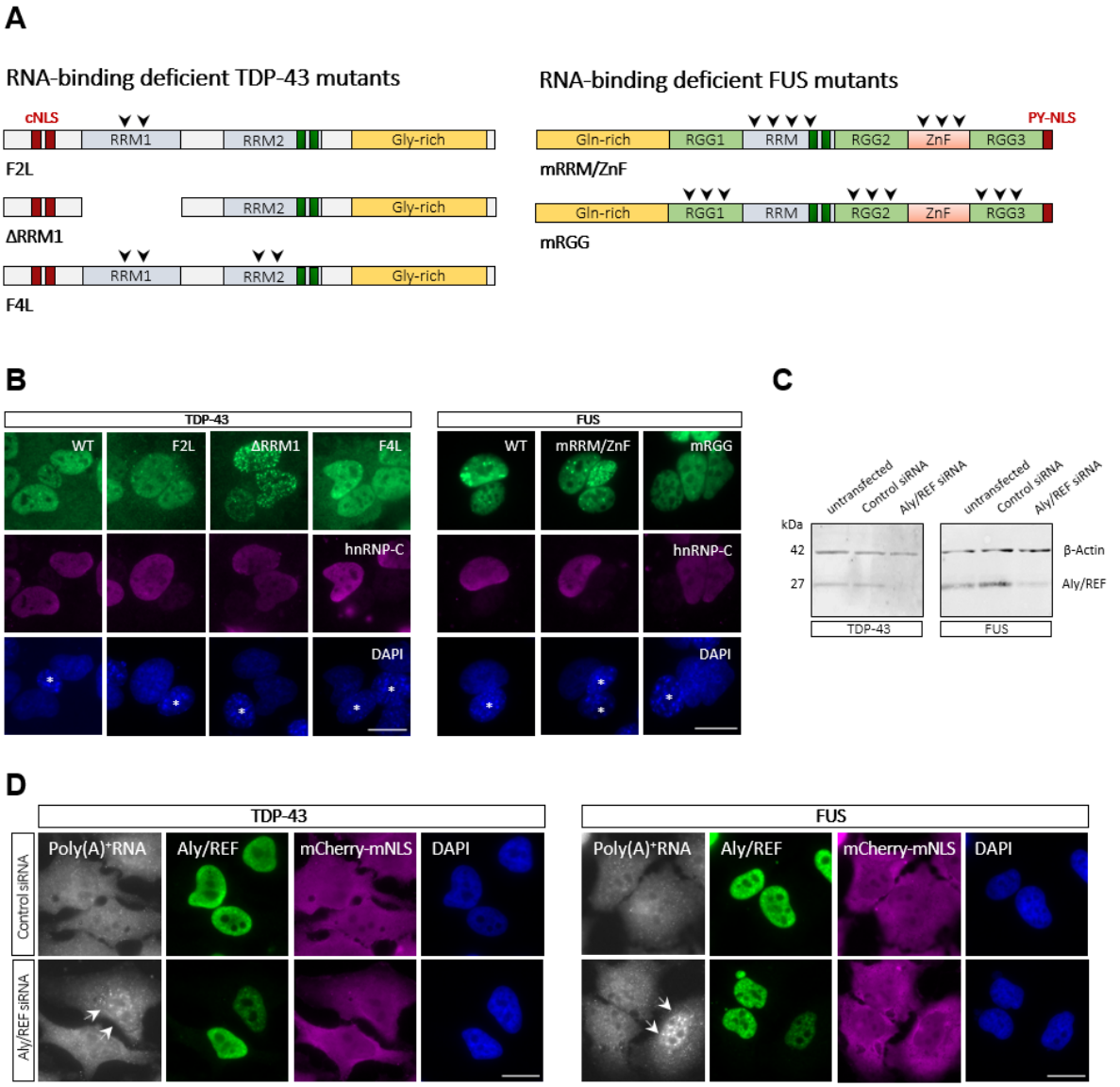


Figure 9. Nuclear export of TDP-43 and FUS does not dependent on RNA binding or the mRNA export route

A. Graphic scheme of RNA-binding deficient mutants of TDP-43 and FUS. F2L (F147L/F149L) and F4L (F147L/F149L/F229L/F231L) RNA-binding deficient TDP-43 mutants contain either 2 or 4 amino acid substitutions, respectively. The mRRM/mZnF mutant of FUS represents a combination of overall 13 point mutations within these two domains, while all arginines within the three RGG domains were replaced by lysine in the mRGG mutant.

B. RNA-binding deficient TDP-43 and FUS mutants in the interspecies heterokaryon assay. RNA-binding deficient mutants of flag-tagged TDP-43 or GFP-tagged FUS undergo nuclear export as they accumulate in MEF nuclei 2h (TDP-43) or 3h (FUS) post-fusion (marked asterisks in DAPI channel). Scale bars: 20μm.

C and D. siRNA-mediated knockdown of Aly/REF efficiently reduced Aly/REF protein levels detected via Western blotting using an Aly/REF-specific antibody and β-actin as loading control (C). In addition to the immunocytochemistry evaluation of TDP-43 or FUS NLS-mutants (pink) either treated with control or Aly/REF-targeting siRNA (green), mutants were analyzed for polyA+ RNA (white). While the reduction of Aly/REF enhances the polyA+ RNA signal in the nucleus (indicated by arrows in the polyA+ RNA channel), it shows no effect on the cytoplasmic distribution of mutant TDP-43 or FUS. Scale bars: 20μm.

5. Alternative export factors in nuclear export of TDP-43 and FUS

In addition to testing the role of Exportin-1/CRM1 and the mRNA export route in nuclear export of TDP-43 and FUS, I wanted to determine whether alternative receptors influence nuclear export of TDP-43 and FUS. Therefore, a siRNA-library that targeted alternative export receptors and different candidates of the mRNA export machinery was designed (see Figure 10A) and screened in HeLa cells stably expressing mCherry-tagged TDP-43 and FUS NLS-mutants. However, siRNA-mediated depletion of tested export factors did not change subcellular localization of TDP-43 and FUS NLS-mutants (data not shown).

Of particular interest in this systematic screening was the export receptor Exportin-5 (XPO5), as the yeast homologue of Exportin-5 was shown to enhance TDP-43 toxicity when overexpressed (Kim, Raphael et al. 2014). I therefore silenced Exportin-5 expression using XPO5-specfic siRNAs in HeLa cells expressing mCherry-tagged TDP-43 and FUS NLS-mutants. Even though, the protein levels of XPO5 were strongly reduced after a 72h-incubation with control or XPO5-targeting siRNA (see Figure 10B), the depletion of XPO5 had no effect on the cytoplasmic localization of mCherry-tagged TDP-43 and FUS NLS-mutants (see Figure 10C), suggesting that XPO5 is not the main export factor of TDP-43 and FUS. Taking together, nuclear export of TDP-43 and FUS appears independent of all examined alternative export receptors.

A

Exportins	mRNA export factors
XPO1	Aly/REF
CSE1L/CAS (XPO2)	NXF1/TAP
XPO4	NXF2
XPO5	NXF3
XPO6	NXF5
XPO7	NXT1
XPOT	NXT2

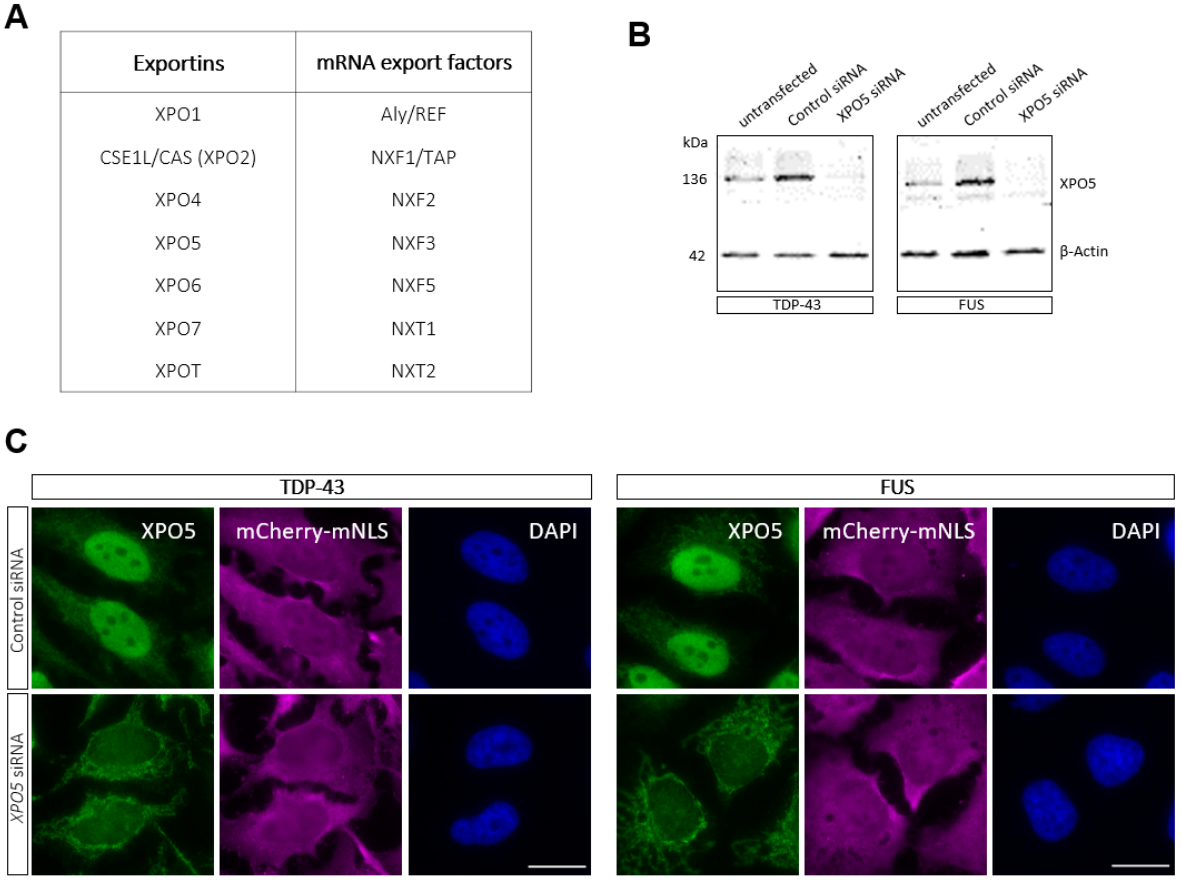


Figure 10. The role of alternative factors in nuclear export of TDP-43 and FUS

A. Table of complementary factors potentially involved in nuclear export of TDP-43 and FUS. Factors were divided into two functional groups: (a) alternative export receptors (exportins) and (b) additional factors associated with the mRNA export route.

B and C. siRNA-mediated knockdown efficiency of XPO5 depletion was evaluated by Western blotting, analyzing the protein levels with an XPO5-specific antibody; β-actin served as a loading control (B). The 72h-incubation of HeLa cells stably expressing mCherry-tagged TDP-43 or FUS NLS-mutants with control or XPO5-targeting siRNA does not cause a nuclear retention of TDP-43 or FUS (C). Scale bars: 20µm.

6. Potential domains involved in nuclear export of TDP-43 and FUS

In addition to the analysis of various export factors, I wanted to determine which domains of TDP-43 and FUS are required for nuclear exit of the two proteins. The idea was that identifying the domain(s) or signal(s) required for nuclear export of TDP-43 and FUS may give us a hint at the utilized export route. Thus, I made use of TDP-43 and FUS domain mutants in which different functional domains were deleted and analyzed them in the interspecies heterokaryon assay.

For TDP-43, I utilized mutants lacking the RRM2 domain (ΔRRM2) (Buratti and Baralle 2001) and the C-terminus (ΔC) (Bentmann, Neumann et al. 2012) (see Figure 11A). Both deletion mutants of TDP-43 shuttle from HeLa to the mouse nuclei (marked by asterisks in the DAPI channel) in heterokaryons, showing that nuclear export is not disrupted by lack of the RRM2 or the C-terminal Gly-rich domain (Figure 11B).

For FUS, I analyzed the following deletion mutants: (1) the ΔQ mutant lacking amino acids 1 to 160 of the N-terminal Gln-rich low complexity domain, (2) the ΔQ/RGG1 mutant lacking the low complexity and the first RGG domain, (3) the ΔQ/RGG1/RRM mutant lacking the low complexity domain, the first RGG domain and the RNA-recognition motif domain, (4) the ΔRGG2 mutant lacking the second RGG domain, (5) the ΔZnF mutant missing the Zink finger motif and (6) the ΔRGG3 mutant lacking the third RGG domain (see Figure 11C). In the heterokaryon assay, all FUS deletion mutants were able to exit the HeLa and accumulate in the mouse nuclei as efficiently as wild-type FUS (Figure 11D), suggesting that nuclear export of FUS is not mediated by the tested functional domains.

Summarizing these results, nuclear export of TDP-43 and FUS occurs dispensable of analyzed functional domain. However, it is still possible that nuclear export of TDP-43 is mediated by signals or domains that were not deleted (*e.g.* N-terminus or the region spacing RRM1 and RRM2). Nuclear export of FUS, on the other hand, takes place independent of domains or potential signals within the domains, suggesting an alternative route, such as passive diffusion, in order to leave the nucleus.

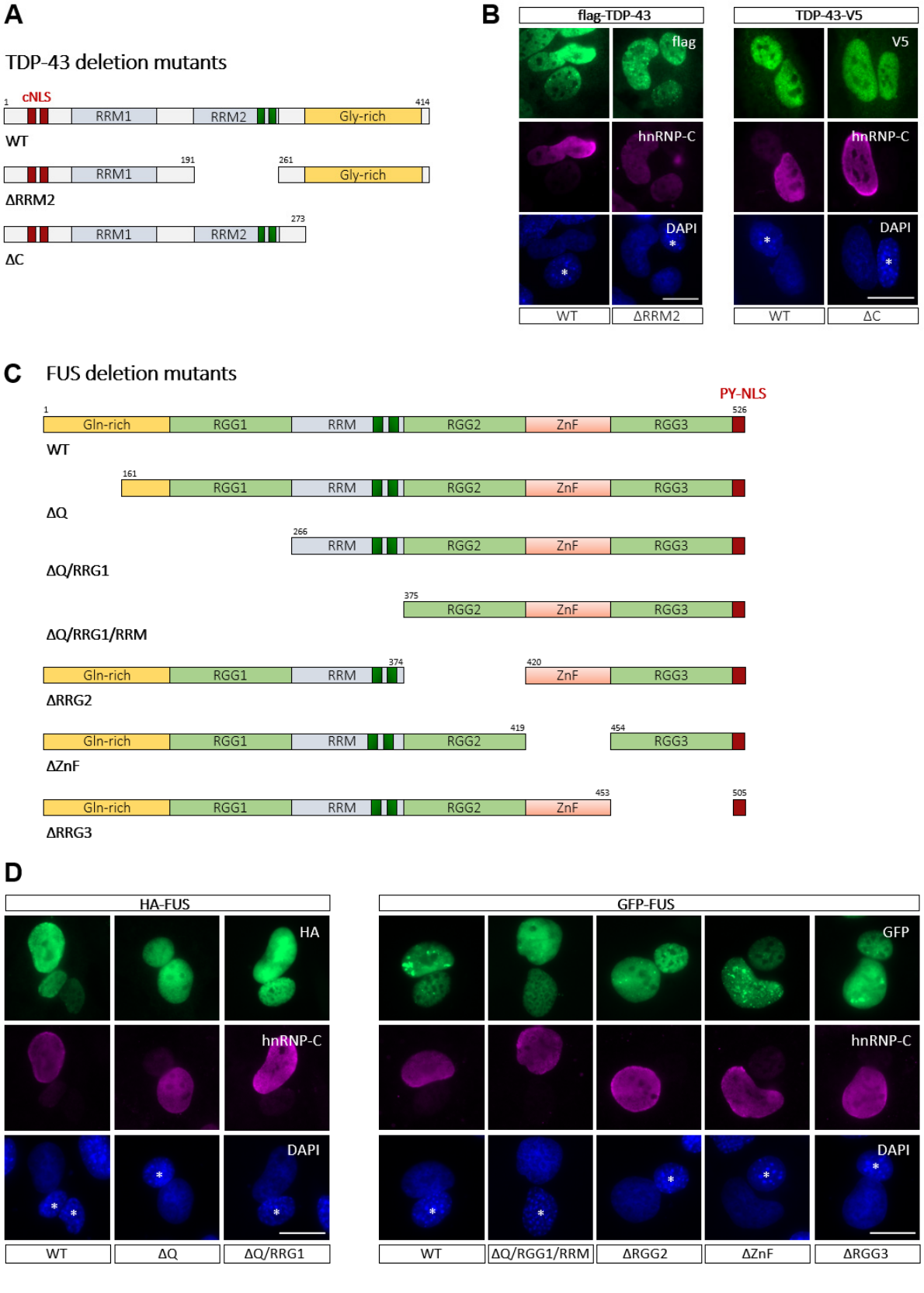

A
TDP-43 deletion mutants
cNLS
1
414
RRM1
RRM2
Gly-rich
WT
191
261
ΔRRM2
273
ΔC
B
flag-TDP-43
TDP-43-V5
flag
V5
hnRNP-C
DAPI
WT
ΔRRM2
WT
ΔC
C
FUS deletion mutants
PY-NLS
1
526
Gln-rich
RGG1
RRM
RGG2
ZnF
RGG3
WT
161
ΔQ
266
ΔQ/RRG1
375
ΔQ/RRG1/RRM
374
420
ΔRRG2
419
454
ΔZnF
453
505
ΔRRG3
D
HA-FUS
GFP-FUS
HA
GFP
hnRNP-C
DAPI
WT
ΔQ
ΔQ/RRG1
WT
ΔQ/RGG1/RRM
ΔRGG2
ΔZnF
ΔRGG3

Figure 11. The role of functional domains in nuclear export of TDP-43 and FUS
A. Graphic scheme of functional domains deleted in TDP-43.
B. HeLa cells expressing the indicated mutant constructs were analyzed in the interspecies heterokaryon assay. Both wild-type (WT) and deletion mutants of TDP-43 undergo nuclear export seen by accumulation in the murine nuclei. Visualization of TDP-43 (green) was achieved using a flag-, or V5-specific antibody, respectively; hnRNP-C (pink) was stained with a human-specific hnRNP-C antibody and nuclei with DAPI (blue). Scale bars: 20µm.
C. Graphic scheme of functional domains deleted in FUS.
D. Transiently transfected HeLa cells expressed either HA-tagged FUS deletion mutants (ΔQ, ΔQ/RGG1) or GFP-tagged ΔQ/RGG1/RRM-, ΔRGG2-, ΔZnF-, and ΔRGG3-mutant versions of FUS were used in the interspecies heterokaryon assay. All tested FUS deletion mutants shuttle from the HeLa to MEF nuclei of heterokaryons. HA-, and hnRNP-C-specific antibody was used to visualize respective proteins, while the GFP-tagged constructs were directly detected via fluorescence microscopy; nuclei were stained with DAPI (blue). Scale bars: 20µm.

7. Enlargement of TDP-43 and FUS impairs their nuclear egress

As nuclear export of TDP-43 and FUS does not seem to depend on export receptors, such as Exportin-1/CRM1, or the mRNA export pathway, the previously discussed passive diffusion might represent a likely alternative route for the exit of both proteins out of the nucleus (see “An alternative possibility: passive diffusion as a potential export route for TDP-43 and FUS”, p. 22).

To address this hypothesis, I utilized the hormone-inducible nuclear transport assay adopted from Love and colleagues (Love, Sweitzer et al. 1998). In this assay, a protein-of-interest is fused to two hormone-binding domains of the glucocorticoid receptor (GR_2, 65 kDa) and two fluorescent proteins (GFP_2, 54 kDa). In the absence of a steroid hormone, the GR domain anchors the NLS-containing protein-of-interest in the cytoplasm. Upon addition of a steroid hormone*, e.g.* dexamethasone (+ DEX), a conformational change abrogates cytosolic anchoring via GR and the protein-of-interest is imported into the nucleus. This assay also allows monitoring nuclear export of the protein-of-interest, as once dexamethasone is removed, the protein-of-interest can be exported and re-anchored in the cytoplasm (DEX removal, see schematic diagram in Figure 12A). If the protein-of-interest contains a functional NES that interacts with an export receptor, nuclear export is a rapid and temperature-sensitive process that is paralyzed at low temperatures (Gama-Carvalho and Carmo-Foncesa. 2006). If the protein-of-interest does not contain an NES and is not exported via an export receptor, but simply can passively diffuse out of the nucleus, nuclear egress of GR_2-GFP_2-fusion proteins is slow and insensitive to low temperature, as passive diffusion also occurs at low temperatures and decelerates with the increasing size of diffusing proteins.

I implemented the hormone-inducible nuclear transport assay to compare nuclear export of enlarged TDP-43 (GR_2-GFP_2-TDP-43, 162 kDa) and FUS (GR_2-GFP_2-FUS, 172 kDa) reporter proteins to export of a Rev (GR-GFP-Rev) reporter protein. Rev is a known Exportin-1/CRM1 cargo (Neville, Stutz et al. 1997) and was used as a positive control for rapid receptor-mediated export in this assay. In the absence of dexamethasone (- DEX), all reporter proteins were mainly localized in the cytoplasm, but shifted to the nucleus upon dexamethasone treatment (+ DEX for 20 min at 37°C, 0h). Removal of dexamethasone led to complete cytoplasmic relocalization of the Rev reporter over a time course of 5 hours, but hardly caused any relocalization of TDP-43 and FUS reporter proteins (Figure 12B). This suggests that enlargement of TDP-43 and FUS strongly decelerates their nuclear egress. Furthermore, analysis of export at low temperature (5h, 4°C) displayed inhibition of the temperature-sensitive active export of GR-GFP-Rev, while the localization of GR_2-GFP_2-TDP-43 and -FUS was virtually indifferent compared to their distribution after 5h at 37°C (see Figure 12B). As nuclear egress of TDP-43- and FUS-fusion proteins was slow and unaffected by low temperatures, it most likely occurred independent of export receptors and rather occurred by slow, passive diffusion across nuclear pores.

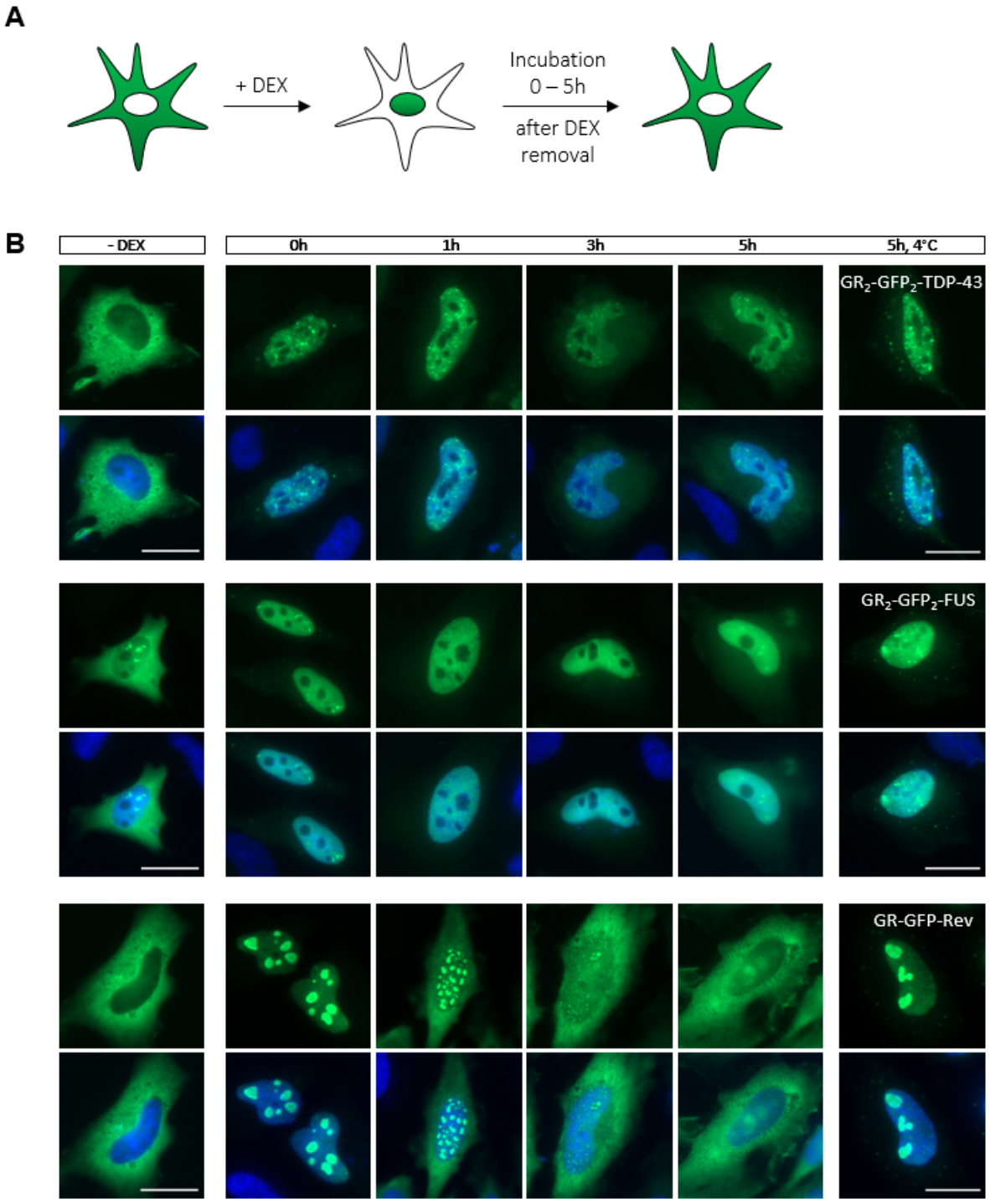


Figure 12. Addressing nuclear export of TDP-43 and FUS in the hormone-inducible nuclear transport assay
A. Graphic scheme of the hormone-inducible nuclear transport assay. GR_2-GFP_2-enlarged TDP-43 and FUS (represented by green color) predominately localize to the cytoplasm until addition of dexamethasone (+ DEX) leads to nuclear import caused by the endogenous NLS of TDP-43 and FUS. If TDP-43 or FUS are exported via active, receptor-mediated export, removal of dexamethasone would be expected to lead to a rapid cytoplasmic redistribution that is sensitive to reduced temperatures. In contrast, if nuclear egress of TDP-43 and FUS simply depends on passive diffusion, removal of dexamethasone would result in very slow, inefficient relocalization of TDP-43 or FUS fusion proteins over an incubation time of 5 hours.

B. HeLa cells were transiently transfected with GR-GFP-Rev, GR_2-GFP_2-TDP-43 and -FUS fusion proteins and their localization was observed in the absence of dexamethasone (-DEX) or at various time points after dexamethasone washout (1, 3, 5h) and after a 20min-incubation with dexamethasone at 37°C labeled as 0h. Steady state localization (- DEX) of all three fusion proteins is predominately cytoplasmic, but shifts to the nucleus upon dexamethasone addition (0h). Removal of dexamethasone leads to an efficient relocalization of GR-GFP-Rev from the nucleus to the cytoplasm over the course of 5 hours, while GR_2-GFP_2-TDP-43 and -FUS remain mainly nuclear with only small amounts present in the cytoplasm after 5h. If the export reaction is carried out at 4°C, GR-GFP-Rev remains nuclear, confirming that active receptor-mediated export is temperature-sensitive and thus is inhibited by low temperature. Scale bars: 20µm.

To further substantiate the idea that TDP-43 and FUS leave the nucleus by passive diffusion the NEX-TRAP (Nuclear Export Trapped by RAPamycin) assay was utilized (see schematic diagram in Figure 13A). This assay depends on the dimerization of FRB (FK506-rapamycin (FR)-binding domain) and FKBP (FK506-binding protein-12) upon addition of rapamycin. A FKBP-trimer ($FKBP_3$) is fused to an integral membrane protein (gM) located at the trans Golgi network (TGN), exposing the resulting reporter protein to the cytoplasm. FRB is fused to the potential nuclear export cargo, while EYFP and a SV40-derived NLS are attached for visualization and constitutive nuclear import, respectively (see Figure 13B). Due to the attached NLS for constitutive nuclear import, the EYFP-NLS-FRB fusion protein is localized in the nucleus at steady state, but if the attached protein-of-interest undergoes active receptor-mediated nuclear export, the fusion protein will be exported to the cytoplasm where it can dimerize with the gM-$FKBP_3$ reporter, thus permanently anchoring the fusion protein at the TGN (Raschbichler, Lieber et al. 2012).

TDP-43 and FUS were enlarged by the fusion to EYFP-NLS-FRB (43 kDa, further referred to as EYFP-TDP-43-WT and -FUS-WT) and nuclear export activity was compared to the EYFP-NLS-FRB-pUL4 (a HSV-1 protein with a known NES, further referred to as EYFP-pUL4) control protein. The control protein EYFP-pUL4 relocalized from the nucleus and dimerized with the gM-$FKBP_3$ reporter protein (further referred to as gM) at the TGN in the cytoplasm upon rapamycin treatment (2h, see bottom panels of Figure 13C), while EYFP-TDP-43-WT and -FUS-WT remained nuclear despite rapamycin addition (see top and middle panels of Figure 13C).

The use of NLS-FRB-TDP-43 or -FUS fusion proteins in the NEX-TRAP assay supports the hypothesis that TDP-43 and FUS exit the nucleus via slow passive diffusion and not by a rapid, receptor-mediated route. This is in line with the observed export deceleration due to increased size of TDP-43 and FUS observed in the hormone-induced nuclear transport assay.

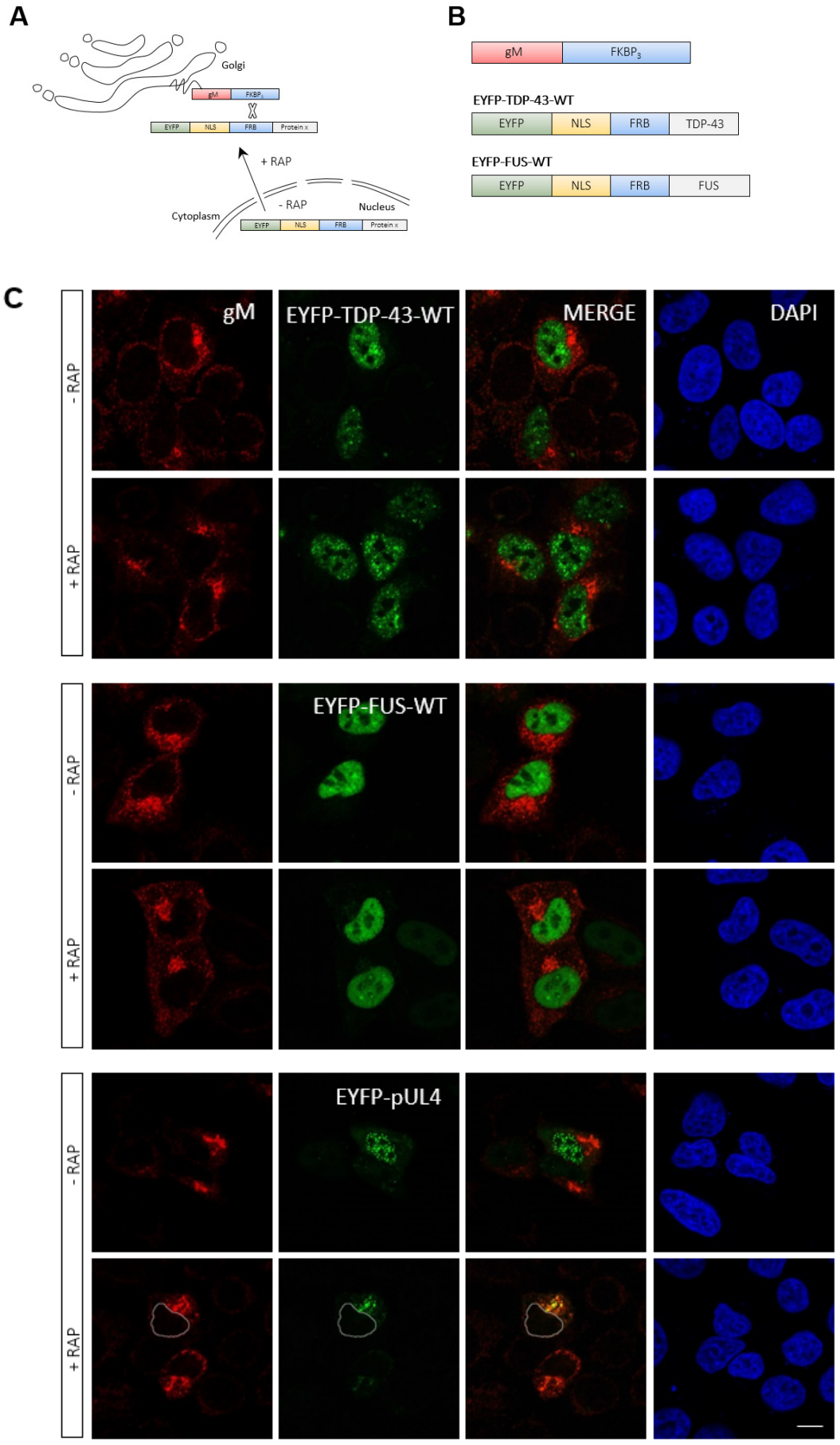

A
Golgi
gM
FKBP3
EYFP
NLS
FRB
Protein x
+ RAP
- RAP
Cytoplasm
Nucleus
B
gM
FKBP3
EYFP-TDP-43-WT
EYFP
NLS
FRB
TDP-43
EYFP-FUS-WT
EYFP
NLS
FRB
FUS
C
- RAP
+ RAP
gM
EYFP-TDP-43-WT
MERGE
DAPI
EYFP-FUS-WT
EYFP-pUL4

Figure 13. Nuclear export of TDP-43 and FUS in the NEX-TRAP assay

A. Graphical scheme of the Nuclear Export Trapped by RAPamycin (NEX-TRAP) assay. EYFP-NLS-FRB-Protein x (x = protein-of-interest with unknown export activity) is nuclear in the absence of rapamycin. Addition of rapamycin induces dimerization of EYFP-NLS-FRB-Protein x with the gM-$FKBP_3$ reporter protein and thus traps the fusion protein at the Golgi apparatus in the cytoplasm.

B. Graphical scheme of fusion proteins used in the NEX-TRAP assay.

C. HeLa cells were transiently co-transfected with the gM-$FKBP_3$ (gM) reporter protein and pEYFP-NLS-FRB-TDP-43 (EYFP-TDP-43-WT), pEYFP-NLS-FRB-FUS (EYFP-FUS-WT) or EYFP-NLS-FRB-pUL4 (EYFP-pUL4) and their nuclear export was analyzed. After the transfection (20h-incubation period), cells were treated with the protein synthesis inhibitor anisomycin (10min) and rapamycin was added (+ RAP for 2h). EYFP-pUL4 translocates to the cytoplasm and co-localizes with gM reporter protein (MERGE-panel) after rapamycin treatment, while EYFP-TDP-43 and –FUS-WT remain nuclear and are not trapped in the cytoplasm, indicating lack of nuclear export. TDP-43, FUS and pUL4 were visualized by EYFP (green) and gM by using a gM-specific antibody (red); nuclei were stained with DAPI (blue). Scale bar: 10µm.

8. Nuclear egress of TDP-43 is promoted upon decreased transcription or RNA-binding of TDP-43

Nuclear egress of TDP-43 and FUS seems to be a diffusion-limited process, as indicated by studying enlarged fusion proteins in the hormone-inducible nuclear transport and NEX-TRAP assays. Thus, their exit from the nucleus is expected to be limited by nuclear interactions (*e.g.* with DNA/RNA or other proteins). As TDP-43 and FUS have well-described functions in regulating nuclear RNA processing (see "From pre-mRNA to microRNA: functions of TDP-43 and FUS in the nucleus", p.13-15), I addressed a potential role of newly-synthesized RNA in restricting passive diffusion of TDP-43 or FUS out of the nucleus.

Upon inhibition of transcription by actinomycin D (Act D) treatment, endogenous TDP-43 partially translocated to the cytoplasm, while FUS remained predominantly nuclear (see Figure 14A). Efficient transcriptional inhibition by actinomycin D under these experimental conditions was demonstrated by the relocalization of coilin into nucleolar caps as previously reported (Shav-Tal, Blechman et al. 2005), (see coilin staining in Figure 14A). Moreover, I analyzed the subcellular localization of TDP-43 and FUS by nuclear and cytoplasmic fractionation of untreated and Act D-treated cells. Transcriptional inhibition by Act D treatment caused significantly increased levels of TDP-43, but not FUS, in the cytoplasmic fraction (see Figure 14B and C), indicating that newly synthesized RNA retains TDP-43 in the nucleus and limits is passive diffusion through nuclear pores. In addition, the RNA-binding deficient TDP-43-F4L mutant showed significantly increased levels in the cytoplasmic fraction compared to wild-type TDP-43, substantiating the nuclear retention capability of RNA on TDP-43 (see Figure 14D and E). Interestingly, comparison of the subcellular localization of wild-type and TDP-43-F4L mutant showed nuclear localization of both proteins (see Figure 14F). However, the RNA-binding deficient TDP-43-F4L mutant accumulates in nuclear foci; a phenomenon previously observed when RNA-binding is disrupted (Ayala, Zago et al. 2008). Thus, the increased levels of TDP-43-4FL in the cytoplasmic fraction are due to a higher extractability of the mutant protein, compared to DNA/RNA-bound wild-type TDP-43.

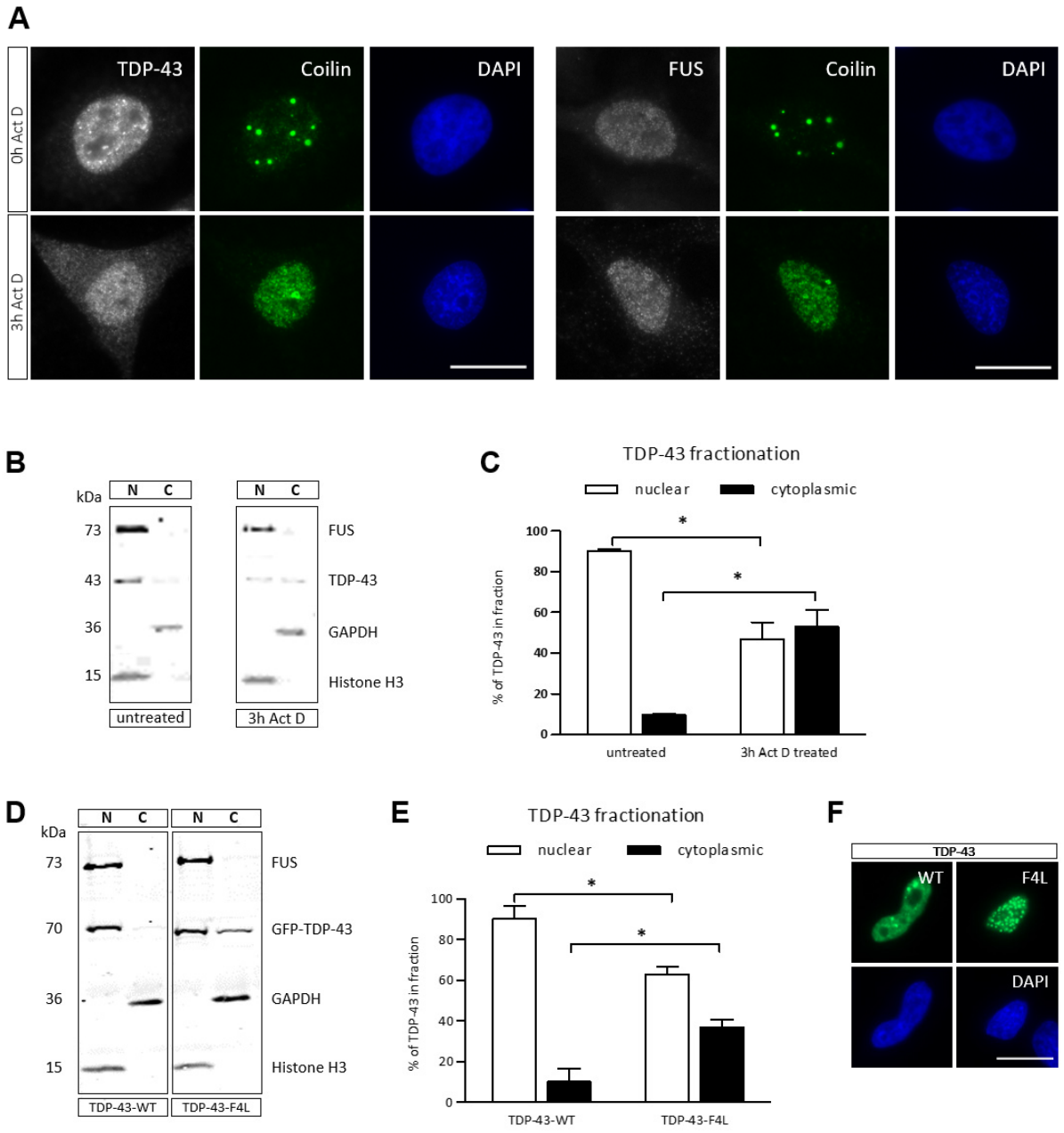


Figure 14. Transcriptional inhibition and disrupted RNA-binding causes redistribution of TDP-43 to the cytoplasm
A. Transcriptional inhibition by actinomycin D (Act D) primarily affects the localization of TDP-43 but not FUS. The incubation of HeLa cells with Act D (5µg/mL, 3h) leads to an enhanced localization of TDP-43 in the cytoplasm, while FUS remains predominantly nuclear. Coilin served as a positive control for the efficient transcription inhibition by Act D, as it redistributed into nuclear caps upon Act D treatment (green, stained with a coilin-specific antibody). TDP-43 and FUS were visualized using antibodies for endogenous TDP-43 or FUS (white) and nuclei were stained with DAPI (blue). Scale bars: 20µm.
B. Nuclear/cytoplasmic fractionation analysis of untreated and Act D-treated cells. Act D-treated HeLa cells (5µg/mL, 3h) display higher TDP-43 levels in the cytoplasmic (C) fraction, while FUS is found predominantly in the nuclear (N) fraction in both untreated and Act D-treated condition. GAPDH was used as a cytosolic and Histone H3 as a nuclear marker protein. Protein levels were detected via Western blotting using TDP-43-, FUS-, GAPDH and Histone H3-specific antibodies.

C. The levels of TDP-43 in the cytoplasmic and nuclear fraction were quantitatively analyzed in immunoblots from three independent fractionation experiments. Error bars represent standard error of the means and p-values (≤ 0.05) were calculated with Wilcoxon rank-sum test for two paired samples.
D. Nuclear/cytoplasmic fractionation analysis of RNA-binding deficient TDP-43 versus wild-type TDP-43. GFP-tagged TDP-43-WT or RNA-binding deficient TDP-43-F4L mutant were transiently transfected into HeLa cells and subsequently fractionated into a nuclear (N) and cytoplasmic (C) fraction. Mutant TDP-43 is partially found in the cytoplasmic fraction, whereas the WT protein is predominantly found in the nuclear fraction. FUS always stays within the nuclear fraction. Like above, protein levels were detected via Western blotting using GFP-, FUS-, GAPDH and Histone H3-specific antibodies. GAPDH was used as a cytosolic and Histone H3 as a nuclear marker.
E. Quantification of TDP-43 levels in the nuclear and cytoplasmic fraction was performed on three independent experiments. Error bars represent standard error of the means and p-values (≤ 0.05) were calculated with Wilcoxon rank-sum test for two paired samples.
F. Both TDP-43-WT and RNA-binding deficient TDP-43-F4L mutant (GFP-tagged) display a nuclear localization in HeLa cells, with TDP-43-4FL displaying nuclear foci, possibly indicating oligomerization/aggregation of mutant TDP-43. Scale bar: 20µm.

Furthermore, I analyzed the effect of transcriptional inhibition on nuclear export rates of EGFP-TDP-43 in living cells using FLIP (Fluorescence loss in photobleaching) analysis; whereby the export rates were assessed by the fluorescence loss in the nucleus that occurred while 3 defined areas in the cytoplasm were continuously bleached over a defined time period (see schematic diagram in Figure 15A). Comparison of export rates in untreated or ActD-treated HeLa cells, expressing EGFP-tagged TDP-43, displayed an accelerated export of EGFP-TDP-43 in cells with impaired *de novo* RNA synthesis (see Figure 15B and C). Thus, nuclear egress of TDP-43 is indeed stimulated when *de novo* RNA synthesis is inhibited and hence the interaction of TDP-43 with RNA is reduced. Together, my data suggests that RNA “anchors” TDP-43 in the nucleus and limits in passive diffusion through nuclear pores.

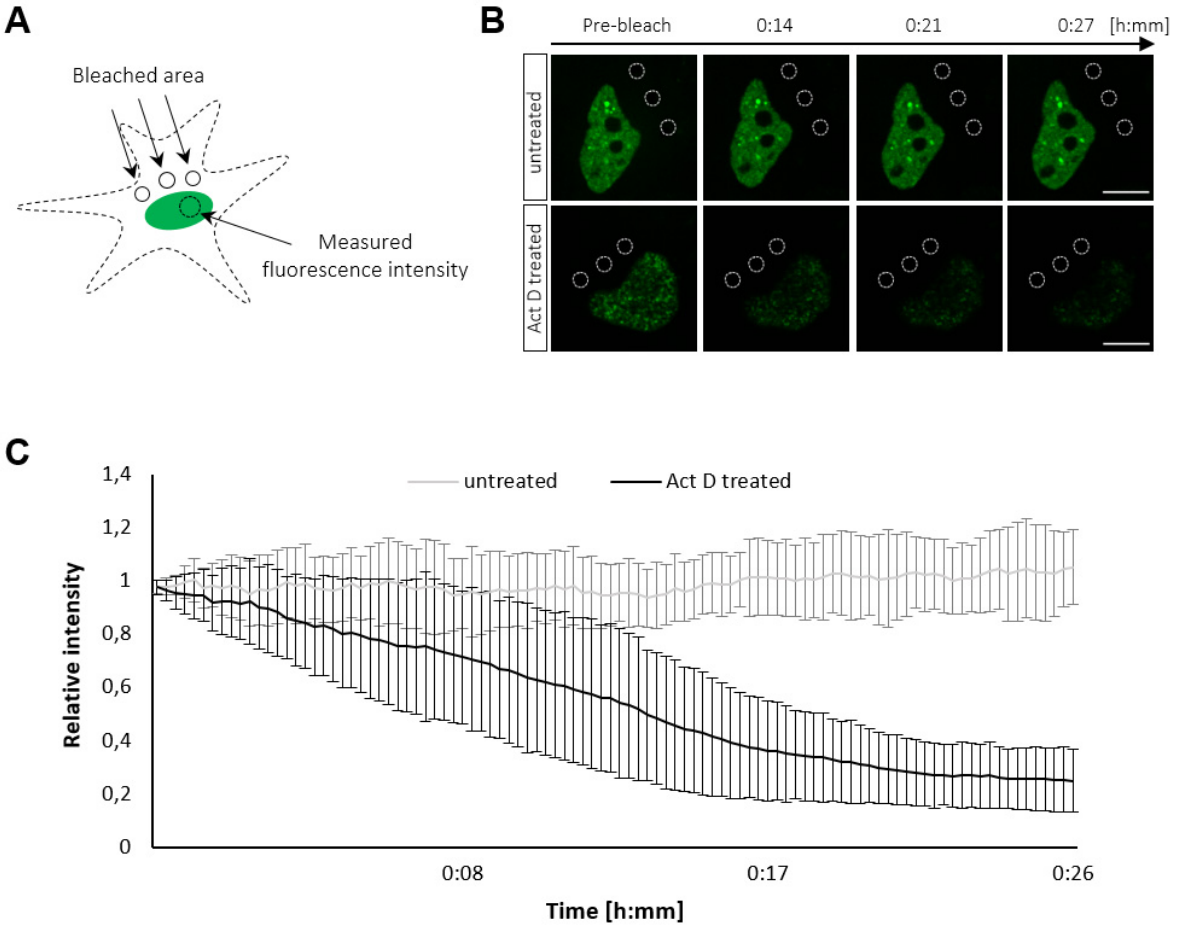


Figure 15. Investigating TDP-43 egress in living cells upon transcription inhibition

A. Graphic scheme of FLIP analysis. HeLa cells were transiently transfected with EGFP-tagged TDP-43-WT (represented by green color) and were either left untreated or were incubated with Act D for 3h. Afterwards the nuclear fluorescence intensity in both conditions was measured over time, while three defined areas in the cytoplasm were continuously bleached.

B. Representative fluorescence images of untreated and Act D-treated HeLa cells demonstrating the accelerated nuclear egress of TDP-43 when transcription is inhibited. Scale bars: 10µm.

C. Quantification of FLIP analysis. Loss of relative EGFP-fluorescence intensity over time was observed in Act D-treated, but not in untreated cells. This documents a quantifiable acceleration of TDP-43 nuclear egress upon inhibition of RNA synthesis.

III. DISCUSSION

DECLARATION OF COPYRIGHT

This section of the Ph.D. thesis was prepared in parallel to the manuscript "Nuclear egress of TDP-43 and FUS is a diffusion-limited process", submitted to Scientific Reports (in revision, but not yet accepted). Therefore, structure and text of this Ph.D. thesis and the manuscript will partially overlap in this regard.

1. Transport defects in ALS and FTD

Defective import is known to critically contribute to ALS and FTD-associated cytoplasmic inclusions of FUS and represents a pathomechanism in the neurodegeneration of ALS and FTD (see p. 10-11 for further information). The role of defects in nuclear export of TDP-43 or FUS in the pathogenesis of ALS and FTD, on the other hand, is poorly understood and it is unclear whether dysfunctional nuclear export is responsible for nuclear aggregation of TDP-43 or FUS. However, there is emerging evidence that the nucleocytoplasmic transport machinery in general, regulating nuclear import and export, harbors key components that may contribute to pathogenesis of neurodegenerative diseases, such as ALS and FTD.

A first link between disturbed factors in nuclear export and neuronal diseases came from work on nucleoporin GLE1, a key component of the mRNA export route. Nousiainen and colleagues found GLE1 to be the underlying cause for the motor neuron syndrome LCCS1 (Lethal congenital contracture syndrome 1). Their study revealed defects in motoneuron development within the spinal cord caused by mutations in GLE1 and suggested that mRNA processing represents a key mechanism for the proper maturation and survival of motoneurons (Nousiainen, Kestila et al. 2008). More recently, a screen mapped mutant GLE1 in ALS patients demonstrating that the identified mutations deplete nuclear pore complexes of the GLE1 protein in HeLa cells. This finding substantiated the hypothesis that RNA metabolism might represent a central component in ALS pathology, albeit through a different mechanism than in the context of LCCS1 (Kaneb, Folkmann et al. 2015). Moreover, Freibaum and colleagues found GLE1 to be an enhancer of hexanucleotide repeat expansion GGGGCC (G_4C_2)-associated toxicity in transgenic fly models for C9orf72-linked ALS. Shortly, C9orf72 (Chromosome 9 open reading frame 72) mutations are known to be the most common genetic cause for ALS and FTD (often referred to as c9ALS/FTD). Patients with C9orf72-associated ALS/FTD display a massive expansion of hexanucleotide repeats (up to thousands of repeats) in a non-coding region of the C9orf72 gene. Currently, three hypotheses exist to illustrate the pathogenesis of C9orf72 repeat expansions. Loss of physiological C9orf72 functions potentially caused by the massive hexanucleotide repeat expansion (HRE) that in turn downregulates the C9orf72 gene expression, represents the first

hypothesis. The second hypothesis includes toxic G_4C_2 RNA foci that might sequester RNA-binding proteins, while the third hypothesis considers the presence of dipeptide-repeat proteins (DPRs), translated from repeat-containing transcripts, as the pathogenic HRE feature (Gitler and Tsuiji 2016, Haeusler, Donnelly et al. 2016).

In addition to GLE1, further components of the nucleocytoplasmic transport machinery, including various nucleoporins (Nups, *e.g.* Nup107 and Nup160) or further mRNA export factors (*e.g.* Aly/REF and NXF1) were identified to modify the toxicity in the Drosophila models suggesting that nucleocytoplasmic transport might play a crucial role in neurodegeneration (Freibaum, Lu et al. 2015). Another C9orf72-related study, published in the same year as Freibaum and colleagues, supported this hypothesis as they independently reported about nucleocytoplasmic transport defects in C9orf72 fly and human cell models (Zhang, Donnelly et al. 2015). Interestingly, they also noted that nuclear levels of the TDP-43 fly homologue TBPH were reduced in G_4C_2-expressing cells hence arguing that nuclear import for proteins with a classical NLS might be disrupted by hexanucleotide repeat expansions (Zhang, Donnelly et al. 2015).

These findings suggest that the nucleocytoplasmic shuttling machinery indeed plays an important role in the pathogenesis of neurodegenerative diseases, such as ALS and FTD. As nuclear export represents a central mechanism of the nucleocytoplasmic shuttling machinery, these findings furthermore support the hypothesis that nuclear export could be altered in ALS and FTD and thus represent a potential pathomechanism. However, how and to which extend nuclear egress of TDP-43 and FUS is affected in the pathogenesis of ALS and FTD and whether altered nuclear egress results in intranuclear TDP-43 and FUS inclusions of ALS and FTD patients requires further work.

2. Export via Exportin-1/CRM1 – a misconception for TDP-43 and FUS

In my thesis work, I focused on identifying the nuclear export routes of TDP-43 and FUS and could show, contrary to the previous predictions, that both proteins neither implement the predicted putative nuclear export signals within the proteins, nor use Exportin-1/CRM1 as the corresponding export receptor.

Addressing the NES activity of predicted leucine-rich NESs in TDP-43 and FUS in a *bona fide* nuclear export assay showed that TDP-43 and FUS exit the nucleus despite the replacement of key hydrophobic residues in the predicted NESs thus suggesting that the predicted leucine-rich NESs are dispensable for the nuclear export of TDP-43 and FUS.

With regard to TDP-43, I reproduced and tested the putative TDP-43 ΔNES2 mutant (L248A/I249A/I250A) introduced by Winton and colleagues (Winton, Igaz et al. 2008) in the interspecies heterokaryon assay (data not shown) . Like the TDP-43 NES-mutants presented in this

work (see p. 28-29), the ΔNES2 mutant had no effect on nuclear export of TDP-43 in this *bone fide* nuclear export assay, further substantiating the hypothesis that the NES predicted by Winton and colleagues and my own bioinformatical prediction is not functional.

I cannot completely exclude the presence of alternative functional TDP-43 and FUS NESs that were not predicted by the available and implemented prediction tools. However, the analysis of various functional domains and their impact on nuclear export of TDP-43 and FUS demonstrated that nuclear export, especially of FUS, occurred independent of tested domains or potential alternative export signals within them. These results reinforce the hypothesis that predicted NESs of FUS are not functional and further suggest that nuclear export of FUS is not mediated by certain domains or signals. In addition, the pharmacological inhibition of Exportin-1/CRM1 with the widely used Exportin-1/CRM1 inhibitor leptomycin B (Kudo, Matsumori et al. 1999, Sun, Carrasco et al. 2013) and the siRNA-mediated depletion of Exportin-1/CRM1 as well as a combination of both clearly demonstrated an Exportin-1/CRM1-autonomous nuclear export route for TDP-43 and FUS contradicting the presence of functional NESs that interact with Exportin-1/CRM1.

Support for an Exportin-1/CRM1-autonomous export route for TDP-43 is provided by the abstract "The role of nuclear export in TDP-43-mediated neurodegeneration" by Hilary C. Archbold (University of Michigan), presented at the Society for Neuroscience (SfN) meeting 2015 in Chicago: This study addressed the effect of selective inhibitors of nuclear export (SINE^{TM}) compounds KPT335 and KPT350, which selectively inhibit Exportin-1/CRM1-dependent export, on the subcellular localization of TDP-43. In line with my data, they did not detect a shift of nuclear TDP-43 in HEK 293T cells or primary cortical neurons of rats, hence excluding Exportin-1/CRM1as key export factor of TDP-43.

Moreover, my results on the Exportin-1/CRM1-independent nuclear export of TDP-43 and FUS are in line with quantitative mass spectrometry screens of Exportin-1/CRM1 cargoes that did not identify TDP-43 and FUS among potential Exportin-1/CRM1 cargoes (Thakar, Karaca et al. 2013, Kirli, Karaca et al. 2015).

Considering an Exportin-1/CRM1-autonomous nuclear export of TDP-43, the question arises what led to the granular expression pattern of Winton´s putative TDP-43 ΔNES2 mutant (Winton, Igaz et al. 2008). As the putative NES resides within the RRM2 domain, the introduced mutations could disrupt a proper folding of the RRM2 domain and thus disturb RNA binding. In line with this hypothesis is a study by Ayala and colleagues who observed nuclear aggregation of RNA-binding deficient TDP-43 (Ayala, Zago et al. 2008, Winton, Igaz et al. 2008) as well as additional work on C-terminal fragments of TDP-43. These C-terminal fragments, containing the RRM2, were shown to remain soluble in the cytoplasmic cellular environment but aggregated into foci upon RNase treatment, suggesting that the solubility of C-terminal TDP-43 fragments is stabilized by RNA binding and that aggregation might be prevented by interaction with RNA (Pesiridis, Tripathy et al. 2011).

The presence of a putative NES in FUS was suggested by Kino and colleagues who observed a predominantly cytoplasmic redistribution of the GFP-tagged FUS RRM and attributed this relocalization to the putative NES (VQGLGENVTI) within this domain, identified via the bioinformatical prediction tool NetNES1.1. However, they observed an evenly distributed localization pattern when the RRM domain was fused to the first RGG domain and hence hypothesized about an inhibitory regulation of the NES activity by the neighboring RGG domain (Kino, Washizu et al. 2010). Like in the case of TDP-43, the putative NES of FUS is located within the RRM domain. Therefore, it seems likely that Kino and colleagues observed a physiological localization of the FUS RRM domain, analogous to cytoplasmic distribution of above-mentioned C-terminal TDP-43 fragments containing RRM2, rather than NES-mediated relocalization. Furthermore, recently published work of Ozdilek and colleagues on RGG/RG domains as the key sequences in RNA binding of FUS could explain that the additionally attached RGG domain strengthened the RNA binding capability of the RRM domain, hence leading to more nuclear localization upon binding to nuclear RNAs (Ozdilek, Thompson et al. 2017).

3. Of alternative routes and factors: TDP-43 and FUS seem to leave the nucleus via passive diffusion

As well-defined RNA-binding proteins, TDP-43 and FUS seem to be likely candidates for the mRNA export pathway, alongside with bound mRNA targets. Nevertheless, the examination of this route showed that nuclear export of TDP-43 and FUS occurs independent of RNA-binding and does not require the mRNA export factor Aly/REF. Previous work by Sugiura and colleagues had implied that FUS is part of the DDX39, ALY and CIP29 complex that is thought to have functions in splicing, transcription and mRNA export, but did not experimentally address whether FUS is exported via the mRNA export pathway or whether it functions in mRNA export (Sugiura, Sakurai et al. 2007). Even though my data argue against FUS being exported along with bound mRNAs or the Aly/REF-dependent mRNA export pathway, it is still possible that DDX39, ALY and CIP29 interact with FUS and that FUS has an active role in mRNA export by interacting with these factors.

The list of newly identified cargoes, interacting with export receptors is constantly growing. Exportin-5 and Exportin-t are known to facilitate the nuclear export of different RNA types: Exportin-5 mediates export of *e.g.* pre-miRNA (Bohnsack, Czaplinski et al. 2004) but also of double-stranded RNA-binding proteins like Staufen 2 (Macchi, Brownawell et al. 2004), while Exportin-t was shown to export mature tRNAs (Arts, Kuersten et al. 1998). Profilin-actin complexes, in turn, are cargoes of Exportin-6 (Stuven, Hartmann et al. 2003). Smad 3 was identified as a cargo of Exportin-4, and the MH2 domain was determined as the corresponding export-mediating sequence (Kurisaki, Kurisaki et al. 2006). Interestingly, a multipartite export signal consisting of three regions within p50RhoGAP, an export

substrate of Exportin-7 was discussed, suggesting that nuclear export of certain cargoes might rely on complex export signals represented by multiple functional domains (Mingot, Bohnsack et al. 2004).

In my thesis, I addressed the role of different export receptors using a systematic knockdown approach (see Figure 10) and tested whether individual functional domains of TDP-43 and FUS serve as export signals (see Figure 11). However, my experiments did not unravel any functional domain that was essential for nuclear export of TDP-43 and FUS, nor did the knockdown studies provide proof for a particular export factor. Since I did not analyze the role of the N-terminal domain and the sequence spacing RRM1 and RRM2 as well as the sequence spacing RRM2 and the C-terminus in nuclear export of TDP-43, I cannot exclude a potential function of these particular sequences in nuclear export of TDP-43. Moreover, it could be possible that nuclear export of cargos, such as TDP-43 and FUS is mediated by multiple export receptors (see analogous hypothesis on import receptors in the Side project, p. 61-62), therefore it should be noted that knockdown of a single export receptor or factor might not be sufficient enough to abolish the complete nuclear export of interacting cargos.

Nevertheless, Exportin-5 seemed a promising candidate, as a genetic screen in yeast found that the yeast orthologue of this export receptor elevates TDP-43-induced toxicity (Kim, Raphael et al. 2014). Since the nucleocytoplasmic transport machinery is conserved among species, this export receptor could indeed represent a potential candidate mediating export of TDP-43 and probably FUS (Pemberton and Paschal 2005). However, in my experiments siRNA-mediated knockdown of Exportin-5 did not affect export of both TDP-43 and FUS.

Only the significant enlargement of TDP-43 and FUS had an impact on nuclear egress of both proteins, suggesting that they exit the nucleus via passive diffusion rather than via an active, receptor-facilitated pathway. Recent work on passive diffusion proposes a “soft barrier” model whereby passive diffusion rates become gradually inefficient with the increasing size of molecules (Mohr, Frey et al. 2009, Timney, Raveh et al. 2016). It was shown, that small molecules translocate through the nuclear pore complexes within minutes, macromolecules with a size of up to 230 kDa, on the contrary, needed hours to pass NPCs (Wang and Brattain 2007, Popken, Ghavami et al. 2015). Nuclear egress of TDP-43 and FUS goes in line with this model as their artificial enlargement decelerated their diffusion in two independent assays (see Figures 12 and 13).

As the exact mechanism of passive diffusion is still under debate, several models were proposed as potential explanations. One model suggests that active transport of macromolecules happens along the walls of nuclear pore complexes, while a narrow tube in the center of the nuclear pore is reserved for the passive transit of cargoes (Peters 2005). Another model, in contrast, proposes passive diffusion through peripheral channels surrounding the central pore of nuclear pore complexes (Hinshaw, Carragher et al. 1992, Naim, Brumfeld et al. 2007). Either way, the translocation of actively transported or passively diffusing proteins share the passage of nuclear pore complexes as common

ground. Nuclear pore complexes are macromolecular structures made up of 30 different Nups (Beck and Hurt 2017) that not only form the permeability barrier in the nuclear envelope, but also can actively affect the transport of certain cargos, *e.g.* Nup98 or Nup 358 (Walde and Kehlenbach 2010). There is indeed evidence that Nups are downregulated or damaged in the aging brain, indicating that defective Nups could contribute to nuclear transport defects in late-onset neurodegenerative diseases, such as ALS and FTD (D'Angelo, Raices et al. 2009). In fact, multiple Nups were identified as modifiers of hexanucleotide repeat expansion-induced toxicity in genetic fly and yeast screens (Freibaum, Lu et al. 2015, Jovicic, Mertens et al. 2015, Boeynaems, Bogaert et al. 2016). Thus, defects in nucleoporins might impair nucleocytoplasmic transport, possibly resulting in a global dysfunction of nuclear import and export as well as receptor-mediated transport and passive diffusion. This global impairment of nucleocytoplasmic transport represents one possible scenario how passive diffusion can be altered in neurodegeneration. Simultaneously, it opens the question which implications passive diffusion could have in TDP-43 and FUS pathology. Moreover, whether and how aberrant passive diffusion could cause cytoplasmic mislocalization and cytoplasmic or intranuclear aggregates, observed in TDP-43 and FUS pathology.

One possibility is that defects in RNA binding or reduction in RNA synthesis can influence passive egress of TDP-43 and FUS. Additionally, intranuclear interactions or self-interactions of TDP-43 and FUS could impact passive diffusion of both proteins. Interestingly, the last point has recently been addresses by Afroz and colleagues reporting that physiological nuclear TDP-43 forms homo-oligomers in mouse and human brain that could even antagonize pathologic aggregate formation (Afroz, Hock et al. 2017). Self-assembly and oligomerization on chromatin-associated RNAs of FUS have also been proposed (Yang, Gal et al. 2014, Yang, Zhang et al. 2015). Taking these findings into account, it seems likely that nuclear egress of TDP-43 and FUS is influenced by self-assembly and physiological oligomerization, especially when these intranuclear interactions/self-interactions result in the formation of large TDP-43 and FUS species that transit the NPCs less efficiently. In this regard, it would be interesting to address the question whether oligomerization-deficient mutants leave the nucleus more rapidly and whether increased nuclear egress, *e.g.* contributes to cytoplasmic aggregation of oligomerization-deficient TDP-43 (Afroz, Hock et al. 2017). It furthermore proposes that physiological self-interactions, such as oligimerization, could represent a therapeutic target in order to prevent or counteract cytoplasmic mislocalization and aggregation.

Finally, passive diffusion of TDP-43 was influenced by *de novo* synthesis of RNA as inhibition of RNA polymerase II upon actinomycin D treatment led to an accelerated exit of TDP-43 from the nucleus. The subcellular relocalization upon interference with RNA synthesis represents a phenomenon already ascribed to further hnRNPs, such as hnRNP-A1 (Lichtenstein, Guo et al. 2001) and reported for TDP-43 (Ayala, Zago et al. 2008) but no underlying mechanism has been proposed yet.

This observed nuclear retention by RNA binding could represent an additional physiological mechanism for mediating and ensuring nuclear localization of TDP-43 in order to maintain nuclear functions in mRNA splicing (Boelens, Palacios et al. 1995). Moreover, loss of nuclear retention upon disrupted RNA homeostatis could represent a novel pathomechanism contributing to mislocalization of TDP-43 in ALS and FTD (Ling, Polymenidou et al. 2013).

Identification of additional import factors regulating nuclear localization of TDP-43 and FUS

I. BACKGROUND & PRELIMINARY RESULTS

DECLARATION OF CONTRIBUTIONS

Udo Müller contributed to handling of the automated imaging and the acquisition of raw data.

Several independent studies on C9orf72 hexanucleotide repeat expansion-induced toxicity have identified numerous factors of the nucleocytoplasmic transport machinery as modulators of C9orf72 repeat-induced toxicity (Freibaum, Lu et al. 2015, Jovicic, Mertens et al. 2015, Zhang, Donnelly et al. 2015, Boeynaems, Bogaert et al. 2016). The identified modulating factors comprised nuclear import and export receptors (importins and exportins), proteins of the NPC (nucleoporins or Nups), as well as factors involved in RNA export and the Ran-GTP machinery. Interestingly, some nucleoporins (*e.g.* Nup98 and 358) are able to specifically affect transport of certain cargos and are known to be downregulated or damaged in the aging brain (D'Angelo, Raices et al. 2009, Walde and Kehlenbach 2010). Therefore, Nups may represent potential factors that control localization as well as intracellular trafficking pathways of TDP-43 and FUS, and it can be speculated that defective Nups may contribute to nuclear transport defects in late-onset neurodegenerative diseases, such as ALS and FTD. Taking these findings into account, dysfunctions of the nucleocytoplasmic transport machinery may critically contribute to neurodegeneration and represent potential pathomechanisms in ALS and FTD.

In order to identify addition factors of the nucleocytoplasmic transport machinery that are important for proper nuclear localization of TDP-43 and FUS, a custom-made siRNA library was designed (Dharmacon Cherry-pick RNAi library). The siRNA library included not only the previously introduced export factors (see "5. Alternative export factors in nuclear export of TDP-43 and FUS, p. 35-36) but also nuclear import receptors, Nups, and components of the Ran system (see Table 2). I used the siRNA library to screen for altered TDP-43 and FUS localization in HeLa cells stably expressing mCherry-tagged wild-type TDP-43 and FUS in an automated high content imaging system. For this purpose, the HeLa cells stably expressing mCherry-tagged wild-type TDP-43 and FUS were transiently transfected with siRNA targeting the factors of the siRNA library and incubated for 72 hours (see schematic diagram in Figure 16A). Post-transfection, the nuclear and cytoplasmic mCherry signal was measured (nuclei defined by DAPI staining) and nuclear/cytoplasmic signal ratios were calculated for each knockdown condition from two technical replicates in two independent experiments.

Positive controls in the screen were Importin β (KPNB1) and Transportin 1 (TNPO1), previously identified as the main nuclear import receptors for TDP-43 (Winton, Igaz et al. 2008, Winton, Van Deerlin et al. 2008) and FUS (Dormann, Rodde et al. 2010), respectively. Indeed, in my screen I observed a 17% (TDP-43) or 23% (FUS) reduction of the steady state nuclear mCherry signal upon knockdown of KPNB1 and TNPO1, respectively (see Figure 16B and C). Additionally, my screen confirmed the previously reported reduced nuclear localization of TDP-43 upon CAS (CSE1L) protein knockdown (Nishimura, Zupunski et al. 2010). CSE1L/CAS re-exports Importin α into the cytoplasm after cargo dissociation in the nucleus (Kutay, Bischoff et al. 1997). As Importin α acts as the adaptor protein between TDP-43 and Importin β, reduced CSE1L/CAS levels impair Importin α/β-dependent nuclear import of TDP-43.

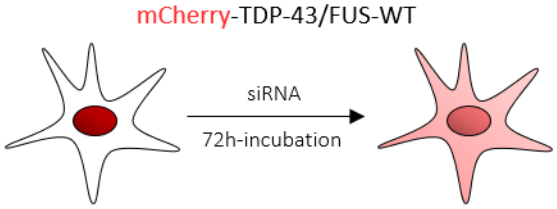
Besides these factors known to be important for proper nuclear localization of TDP-43 and FUS, I observed a cytoplasmic shift of TDP-43 and FUS when nucleoporins NUP54 (TDP-43 and FUS), NUP188 (FUS) and NUP43 (TDP-43) were reduced. Moreover, knockdown of Importin 9 (IPO9) and Importin 11 (IPO11) also strongly reduced the steady state nuclear localization of both TDP-43 (IPO9 and IPO11) and FUS (IPO9, see Figure 16B and C). While reduction of ENY2 (Enhancer Of Yellow 2 Transcription Factor Homolog) led to cytoplasmic shift of TDP-43, FUS redistributed toward the cytoplasm upon siRNA-mediated knockdown of RanBP3 (RAN Binding Protein 3) and DDX19B (DEAD Box Polypeptide 19B). DDX19 was also shown to be involved in nuclear import of certain proteins (Rajakyla, Viita et al. 2015).

Import factors	Export factors	Ran-binding proteins	Nucleoporins/ Nup-like	Other
IPO4	XPO1	RAN	Aladin/AAAS	CASC3/Barentsz
IPO5	CSE1L/CAS (XPO2)	RANGAP1	AGFG1/HRB	CHTOP
IPO7	XPO4	RCC1	GLE1	DDX19A
IPO8	XPO5	RanBP1	NUP35	DDX19B
IPO9	XPO6	RanBP3	NUP37	EIF4E
IPO11	XPO7	RanBP6	NUP43	ENY2
IPO13	XPOT	RanBP17	NUP50	MAP2K1/MEK1
KPNA1	Aly/REF		NUP54	THOC3
KPNA2	NXF1/TAP		NUP62	
KPNA3	NXF2		NUP85	
KPNA4	NXF3		NUP88	
KPNA5	NXF5		NUP93	
KPNA6	NXT1		NUP98	
KPNB1	NXT2		NUP107	
TNPO1/KPNB2			NUP133	
TNPO2			NUP153	
TNPO3			NUP155	
			NUP160	
			NUP188	
			NUP205	
			NUP210	
			NUP214	
			NUP358/RANBP2	
			POM121	
			RAE1	
			SEC13	
			SEH1L	
			NDC1/TMEM48	
			TPR	
			NUPL1	
			NUPL2	

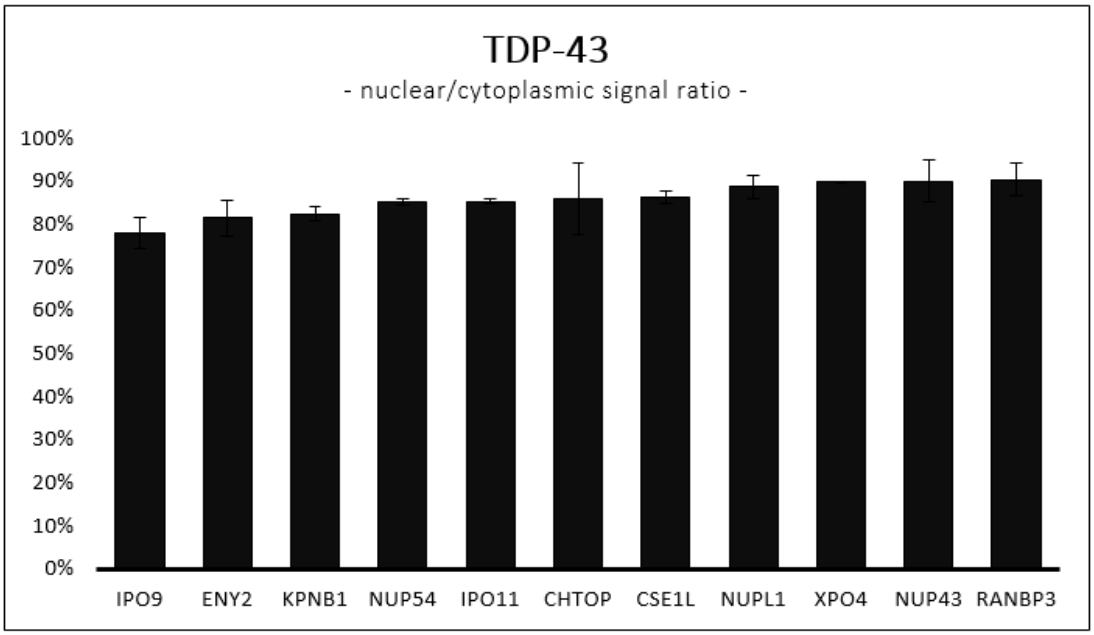
Table 2. Summary of nucleocytoplasmic transport factors used in the systematic siRNA screen

In a preliminary follow-up experiment, I could additionally validate the role of IPO11 on nuclear import of TDP-43 by utilizing the hormone-inducible nuclear transport assay. As explained in "7. Enlargement of TDP-43 and FUS impairs their nuclear egress" (see p. 40), in this assay GR_2-GFP_2-TDP-43 and –FUS fusion proteins are anchored in the cytoplasm in the absence of steroid hormones, but cytoplasmic anchoring is released and the fusion proteins are imported into the nucleus upon addition of the steroid hormone dexamethasone. I could demonstrate that siRNA-mediated interference with Importin β (KPNB1) and IPO11 expression (72 h) led to an enhanced cytoplasmic retention of wild-type GR_2-GFP_2-TDP-43 but not GR_2-GFP_2-FUS (see Figure 17A), suggesting that nuclear import of TDP-43 is disturb when these import receptors are reduced. A preliminary quantification of the nuclear/cytoplasmic ratio of the GR_2-GFP_2-TDP-43 signals showed that knockdown of Importin β and IPO11 cause a similar decrease in nuclear localization of TDP-43 (6% for KPNB1, 9% for IPO11). This suggests that IPO11, besides Importin β, might be utilized as additional, so far unknown, nuclear import receptor of TDP-43.

A


mCherry-TDP-43/FUS-WT
siRNA
72h-incubation

B


TDP-43
- nuclear/cytoplasmic signal ratio -
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
IPO9
ENY2
KPNB1
NUP54
IPO11
CHTOP
CSE1L
NUPL1
XPO4
NUP43
RANBP3

C

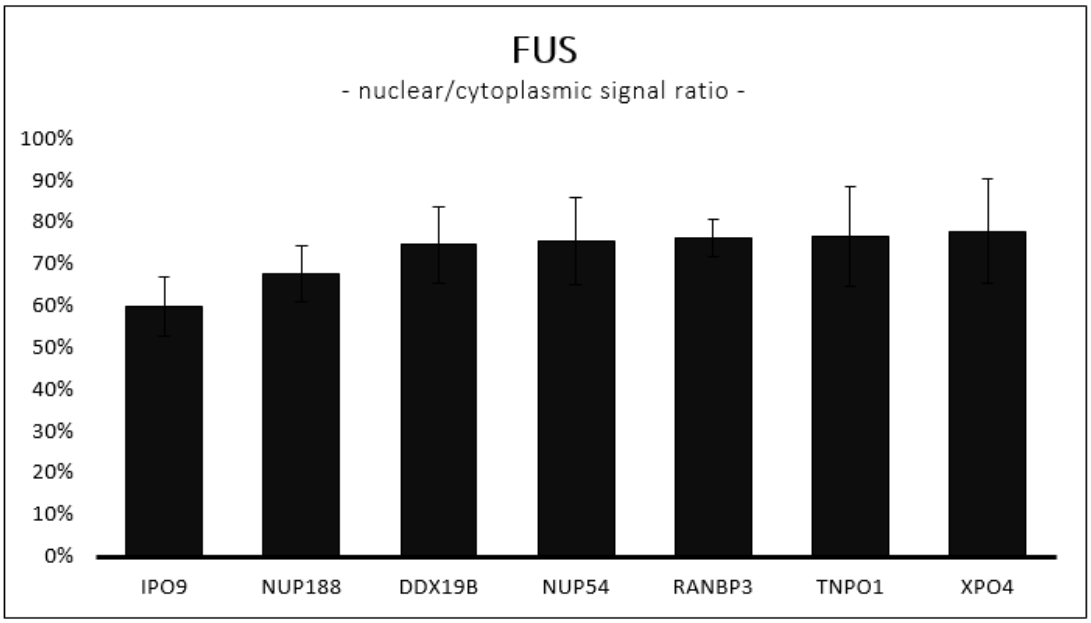

FUS
- nuclear/cytoplasmic signal ratio -
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
IPO9
NUP188
DDX19B
NUP54
RANBP3
TNPO1
XPO4

Figure 16. Top hit of systematic siRNA screen

A. Graphic scheme of siRNA screen in mCherry-tagged wild-type TDP-43 and FUS. At steady state, TDP-43 and FUS are localized predomcinantly in the nucleus (represented by red color), but are expected to partially relocalize to the cytoplasm upon siRNA-mediated knockdown of relevant nucleocytoplasmic transport factors involved in nuclear import of TDP-43 and FUS (72 hours after siRNA transfection).

B and C. Top hits from siRNA screen in HeLa cells stably expressing mCherry-tagged wild-type TDP-43 and FUS. Analysis of nuclear/cytoplasmic ratios normalized to the ratio of control siRNA treated cells and compared to known import factors KPNB1 (TDP-43) and TNPO1 (FUS) suggest that additional factors are required for proper nuclear localization of TDP-43 and FUS.

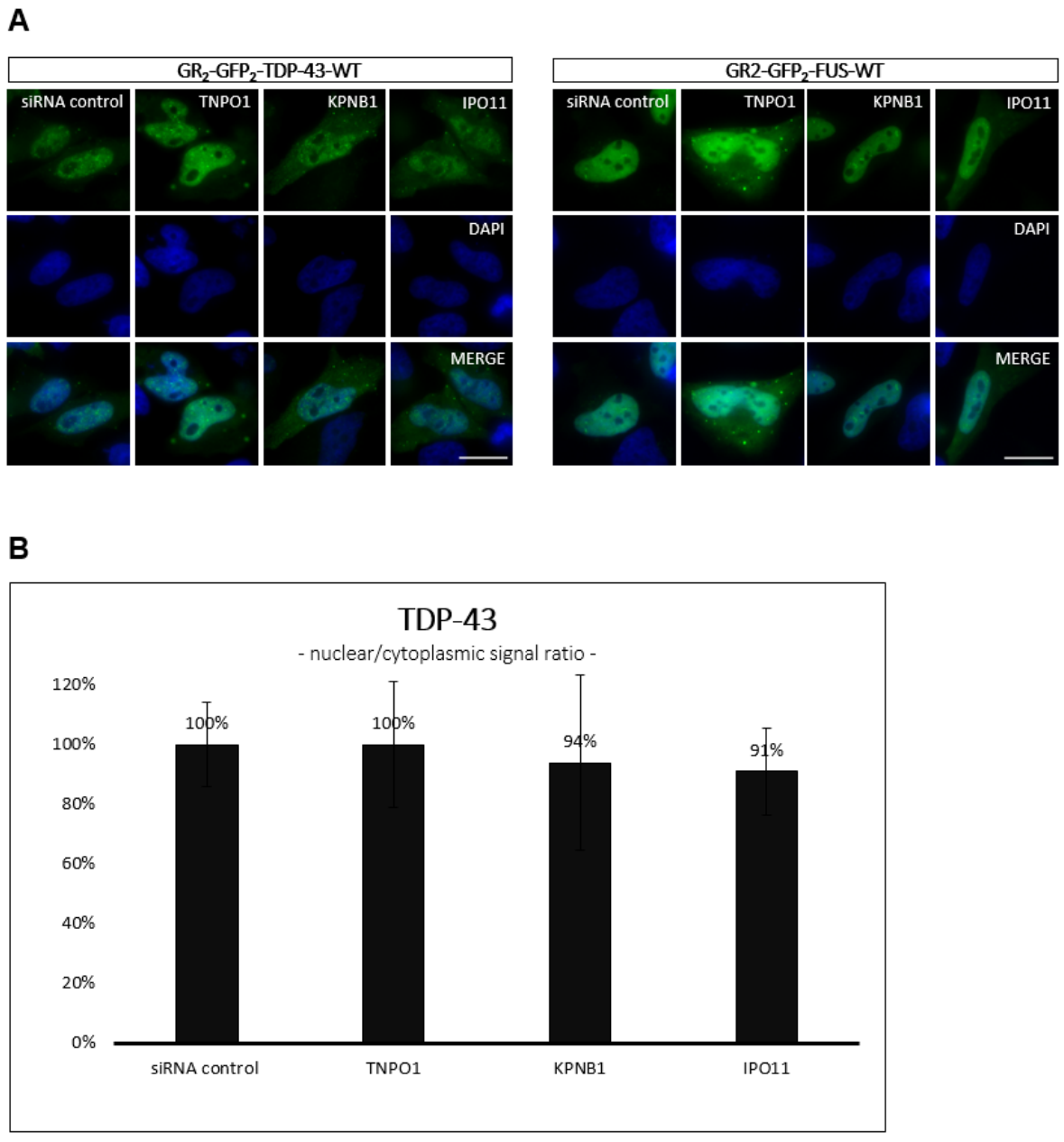


Figure 17. Validating Importin 11 (IPO11) as potentially novel nuclear import receptor for TDP-43 in the hormone-inducible nuclear transport assay

A. siRNA-mediated knockdown of TNPO1, KPNB1 or IPO11 in HeLa cells stably expressing mCherry-tagged wild-type TDP-43 or FUS revealed reduced nuclear import of GR_2-GFP_2-TDP-43 upon knockdown of both KPNB1 and IPO11, but not Transportin 1 (TNPO1).
B. Quantification of nuclear/cytoplasmic GR_2-GFP_2-TDP-43 signals in the hormone-inducible nuclear transport assay. Knockdown of KPNB1 and IPO11 both slightly decreases the nuclear/cytoplasmic signal ratio of the GR_2-GFP_2-TDP-43 reporter protein in comparison to control siRNA or TNPO1 siRNA transfected cells.

II. OUTLOOK

The preliminary results of the systematic siRNA screen targeting various factors of the nucleocytoplasmic transport machinery revealed several candidates that may influence the steady state localization of TDP-43 and FUS. These findings indicate that the identified candidate factors might be involved in nuclear import of TDP-43 or FUS. Cytoplasmic mislocalization of TDP-43 and FUS is a major hallmark of ALS and FTD pathology and it is known that mutations within or deletions of the NLS of FUS (Dormann, Rodde et al. 2010, Bentmann, Neumann et al. 2012, Dormann, Madl et al. 2012) are partly responsible for the mislocalization. However, it is possible that defects in other components of the nucleocytoplasmic transport machinery may furthermore impair nuclear import of TDP-43 and FUS in ALS and FTD patients. Moreover, it is poorly defined how TDP-43 or FUS are mislocalized to the cytoplasm in sporadic ALS and FTD patients that do not carry NLS mutations. The results of my side project propose the involvement of additional nuclear transport factors in proper nuclear import of both TDP-43 and FUS. Additionally, these factors might represent novel candidates contributing to the pathomechanisms in ALS and FTD.

Interestingly, Nishimura and colleagues already reported NUP54, NUPL1 and CSE1/CAS as modifier of TDP-43 nuclear import (Nishimura, Zupunski et al. 2010). While they did not further examine NUP54 and NUPL1 by arguing that knockdown of these central nuclear pore components (Strambio-De-Castillia, Niepel et al. 2010) might represent an unspecific global effect on import, they continued to analyze the role of CAS protein in nuclear import of TDP-43. Alongside knockdown studies in different cell lines (*e.g.* SHSY-5Y) and mouse cortical neurons, they even found a significant decrease of CAS protein levels in FTD-TDP patient samples, linking that defects in the Importin α recycling factor CAS might contribute to impaired nuclear import of TDP-43 and thus FTD pathogenesis. The siRNA screen data presented here independently recapitulates the finding of Nishimura about a crucial role of CAS in TDP-43 import and strengthens the hypothesis that multiple import factors are involved in nuclear import of TDP-43 and FUS.

The concept of multiple import receptors facilitating nuclear import was already introduced by Jakel and Gorlich who demonstrated that nuclear import of ribosomal proteins can be mediated by four import receptors, namely Importin β, Transportin, RanBP5 (IPO5) and RanBP7 (IPO7) (Jakel and Gorlich

1998). Shortly after, Jakel and colleagues reported their results on Importin β and Importin 7 acting as a heterodimeric import receptor for linker histone H1 (Jakel, Albig et al. 1999). By investigating the import capacities of both importins individually as well as in an interacting heterodimer state, they proposed a dispensable helper function of Importin 7 as an adaptor protein. Additional evidence that multiple receptors can be involved in nuclear import of one protein came from work on Rev protein of the Human Immunodeficiency Virus type 1 (Arnold, Nath et al. 2006) and histone H3 and H4 tails (Soniat, Cagatay et al. 2016); demonstrating that Importin β, Importin 5 and 7 and Transportin can mediate nuclear import of the Rev protein and that seven different import receptors (*e.g.* Importin 7 and Importin 9) can mediate import of histone H3 and H4 tails.

The preliminary results of my siRNA screen now suggest that nuclear import of TDP-43 and FUS might also rely on more than one import receptors or might be regulated by addition factors of the nucleocytoplasmic transport machinery, such as nucleoporins. In order to substantiate this hypothesis, further experiments are required: *e.g.* validation of top hits unraveled by the siRNA screen with alternative siRNAs as well as the analysis of effects on endogenous TDP-43 and FUS localization upon siRNA-mediated knockdown of potential factors. Subsequently, the hormone-inducible nuclear transport assay could be implemented in order to assess the role of validated candidates on nuclear import of TDP-43 and FUS (like in Figure 17A).

Which signals (*e.g.* NLS) and mechanisms these additional import factors might use to interact with TDP-43 and FUS or associated partner proteins require further investigation. Moreover, it will be interesting to investigate whether some of the candidate transport factors function as both import and export receptors of TDP-43 or FUS, *e.g.* the mRNA export factors ENY2, CHTOP or DDX19B that are also able to participate in import. Furthermore, it would be interesting to examine whether there is a hierarchy in the usage of TDP-43 and FUS import receptors, with the already known Importin β and Transportin 1, respectively, as main import receptors and the other receptors being used as assisting or redundant receptors that *e.g.* are activated upon stress conditions.

EXPERIMENTAL PROCEDURES

DECLARATION OF COPYRIGHT AND CONTRIBUTIONS

This section of the Ph.D. thesis was prepared in parallel to the manuscript "Nuclear egress of TDP-43 and FUS is a diffusion-limited process", submitted to Scientific Reports (in revision, but not yet accepted). Therefore, structure and text of this Ph.D. thesis and the manuscript will partially overlap in this regard.

Christina Funk and Susanne M. Bailer wrote the methodical explanation of the NEX-TRAP assay.

1. Automated-imaging screen and analysis

Analysis of the systematic siRNA-mediated knockdown of transport factors in HeLa cells stably expressing mCherry-tagged wild-type TDP-43 and FUS was achieved using the Operetta automated imaging system equipped with a 40x high NA objective (Muller, Bauer et al. 2014). Cells were platted into a 96-well plate, transfected according to Table 3, following the "siRNA-mediated knockdown"-protocol (described on p. 69). For the screen, 12 fields per well were imaged, cells within the fields counted and quantified via the Harmony 3.6 software (PerkinElmer). Counted cells were divided into nuclei and cytoplasm based on DAPI staining and the mCherry signal.

Quantification is based on two independent experiments with two technical replicates each. Mean grey values in the nucleus and cytoplasm were measured and calculated as relative nuclear to cytoplasmic (nuclear/cytoplasmic) ratios. Relative nuclear/cytoplasmic ratios were then normalized to the relative nuclear/cytoplasmic ratio of control cells. Subsequently, ratio means were converted into percentage values and calculated as percentage means of the two independent experiments. Indicated error bars represent standard deviations.

2. Cell culture, transfection and drug treatment

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Life Technologies) supplemented with 10% (vol/vol) foetal calf serum (FCS, Life Technologies) and Gentamycin (10µg/mL, Invitrogen). HeLa cells used for the hormone-induced nuclear transport assay were cultured in Dulbecco's modified Eagle's medium with Glutamax (Life Technologies) supplemented with 10% dialyzed fetal bovine serum (FBS, ThermoFisher). Transfections were carried out with Lipofectamine 2000 (Invitrogen) according to Table 3.

Actinomycin D treatment: Actinomycin D (5µg/mL, Sigma-Aldrich) was added to the culture medium and incubated for 3 hours at 37°C.

Dexamethasone treatment: Cells were incubated for 20min with dexamethasone (5µM, Sigma-Aldrich) in order to induce nuclear import of GR_2-GFP_2-fusion proteins.

Emetine, cycloheximide and harringtonine treatment: Emetine (10µg/mL, Sigma-Aldrich), cycloheximide (75µg/mL, Roth) or harringtonine (10µg/mL, Biomol) was added to the cell culture medium and incubated for indicated time points.

Leptomycin B treatment: Incubation with leptomycin B (20nM, Santa Cruz Biotechnology) was carried out by supplementing the culture medium for 2 up to 3 hours, respectively at 37°C.

Purpose	Dishes	Surface area (mm^2)	Seeding density	Growth medium	DNA/RNA amount	Lipofectamine 2000
24h DNA expression	35mm glass bottom dish	962	$1.8x10^5$/well	2mL/dish	2µg/dish in 100µL OptiMEM	5µL/dish in 100µL OptiMEM
24h DNA expression	12-well	401	$1x10^5$/well	1mL/well	1µg/dish in 50µL OptiMEM	2µL/well in 50µL OptiMEM
24h DNA expression	24-well	200	$1x10^5$/well	0.5mL/well	max. 1µg/well in 25µL OptiMEM*	1µL/well in 25µL OptiMEM
48h DNA expression	24-well	200	$5x10^4$/well	0.5mL/well	max. 1µg/well in 25µL OptiMEM*	1µL/well in 25µL OptiMEM
72h siRNA expression	24-well	200	$3x10^4$/well	0.5mL/well	25nM in 25µL OptiMEM	1µL/well in 25µL OptiMEM
72h siRNA expression	96-well	34	$3x10^3$/well	0.1mL/well	50nM in 10µL OptiMEM	0.25µL/well in 10µL OptiMEM

Table 3. Transfection protocol according to expression purpose

* Dependent on expression construct; DNA amount should be adjusted according to expression level.

3. Cell fractionation and analysis

HeLa cells were cultured in 12-well plates, harvested in trypsin/EDTA (Sigma-Aldrich) and washed twice with ice-cold PBS. Cells were incubated in mild cell lysis buffer (20mM Tris, pH 7.4, 10mM KCl, 3mM $MgCl_2$, 0.1% NP-40, 10% Glycerol and complete protease inhibitor cocktail (Roche)) for 10min on ice and centrifuged (2000g) for 10min at 4°C. Fourfold SDS-PAGE buffer was added to the supernatant (cytoplasmic fraction), while the pellet was resuspended (nuclear fraction) in mild cell lysis buffer and fourfold SDS-PAGE buffer was added. Equal volumes of the cytoplasmic and nuclear fractions were analyzed via immunoblotting with antibodies summarized in Table 6 (see Appendix).

Quantification is based on the analysis of immunoblots from three independent cell fractionation experiments. Regions-of-interest (ROI) bordering the protein bands were defined and measured as mean grey values using Fiji/ImageJ software. Mean grey values were calculated and converted into percentage values. Means of the three independent experiments were calculated and standard error

of the mean (SEM) indicated by error bars. The Wilcoxon rank-sum test for two paired samples was used for statistical analysis.

4. Cloning and cDNA constructs

TDP-43 and FUS carrying mutations in putative nuclear export signals (mNES/double-mNES) were generated by QuikChange mutagenesis (Stratagene) using pcDNA6-TDP-43-V5 (Dormann, Capell et al. 2009) and pcDNA3.1/Hygro(-)-HA-FUS (Dormann, Rodde et al. 2010) as templates. TDP-43 mNES-222 and FUS mNES-289 were used as templates to generate double-mNES mutants; primers used to generate mNES and double-mNES constructs are indicated in Tables 4 and 5.

FUS RRM/mZnF and RGG mutants (mRRM/ZnF and mRGG) were commercially synthesized (Genscript) and cloned by enzyme restriction digest into the HindIII and BamHI sites of pEGFP-C2 mammalian expression vector (Clontech).

Flag-TDP-ΔRRM2 was PCR amplified from the GST-TDP-43-ΔRRM2 template and cloned by enzyme restriction digest into the HindIII and BamHI sites of pFLAG-CMV2 mammalian expression vector (Buratti and Baralle 2001). The EGFP-TDP-43-F4L construct was generated by PCR amplification of TDP-43-4FL and after restriction digest cloned into the HindIII and BamHI sites of pEGFP-C3 mammalian expression vector (Clontech).

TDP-43-WT and FUS-WT were PCR amplified in order to introduce EcoRV and BamHI restriction sites. Subsequently, TDP-43-WT and FUS-WT were cloned by enzyme restriction digest into the EcoRV and BamHI sites of the modified pEGFP-C1 mammalian expression vector that contained the GR_2-GFP_2 cassette, resulting in GR_2-GFP_2-TDP-43-WT and GR_2-GFP_2-FUS-WT (Hutten, Flotho et al. 2008). The integrity of all constructs was verified by sequencing

cDNA constructs with respective templates and primer pairs are summarized in Table 4 and 5; detailed primer sequences are attached under “Primer sequences” in Tables 12 and 13 (see Appendix).

Name	Template	Forward primer	Reverse primer
TDP-43-mNES-222	pcDNA6-TDP-43-V5	TDP-mNES-222 forward	TDP-mNES-222 reverse
TDP-43-mNES-239	pcDNA6-TDP-43-V5	TDP-mNES-239 forward	TDP-mNES-239 reverse
TDP-43 double-mNES	pcDNA6-TDP-43-mNES-222-V5	TDP-mNES-239 forward	TDP-mNES-239 reverse
TDP-43-ΔRRM2	pGEX-3X	TDP-43-RRM2 forward	TDP-43-RRM2 reverse
EGFP-TDP-43-F4L	pFLAG-TDP-43-F4L	TDP-43-RRM2 forward	TDP-43-RRM2 reverse
GR_2-GFP_2-TDP-43-WT	pcDNA6-TDP-43-V5	TDP-43-GR_2-GFP_2 forward	TDP-43-RRM2 reverse

Table 4. PCR-amplified TDP-43 constructs with respective templates and primers

Name	Template	Forward primer	Reverse primer
FUS-mNES-289	pcDNA3.1-Hygro(-)-HA-FUS	FUS-mNES-289 forward	FUS-mNES-289 reverse
FUS-mNES-301	pcDNA3.1-Hygro(-)-HA-FUS	FUS-mNES-301 forward	FUS-mNES-301 reverse
FUS double-mNES	pcDNA3.1-Hygro(-)-HA-FUS-mNES-289	FUS-mNES-301 forward	FUS-mNES-301 reverse
GR_2-GFP_2-FUS-WT	pcDNA3.1-Hygro(-)-HA-FUS	FUS-GR_2-GFP_2 forward	FUS-WT reverse

Table 5. PCR-amplified FUS constructs with respective templates and primers

5. Fluorescence Loss In Photobleaching (FLIP) and analysis

HeLa cells were cultured in µ-Dish 35 mm, high Glass Bottom (ibidi) and transiently transfected with EGFP-TDP-43-WT. 24h post-transfection cells were treated with actinomycin D (5µg/mL) for 3 hours or left untreated. Images were acquired with an inverted Zeiss Axio Observer.Z1 microscope with a 63/1.4NA oil immersion lens equipped with a confocal spinning disc (CSU-X1, Japan) and a Rapp OptoElectronic laser-scanning device (UGA-42, Germany). Before bleaching, 5 images were taken in streaming mode with a 488nm 50mW laser. For bleaching, 3 circular ROIs of 6µm each were repeatedly photobleached in the cytoplasm using a 473nm diode laser (DL-473/75, Rapp OptoElectronic) with full laser power with an iteration of 100 and duration of 200ms per bleach event. An image was acquired before and after each bleach event with the same settings described for the pre-bleaching image mode and with 15s intervals between each bleach event.

The fluorescence loss of a defined area in the nucleus of the bleached cell was measured over time, and corrected for bleaching by acquisition and background noise as follows using the Fiji/ImageJ macro “TimeSeries Analyzer”:

$$I(t) = [ROI1(t)–ROI3(t)]/[ROI2(t)–ROI3(t)]$$

ROI1 is defined as the average grey value of an area in the nucleus of the cells which cytoplasm was repeatedly photobleached. A corresponding area of a non-photobleached cell in the same field of view served as control for bleaching due to image acquisition and is represented by the average grey value of ROI2. ROI3 is the defined average grey value of the background. Furthermore, average grey values were normalized to the mean grey value of the 5 pre-bleach images (set to 1).

6. Hormone-inducible nuclear transport assay

HeLa cells were transiently transfected with indicated siRNAs according to Table 3 following the “siRNA-mediated knockdown”-protocol (described on p. 69). 48h post-transfection, cells were additionally transiently transfected with GR_2-GFP_2-TDP-43-WT or GR_2-GFP_2-FUS-WT reporter constructs, respectively. After additional 24 hours of expression, cells were treated for 20min with

dexamethasone in order to induce nuclear import of GR_2-GFP_2-TDP-43-WT or GR_2-GFP_2-FUS-WT reporter proteins (see also “Cell culture, transfection and drug treatment”, p. 63-64) and analyzed by direct visualization of GFP fluorescence and DAPI staining.

7. Image acquisition and analysis

Images were acquired with an inverted Zeiss Axio Observer.Z1 wide-field fluorescence microscope with a 63/1.4NA oil immersion lens and an AxioCam506. Images were analyzed with Zen software (Zeiss) and optionally post-processed (linear enhancement of brightness and contrast) for printing reasons. Images presenting the NEX-TRAP results were taken using a confocal laser-scanning microscope (LSM710, Zeiss) and processed using Adobe Photoshop CS3 by linear enhancement of brightness and contrast.

Preliminary quantification of siRNA-mediated knockdown of IPO11, KPNB1, TNPO1 and control siRNA in GR_2-GFP_2-TDP-43-WT-transfected cells is based on grey values measured in the nucleus and cytoplasm using Fiji/ImageJ and calculated as relative nuclear to cytoplasmic (nuclear/cytoplasmic) ratios. Relative nuclear/cytoplasmic ratios were then normalized to the relative nuclear/cytoplasmic ratio of control siRNA-treated cells and converted into percentage values. Indicated error bars represent standard deviations.

8. Immunocytochemistry

All steps were carried out at room temperature. Cells were fixed for 10min in 4% formaldehyde in PBS, permeabilized for 5min in 0.2% Triton X-100 supplemented with 50mM NH4Cl and blocked for 30min with 0.1% saponine in PBS supplemented with 5% goat or donkey serum. Primary and secondary antibodies diluted in 0.1% saponine in PBS were applied for 45min each and washed with 0.1% saponine in PBS. Coverslips were mounted onto glass slides using ProLong Diamond Antifade Reagent with DAPI (Invitrogen) and dried at room temperature overnight.

9. Interspecies heterokaryon assay

HeLa cells were seeded onto coverslips in a 12-well-plate and transfected according to protocol in Table 3. Following 5 hours of incubation, medium was removed and mouse embryonic fibroblasts (MEFs) were added. The fusion of HeLa cells and MEFs was carried out by a 2min-incubation with polyethylene glycol (PEG 1500, Roche) 24h after transfection at room temperature. PEG was removed by 3 washing steps with PBS supplemented with 0.1% Glucose (Merck). Post-fusion, cells were incubated in antibiotic-free DMEM supplemented with the protein synthesis inhibitor cycloheximide

(75µg/mL, ROTH) before cells were processed by immunocytochemistry with antibodies summarized in Table 7 (see Appendix).

10. NEX-TRAP assay

The NEX-TRAP assay was performed as described (Raschbichler, Lieber et al. 2012). Briefly, HeLa cells were co-transfected for 20 hours with the plasmid pCR3-N-HA-UL10/gM-$FKBP_3$ and either pEYFP-NLS-FRB-TDP-43 or pEYFP-NLS-FRB-FUS. The EYFP-NLS-FRB-pUL4 plasmid was used as control. Following a 10min-incubation with anisomycin (50µM), HeLa cells were treated with rapamycin (150ng/mL) or without for 2 hours. Protein localization was visualized by immunofluorescence staining using an anti-gM-specific antibody (Rabbit anti-gM, kind gift of Thomas Mettenleiter) (Kopp, Granzow et al. 2003). EYFP-tagged proteins were visualized directly and nuclei by DAPI staining.

11. Poly(A)+RNA in situ hybridization

All reagents were treated with 0.1% diethyl pyrocarbonate (DEPC, Sigma-Aldrich). Cells grown on 12mm coverslips were fixed for 15min at room temperature in 4% formaldehyde in PBS, permeabilized on ice for 5min in 0.2% Triton X-100 in PBS and equilibrated on ice for 5min with 2x SSC (saline sodium citrate, Ambion) and 25% formamide supplemented PBS. Cells were then hybridized for 2 hours at 37°C with 1ng/mL digoxigenin labeled oligo(dT) ($Dig(T)_{40}$; 40mer, Eurofins) in 0.02% bovine serum albumin (BSA), 5% dextran sulfate, 25% formamide, 2mM ribonucleoside vanadyl complex (RVC, Sigma-Aldrich), 2x SSC and 1mg/mL tRNA. Subsequently, cells were washed twice with 2x SSC and 25% formamide supplemented PBS, followed by one washing step with 0.5x SSC in PBS and another washing step with 0.5% Triton X-100 in PBS. Cells were then re-fixed in 4% formaldehyde in PBS and immunocytochemistry was carried out using a FITC-coupled anti-digoxigenin Fab fragment (Roche) for detection of the labeled oligonucleotide and antibodies specific for indicated proteins summarized in Table 7 (see Appendix).

12. Preparation of cell lysates and immunoblotting

Cells were harvested in trypsin/EDTA (Sigma-Aldrich), washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with complete protease inhibitor cocktail (Roche). Protein concentrations of sonicated lysates were identified by BCA protein assay (Pierce). Fourfold SDS-PAGE buffer was added and samples were boiled for 5min at 95°C; subsequently, proteins were separated by SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham™ Protran™ 0.2µm NC, GE

Healthcare Life Sciences). The membrane was blocked in Tris-buffered saline supplemented with Tween-20 (TBS-T) and 5% milk powder and incubated primary antibodies and secondary antibodies diluted in blocking buffer summarized in Table 6 (see Appendix), followed by 3 washes in TBS-T. Bound antibodies were visualized using the LI-COR fluorescent immunoblotting system (Odyssey CLx Imaging system).

13. siRNA-mediated knockdown

Knockdown of transport factors summarized in the siRNA library (see Table2, p. 58 and Appendix for detailed listing) was achieved using Dharmacon siGENOME SMARTpools composed of 4 different siRNAs. siRNA transfections were carried out with Lipofectamine 2000 and according to Table 3. Medium containing siRNA was removed 5 hours post-transfection and replaced by fresh medium. Knockdown was analyzed 72 hours post-transfection by immunoblotting and/or immunocytochemistry. Aly/REF, Exportin-1/CRM1 and XPO5 were screened in HeLa cells stably expressing mCherry-tagged TDP-43 and FUS NLS-mutant using immunoblotting (see “Preparation of cell lysates and immunoblotting” on p. 68) and immunocytochemistry with antibodies specific for proteins summarized in Table 7 (see Appendix).

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APPENDIX

1. Antibodies

Antigen	1. Antibody	2. Antibody
Aly/REF	Mouse anti-Aly/REF (Monoclonal, 11G5, Abcam)	IRDye® 680RD Donkey anti-Mouse IgG (LI-COR)
EGFP-TDP-43	Rabbit anti-GFP (A1122, Thermo Scientific)	IRDye® 800CW Donkey anti-Rabbit IgG (LI-COR)
Exportin-1/CRM1	Goat anti-Exportin-1/CRM1 (Kind gift of R. H. Kehlenbach)	IRDye® 680RD Donkey anti-Goat IgG (LI-COR)
FUS	Rabbit anti-FUS (A300-302A, Bethyl)	IRDye® 800CW Donkey anti-Rabbit IgG (LI-COR)
GAPDH	Rat anti-GAPDH (10F4) (Kind gift of Helmholtz Center Munich Antibody Core Facility)	IRDye® 680RD Goat anti-Rat IgG (LI-COR)
Histone H3	Rabbit anti-Histone H3 (Abcam)	IRDye® 800CW Donkey anti-Rabbit IgG (LI-COR)
TDP-43	Rabbit anti-TDP-43 (TIP-TD-P09, Cosmo)	IRDye® 800CW Donkey anti-Rabbit IgG (LI-COR)
XPO5	Mouse anti-XPO5 (Monoclonal, ab57491, Abcam)	IRDye® 680RD Donkey anti-Mouse IgG (LI-COR)
β-Actin	Mouse anti-β-Actin (Monoclonal, AC-74, Sigma-Aldrich)	IRDye® 680RD Donkey anti-Mouse IgG (LI-COR)

Table 6. Antibody combinations used for immunoblotting

Antigen	1. Antibody	2. Antibody
Aly/REF	Mouse anti-Aly/REF (Monoclonal, 11G5, Abcam)	Alexa Fluor® 647 Donkey anti-mouse (Invitrogen)
Exportin-1/CRM1	Goat anti-Exportin-1/CRM1 (Kind gift of R. H. Kehlenbach)	Alexa Fluor® 488 Donkey anti-Goat (Invitrogen)
Flag	Rabbit anti-FLAG (F7425, Sigma-Aldrich)	Alexa Fluor® 488 Donkey anti-Rabbit (Invitrogen)
HA	Rat anti-HA (Monoclonal, 3F10, Roche)	Alexa Fluor® 488 Donkey anti-Rat (Invitrogen)
Human hnRNP-C1/C2	Mouse anti-hnRNP-C1/C2 (Monoclonal, 4F4, Abcam)	Alexa Fluor® 555 Donkey anti-Mouse (Invitrogen)
mCherry	Rabbit anti-mCherry (ab167453, Abcam)	Alexa Fluor® 555 Donkey anti-Rabbit (Invitrogen)
V5	Rabbit anti-V5 (Polyclonal, AB3792, Merck)	Alexa Fluor® 488 Donkey anti-Rabbit (Invitrogen)
XPO5	Mouse anti-XPO5 (Monoclonal, ab57491, Abcam)	Alexa Fluor® 488 Donkey anti-Mouse (Invitrogen)

Table 7. Antibody combinations used for immunocytochemistry

2. cDNA constructs

TDP-43

Name	Vector	Modification	Tag	Resistance	Source
TDP-43-V5-WT	pcDNA6/V5-His B (Invitrogen)	Insertion of V5 epitope tag	V5 epitope tag	Ampicillin, Blasticidin	D. Dormann
TDP-43-V5-ΔC	pcDNA6/V5-His B (Invitrogen)	Deletion of aa 274-414	V5 epitope tag	Ampicillin, Blasticidin	D. Dormann
TDP-43-mNES-222	pcDNA6/V5-His B (Invitrogen)	F226A, F229A point mutations	V5 epitope tag	Ampicillin, Blasticidin	This Ph.D. thesis
TDP-43-mNES-239	pcDNA6/V5-His B (Invitrogen)	L243A, L248A point mutations	V5 epitope tag	Ampicillin, Blasticidin	This Ph.D. thesis
TDP-43-double-mNES	pcDNA6/V5-His B (Invitrogen)	F226A, F229A, L243A, L248A point mutations	V5 epitope tag	Ampicillin, Blasticidin	This Ph.D. thesis
FLAG-TDP-43-WT	pFLAG-CMV 2 (Sigma-Aldrich)	Insertion of TDP-43	FLAG epitope tag	Ampicillin	Kind gift of E. Buratti
FLAG-TDP-43-F2L	pFLAG-CMV 2 (Sigma-Aldrich)	F147L, F149L point mutations	FLAG epitope tag	Ampicillin	Kind gift of E. Buratti
FLAG-TDP-43-F4L	pFLAG-CMV 2 (Sigma-Aldrich)	F147L, F149L, F229L, F231L point mutations	FLAG epitope tag	Ampicillin	Kind gift of E. Buratti
FLAG-TDP-43-ΔRRM1	pFLAG-CMV 2 (Sigma-Aldrich)	Deletion of RRM1 domain	FLAG epitope tag	Ampicillin	Kind gift of E. Buratti
FLAG-TDP-43-ΔRRM2	pFLAG-CMV 2 (Sigma-Aldrich)	Deletion of RRM2 domain	FLAG epitope tag	Ampicillin	This Ph.D. thesis
GFP-TDP-43-WT	pEGFP-C1 (Clontech)	Insertion of TDP-43	EGFP	Kanamycin, Neomycin	D. Dormann
GFP-TDP-43-F4L	pEGFP-C3 (Clontech)	Insertion of TDP-43-ΔRRM2	EGFP	Kanamycin, Neomycin	This Ph.D. thesis
GR_2-GFP_2-TDP-43-WT	pEGFP-C1 (Clontech)	Insertion of GR-tandem, additional EGFP and TDP-43-WT	EGFP	Kanamycin, Neomycin	This Ph.D. thesis

Table 8. Summary of used TDP-43 cDNA constructs

FUS

Name	Vector	Modification	Tag	Resistance	Source
HA-FUS-WT	pcDNA3.1-Hygro(-) (Invitrogen)	Insertion of HA tag	HA epitope tag	Ampicillin, Hygromycin	D. Dormann
FUS-mNES-289	pcDNA3.1-Hygro(-) (Invitrogen)	L292A, V296A point mutations	HA epitope tag	Ampicillin, Hygromycin	This Ph.D. thesis
FUS-mNES-301	pcDNA3.1-Hygro(-) (Invitrogen)	F305A, I308A point mutations	HA epitope tag	Ampicillin, Hygromycin	This Ph.D. thesis
FUS-double-mNES	pcDNA3.1-Hygro(-) (Invitrogen)	L292A, V296A, F305A, I308A point mutations	HA epitope tag	Ampicillin, Hygromycin	This Ph.D. thesis
HA-FUS-ΔQ	pcDNA3.1-Hygro(-) (Invitrogen)	Deletion of aa 1-160	HA epitope tag	Ampicillin, Hygromycin	R. Rodde
HA-FUS-ΔQ/RGG1	pcDNA3.1-Hygro(-) (Invitrogen)	Deletion of aa 1-265	HA epitope tag	Ampicillin, Hygromycin	R. Rodde
GFP-FUS-WT	pEGFP-C1 (Clontech)	Insertion of FUS	GFP	Kanamycin, Neomycin	D. Dormann
GFP-FUS-ΔQ/RGG1/RRM	pEGFP-C1 (Clontech)	Deletion of aa 1-375	EGFP	Kanamycin, Neomycin	R. Rodde
GFP-FUS-ΔRGG2	pEGFP-C1 (Clontech)	Deletion of RGG2 domain	EGFP	Kanamycin, Neomycin	R. Rodde
GFP-FUS-ΔRGG3	pEGFP-C1 (Clontech)	Deletion of RGG3 domain	EGFP	Kanamycin, Neomycin	R. Rodde
GFP-FUS-ΔZnF	pEGFP-C1 (Clontech)	Deletion of ZnF domain	EGFP	Kanamycin, Neomycin	R. Rodde
GFP-FUS-mRRM/mZnF	pEGFP-C2 (Clontech)	Points mutations in F305L/K312A/K315A/ K316A/F341L/F359L/ F368L/D425A/N435A/ F438A/W440A/R441A/ N445A	EGFP	Kanamycin, Neomycin	This Ph.D. thesis
GFP-FUS-mRGG	pEGFP-C2 (Clontech)	RGG to KGGpoint mutations	EGFP	Kanamycin, Neomycin	This Ph.D. thesis
GR_2-GFP_2-FUS-WT	pEGFP-C1 (Clontech)	Insertion of GR-tandem, additional EGFP and FUS-WT	EGFP	Kanamycin, Neomycin	This Ph.D. thesis

Table 9. Summary of used FUS cDNA constructs

3. Cell lines

HeLa cells stably expressing HA-FUS were described in Dormann et al., EMBO J 2012 (Dormann, Madl et al. 2012). HeLa cells stably expressing TDP-43-V5, mCherry-TDP-43-WT, mCherry-FUS-WT, mCherry-TDP-43-83AAA and mCherry-FUS-P525L were generated by lentiviral transduction (pCDH-Ef1-MCS-IRES-Puro vector, System Biosciences) as described in Kuhn et al., EMBO J 2010 (Kuhn, Wang et al. 2010), followed by selection with 0.5µg/ml puromycin (Sigma-Aldrich). Monoclonal HeLa cells stably

expressing mCherry-TDP-43-WT, mCherry-FUS-WT, mCherry-TDP-43-83AAA and mCherry-FUS-P525L cell lines were generated using Fluorescence-Activated Cell Sorting (FACS); mCherry-tagged single cells were sorted into 96-well-plates, screened for mCherry expression and expanded as monoclonal cell lines.

Cell line	Tag	Features	Source
HeLa WT	-	Commercial cell line	D. Dormann
HeLa-TDP-43-V5-WT	V5 (C-terminal)	Polyclonal	E. Funk
HeLa-HA-FUS-WT	HA (N-terminal)	Polyclonal	E. Funk
HeLa-mCherry-TDP-43-WT	mCherry (N-terminal)	Monoclonal	This Ph.D. thesis
HeLa-mCherry-FUS-WT	mCherry (N-terminal)	Monoclonal	This Ph.D. thesis
HeLa-mCherry-TDP-43-83AAA	mCherry (N-terminal)	Monoclonal	This Ph.D. thesis
HeLa-mCherry-FUS-P525L	mCherry (N-terminal)	Monoclonal	This Ph.D. thesis
MEF WT	-	Commercial cell line	D. Dormann

Table 10. Summary of used cell lines

4. Drugs

Name	Abbreviation	Company	Working concentration
Actinomycin D	ActD	Sigma-Aldrich	5µg/mL
Cycloheximide	CHX	Roth	75µg/mL
Dexamethasone	DEX	Sigma-Aldrich	5µM
Emetine dihydrochloride	Emetine	Sigma-Aldrich	10µg/mL
Gentamycin	Genta	Invitrogen	10µg/mL,
Leptomycin B	LMB	Santa Cruz Biotechnology	20nM
Harringtonine	HR	Biomol	10µg/mL
Polyethylene glycole	PEG	Roche	150µL (for 12-well plate with surface area of 401 mm^{2}/well)

Table 11. Summary of used drugs

5. Primes sequences

TDP-43

Name	Sequence
TDP-mNES-222 forward	TTCATCCCCAAGCCAGCCAGGGCCGCTGCCTTTGTTACATTTGCAGATG
TDP-mNES-222 reverse	CATCTGCAAATGTAACAAAGGCAGCGGCCCTGGCTGGCTTGGGGATGAA
TDP-mNES-239 forward	ATTGCGCAGTCTGCTTGTGGAGAGGACGCGATCATTAAAGGAATCAGCGTTC
TDP-mNES-239 reverse	GAACGCTGATTCCTTTAATGATCGCGTCCTCTCCACAAGCAGACTGCGCAAT
TDP-43-RRM2 forward (HindIII)	ATTAAGCTTACCATGTCTGAATATATTCGGG
TDP-43-RRM2 reverse (BamHI)	TTTGGATCCCTACATTCCCCAGCCAGAAG
TDP-43-GR_2-GFP_2 forward (EcoRV)	AATCCGATATCCCATGTCTGAATATATTCGGG

Table 12. Summary of primer sequences used to generate TDP-43 cDNA constructs

FUS

Name	Sequence
FUS-mNES-289 forward	TCTTTGTGCAAGGCGCGGGTGAGAATGCTACAATTGAGTCTGTGGCTGATTAC
FUS-mNES-289 reverse	GTAATCAGCCACAGACTCAATTGTAGCATTCTCACCCGCGCCTTGCACAAAGA
FUS-mNES-301 forward	TCTGTGGCTGATTACGCCAAGCAGGCTGGTATTATTAAGACAAACAAGAAAAC
FUS-mNES-301 reverse	GTTTTCTTGTTTGTCTTAATAATACCAGCCTGCTTGGCGTAATCAGCCACAGA
FUS-GR_2-GFP_2 forward (EcoRV)	AATTCGATATCCCATGGCCTCAAACGATTATACCCAACAAG
FUS-WT reverse (BamHI)	CGGGATCCTTAATACGGCCTCTCCCTGCGATCC

Table 13. Summary of primer sequences used to generate FUS cDNA constructs

6. siRNA library

AAAS	Aladin/adracalin: component of nuclear pore complex The protein encoded by this gene is a member of the WD-repeat family of regulatory proteins and may be involved in normal development of the peripheral and central nervous system. The encoded protein is part of the nuclear pore complex and is anchored there by NDC1. Defects in this gene are a cause of achalasia-addisonianism-alacrima syndrome (AAAS), also called triple-A syndrome or Allgrove syndrome. Two transcript variants encoding different isoforms have been found for this gene.
CSE1L/CAS	Exportin-2 Proteins that carry a nuclear localization signal (NLS) are transported into the nucleus by the importin-alpha/beta heterodimer. Importin-alpha binds the NLS, while importin-beta mediates translocation through the nuclear pore complex. After translocation, RanGTP binds importin-beta and displaces importin-alpha. Importin-alpha must then be returned to the cytoplasm, leaving the NLS protein behind. The protein encoded by this gene binds strongly to NLS-free importin-alpha, and this binding is released in the cytoplasm by the combined action of RANBP1 and RANGAP1. In addition, the encoded protein may play a role both in apoptosis and in cell proliferation. Alternatively spliced transcript variants have been found for this gene.
DDX19A	DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 19A ATP-dependent RNA helicase involved in mRNA export from the nucleus. Rather than unwinding RNA duplexes, DDX19 functions as a remodeler of ribonucleoprotein particles, whereby proteins bound to nuclear mRNA are dissociated and replaced by cytoplasmic mRNA binding proteins.
DDX19B	DEAD (Asp-Glu-Ala-Asp) Box Polypeptide 19B DEAD box proteins, characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), are putative RNA helicases. They are implicated in a number of cellular processes involving alteration of RNA secondary structure such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division. This gene encodes a DEAD box protein, which exhibits RNA-dependent ATPase and ATP-dependent RNA-unwinding activities. This protein is recruited to the cytoplasmic fibrils of the nuclear pore complex, where it participates in the export of mRNA from the nucleus. Multiple alternatively spliced transcript variants encoding different isoforms have been found for this gene.
ENY2	Enhancer Of Yellow 2 Transcription Factor Homolog Involved in mRNA export coupled transcription activation by association with both the TREX-2 and the SAGA complexes.
GLE1	Nucleoporin Gle1 RNA Export Mediator (Enhancer Freibaum) Required for the export of mRNAs containing poly(A) tails from the nucleus into the cytoplasm. May be involved in the terminal step of the mRNA transport through the nuclear pore complex (NPC).

IPO4	Importin 4/Ran-Binding Protein 4 Functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism.
IPO5	Importin 5/Karyopherin (Importin) Beta 3 Nucleocytoplasmic transport, a signal- and energy-dependent process, takes place through nuclear pore complexes embedded in the nuclear envelope. The import of proteins containing a nuclear localization signal (NLS) requires the NLS import receptor, a heterodimer of importin alpha and beta subunits also known as karyopherins. Importin alpha binds the NLS-containing cargo in the cytoplasm and importin beta docks the complex at the cytoplasmic side of the nuclear pore complex. In the presence of nucleoside triphosphates and the small GTP binding protein Ran, the complex moves into the nuclear pore complex and the importin subunits dissociate. Importin alpha enters the nucleoplasm with its passenger protein and importin beta remains at the pore. Interactions between importin beta and the FG repeats of nucleoporins are essential in translocation through the pore complex. The protein encoded by this gene is a member of the importin beta family.
IPO7	Importin 7/ Ran-Binding Protein 7 The importin-alpha/beta complex and the GTPase Ran mediate nuclear import of proteins with a classical nuclear localization signal. The protein encoded by this gene is a member of a class of approximately 20 potential Ran targets that share a sequence motif related to the Ran-binding site of importin-beta. Similar to importin-beta, this protein prevents the activation of Ran's GTPase by RanGAP1 and inhibits nucleotide exchange on RanGTP, and also binds directly to nuclear pore complexes where it competes for binding sites with importin-beta and transportin. This protein has a Ran-dependent transport cycle and it can cross the nuclear envelope rapidly and in both directions. At least four importin beta-like transport receptors, namely importin beta itself, transportin, RanBP5 and RanBP7, directly bind and import ribosomal proteins.
IPO8	Importin 8/ Ran-Binding Protein 8 The importin-alpha/beta complex and the GTPase Ran mediate nuclear import of proteins with a classical nuclear localization signal. The protein encoded by this gene is a member of a class of approximately 20 potential Ran targets that share a sequence motif related to the Ran-binding site of importin-beta. This protein binds to the nuclear pore complex and, along with RanGTP and RANBP1, inhibits the GAP stimulation of the Ran GTPase. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.
IPO9	Importin 9/Ran-Binding Protein 9 Functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to the importin, the importin/substrate complex dissociates and importin is re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus (By similarity). Mediates the nuclear import of H2B histone (By similarity), RPS7 and RPL18A. Prevents the cytoplasmic aggregation of RPS7 and RPL18A by shielding exposed basic domains. May also import H2A, H3, H4 histones (By similarity), RPL4 and RPL6.
IPO11	Importin 11/Ran-Binding Protein 11 Functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to the importin, the importin/substrate complex dissociates and importin is re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran. The directionality of nuclear import is thought

	to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus (By similarity). Mediates the nuclear import of UBE2E3, and of RPL12 (By similarity).
IPO13	Importin 13/Ran-Binding Protein 13 Functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to the importin, the importin/substrate complex dissociates and importin is re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus (By similarity). Mediates the nuclear import of UBC9, the RBM8A/MAGOH complex, PAX6 and probably other members of the paired homeobox family. Also mediates nuclear export of eIF-1A, and the cytoplasmic release of eIF-1A is triggered by the loading of import substrates onto IPO13.
KPNA1	Karyopherin Alpha 1 (Importin Alpha 5) Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1. Binds specifically and directly to substrates containing either a simple or bipartite NLS motif.
KPNA2	Karyopherin Alpha 2 (RAG Cohort 1, Importin Alpha 1) Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1. Binds specifically and directly to substrates containing either a simple or bipartite NLS motif.
KPNA3	Karyopherin Alpha 3 (Importin Alpha 4) Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1.
KPNA4	Karyopherin Alpha 4 (Importin Alpha 3) Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1. Binds specifically and directly to substrates containing either a simple or bipartite NLS motif. Docking of the importin/substrate complex to the nuclear pore complex (NPC) is mediated by KPNB1 through binding to nucleoporin FxFG repeats and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to importin-beta and the three components separate and importin-alpha and -beta are re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran from importin. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus. In vitro, mediates the nuclear import of human cytomegalovirus UL84 by recognizing a non-classical NLS. In vitro, mediates the nuclear import of human cytomegalovirus UL84 by recognizing a non-classical NLS.
KPNA5	Karyopherin Alpha 5 (Importin Alpha 6) Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1. Binds specifically and directly to substrates containing either a simple or bipartite NLS motif. Docking of the importin/substrate complex to the nuclear pore complex (NPC) is mediated by KPNB1 through binding to nucleoporin FxFG repeats and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to importin-beta and the three components separate and importin-alpha and -beta are re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran from importin. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus. Mediates nuclear import of STAT1 homodimers and STAT1/STAT2 heterodimers by recognizing non-classical NLSs of STAT1 and STAT2 through ARM repeats 8-9. Recognizes influenza A virus nucleoprotein through ARM repeat 7-9 In vitro, mediates the nuclear import of human cytomegalovirus UL84 by recognizing a non-classical NLS.
KPNA6	Karyopherin Alpha 6 (Importin Alpha 7) Functions in nuclear protein import as an adapter protein for nuclear receptor KPNB1. Binds specifically and directly to substrates containing either a simple or bipartite NLS motif. Docking of the importin/substrate complex to the nuclear pore complex (NPC) is mediated by KPNB1 through binding to nucleoporin FxFG repeats and the complex is subsequently translocated

	through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to importin-beta and the three components separate and importin-alpha and -beta are re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran from importin. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus.
KPNB1	Karyopherin (Importin) Beta 1 Functions in nuclear protein import, either in association with an adapter protein, like an importin-alpha subunit, which binds to nuclear localization signals (NLS) in cargo substrates, or by acting as autonomous nuclear transport receptor. Acting autonomously, serves itself as NLS receptor. Docking of the importin/substrate complex to the nuclear pore complex (NPC) is mediated by KPNB1 through binding to nucleoporin FxFG repeats and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism.
TNPO1	Transportin 1/KPNB2 (toxicity enhancer Freibaum) Functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to the importin, the importin/substrate complex dissociates and importin is re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus (By similarity). Involved in nuclear import of M9-containing proteins. In vitro, binds directly to the M9 region of the heterogeneous nuclear ribonucleoproteins (hnRNP), A1 and A2 and mediates their nuclear import. Appears also to be involved in hnRNP A1/A2 nuclear export. Mediates the nuclear import of ribosomal proteins RPL23A, RPS7 and RPL5. Binds to a beta-like import receptor binding (BIB) domain of RPL23A. In vitro, mediates nuclear import of H2A, H2B, H3 and H4 histones, and SRP19. In case of HIV-1 infection, binds and mediates the nuclear import of HIV-1 Rev. Mediates nuclear import of ADAR/ADAR1 (isoform 5) in a RanGTP-dependent manner.
TNPO2	Transportin 2 Probably functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to the importin, the importin/substrate complex dissociates and importin is re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus (By similarity).
TNPO3	Transportin 3 Seems to function in nuclear protein import as nuclear transport receptor. In vitro, mediates the nuclear import of splicing factor SR proteins RBM4, SFRS1 and SFRS2, by recognizing phosphorylated RS domains.
XPO1	Exportin 1/CRM1 (toxicity enhancer Freibaum) Mediates the nuclear export of cellular proteins (cargos) bearing a leucine-rich nuclear export signal (NES) and of RNAs. In the nucleus, in association with RANBP3, binds cooperatively to the NES on its target protein and to the GTPase RAN in its active GTP-bound form (Ran-GTP). Docking of this complex to the nuclear pore complex (NPC) is mediated through binding to nucleoporins. Upon transit of a nuclear export complex into the cytoplasm, disassembling of the complex and hydrolysis of Ran-GTP to Ran-GDP (induced by RANBP1 and RANGAP1, respectively) cause release of the cargo from the export receptor. The directionality of nuclear export is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus. Involved in U3 snoRNA transport from Cajal bodies to nucleoli.

XPO4	Exportin 4 Mediates the nuclear export of proteins (cargos) with broad substrate specificity. In the nucleus binds cooperatively to its cargo and to the GTPase Ran in its active GTP-bound form. Docking of this trimeric complex to the nuclear pore complex (NPC) is mediated through binding to nucleoporins. Upon transit of a nuclear export complex into the cytoplasm, disassembling of the complex and hydrolysis of Ran-GTP to Ran-GDP (induced by RANBP1 and RANGAP1, respectively) cause release of the cargo from the export receptor. XPO4 then return to the nuclear compartment and mediate another round of transport. The directionality of nuclear export is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus.
XPO5	Exportin 5/Ran-Binding Protein 21 Mediates the nuclear export of proteins bearing a double-stranded RNA binding domain (dsRBD) and double-stranded RNAs (cargos). XPO5 in the nucleus binds cooperatively to the RNA and to the GTPase Ran in its active GTP-bound form. Proteins containing dsRBDs can associate with this trimeric complex through the RNA. Docking of this complex to the nuclear pore complex (NPC) is mediated through binding to nucleoporins. Upon transit of a nuclear export complex into the cytoplasm, hydrolysis of Ran-GTP to Ran-GDP (induced by RANBP1 and RANGAP1, respectively) cause disassembly of the complex and release of the cargo from the export receptor. XPO5 then returns to the nuclear compartment by diffusion through the nuclear pore complex, to mediate another round of transport. The directionality of nuclear export is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus. Overexpression may in some circumstances enhance RNA-mediated gene silencing (RNAi). Mediates nuclear export of isoform 5 of ADAR/ADAR1 in a RanGTP-dependent manner. Mediates the nuclear export of micro-RNA precursors, which form short hairpins. Also mediates the nuclear export of synthetic short hairpin RNAs used for RNA interference, and adenovirus VA1 dsRNA. In some circumstances can also mediate the nuclear export of deacylated and aminoacylated tRNAs. Specifically recognizes dsRNAs that lack a 5-overhang in a sequence-independent manner, have only a short 3-overhang, and that have a double-stranded length of at least 15 base pairs. Binding is dependent on Ran-GTP.
XPO6	Exportin 5/Ran-Binding Protein 20 Mediates the nuclear export of actin and profilin-actin complexes in somatic cells.
XPO7	Exportin 7/RAN Binding Protein 16 Mediates the nuclear export of proteins (cargos) with broad substrate specificity. In the nucleus binds cooperatively to its cargo and to the GTPase Ran in its active GTP-bound form. Docking of this trimeric complex to the nuclear pore complex (NPC) is mediated through binding to nucleoporins. Upon transit of a nuclear export complex into the cytoplasm, disassembling of the complex and hydrolysis of Ran-GTP to Ran-GDP (induced by RANBP1 and RANGAP1, respectively) cause release of the cargo from the export receptor. XPO7 then return to the nuclear compartment and mediate another round of transport. The directionality of nuclear export is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus.
XPOT	Exportin tRNA Mediates the nuclear export of aminoacylated tRNAs. In the nucleus binds to tRNA and to the GTPase Ran in its active GTP-bound form. Docking of this trimeric complex to the nuclear pore complex (NPC) is mediated through binding to nucleoporins. Upon transit of a nuclear export complex into the cytoplasm, disassembling of the complex and hydrolysis of Ran-GTP to Ran-GDP (induced by RANBP1 and RANGAP1, respectively) cause release of the tRNA from the export receptor. XPOT then return to the nuclear compartment and mediate another round of transport. The directionality of nuclear export is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and nucleus.
RAN	(toxicity enhancer Freibaum) GTP-binding protein involved in nucleocytoplasmic transport. Required for the import of protein into the nucleus and also for RNA export. Involved in chromatin condensation and control of cell cycle.

RANGAP1	Ran GTPase Activating Protein 1 This gene encodes a protein that associates with the nuclear pore complex and participates in the regulation of nuclear transport. The encoded protein interacts with Ras-related nuclear protein 1 (RAN) and regulates guanosine triphosphate (GTP)-binding and exchange. GTPase activator for the nuclear Ras-related regulatory protein Ran, converting it to the putatively inactive GDP-bound state.
RCC1	Regulator Of Chromosome Condensation 1 Guanine-nucleotide releasing factor that promotes the exchange of Ran-bound GDP by GTP. Involved in the regulation of onset of chromosome condensation in the S phase. Binds both to the nucleosomes and double-stranded DNA. RCC1-Ran complex (together with other proteins) acts as a component of a signal transmission pathway that detects unreplicated DNA. Plays a key role in nucleo-cytoplasmic transport, mitosis and nuclear-envelope assembly.
RanBP1	RAN Binding Protein 1 Inhibits GTP exchange on Ran. Forms a Ran-GTP-RANBP1 trimeric complex. Increase GTP hydrolysis induced by the Ran GTPase activating protein RANGAP1. May act in an intracellular signaling pathway which may control the progression through the cell cycle by regulating the transport of protein and nucleic acids across the nuclear membrane.
RanBP3	RAN Binding Protein 3 Acts as a cofactor for XPO1/CRM1-mediated nuclear export, perhaps as export complex scaffolding protein. Bound to XPO1/CRM1, stabilizes the XPO1/CRM1-cargo interaction. In the absence of Ran-bound GTP prevents binding of XPO1/CRM1 to the nuclear pore complex. Binds to CHC1/RCC1 and increases the guanine nucleotide exchange activity of CHC1/RCC1. Recruits XPO1/CRM1 to CHC1/RCC1 in a Ran-dependent manner. Negative regulator of TGF-beta signaling through interaction with the R-SMAD proteins, SMAD2 and SMAD3, and mediating their nuclear export.
RanBP6 IPO5 paralog	RAN Binding Protein 6 (Nishimura) May function in nuclear protein import as nuclear transport receptor.
RanBP17	RAN Binding Protein 17 (Nishimura) The transport of protein and large RNAs through the nuclear pore complexes (NPC) is an energy-dependent and regulated process. The import of proteins with a nuclear localization signal (NLS) is accomplished by recognition of one or more clusters of basic amino acids by the importin-alpha/beta complex; see MIM 600685 and MIM 602738. The small GTPase RAN (MIM 601179) plays a key role in NLS-dependent protein import. RAN-binding protein-17 is a member of the importin-beta superfamily of nuclear transport receptors.May function as a nuclear transport receptor.
RANBP2/Nup358	RAN Binding Protein 2/Nucleoporin 358 E3 SUMO-protein ligase which facilitates SUMO1 and SUMO2 conjugation by UBE2I. Involved in transport factor (Ran-GTP, karyopherin)-mediated protein import via the F-G repeat-containing domain which acts as a docking site for substrates. Binds single-stranded RNA (in vitro). May bind DNA. Component of the nuclear export pathway. Specific docking site for the nuclear export factor exportin-1. Sumoylates PML at Lys-490 which is essential for the proper assembly of PML-NB.
NUP35	Nucleoporin 35 kDA Functions as a component of the nuclear pore complex (NPC). NPC components, collectively referred to as nucleoporins (NUPs). Can play the role of both NPC structural components and of docking or interaction partners for transiently associated nuclear transport factors. May play a role in the association of MAD1 with the NPC.
NUP37	Nucleoporin 37 kDA Bidirectional transport of macromolecules between the cytoplasm and nucleus occurs through nuclear pore complexes (NPCs) embedded in the nuclear envelope. NPCs are composed of subcomplexes, and NUP37 is part of one such subcomplex, Nup107-160. Component of the Nup107-160 subcomplex of the nuclear pore complex (NPC). The Nup107-160 subcomplex is required for the assembly of a functional NPC. The Nup107-160 subcomplex is also required for normal kinetochore microtubule attachment, mitotic progression and chromosome segregation.

NUP43	Nucleoporin 43 kDA Bidirectional transport of macromolecules between the cytoplasm and nucleus occurs through nuclear pore complexes (NPCs) embedded in the nuclear envelope. NPCs are composed of subcomplexes, and NUP43 is part of one such subcomplex, Nup107-160. Component of the Nup107-160 subcomplex of the nuclear pore complex (NPC). The Nup107-160 subcomplex is required for the assembly of a functional NPC. The Nup107-160 subcomplex is also required for normal kinetochore microtubule attachment, mitotic progression and chromosome segregation.
NUP50	Nucleoporin 50kDA (toxicity enhancer Freibaum) Component of the nuclear pore complex that has a direct role in nuclear protein import. Actively displaces NLSs from importin-alpha, and facilitates disassembly of the importin-alpha:beta-cargo complex and importin recycling. Interacts with multiple transport receptor proteins including CDKN1B. This interaction is required for correct intracellular transport and degradation of CDKN1B.
NUP54	Nucleoporin 54 kDA Component of the nuclear pore complex, a complex required for the trafficking across the nuclear membrane.
NUP62	Nucleoporin 62 kDA (Kinoshita) Essential component of the nuclear pore complex. The N-terminal is probably involved in nucleocytoplasmic transport. The C-terminal is probably involved in protein-protein interaction via coiled-coil formation and may function in anchorage of p62 to the pore complex.
NUP85	Nucleoporin 85 kDA Essential component of the nuclear pore complex (NPC) that seems to be required for NPC assembly and maintenance. As part of the NPC Nup107-160 subcomplex plays a role in RNA export and in tethering NUP98/Nup98 and NUP153 to the nucleus. The Nup107-160 complex seems to be required for spindle assembly during mitosis. NUP85 is required for membrane clustering of CCL2-activated CCR2. Seems to be involved in CCR2-mediated chemotaxis of monocytes and may link activated CCR2 to the phosphatidyl-inositol 3-kinase-Rac-lammellipodium protrusion cascade.
NUP88	Nucleoporin 88 kDA (Kinoshita) Essential component of nuclear pore complex.
NUP93	Nucleoporin 93 kDA Plays a role in the nuclear pore complex (NPC) assembly and/or maintenance. May anchor nucleoporins, but not NUP153 and TPR, to the NPC.
NUP98	Nucleoporin 98 kDA (toxicity suppressor Freibaum) Plays a role in the nuclear pore complex (NPC) assembly and/or maintenance. Nup98 and Nup96 are involved in the bidirectional transport across the NPC. May anchor NUP153 and TPR to the NPC.
NUP107	Nucleoporin 107 kDA (Zhang) (toxicity suppressor Freibaum) Plays a role in the nuclear pore complex (NPC) assembly and/or maintenance. Required for the assembly of peripheral proteins into the NPC. May anchor NUP62 to the NPC.
NUP133	Nucleoporin 133 kDA Involved in poly(A)+ RNA transport.
NUP153	Nucleoporin 153 kDA (Kinoshita) (toxicity enhancer Freibaum) Component of the nuclear pore complex (NPC), a complex required for the trafficking across the nuclear envelope. Functions as a scaffolding element in the nuclear phase of the NPC essential for normal nucleocytoplasmic transport of proteins and mRNAs. Involved in the quality control and retention of unspliced mRNAs in the nucleus; in association with TPR, regulates the nuclear export of unspliced mRNA species bearing constitutive transport element (CTE) in a NXF1- and KHDRBS1-independent manner. Mediates TPR anchoring to the nuclear membrane at NPC. The repeat-containing domain may be involved in anchoring other components of the NPC to the pore membrane. Possible DNA-binding subunit of the nuclear pore complex (NPC).
NUP155	Nucleoporin 155 kDA Essential component of nuclear pore complex. Could be essessential for embryogenesis. Nucleoporins may be involved both in binding and translocating proteins during

	nucleocytoplasmic transport.
NUP160	Nucleoporin 160 kDA (toxicity suppressor Freibaum) Involved in poly(A)+ RNA transport.
NUP188	Nucleoporin 188 kDA May function as a component of the nuclear pore complex (NPC).
NUP205	Nucleoporin 205 kDA (Zhang) Plays a role in the nuclear pore complex (NPC) assembly and/or maintenance. May anchor NUP62 and other nucleoporins, but not NUP153 and TPR, to the NPC.
NUP210	Nucleoporin 210 kDA Nucleoporin essential for nuclear pore assembly and fusion, nuclear pore spacing, as well as structural integrity.
NUP214	Nucleoporin 214 kDA May serve as a docking site in the receptor-mediated import of substrates across the nuclear pore complex.
NUPL1	Nucleoporin-like protein Component of the nuclear pore complex, a complex required for the trafficking across the nuclear membrane.
NUPL2	Nucleoporin like 2/Nucleoporin-like protein 1 (Nishimura) Required for the export of mRNAs containing poly(A) tails from the nucleus into the cytoplasm. In case of infection by HIV-1, it may participate in the docking of viral Vpr at the nuclear envelope.
NUTF2	Nuclear Transport Factor 2 Facilitates protein transport into the nucleus. Interacts with the nucleoporin p62 and with Ran. Acts at a relatively late stage of nuclear protein import, subsequent to the initial docking of nuclear import ligand at the nuclear envelope. Could be part of a multicomponent system of cytosolic factors that assemble at the pore complex during nuclear import.
POM121	POM121 Transmembrane Nucleoporin Essential component of the nuclear pore complex (NPC). The repeat-containing domain may be involved in anchoring components of the pore complex to the pore membrane. When overexpressed in cells induces the formation of cytoplasmic annulate lamellae (AL).
SEC13	EC13 Homolog (S. Cerevisiae) Functions as a component of the nuclear pore complex (NPC) and the COPII coat. At the endoplasmic reticulum, SEC13 is involved in the biogenesis of COPII-coated vesicles. As a component of the GATOR2 complex, inhibits GATOR1 complex, an inhibitor of the amino acid-sensing branch of the TORC1 pathway.
SEH1L	SEH1-Like (S. Cerevisiae) Component of the Nup107-160 subcomplex of the nuclear pore complex (NPC). The Nup107-160 subcomplex is required for the assembly of a functional NPC. The Nup107-160 subcomplex is also required for normal kinetochore microtubule attachment, mitotic progression and chromosome segregation. This subunit plays a role in recruitment of the Nup107-160 subcomplex to the kinetochore. As a component of the GATOR2 complex, inhibits GATOR1 complex, an inhibitor of the amino acid-sensing branch of the TORC1 pathway.
TMEM48	NDC1 Transmembrane Nucleoporin/ Transmembrane Protein 48 Component of the nuclear pore complex (NPC), which plays a key role in de novo assembly and insertion of NPC in the nuclear envelope. Required for NPC and nuclear envelope assembly, possibly by forming a link between the nuclear envelope membrane and soluble nucleoporins, thereby anchoring the NPC in the membrane.
TPR	Translocated Promoter Region, Nuclear Basket Protein Component of the nuclear pore complex (NPC), a complex required for the trafficking across the nuclear envelope. Functions as a scaffolding element in the nuclear phase of the NPC essential for normal nucleocytoplasmic transport of proteins and mRNAs, plays a role in the establishment of nuclear-peripheral chromatin compartmentalization in interphase, and in the mitotic spindle checkpoint signaling during mitosis.
Aly/REF	Export factor Export adapter involved in nuclear export of spliced and unspliced mRNA. Binds mRNA which is thought to be transferred to the NXF1-NXT1 heterodimer for export (TAP/NFX1 pathway).

	Component of the TREX complex which is thought to couple mRNA transcription, processing and nuclear export, and specifically associates with spliced mRNA and not with unspliced pre-mRNA.
NXF1/TAP	Nuclear RNA export factor 1 (toxicity enhancer Freibaum) Involved in the nuclear export of mRNA species bearing retroviral constitutive transport elements (CTE) and in the export of mRNA from the nucleus to the cytoplasm (TAP/NFX1 pathway). The NXF1-NXT1 heterodimer is involved in the export of HSP70 mRNA in conjunction with ALYREF/THOC4 and THOC5 components of the TREX complex. ALYREF/THOC4-bound mRNA is thought to be transferred to the NXF1-NXT1 heterodimer for export.
NXF2	Nuclear RNA Export Factor 2 Involved in the export of mRNA from the nucleus to the cytoplasm.
NXF3	Nuclear RNA Export Factor 3/TAP-Like Protein 3 May function as a tissue-specific nuclear mRNA export factor.
NXF5	Nuclear RNA Export Factor 5/TAP-Like Protein 1 Could be involved in the export of mRNA from the nucleus to the cytoplasm. Could also have a role in polarized cytoplasmic transport and localization of mRNA in neurons.
NXT1	Nuclear Transport Factor 2-Like Export Factor 1 Stimulator of protein export for NES-containing proteins. Also plays a role in the nuclear export of U1 snRNA, tRNA, and mRNA. The NXF1-NXT1 heterodimer is involved in the export of HSP70 mRNA in conjunction with ALYREF/THOC4 and THOC5.
NXT2	Nuclear Transport Factor 2-Like Export Factor 2 Regulator of protein export for NES-containing proteins. Also plays a role in mRNA nuclear export.
RAE1	Ribonucleic Acid Export 1 Binds mRNA. May function in nucleocytoplasmic transport and in directly or indirectly attaching cytoplasmic mRNPs to the cytoskeleton.
MEK1	Mitogen-Activated Protein Kinase Kinase 1 mitogen-activated protein kinase kinase 1,nucleocytoplasmic shuttling protein, involved in signal transduction
CHTOP	Chromatin Target Of PRMT1 Required for effective mRNA nuclear export and is a component of the TREX complex which is thought to couple mRNA transcription, processing and nuclear export, and specifically associates with spliced mRNA and not with unspliced pre-mRNA. TREX is recruited to spliced mRNAs by a transcription-independent mechanism, binds to mRNA upstream of the exon-junction complex (EJC) and is recruited in a splicing- and cap-dependent manner to a region near the 5 end of the mRNA where it functions in mRNA export to the cytoplasm via the TAP/NFX1 pathway.
AGFG1/HRB	ArfGAP With FG Repeats 1 (Nishimura) Required for vesicle docking or fusion during acrosome biogenesis (By similarity). May play a role in RNA trafficking or localization. In case of infection by HIV-1, acts as a cofactor for viral Rev and promotes movement of Rev-responsive element-containing RNAs from the nuclear periphery to the cytoplasm. This step is essential for HIV-1 replication.
CASC3/Barentsz	Cancer Susceptibility Candidate 3 (Nishimura) Core component of the splicing-dependent multiprotein exon junction complex (EJC) deposited at splice junctions on mRNAs. The EJC is a dynamic structure consisting of core proteins and several peripheral nuclear and cytoplasmic associated factors that join the complex only transiently either during EJC assembly or during subsequent mRNA metabolism. The EJC marks the position of the exon-exon junction in the mature mRNA for the gene expression machinery and the core components remain bound to spliced mRNAs throughout all stages of mRNA metabolism thereby influencing downstream processes including nuclear mRNA export, subcellular mRNA localization, translation efficiency and nonsense-mediated mRNA decay (NMD). Stimulates the ATPase and RNA-helicase activities of EIF4A3. Plays a role in the stress response by participating in cytoplasmic stress granules assembly and by favoring cell recovery following stress. Component of the dendritic ribonucleoprotein particles (RNPs) in hippocampal neurons. May play a role in mRNA transport. Binds spliced mRNA in sequence-

	independent manner, 20-24 nucleotides upstream of mRNA exon-exon junctions. Binds poly(G) and poly(U) RNA homopolymer.
EIF4E Transporter	Eukaryotic Translation Initiation Factor 4E (Nishimura) Interacts with EIF4E. Interacts with importin beta only in the presence of importin alpha, suggesting a direct interaction with importin alpha. Interacts with APOBEC3G in an RNA-dependent manner.
THOC3 = part of TREX complex	Tho Complex 3 (Nishimura) Required for efficient export of polyadenylated RNA and spliced mRNA. Acts as component of the THO subcomplex of the TREX complex which is thought to couple mRNA transcription, processing and nuclear export, and which specifically associates with spliced mRNA and not with unspliced pre-mRNA. TREX is recruited to spliced mRNAs by a transcription-independent mechanism, binds to mRNA upstream of the exon-junction complex (EJC) and is recruited in a splicing- and cap-dependent manner to a region near the 5 end of the mRNA where it functions in mRNA export to the cytoplasm via the TAP/NFX1 pathway. The TREX complex is essential for the export of Kaposis sarcoma-associated herpesvirus (KSHV) intronless mRNAs and infectious virus production.

Table 17. Summary of siRNA library targeting the nucleocytoplasmic transport machinery based on genecards.org and uniprot.org

PUBLICATION RECORD

Ederle, H., Funk, C., Abou-Ajram, C., Hutten, S., Funk, E., Kehlenbach, R. H., Bailer, S. M. and Dormann, D. Nuclear egress of TDP-43 and FUS is a diffusion-limited process. Submitted to Scientific Reports (under revisions)

Ederle, H. and Dormann, D. TDP-43 and FUS en route from the nucleus to the cytoplasm. FEBS Lett 591 (11) 2017. (Ederle and Dormann 2017)

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Khosravi, B., Hartmann, H., May, S., Mohl, C., Ederle, H., Michaelsen, M., Schludi, M. H., Dormann, D. and Edbauer, D. Cytoplasmic poly-GA aggregates impair nuclear import of TDP-43 in C9orf72 ALS/FTLD. Hum Mol Genet 26 (4) 2017. (Khosravi, Hartmann et al. 2017).

Curriculum Vitae

RÉSUMÉ

PhD student in neuroscience | International experience through stays in the U.K. & U.S. | Industrial internship at ROCHE DIAGNOSTICS | Fluent in ENGLISH & GERMAN

EDUCATION (Grades are based on the German grading system)

09/2014 – present | PhD student in neuroscience | Biomedical Center (BMC) | LMU Munich, Germany
10/2011 – 08/2014 | Master of Science (M.Sc.) in molecular biosciences | Ruprecht-Karls-University Heidelberg, Germany | Grade 1.4
10/2007 – 07/2010 | Bachelor of Science (B.Sc.) in biology | University of Ulm, Germany | Grade 1.6
09/1998 – 06/2007 | General qualification for university entrance | Maria-Stern Gymnasium Augsburg, Germany | Grade 2.1

INTERNATIONAL & PRACTICAL EXPERIENCE

09/2013 – 07/2014 | Cold Spring Harbor Laboratory, New York, U.S. | Internship and master thesis
04 – 08/2013 | Roche Diagnostics GmbH, Penzberg, Germany | Industrial internship
09 – 11/2012 | Department of Psychiatry, Oxford University, U.K. | Erasmus stipend internship
09/2010 – 03/2011 | Max Planck Institute of Psychiatry, Munich, Germany | Internship

HONORS & AWARDS

2016/2017 | 2 SyNergy (LMU Munich) travel awards for scientific conferences
01/2016 – present | PhD stipend of the Hans- and Ilse Breuer foundation
09/2014 – present | PhD student of the Graduate School of Systemic Neurosciences (GSN), LMU Munich, Germany
09/2013 – 07/2014 | Master stipend of the Rotary International foundation for international stays

EXTRACURRICULAR ACTIVITIES

Member of the GSN retreat organization committee
Mentor for the „Change in“-project, Augsburg, Germany
Ambassador for „Bündnis für Augsburg“-project, Augsburg, Germany

ADDITIONAL LANGUAGES

English (business fluent) I Russian (mother tongue)

INTERESTS | REFERENCES

Art history & Drawing I Countries & Cultures I Movies I Pilates & Yoga

REFERENCES

Available upon request

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"And, of all the things upon earth, I hold that a faithful friend is the best."

Edward Bulwer-Lytton

… to Dorothee, my π, for her advice and support and all the opportunities she provided during my Ph.D. work. ∞

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… to Paul, passionate cineaste, for a long time friendship and the effort to read my fabulous work. □

… to those I forgot in writing but surely acknowledged in person.

AFFIDAVIT/EIDESSTATTLICHE VERSICHERUNG

I hereby confirm that the dissertation “Identification of nuclear pathways of TDP-43 and FUS” is the result of my own work and that I have only used sources and materials listed and specified in the dissertation.

Munich, 21/12/2017 _____

Helena Ederle

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation “Identification of nuclear pathways of TDP-43 and FUS” selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annährend übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

München, 21/12/2017 _____

Helena Ederle

DECLARATION OF COPYRIGHT AND CONTRIBUTIONS

Helena Ederle wrote the manuscript text, performed most experiments and prepared most figures (for detailed listing of exceptions see “Main project – II. Results – Declaration of contributions”, p. 24 and “Side project – I. Background & Results – Declaration of contributions”, p. 56).

Munich, 21/12/2017 _____

Claudia Abou-Ajram, technician of research group of Dorothee Dormann, conducted immunoblotting experiments and cell fractionations (for detailed listing see “Main project – II. Results – Declaration of contributions”, p. 24).

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Dorothee Dormann, supervisor and lab head, designed the main and side project and coordinated involved collaborations (*e.g.* communication with collaborators, acquisition of material such as expression vectors). As Dorothee Dormann is also the senior/corresponding author for the in parallel to Scientific Reports submitted manuscript “Nulcear egress of TDP-43 and FUS is a diffusion-limited process” based on the majority results presented in the main project-section, she functions as a representative for contributing co-authors and a former technician of her laboratory:

1. Emanuele Buratti and Francisco Baralle who kindly provided Flag-TDP-43-WT and RNA-binding-deficient mutants F2L/ΔRRM1/F4L as well as bacterial expression vector used to generate Flag-TDP-43-ΔRRM2.
2. Eva Funk, former PhD student of Dorothee Dormann, who generated polyclonal HeLa cells stably expressing mCherry-tagged TDP-43-83AAA and FUS-P525L NLS-mutants.

   * Note: Eva Funk also generated polyclonal HeLa cells stably expressing mCherry-tagged TDP-43-WT and mCherry-tagged FUS-WT that were used for the siRNA-mediated screen in the side project.
3. Christina Funk and Susanne M. Bailer who generated pEYFP-NLS-FRB-TDP-43 and pEYFP-NLS-FRB-FUS used in the NEX-TRAP assay (see “Main project – II. Results, 7. Enlargement of TDP-43 and FUS impairs their nuclear egrass”, p. 42-44). They also conduced and analyzed the assay and contributed experimental explanation used in the Material and Methods section (see “10. NEX-TRAP assay, p. 67-68)

4. Ramona Rodde, former technician of research group of Dorothee Dormann, kindly provided cDNA constructs used in Figure 10C (for detailed listing see “Main project – II. Results – Declaration of contributions”, p. 24).

Munich, 21/12/2017 _____