POLY-GA TRIGGERS TDP-43 PATHOLOGY BY INHIBITING THE PROTEASOME AND NUCLEOCYTOPLASMIC IMPORT IN *C9ORF72* ALS/FTD

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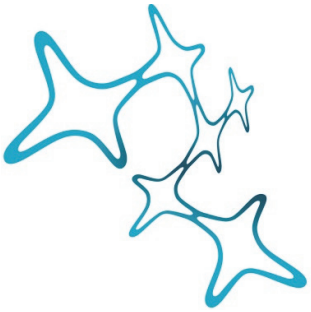

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1. List of abbreviations

This list of abbreviations is independent of abbreviation lists in the research articles.

AD	Alzheimer's disease
AD*	autosomal dominant
ALS	amyotrophic lateral sclerosis
ALS2	amyotrophic lateral sclerosis 2
ALS-bi	ALS with behavioral impairment
ALS-cbi	ALS with combined cognitive and behavioral impairment
ALS-ci	ALS with cognitive impairment
ANG	angiogenin
ANXA11	Annexin-11
AR	autosomal recessive
ATXN2	ataxin 2
Aβ	amyloid-β peptide
cAMP	Cyclic adenosine monophosphate
C9orf72	chromosome 9 open reading frame 72
CCNF	G2/Mitotic-Specific Cyclin-F
CCTα	cytidylyltransferase
CHCHD10	coiled-coil-helix-coiled-coil-helix domain containing protein 10
CHMP2B	charged multivesicular body protein 2B
CHMP2B	charged multivesicular body protein 2B
CMA	chaperone-mediated autophagy
cryo-ET	cryo-electron tomography
CSE1L/CAS	chromosome Segregation 1 Like/cellular apoptosis susceptibility

DNA	Deoxyribonucleic acid
DPRs	dipeptide repeat proteins
DSB	DNA double-strand break
eIF2α	eukaryotic translation initiation factor 2A
ERBB4	Neuregulin-receptor tyrosine kinase
fALS	familial ALS
fFTD	familial FTD
FIG4	phosphoinositide 5-phosphatase
FTD	frontotemporal dementia
FTD-ALS	frontotemporal dementia-amyotrophic lateral sclerosis
FUS	fused in sarcoma
G3BP	Ras GTPase-activating protein-binding protein 1
GA	glycine (gly), alanine (ala)
G_4C_2	4x guanine, 2x cytosine
GLT8D1	Glycosyltransferase 8 Domain Containing 1
GP	glycine (gly), proline (pro)
GR	glycine (gly), arginine (arg)
GRN	granulin precursor
HD	Huntington's disease
hnRNPA1	heterogeneous nuclear ribonucleoprotein A1
hnRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1
hnRNPA3	heterogeneous nuclear ribonucleoprotein A3
HR23	Ubiquitin receptor RAD23
HRE	hexanucleotide repeat expansion
Htt	huntingtin
IN	induced neuron
iPSCs	induced pluripotent stem cells

K95	lysine 95
KIF5A	Kinesin Heavy Chain Neuron-Specific 1
LCD	low complexity sequence domain
LLPS	liquid-liquid phase separation
MAPT	microtubule-associated protein tau
MATR3	Matrin 3
MG132	benzyloxycarbonyl-l-leucyl-l-leucyl-l-leucinal
MND	motor neuron disease
mRNA	messenger ribonucleic acid
N2a	Neuro-2a
NEK1	NIMA (Never In Mitosis Gene A)-Related Kinase 1
NES	nuclear export signal
NFκB	nuclear factor kappa B
NLS	nuclear localization signal
NMDA	*N*-methyl-D-aspartate
NPC	nuclear pore complex
NSC-34	the spinal cord neuron × neuroblastoma hybrid cell line
NUP	Nucleoporin
NUP54	Nucleoporin p54 kDa
NUP62	Nuclear pore glycoprotein p62 kDa
OPTN	optineurin
P53	tumor protein p53
p62	nucleoporin 62
PD	Parkinson's disease
PDE4	phosphodiesterase 4
PFN1	profilin 1

PI31	proteasome inhibitor of 31kD
PKA	cAMP-dependent protein kinase
PR	proline (pro), arginine (arg)
PSMC4	proteasome 26S subunit ATPase 4/regulatory subunit 6B
PSMD11	26S proteasome non-ATPase regulatory subunit D11
Rab39b	Ras-Related Protein Rab-39B
Rab8a	Ras-related protein Rab-8A
RAN	repeat-associated non-AUG translation
RanGAP1	Ran GTPase-activating protein 1
RanGEF	Ran guanine nucleotide exchange factor
Ras	rat sarcoma
RBPs	RNA-binding proteins
RCC1	Regulator of chromosome condensation 1
Rpn6	regulatory particle non-ATPase 6
RNA	ribonucleic acid
sALS	sporadic ALS
Ser-409/410	serine 409/410
SETX	senataxin
sFTD	sporadic FTD
SG	stress granule
SIGMAR1	Sigma Non-Opioid Intracellular Receptor 1
SMCR8	Smith-Magenis chromosome region 8
SOD1	superoxide dismutase 1
SPG11	spatacsin (Spastic Paraplegia 11)
SQSTM1/p62	sequestosome 1
TAR	transactive response

TARDBP	TAR DNA-binding protein 43
TBK1	TANK-Binding Kinase 1
TDP-43	transactive response DNA binding protein 43 kDa
ThT	Thioflavin T
TTX	Tetrodotoxin
TUBA4A	tubulin, alpha 4A
UBQLN2	ubiquilin-2
Unc119	Unc-119 Lipid Binding Chaperone
UPS	ubiquitin-proteasome system
VAPB	VAMP (Vesicle-Associated Membrane Protein)-Associated Protein B And C
VCP	valosin-containing protein
WD	glycine-histidine and tryptophan-aspartate
WDR41	WD repeat-containing protein 41
X-LD	X-linked inheritance
α-syn	α-synuclein

2. Abstract

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are two fatal neurodegenerative disorders that share overlapping clinical and pathological features (Kato, Hayashi et al. 1993, Lomen-Hoerth, Anderson et al. 2002). Although both diseases occur mostly sporadically, several disease-associated mutations have been identified in >25 different genes, many of which are encoding RNA binding proteins (RBP), components of the ubiquitin proteasome system (UPS), and the autophagy pathway (Kapeli, Martinez et al. 2017, Bartoletti, Bosco et al. 2019). Cytoplasmic inclusions of the multifunctional RNA-binding protein TDP-43 (TAR DNA-binding protein) that normally resides predominantly in the nucleus are found in ~90% of ALS and ~45% of FTD cases. TDP-43 dyshomeostasis including aberrations in its nucleocytoplasmic shuttling and aggregation has been shown to induce toxicity, explaining a crucial role in the process of neurodegeneration (Araki, Minegishi et al. 2014, Leibiger, Deisel et al. 2018, Prasad, Bharathi et al. 2019).

The most common pathogenic mutation found in ~10% of ALS/FTLD patients is the massive expansion of a hexanucleotide repeat $(G_4C_2)_n$ in the first intron of *C9orf72* gene (DeJesus-Hernandez, Mackenzie et al. 2011, Renton, Majounie et al. 2011). *C9orf72* patients show, in addition to typical TDP-43 pathology, nuclear RNA foci containing the repeat RNA from both sense and antisense transcripts (DeJesus-Hernandez, Mackenzie et al. 2011) and unique inclusions of five different species of dipeptide repeat (DPR) proteins (poly-GA/-GP/-GR/-PA and -PR). DPR inclusions are derived from an unconventional non-AUG translation of the intronic repeat RNA in all reading frames (Ash, Bieniek et al. 2013, Gendron, Bieniek et al. 2013, Mori, Arzberger et al. 2013, Mori, Weng et al. 2013, Zu, Liu et al. 2013). Nearly all inclusions are poly-GA positive and often contain also poly-GP/-GR and far less frequently poly-PA/-PR. *In vitro*, poly-GA sequesters large amounts of stalled proteasomes suggesting a deleterious effect on cellular proteostasis (Guo, Lehmer et al. 2018). DPR pathology has been shown to precede TDP-43 pathology in patients, thereby considered as a major driver of neurodegeneration associated with *C9ORF72* expansion (Baborie et al. 2015; Mann 2015; Mori, Arzberger, et al. 2013; Mori, Weng, et al. 2013; Proudfoot et al. 2014). **How *C9orf72*-specific pathology triggers TDP-43 pathology, despite not being spatially correlated in human studies (Schludi et al. 2017), was the primary objective of my PhD studies.**

When I started my PhD project, several groups reported impaired global nucleocytoplasmic transport possibly through a direct effect on the nuclear pore complex (NPC) in different *C9orf72* models and connected it to poly-GR/PR (Jovicic, Mertens et al. 2015), repeat RNA (Zhang, Donnelly et al. 2015), or both (Freibaum, Lu et al. 2015), however in these studies trafficking of TDP-43 itself was not analyzed. Furthermore, artificial aggregating β-sheet

proteins were shown to inhibit nucleocytoplasmic transport related to sequestration of the THOC complex and RNA binding proteins (Woerner, Frottin et al. 2016). Since GA_{15} peptides, but not 15-mers of the other DPR species, form amyloid-like fibrils (Chang, Jeng et al. 2016), I asked whether poly-GA may also impair nucleocytoplasmic transport. I therefore compared the impact of individual expression of poly-GA, poly-GR and poly-PR on the nuclear import, specifically of TDP-43 to understand the link between the *C9orf72* mutation and TDP-43 pathology. I quantitatively analyzed cytoplasmic mislocalization of endogenous TDP-43 and of a reporter containing the established bipartite classical nuclear localization signal (NLS) of TDP-43. Interestingly, poly-GA blocked nuclear import of TDP-43 more robustly than poly-GR/PR, while none of the DPR proteins affected the localization of a reporter containing a transportin-dependent PY-NLS in our *in vitro* models arguing DPR proteins mainly impair nuclear transport through the classical importin α/β pathway mediating TDP-43 import. In addition, I found that overexpression of two NPC components (NUP54 and NUP62) can fully rescue nuclear localization of the TDP-43 reporter. NUP54 and NUP62 were interestingly shown to be essential for nuclear import of TDP-43 (Nishimura, Zupunski et al. 2010) and NUP62 knockdown enhances $(PR)_{25}$ toxicity in flies (Boeynaems, Bogaert et al. 2016). Thus, **inhibition of nuclear import of TDP-43 by poly-GA may link the *C9orf72* mutation to TDP-43 pathology** in *C9orf72* ALS/FTD cases.

Since poly-GA precedes symptom onset by many years (Vatsavayai, Yoon et al. 2016) and rarely co-localize with TDP-43 inclusions, chronic toxicity and possibly non-cell-autonomous effects were proposed (Edbauer and Haass 2016). For example, cell-to-cell transmission of cytoplasmic Tau and α-synuclein aggregates results in stereotypic spreading during the progression of Alzheimer's and Parkinson's disease, respectively (Jucker and Walker 2018). Thus, I analyzed non-cell-autonomous effects of DPRs as a potential trigger of TDP-43 pathology using co-culture assays and antibody treatment experiments to inhibit poly-GA cell-to-cell transmission. Importantly, I discovered that poly-GA transmission inhibits the proteasome even in neighboring cells. Activating the proteasome pharmacologically (with the PDE4 inhibitor rolipram) or genetically (using PSMD11 overexpression) rescues nuclear import of the TDP-43 NLS reporter. Therefore, I hypothesized that poly-GA inclusions may block the import of TDP-43 via ubiquitination directly within its NLS, which accumulate through impaired proteostasis. I could show that mutagenizing lysine 95 in the NLS largely prevents ubiquitination of TDP-43 and did not impair basal nuclear import, but completely prevented poly-GA mediated inhibition of TDP-43 import. In contrast, mutation of lysine 84 severely blocks interactions with nuclear import receptors such as importin-α5/KPNA1, independent of poly-GA expression, which is in consistence with previous reports (Hans, Eckert et al. 2018). Importantly, poly-GA antibodies reduce poly-GA transmission and cytoplasmic TDP-43

mislocalization in HeLa cells and primary neurons, suggesting poly-GA antibodies could be potentially used to treat *C9orf72* ALS/FTD.

Taken together, my work shows that poly-GA promotes cytoplasmic mislocalization of TDP-43 cell- and non-cell-autonomously due to impaired proteasomal clearance of TDP-43 ubiquitinated within its NLS at lysine 95. My work also indicates that pharmacologically boosting proteasome activity (e.g. by rolipram) or inhibiting poly-GA transmission using antibodies are promising therapeutic approaches for *C9orf72* ALS/FTD. Indeed, our group (Zhou, Mareljic et al. 2020) and others (Nguyen, Montrasio et al. 2020) have reported promising results using anti-GA antibodies or vaccination in mouse models confirming my data *in vivo*.

3. Introduction

3.1. Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) terminologies

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are two clinically distinct devastating neurodegenerative diseases with overlapping pathology and genetics, that are now recognized as two extremes of a disease spectrum (Van Langenhove, van der Zee et al. 2012, Ling, Polymenidou et al. 2013).

3.1.1. Clinical and pathological features of ALS, FTLD, and ALS/FTD

3.1.1.1. ALS

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, and Charcot disease, was first described in 1874 and is the most common form of motor neuron disease (MND) (Ferraiuolo, Kirby et al. 2011). ALS is a fatal neuromuscular disorder caused by the degeneration of both upper (UMNs) and lower (LMNs) motor neurons, which leads to hyperreflexia, spasticity, fasciculations, and muscular atrophy and eventually death (Van Langenhove, van der Zee et al. 2012, Babic Leko, Zupunski et al. 2019). Most cases of ALS are considered ‘sporadic’ since they occur without a known cause or a family history. However, about 5–10% of cases are due to genetic mutations, typically with dominant inheritance (Brown and Al-Chalabi 2017). The disease onset mostly occurs in the range of 54–67 years of age with a mean survival time of 3-5 years after diagnosis (Chio, Logroscino et al. 2013). The incidence of ALS is about 1–2 cases per 100,000 per year (Rowland and Shneider 2001, Worms 2001, Brown and Al-Chalabi 2017, Marin, Boumediene et al. 2017, Babic Leko, Zupunski et al. 2019). Sixty-two percent of patients with familial ALS and 48.5% with sporadic ALS show cognitive impairment (Wheaton, Salamone et al. 2007). According to the type and severity of neuropsychological deficits, ALS patients can be divided into several subgroups: pure ALS with no impairment, ALS with behavioral impairment (ALS-bi), ALS with cognitive impairment (ALS-ci), ALS with combined cognitive and behavioral impairment (ALS-cbi), and ALS/FTLD. (Strong, Abrahams et al. 2017).

3.1.1.2. FTLD

Frontotemporal lobar degeneration (FTLD), in patients younger than 65 years of age, is the most common cause of dementia after Alzheimer’s disease (Onyike and Diehl-Schmid 2013, Warren, Rohrer et al. 2013), accounting for about 10% of the cases (Irwin, Cairns et al. 2015, Van Mossevelde, Engelborghs et al. 2018, Thathiah 2020). FTLD is characterized by predominant degeneration of the frontal and anterior temporal lobes (Gorno-Tempini, Hillis et

al. 2011, Rascovsky, Hodges et al. 2011), resulting in behavioral changes, apathy, dementia, and loss of executive functions or deterioration of language functions during the later stages of the disease (Ash, Moore et al. 2009, Knibb, Woollams et al. 2009, Wilson, Henry et al. 2010, Thompson, Lukic et al. 2012). The prevalence of FTLD is about 15-22 cases per 100,000 per year (Ratnavalli, Brayne et al. 2002, Knopman and Roberts 2011).The clinicopathological spectrum of FTLD consists of frontotemporal dementia (FTD), FTD with motor neuron disease (FTD–MND), progressive subcortical gliosis (PSG), primary progressive aphasia, cortico-basal degeneration (CBD), semantic dementia, and progressive supranuclear palsy (PSP) (Bugiani 2007). In addition, FTLD has been subcategorized into specific proteinopathies based on the major accumulated protein component, including 45% FTLD-tau (with inclusions of hyperphosphorylated tau protein) (Mackenzie, Rademakers et al. 2010), 45% FTLD-TDP (with inclusions of response (TAR) DNA-binding protein 43 (TDP-43)) (Neumann, Sampathu et al. 2006, Neumann, Kwong et al. 2007, Mackenzie, Neumann et al. 2009), 9% FTLD-FUS (with inclusions of fused-in-sarcoma protein) (Mackenzie, Neumann et al. 2010), and a small number of 1% FTLD-UPS (due to co-localization with markers of the ubiquitin proteasome system) (Mackenzie, Neumann et al. 2009, Dickson 2011). It is important to highlight that the term FTLD refers to pathological conditions that are commonly connected to clinical entities of FTD, which characterized by degeneration of frontal and temporal lobes (Mackenzie, Neumann et al. 2009).

3.1.1.3. ALS/FTD

ALS and FTD for a long time were considered distinct disease entities as ALS patients typically show loss of voluntary motor function and FTD patients exhibit behavioral and speech impairments. Strikingly, several recently identified pathogenic mutations (e.g. in *C9orf72*) can cause ALS, FTD or a mixed disease (Lomen-Hoerth, Anderson et al. 2002, Ringholz, Appel et al. 2005, Burrell, Kiernan et al. 2011, Guerreiro, Bras et al. 2015). These genetic findings prompted clinical research in ALS and FTD cases: About 50% of ALS cases manifest cognitive impairment similar to the type observed in FTD, with around 15% of ALS cases meeting diagnostic criteria for FTD at the time ALS is diagnosed (Ringholz, Appel et al. 2005). Furthermore, about 15% of FTD cases show clinically detectable motor symptoms (Ling, Polymenidou et al. 2013). Ten percent of all ALS, and one third of all FTD cases, seem to have a positive family history with at least one immediate family member showing the disease (Neumann, Sampathu et al. 2006, Ling, Polymenidou et al. 2013). Currently, riluzole, which is thought to inhibit tetrodotoxin (TTX)-sensitive sodium channels, kainate receptors and NMDA receptors (Debono, Le Guern et al. 1993, Song, Huang et al. 1997, Bellingham 2011) and the

antioxidant Edaravone are the only two ALS therapies available, but extend life only by a few months (Moujalled and White 2016, Hardiman and van den Berg 2017, Writing and Edaravone 2017). In addition, riluzole is the only disease-modifying drug that has been approved for any form of FTD in the USA (Tsai and Boxer 2016).

3.1.1. Genetics of ALS and FTD

Considerable efforts have been made in unraveling the genetics of ALS and FTD, and it is now obvious that the genetics of these two neurodegenerative syndromes considerably overlap.

So far mutations in > 25 genes contributing to the etiology of both ALS (Table 1), FTD (Table 2) have been identified. Many of these identified genes encode proteins that function in common biological pathways such as ubiquitin proteasome system (UPS), intracellular trafficking, RNA processing, autophagy and unfolded protein responses. Interestingly, mutations in most of these genes lead to similar pathology including TDP-43 aggregation and neuroinflammation, emphasizing several mechanisms resulting in common downstream implications and neuronal loss. Associated genes with amyotrophic lateral sclerosis and frontotemporal dementia have been listed in Tables 1. and 2. respectively. (Rosen, Siddique et al. 1993, Hadano, Hand et al. 2001, Yang, Hentati et al. 2001, Chen, Bennett et al. 2004, Nishimura, Mitne-Neto et al. 2004, Greenway, Andersen et al. 2006, Parkinson, Ince et al. 2006, Watts, Thomasova et al. 2007, Seelaar, Kamphorst et al. 2008, Sreedharan, Blair et al. 2008, Chow, Landers et al. 2009, Kwiatkowski, Bosco et al. 2009, Vance, Rogelj et al. 2009, Elden, Kim et al. 2010, Felbecker, Camu et al. 2010, Johnson, Mandrioli et al. 2010, Luty, Kwok et al. 2010, Maruyama, Morino et al. 2010, Orlacchio, Babalini et al. 2010, Al-Saif, Al-Mohanna et al. 2011, Chen-Plotkin, Martinez-Lage et al. 2011, DeJesus-Hernandez, Mackenzie et al. 2011, Deng, Chen et al. 2011, Fecto, Yan et al. 2011, Mackenzie, Neumann et al. 2011, Renton, Majounie et al. 2011, Rubino, Rainero et al. 2012, Wu, Fallini et al. 2012, Kim, Kim et al. 2013, Le Ber, Camuzat et al. 2013, Miller, Pestronk et al. 2013, Takahashi, Fukuda et al. 2013, Teyssou, Takeda et al. 2013, Bannwarth, Ait-El-Mkadem et al. 2014, Chaussenot, Le Ber et al. 2014, Johnson, Glynn et al. 2014, Muller, Andersen et al. 2014, Smith, Ticozzi et al. 2014, van der Zee, Van Langenhove et al. 2014, Ajroud-Driss, Fecto et al. 2015, Cirulli, Lasseigne et al. 2015, Dols-Icardo, Nebot et al. 2015, Freischmidt, Wieland et al. 2015, Kurzwelly, Kruger et al. 2015, Ronchi, Riboldi et al. 2015, Zhang, Xi et al. 2015, Brenner, Muller et al. 2016, Genin, Plutino et al. 2016, Nizzardo, Simone et al. 2016, Williams, Topp et al. 2016, Che, Zhao et al. 2017, Perrone, Nguyen et al. 2017, Shen, He et al. 2017, Nicolas, Kenna et al. 2018, Cooper-Knock, Moll et al. 2019).

Most importantly *C9orf72* is by far the biggest contributor explaining 40% of familial ALS (fALS) and 10% of sporadic ALS (sALS) cases (DeJesus-Hernandez, Mackenzie et al. 2011, Renton, Majounie et al. 2011, Wood 2011, Gijselinck, Van Langenhove et al. 2012, Majounie, Renton et al. 2012, Cruts, Gijselinck et al. 2013, Webster, Smith et al. 2016, Nguyen, Van Broeckhoven et al. 2018).

Table 1. Overview of associated genes with amyotrophic lateral sclerosis (ALS).

ALS related genes	Chromosomal location	Onset	Inheritance
C9ORF72	9p21.2	Adult	AD
TARDBP	1p36.22	Adult	AD
SQSTM1	5q35.3	Adult	AD
UBQLN2	Xp11.21	Adult	X-LD
CHCHD10	22q11.23	Adult	AD
SOD1	21q22.11	Adult	AD (AR)
FUS	16p11.2	Adult	AD (AR)
OPTN	10p13	Adult	AD (AR)
TBK1	12q14.2	Adult	AD
hnRNPA1	12q13.13	Adult	AD
TUBA4A	2q35	Adult	AD
ATXN2	12q24.12	Adult	AD
ALS2	2q33.1	Juvenile	AR
VCP	9p13.3	Adult	AD
CHMP2B	3p11.2	Adult	AD
ANG	14q11.2	Adult	AD
SETX	9q34.13	Juvenile	AD
FIG4 (SAC3)	6q21	Adult	AD
SPG11	15q21.1	Juvenile	AR
PFN1	17p13.2	Adult	AD

ERBB4	2q34	Adult	AD
MATR3	5q31.2	Adult	AD
ANXA11	10q22.2	Adult	AD
SIGMAR1	9p13.3	Juvenile	AD and AR
GLT8D1	3p21.1	Adult	AD
NEK1	4q33	Adult	AD
KIF5A	12q13.3	Adult	AD
VAPB	20q13. 32	Adult	AD
CCNF	16p13.3	Adult	AD

Abbreviations: autosomal dominant (**AD***), autosomal recessive (**AR**), X-linked inheritance (**X-LD**), chromosome 9 open reading frame 72 (***C9orf72***), TAR DNA-binding protein 43 (***TARDBP***), sequestosome 1 (***SQSTM1***/***p62***), ubiquilin-2 (***UBQLN2***), coiled-coil-helix-coiled-coil-helix domain containing protein 10 (***CHCHD10***), superoxide dismutase 1 (***SOD1***), fused in sarcoma (***FUS***), optineurin (***OPTN***), TANK-Binding Kinase 1 (***TBK1***), heterogeneous nuclear ribonucleoprotein A1 (***hnRNPA1***), tubulin, alpha 4A (***TUBA4A***), ataxin 2 (***ATXN2***), Amyotrophic lateral sclerosis 2 (***ALS2***), valosin-containing protein (***VCP***), charged multivesicular body protein 2B (***CHMP2B***), angiogenin (***ANG***), Senataxin (***SETX***), phosphoinositide 5-phosphatase (***FIG4***), spatacsin (***SPG11***), profilin 1 (***PFN1***), Neuregulin-receptor tyrosine kinase (***ERBB4***), Matrin 3 (***MATR3***), Annexin-11 (***ANXA11***), Sigma Non-Opioid Intracellular Receptor 1 (***SIGMAR1***), Glycosyltransferase 8 Domain Containing 1 (***GLT8D1***), NIMA (Never In Mitosis Gene A)-Related Kinase 1 (***NEK1***), Kinesin Heavy Chain Neuron-Specific 1 (***KIF5A***), VAMP (Vesicle-Associated Membrane Protein)-Associated Protein B And C (***VAPB***), G2/Mitotic-Specific Cyclin-F (***CCNF***).

Table 2. Overview of associated genes with frontotemporal dementia (FTD).

FTD related genes	Chromosomal location	Onset	Inheritance
C9ORF72	9p21.2	Adult	AD
SQSTM1	5q35.3	Adult	AD
GRN	17q21.31	Adult	AD
VCP	9p13.3	Adult	AD
CHMP2B	3p11.2	Adult	AD
MAPT	17q21.2	Adult	AD
TARDBP	1p36.22	Adult	AD
TBP	6q27	Adult	AD
TBK1	12q14.2	Adult	AD
FUS	16p11.2	Adult	AD (AR)
CHCHD10	22q11.23	Adult	AD
OPTN	10p13	Adult	AD (AR)
TUBA4A	2q35	Adult	AD
ATXN2	12q24.12	Adult	AD
CCNF	16p13.3	Adult	AD
UBQLN2	Xp11.21	Adult	X-LD

Abbreviations: autosomal dominant (**AD***), autosomal recessive (**AR**), X-linked inheritance (**X-LD**), chromosome 9 open reading frame 72 (***C9orf72***), sequestosome 1 (***SQSTM1*/*p62***), granulin precursor (***GRN***), valosin-containing protein (***VCP***), charged multivesicular body protein 2B (***CHMP2B***), Microtubule Associated Protein Tau (***MAPT***), TAR DNA-binding protein 43 (***TARDBP***), TATA Sequence-Binding Protein (***TBP***), TANK-Binding Kinase 1 (***TBK1***), fused in sarcoma (***FUS***), coiled-coil-helix-coiled-coil-helix domain containing protein 10 (***CHCHD10***), optineurin (***OPTN***), tubulin, alpha 4A (***TUBA4A***), ataxin 2 (***ATXN2***), G2/Mitotic-Specific Cyclin-F (***CCNF***), ubiquilin-2 (***UBQLN2***).

3.2. TAR DNA binding protein (TARDBP) gene in ALS/FTD

TDP-43 was first identified in 1995 as an interactor of the transactive response (TAR) DNA element in human immunodeficiency virus 1 (HIV-1), and therefore the gene encoding TDP-43 was named TAR DNA Binding Protein (TARDBP) (Ou, Wu et al. 1995). The *TARDBP* gene is comprised of six exons and is found on chromosome 1p36.22. *TARDBP* mRNA is widely expressed in many different human tissues, particularly pancreas, brain and spinal cord (Zhang, Xu et al. 2007, Wang, Wu et al. 2008).

TARDBP gene encodes a highly conserved 414-amino-acid-protein with the molecular weight of 43 kDa, therefore is named TDP-43 (Ayala, Pantano et al. 2005, Buratti and Baralle 2008). TDP-43 is composed of an N-terminus including a nuclear localization signal (NLS), nuclear export signal (NES), two RNA recognition motifs (RRM1 and RRM2), with a C-terminus containing a glutamine/asparagine-rich (Q/N) domain and a glycine-rich region (Ayala, Zago et al. 2008).

The N-terminal domain has been extensively studied and shown to play an essential role in the dimerization of TDP-43 (Chang, Wu et al. 2012, Afroz, Hock et al. 2017). TDP-43, in order to function as a transcriptional repressor or activator, via mainly RRM1 domain (RRM1 playing a predominant role while RRM2 has more of a supporting role) binds an RNA repeat with UG-repeats (Ayala, Pantano et al. 2005, Forman, Trojanowski et al. 2007) and TG-rich DNA sequences (Kuo, Doudeva et al. 2009, Lukavsky, Daujotyte et al. 2013, Kuo, Chiang et al. 2014). NLS and NES sequences permit TDP-43 to shuttle between the nucleus and the cytosol (Buratti and Baralle 2008, Ederle, Funk et al. 2018), and play a role in interactions between TDP-43 and members of hnRNP family (A1, A2/B1, and A3) (Buratti, Brindisi et al. 2005).

The C-terminus of TDP-43 is largely disordered, interacts with several RNA binding proteins (RBPs) and promotes aggregation of the full length protein of proteolytically processed C-terminal fragments (Johnson, Snead et al. 2009, Fuentealba, Udan et al. 2010). TDP-43 also binds another RBP, Fragile X mental retardation protein (FMRP) to co-inhibit translation (Majumder, Chu et al. 2016). It has been shown that TDP-43 via its C-terminus can also interact with nucleic acids (i.e., ssDNA) in addition to membranes (Lim, Wei et al. 2016). Moreover, TDP-43 associates with ubiquillin-2 via its C-terminal domain (Cassel, McDonnell et al. 2012, Cassel and Reitz 2013).

TDP-43 is an RNA/DNA-binding protein that processes RNA predominantly in the nucleus, therefore is mainly localized in the nucleus with low abundance in the cytosol. However, TDP-43 regulates expression and splicing of >900 distinct genes, revealing an important role for

TDP-43 in maintaining the RNA levels, including mRNAs that are essential for neuronal function (Polymenidou, Lagier-Tourenne et al. 2011), some of which are decreased in human diseases (Tollervey, Curk et al. 2011). Furthermore, patients with TARDBP mutations show characteristic TDP-43 positive neuronal and glial inclusions together with motor neuron loss and presence of so-called Bunina bodies. (Neumann 2009, Polymenidou, Lagier-Tourenne et al. 2011, Colombrita, Onesto et al. 2012, Lagier-Tourenne, Polymenidou et al. 2012). Cytoplasmic TDP-43 aggregation likely causes neurodegeneration through series of direct aggregate toxicity and additional loss of function mechanisms due to striking nuclear clearance of TDP-43 in aggregate-bearing cells (Gendron and Petrucelli 2011, Walker, Tripathy et al. 2015, Ederle and Dormann 2017, Prasad, Bharathi et al. 2019). How pathogenic mutations in other genes, such as *C9orf72* trigger TDP-43 pathology and their link to neurodegeneration remains unclear.

3.3. Chromosome 9 open reading frame 72 gene (*C9orf72*) in ALS/FTD

The discovery of a noncoding hexanucleotide $(G_4C_2)_n$ repeat expansion (HRE) mutation in 5′ of the open reading frame of *C9orf72* in 2011 was a breakthrough in the field, because the mutation explains up to 40% of fALS patients, 25% of familial FTD, and striking 88% of familial patients with both ALS and FTD (Van Mossevelde, van der Zee et al. 2017). The *C9orf72* gene was first found in multiple multigenerational families with FTD-ALS and linked to chromosome 9p21 (DeJesus-Hernandez, Mackenzie et al. 2011, Renton, Majounie et al. 2011, Gijselinck, Van Langenhove et al. 2012).

3.3.1. *C9orf72* link to ALS/FTD

C9orf72-related ALS/FTD is inherited in an autosomal dominant manner, with age-dependent penetrance. Repeat sizes of less than 25 (G_4C_2) are considered normal, while expansions to more than 60 (G_4C_2) hexanucleotide repeats are considered pathogenic, (Majounie, Renton et al. 2012, van der Zee, Gijselinck et al. 2013). However, the exact cut-off is still controversial because most controls have 2-3 repeats and most patients harbor several hundred or thousand repeats, while cases with intermediate repeat size are extremely rare (Cruts, Engelborghs et al. 1993, Xi, Zinman et al. 2012).

3.3.2 *C9orf72* disease mechanisms

In understanding *C9orf72* mutations, three main non-exclusive pathological disease mechanisms have been proposed:

Many studies have shown loss-of-function (LOF) and haploinsufficiency where $(G_4C_2)_n$ repeat expansion in *C9orf72* promoter suppresses expression of the mutant allele, resulting in

reduced mRNA and proteins levels (Figure 1a). This phenomenon had previously been seen in fragile X syndrome and Friedrich's ataxia (Tassone, Pan et al. 2008, Gijselinck, Van Langenhove et al. 2012). Although this proposed mechanism has been under intense debate (Donnelly, Zhang et al. 2013, Fratta, Poulter et al. 2013, Lagier-Tourenne, Baughn et al. 2013, Sareen, O'Rourke et al. 2013), very recently in a mouse model expressing a *C9orf72* transgene with 450 repeats (without producing C9ORF72 protein), suppression of one or both *C9orf72* allele(s) was reported to aggravate cognitive-related deficits potentially via inhibiting autophagy (Zhu, Jiang et al. 2020). Moreover, cryo-electron microscopy revealed the structure of the CSW complex consisting of C9orf72 protein together with Smith-Magenis chromosome region 8 (SMCR8) and WD repeat-containing protein 41 (WDR41) . The CSW complex regulates membrane trafficking by mediating the activity of Rab GTPases (Tang, Sheng et al. 2020). *In vitro*, the CSW complex stimulates the exchange of GDP for GTP in small GTP/GDP-binding proteins such as Ras-related protein Rab-8A (Rab8a) and Ras-related protein Rab-39b (Rab39b) (Sellier, Campanari et al. 2016, Yang, Liang et al. 2016).

Furthermore, expanded sense and antisense RNA transcripts form toxic RNA foci that may sequester essential RNA-binding proteins and inhibit the RNA processing machinery, similar to myotonic dystrophy type 1 (Figure 1b) (Wheeler and Thornton 2007, DeJesus-Hernandez, Mackenzie et al. 2011). However, at least in *Drosophila* the repeat RNA itself has little effect on global RNA processing and neuronal survival (Mizielinska, Gronke et al. 2014, Tran, Almeida et al. 2015).

Last but not least, unconventional repeat-associated non-AUG (RAN) translation of both sense and anti-sense RNA transcripts in all three reading frames leads to 5 different species of aggregating dipeptide repeat proteins (DPRs): poly-GA, poly-GP, poly-GR, poly-PA, and poly-PR inclusions (Figure 1c) (Ash, Bieniek et al. 2013, Mori, Arzberger et al. 2013, Mori, Weng et al. 2013, Zu, Liu et al. 2013, Edbauer and Haass 2016, Frick, Sellier et al. 2018, Saberi, Stauffer et al. 2018). These inclusions are most abundant in the cerebellum, hippocampus, and neocortex (Mori, Arzberger et al. 2013). In postmortem studies of *C9orf72* FTD/ALS brains, poly-GA and poly-GP inclusions are more abundant than poly-GR, while poly-PA and poly-PR inclusions are rare (Mackenzie, Frick et al. 2014, Mackenzie, Frick et al. 2015).

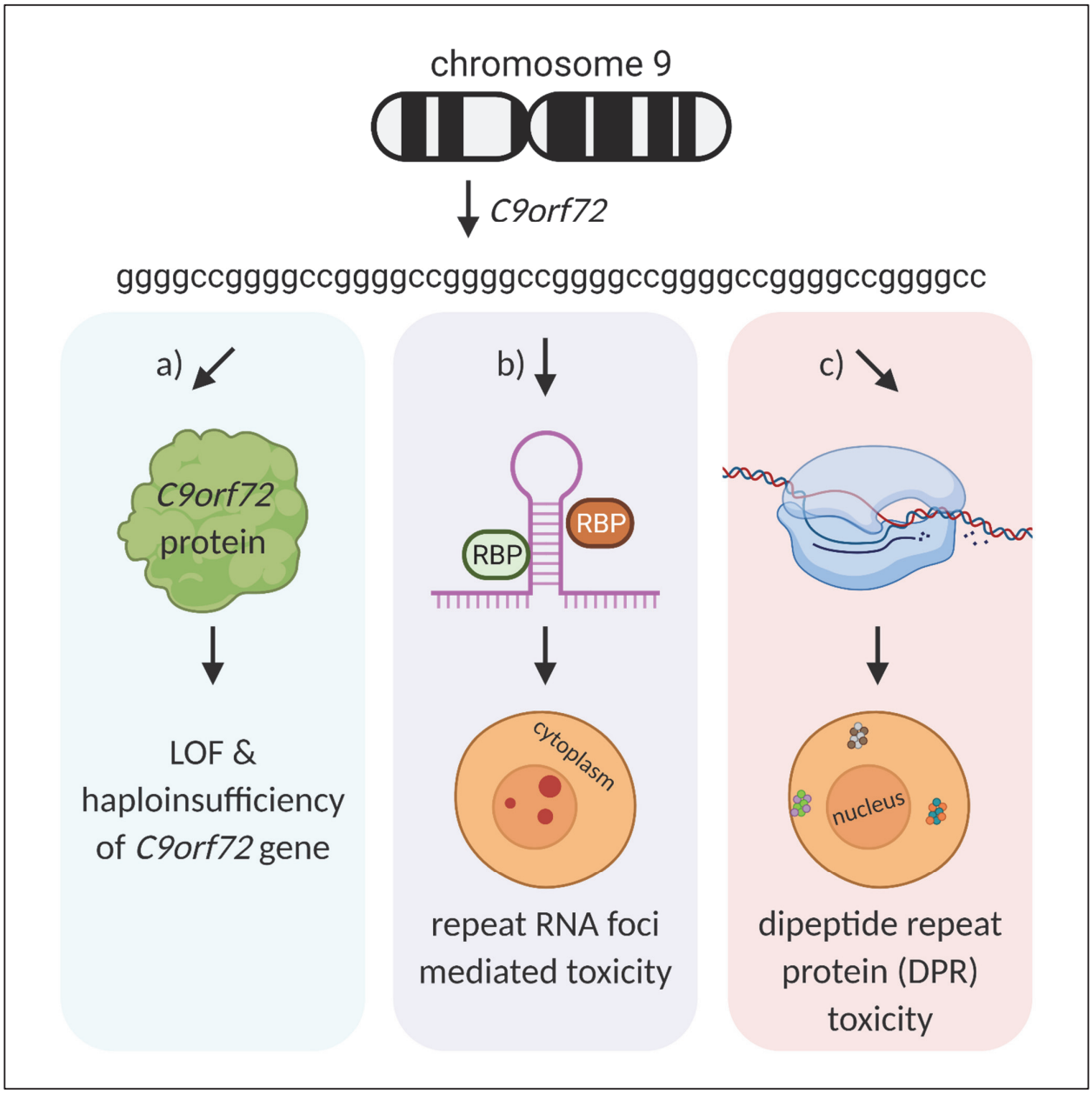

Figure 1. *C9orf72* repeat expansion: three proposed pathomechanisms. The *C9orf72* gene harbors a polymorphic hexanucleotide $(G_4C_2)_n$ repeat in a non-coding region (depending on the transcript either in the promoter region or in the first intron) of the gene. Large expansions of this nucleotide repeat cause ALS, FTD or both. There are currently three major hypotheses to explain pathological disease mechanisms. (a) $(G_4C_2)_n$ repeat expansion reduces *C9orf72* gene expression and could result in *C9orf72* haploinsufficiency. (b) RNA transcripts of expanded $(G_4C_2)_n$ from both sense and anti-sense strands causing an accumulation of toxic RNA foci. These RNA foci further sequester RNA-binding proteins (RBPs), such as splicing factors, and result in impaired pre-mRNA splicing. (c) Dipeptide repeat protein (DPR) inclusions are derived from non-AUG translation of sense and anti-sense transcripts in all reading frames and are abundant in brain and rare in spinal cord of *C9orf72* carriers.

3.3.3 Dipeptide repeat (DPR) proteins pathology

Pathologically, *C9orf72* mutation carriers exhibit aberrant, particular star-shaped or dot-like TDP-43 negative, p62-positive cytoplasmic dipeptide repeat protein (DPRs) inclusions, that are not found in other types of ALS/FTD (Al-Sarraj, King et al. 2011). Most importantly, among all five species of DPRs in patients with *C9orf72* HRE, only poly-GA overexpression in primary neurons mimicked the p62-positive neuronal inclusions (Schludi et al. 2015). RAN-translation was first reported for $(CAG)_n$ repeats in spinocerebellar ataxia type 8 and myotonic dystrophy (Zu, Gibbens et al. 2011). Several groups including us have reported *C9orf72*-derived DPRs show differential toxicity in cell culture models (Zu, Liu et al. 2013, Kwon, Xiang et al. 2014, May, Hornburg et al. 2014, Wen, Tan et al. 2014, Zhang, Jansen-West et al. 2014, Tao, Wang et al. 2015, Yamakawa, Ito et al. 2015, Chang, Jeng et al. 2016, Kanekura, Yagi et al. 2016), animal models including *Drosophila* (Mizielinska, Gronke et al. 2014, Wen, Tan et al. 2014, Freibaum, Lu et al. 2015, Tran, Almeida et al. 2015, Yang, Abdallah et al. 2015, Boeynaems, Bogaert et al. 2016, Lee, Zhang et al. 2016), zebrafish (Ohki, Wenninger-Weinzierl et al. 2017, Swaminathan, Bouffard et al. 2018, Swinnen, Bento-Abreu et al. 2018), and mice (Zhang, Gendron et al. 2016, Schludi, Becker et al. 2017). DPRs can cause neurodegeneration, behavioral deficits and have been linked to impaired nucleocytoplasmic transport (Freibaum, Lu et al. 2015, Jovicic, Mertens et al. 2015, Zhang, Donnelly et al. 2015, Zhang, Gendron et al. 2016, Khosravi, Hartmann et al. 2017, Shi, Mori et al. 2017, Yin, Lopez-Gonzalez et al. 2017).

3.3.4. DPRs transmit between cells

For a long time, disease transmission due to misfolded proteins was considered a unique property of the prion protein. However, several studies have shown underlying ‘prion-like’ mechanisms in pathological spreading of various neurodegenerative diseases (Aguzzi and Rajendran 2009, Brundin, Melki et al. 2010). In fact, ‘non-prion’ protein aggregates, such as tau in AD (Alzheimer's disease), α-synuclein in PD (Parkinson's disease), and Huntingtin in HD (Huntington's disease) can transmit between cells and seed to recruit the endogenous proteins into aggregates through various mechanisms, including phagocytosis, endocytosis, and exocytosis (Kane, Lipinski et al. 2000, Meyer-Luehmann, Coomaraswamy et al. 2006, Frost and Diamond 2009, Krammer, Schatzl et al. 2009, Angot, Steiner et al. 2010, Brundin, Melki et al. 2010, Frost and Diamond 2010, Lee, Desplats et al. 2010, Guest, Silverman et al. 2011, Jucker and Walker 2011, Langer, Eisele et al. 2011, Moreno-Gonzalez and Soto 2011, Dunning, Reyes et al. 2012, Hall and Patuto 2012, Morales, Duran-Aniotz et al. 2012, Costanzo and Zurzolo 2013, Fritschi, Langer et al. 2014, Watts, Condello et al. 2014, Rasmussen, Mahler et al. 2017, Jucker and Walker 2018, Ruiz-Riquelme, Lau et al. 2018).

Cell-to-cell spreading of DPRs via exosome-dependent and exosome-independent pathways has been shown in cell culture experiments, including spinal motor neurons derived from induced pluripotent stem cells from *C9orf72*-ALS cases (Westergard et al. 2016). In addition, $(GA)_{15}$ forms ribbon-type fibrils and is taking up in neuroblastoma N2a cells (Chang, Jeng et al. 2016). Our group also detected cell-to-cell transmission of all hydrophobic DPR species (poly-GA, -GP, and -PA) using co-culture experiments in HEK293 cells. Using rat primary neurons, poly-GA release was detected in conditioned media by immunoassay, in addition poly-GA was shown to transmit between neurons (Zhou et al. 2017). Therefore, I asked how DPR uptake affects neighboring cells, focusing on proteostasis and nucleocytoplasmic transport.

3.3.5. DPRs impair nucleocytoplasmic transport

In eukaryotic cells, a double-membraned nuclear envelope (NE) separates the nucleus from the cytoplasm. Molecules are transported between nucleus and cytoplasm via large gateways called nuclear pore complexes (NPCs). The NPC is the largest macromolecular complex in eukaryotic cells, comprising ~30 distinct proteins (Cronshaw and Matunis 2004). Small molecules can passively cycle in and out of the nucleus through the NPC, while for larger molecules like proteins larger than ~40 kDa, an active transport by recruiting receptors that bind nuclear pore complex proteins (NUPs) is required. Protein import and export is mediated by specific signals, termed nuclear localization sequence (NLS) and nuclear export signal (NES) that are recognized by carrier protein importins and exportins, respectively (Steggerda and Paschal 2002, Corbett and Krebber 2004, Mor, White et al. 2014). Furthermore, compartmentalized distribution of Ran-GTP defines the directionality of nucleocytoplasmic transport (Melchior, Paschal et al. 1993, Moore and Blobel 1993, Gorlich and Mattaj 1996, Sweet and Gerace 1996, Weis, Dingwall et al. 1996, Goldfarb 1997, Gorlich 1997, Nigg 1997). A genetic screen in Drosophila expressing $(G_4C_2)_n$ repeats identified RAN GTPase activating protein (RanGAP) (Drosophila orthologue of human RanGAP1) as an essential regulator of nucleocytoplasmic transport (Zhang, Donnelly et al. 2015). However, RanGAP1 localization with poly-GA inclusions in different mouse models is still under debate (Zhang, Gendron et al. 2016, Schludi, Becker et al. 2017). In addition, artificial aggregating β-sheet proteins have been shown to impair nucleocytoplasmic transport due to sequestration of RNA binding proteins (Woerner, Frottin et al. 2016). Finally, several interactome studies have shown that poly-GR and poly-PR inclusions engage with RNA binding proteins (RBPs) and influence the formation of membrane-less organelles and their liquid-liquid phase separation (LLPS) dynamics mediated by low complexity sequence domains (LCDs) (Lee, Zhang et al. 2016, Lin, Mori et al. 2016, Zhang, Gendron et al. 2018, Moens, Niccoli et al. 2019). Moreover, selective transportation of cargos through the nuclear pore is believed to be via liquid-liquid phase

separation. Therefore, several studies have identified interactions between poly-GR and poly-PR aggregates with RBPs that led to nucleocytoplasmic transport abnormalities (Jovicic, Mertens et al. 2015, Boeynaems, Bogaert et al. 2016, Lee, Zhang et al. 2016). These interactions have been reported to inhibit nucleocytoplasmic transport (Zhang, Donnelly et al. 2015).

3.3.6. DPRs and protein homeostasis

In a typical human cell about 10,000 different proteins are expressed and they must be appropriately folded to exert their biological functions. The term "proteostasis" (protein homeostasis) refers to a dynamic quality control network that maintains proteins in the correct concentration, conformation, and subcellular location (Powers, Morimoto et al. 2009, Labbadia and Morimoto 2015, Kulak, Geyer et al. 2017, Klaips, Jayaraj et al. 2018). During proteostasis, cells are in a dynamic state between protein synthesis/folding and protein degradation. Since proteins control almost every process in the cells, it is crucial to maintain and regulate proteostasis in order for the cells to be functional and able to adapt to new environmental challenges (Balch, Morimoto et al. 2008, Hartl, Bracher et al. 2011). Proteostasis dysfunction fundamentally leads to disease pathogenesis, therefore in order for the cells to prevent protein misfolding and aggregation, they use very efficient and largely sensitive protein quality control mechanisms, involving degradation systems for damaged proteins. Two major intracellular protein degradation pathways are the ubiquitin-proteasome system (UPS) and autophagy.

The ubiquitin-proteasome system (UPS) has been particularly studied in neurodegenerative disorders since it functions as a key player in proteostasis in eukaryotic cells. UPS maintains the cellular environment clear of non-functional, misfolded, and aggregated proteins that have been shown to accumulate in various neurodegenerative disorders (Ciechanover and Brundin 2003, Dantuma and Bott 2014, Hipp, Park et al. 2014, Schmidt and Finley 2014). Degradation of intracellular proteins by UPS is composed of two separate, following steps: ubiquitylation and proteasomal degradation (Hershko and Ciechanover 1998, Kleiger and Mayor 2014).

Ubiquitylation usually results in the conjugation between its carboxy-terminal glycine (G76) and the lysine (Lys) residue, and less frequently, free N-terminus of the substrate (Pickart 2001). This reaction is catalyzed by a consecutive cascade of three enzymes: a ubiquitin activator (E1) forming a thiol ester with the carboxyl group of G76 and activating it for nucleophilic attack, a conjugating enzyme (E2) transferring the activated ubiquitin to the conjugation site as an E2-ubiquitin thiol ester intermediate, and a ligase (E3) carrying activated ubiquitin to the substrate’s lysine residue (Hershko, Heller et al. 1983, Pickart 2001, Goldberg 2003, Deshaies and Joazeiro 2009, Schulman and Harper 2009, Ye and Rape 2009).

Substrate proteins targeted for proteasomal degradation normally bind a poly-ubiquitin chain that has been formed from several rounds of ubiquitylation, in which ubiquitins are linked by lysine 48-glycine 76 (K48-G76) isopeptide bonds (Hershko and Ciechanover 1998).

Moreover, proteasomes are found in eukaryotes, archaea, and some bacteria. Eukaryotic 26S proteasome is a large multi-subunit complex that is responsible for selected degradation of a wide number of cell proteins (Hershko, Heller et al. 1983, Hegde, Goldberg et al. 1993, Hershko and Ciechanover 1998, Walker and LeVine 2000, Pickart 2001, Sherman and Goldberg 2001, Ciechanover and Brundin 2003, Goldberg 2003, Fonseca, Vabulas et al. 2006, Hou, Antion et al. 2006, Karpova, Mikhaylova et al. 2006, Ortega, Diaz-Hernandez et al. 2007, Kabashi, Valdmanis et al. 2008, Rutherford, Zhang et al. 2008, Tai and Schuman 2008, Deshaies and Joazeiro 2009, Schulman and Harper 2009, Ye and Rape 2009, Hegde 2010, van Eersel, Ke et al. 2011, Saeki and Tanaka 2012, Kleiger and Mayor 2014, McKinnon and Tabrizi 2014, Ortega and Lucas 2014, Gupta, Lan et al. 2017, Opoku-Nsiah and Gestwicki 2018, McAlary, Plotkin et al. 2019, Thibaudeau and Smith 2019). It is comprised of a 20S core particle and 19S regulatory particle at one or both ends, a key discriminator gateway for targeted protein substrates (Saeki and Tanaka 2012). The 19S regulatory particle recognizes and assists in deubiquitylation, unfolds and translocates protein substrates into the 20S core particle. 20S particle forms a cavity where the proteolytic site of the proteasome is located, therefore the substrate is degraded into short peptides that are eventually broken down to amino acids by peptidases and can be reused by the cell (Zhang, Zhao et al. 2018). Proteasome's capacity to degrade ubiquitin conjugates and on protein substrate degradation in cells can be modified by several ways, including pharmacological agents (rolipram by increasing cAMP levels) and genetic manipulations (19S proteasome subunit PSMD11/RPN-6) (Vilchez, Boyer et al. 2012, Lokireddy, Kukushkin et al. 2015). Rolipram treatment has been studied in Alzheimer models and was shown to help with clearance of abnormal tau, thereby improving cognition (Myeku, Clelland et al. 2016).

Although various neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), and Amyotrophic lateral sclerosis (ALS) together with frontotemporal dementia (FTD) manifest different clinical symptoms, they all have been shown to be associated with accumulation of aggregating proteins (Walker and LeVine 2000, Sherman and Goldberg 2001, Jellinger 2012, McAlary, Plotkin et al. 2019). In addition, neurons like each post-mitotic cell are very sensitive to proteostasis imbalances because of their unique metabolism and long lifespan (Tai and Schuman 2008). A vast growing range of studies have linked neurodegeneration to mutations in UPS components (Ciechanover and Brundin 2003, Ortega, Diaz-Hernandez et al. 2007, McKinnon and Tabrizi 2014, Ortega and Lucas 2014, Thibaudeau and Smith 2019) and respective decline in proteostasis and

proteasomal dysfunction (Dantuma and Bott 2014, Opoku-Nsiah and Gestwicki 2018).

In ALS research, our group and others reported that poly-GA expression leads to upregulation of p62 and accumulation of ubiquitinated proteins, suggesting impaired UPS activity (May, Hornburg et al. 2014, Zhang, Jansen-West et al. 2014, Yamakawa, Ito et al. 2015). We have shown that poly-GA inclusions sequesters 26S proteasomes that are locked in an otherwise rare substrate-processing state. This leads to impaired proteasome function that is essential for proteostasis maintenance, and responsible for inducing ER stress (Zhang, Jansen-West et al. 2014, Guo, Lehmer et al. 2018). Moreover, we and others have shown poly-GA interacts with components of UPS-related proteins Unc119 and HR23 (May, Hornburg et al. 2014, Zhang, Gendron et al. 2016). Significantly, poly-GA, poly-GP, and poly-GR aggregates were shown to increase levels of TDP-43, a well-known substrate for UPS (Yamakawa, Ito et al. 2015). Furthermore, poly-PR inclusions have been shown to reduce UPS flux, thereby impeding UPS function and induce cell toxicity (Gupta, Lan et al. 2017). Most importantly, reduced proteasome activity has been shown to lead to decreased solubility and fragmentation of TDP-43 (Kabashi, Valdmanis et al. 2008, Rutherford, Zhang et al. 2008), in addition to chemically blocking proteasome activity resulting in induced cytoplasmic accumulation of TDP-43 (van Eersel, Ke et al. 2011).

Since poly-GA inclusions impair proteasome and impaired proteasome leads to cytoplasmic mislocalization of TDP-43, considering the fact that DPRs and TDP-43 inclusions are rarely found in the same cell, I asked whether cell-to-cell transmission of poly-GA could cause TDP-43 pathology (comparing to other DPRs) in neighboring cells, mainly by studying effects on proteasome activity and nucleocytoplasmic transport. Moreover, I focused on proteasomal activity manipulations (pharmacologically and genetically) to see whether impeded or induced proteasome could affect poly-GA and TDP-43 pathology.

In addition, autophagy is a largely conserved catabolic process contributing to maintaining cellular homeostasis (Kaushik and Cuervo 2018, Mizushima 2018). Overwhelming evidence has shown various autophagy receptors are linked to ALS/FTD (Maruyama, Morino et al. 2010, Deng, Chen et al. 2011, Kachaner, Genin et al. 2012, Dillen, Van Langenhove et al. 2013, Rea, Majcher et al. 2014, van der Zee, Van Langenhove et al. 2014).

4. Goals of the study

Although TDP-43 cytoplasmic mislocalization and aggregation strongly correlates with neurodegeneration in ALS and FTD, it is unclear what mechanisms trigger TDP-43 pathology. Genetic forms of ALS/FTD provide an excellent opportunity to explore this outstanding question at least in a subset of patients with the hope to identify mechanisms that are also relevant for sporadic ALS/FTD. The pathogenic $(G_4C_2)_n$ repeat expansion upstream of the coding region of *C9orf72* is found in 5-10% of ALS and FTD cases and virtually all *C9orf72* patients develop TDP-43 pathology (DeJesus-Hernandez, Mackenzie et al. 2011, Renton, Majounie et al. 2011). *C9orf72* patients express five unique species of dipeptide repeat (DPR) proteins (poly-GA/-GP/-GR/-PA and -PR) derived from an unconventional non-AUG translation of the repeat RNA in all reading frames (Ash, Bieniek et al. 2013, Gendron, Bieniek et al. 2013, Mori, Arzberger et al. 2013, Mori, Weng et al. 2013, Zu, Liu et al. 2013). The five DPR species co-aggregate and nearly all DPR inclusions are poly-GA positive and often contain also poly-GP/-GR and far less frequently poly-PA/-PR. DPRs and TDP-43 co-aggregate only occasionally and are not spatially correlated, but poly-GA has been shown to be transmitted from cell to cell similar to aggregating proteins in other neurodegenerative diseases (Zhou, Lehmer et al. 2017, Jucker and Walker 2018). Moreover, poly-GA forms amyloid-like fibrils (Chang, Jeng et al. 2016) and artificial aggregating β-sheet proteins inhibit nucleocytoplasmic transport (Woerner, Frottin et al. 2016).

Based on these findings, the first aim of my thesis was to study whether DPRs and in particular poly-GA may impair nucleocytoplasmic transport of endogenous TDP-43 and fluorescent reporters in cell lines and primary neurons in order to understand the link between the *C9orf72* mutation and TDP-43 pathology.

I could indeed show that poly-GA inhibits the nuclear import of TDP-43 and colleagues in my lab reported that poly-GA inclusions sequester large amounts of proteasomes (Guo, Lehmer et al. 2018). Thus, my second goal was to test whether poly-GA mediated proteasome inhibition impairs TDP-43 trafficking and whether chemical or genetic activation of proteasome function would restore nuclear import of TDP-43.

Moreover, cytoplasmic Tau and α-synuclein inclusions found in Alzheimer's and Parkinson's disease have been shown to cause stereotypic spreading (Jucker and Walker 2018). Accordingly, the third goal of my thesis was to analyze whether non-cell-autonomous effects of DPRs would contribute to TDP-43 pathology and whether this could be inhibited by monoclonal anti-GA antibodies that may serve as future therapies.

5. Research Articles

5.1. Cytoplasmic poly-GA aggregates impair nuclear import of TDP-43 in *C9orf72* ALS/FTLD

TDP-43 inclusion pathology correlates well with regional neurodegeneration in ALS and FTD in sporadic and *C9orf72* cases. However, the cause of TDP-43 aggregation in *C9orf72* ALS/FTD has been largely unclear. The *C9orf72* mutation has been linked to nucleocytoplasmic transport deficits in cell and animal models (Freibaum, Lu et al. 2015, Jovicic, Mertens et al. 2015, Zhang, Donnelly et al. 2015), but the relative contribution of the $(G_4C_2)_n$ RNA and the different DPR species was debated and the effects on TDP-43 itself had not been investigated. By using a reporter containing TDP-43 nuclear localization (NLS) signal, I have shown that cytoplasmic poly-GA inclusions *in vitro* inhibit TDP-43 NLS to a greater extent compared to poly-GR/-PR expression. Furthermore, since only cytosolic artificial β-sheet aggregates (Woerner, Frottin et al. 2016), but not the nuclear ones, had been shown to inhibit nuclear import, we asked whether the localization of poly-GA inclusions plays a role in their implications on TDP-43 import. By fusing poly-GA with the NLS, we could reroute aggregate formation from the cytosol to the nucleus. Interestingly, HeLa cells and neurons with nuclear poly-GA inclusions showed far less cytoplasmic mislocalization of TDP-43 compared to cells with cytoplasmic poly-GA aggregates.

Moreover, I showed that DPRs affect the nuclear import mainly through the classical importin α/β pathway. In contrast, a reporter construct containing the non-classical PY-NLS of hnRNPA1 was not affected by DPRs. Finally, by overexpression importin-α (KPNA3, KPNA4) and nuclear pore components (NUP54, NUP62), we were able to rescue nucleocytoplasmic transport of TDP-43 NLS reporter.

My findings, for the first time, indicate a direct link between poly-GA, the predominant DPR species in *C9orf72* cases, and cytoplasmic TDP-43 pathology. In addition, our study underlines the role of nucleocytoplasmic transport deficits in *C9orf72* pathogenesis.

Author contribution: performed most cell biological and all biochemical experiments, image acquisition, *analyzed HeLa cell experiment data*, *generated reagents, helped designing the study, and writing parts of the manuscript (please see section 11 for further details).*

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ORIGINAL ARTICLE

Cytoplasmic poly-GA aggregates impair nuclear import of TDP-43 in *C9orf72* ALS/FTLD

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Abstract

A repeat expansion in the non-coding region of *C9orf72* gene is the most common mutation causing frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Sense and antisense transcripts are translated into aggregating dipeptide repeat (DPR) proteins in all reading frames (poly-GA,-GP,-GR,-PA and -PR) through an unconventional mechanism. How these changes contribute to cytoplasmic mislocalization and aggregation of TDP-43 and thereby ultimately leading to neuron loss remains unclear. The repeat RNA itself and poly-GR/PR have been linked to impaired nucleocytoplasmic transport. Here, we show that compact cytoplasmic poly-GA aggregates impair nuclear import of a reporter containing the TDP-43 nuclear localization (NLS) signal. However, a reporter containing a non-classical PY-NLS was not affected. Moreover, poly-GA expression prevents TNFα induced nuclear translocation of p65 suggesting that poly-GA predominantly impairs the importin-α/β-dependent pathway. In neurons, prolonged poly-GA expression induces partial mislocalization of TDP-43 into cytoplasmic granules. Rerouting poly-GA to the nucleus prevented TDP-43 mislocalization, suggesting a cytoplasmic mechanism. In rescue experiments, expression of importin-α (KPNA3, KPNA4) or nucleoporins (NUP54, NUP62) restores the nuclear localization of the TDP reporter. Taken together, inhibition of nuclear import of TDP-43 by cytoplasmic poly-GA inclusions causally links the two main aggregating proteins in *C9orf72* ALS/FTLD pathogenesis.

[†]The authors wish it to be known that, in their opinion, the first 2 authors should be regarded as joint First Authors.
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Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are two devastating neurodegenerative diseases with overlapping pathology and genetics (1). The pathogenic *C9orf72* repeat expansion is the most common genetic cause of ALS and FTLD. Upon autopsy, *C9orf72* patients present typical cytoplasmic TDP-43 aggregates that are also seen in other familial and sporadic ALS/FTLD cases (2). Three potential pathomechanisms leading to *C9orf72* ALS/FTLD have been proposed so far. Reduced expression from the mutant *C9orf72* allele may inhibit autophagy and promote neuroinflammation without causing overt neurodegeneration by itself (3–5). The RNA containing hundreds or thousands GGGGCC repeats rather than 2–30 repeats seen in healthy people is thought to sequester a number of RNA-binding proteins in nuclear RNA foci (6). Repeat-associated non-ATG translation (7) of the intronic repeat in all reading frames gives rise to five dipeptide repeat (DPR) proteins. The sense transcript-derived poly-GA, poly-GR and poly-GP are much more abundant than poly-PA and poly-PR derived from the antisense transcript (8–11). The DPR proteins coaggregate in compact inclusions predominantly in the cytoplasm of neurons in the neocortex, cerebellum and thalamus. In patients DPR inclusions likely appear several years prior to TDP-43 pathology (12). TDP-43 pathology correlates better with regional neurodegeneration than DPR pathology (13) and DPR and TDP-43 inclusions appear mostly in distinct cells. If they occur in the same cell, TDP-43 seems to coat the poly-GA aggregates (8). Intercellular spreading of both aggregated poly-GA (14,15) and TDP-43 (16) has been reported.

The cause of TDP-43 aggregation in *C9orf72* ALS/FTLD is still largely unclear (17). We and others have found no obvious effect on TDP-43 upon expression of individual DPR proteins in cell lines (18–21). High level expression of $(GGGGCC)_{66}$ using AAV results in significant TDP-43 aggregation and neurodegeneration, although the TDP-43 inclusions are (unlike in patients) predominantly found within the nucleus (22). Several BAC-transgenic lines replicate DPR and RNA foci pathology (4,23,24), but strangely only one such line additionally showed TDP-43 inclusions and rapid neurodegeneration in a subset of female animals (25).

Recently, several groups have reported impaired nucleocytoplasmic transport in several *C9orf72* models and attributed it to the repeat RNA (26), poly-GR/PR (27) or both (28). The repeat RNA or poly-GR/PR are thought to disrupt the nucleocytoplasmic Ran-GTP gradient that is crucial for correct sorting of most proteins, but the mechanism remains unclear (29). Similarly, altered RanGAP1 localization has been reported in mice with high levels of poly-GA expression, but the functional consequences have not been addressed (30). Recently, it was shown that artificial aggregating β-sheet proteins impair nucleocytoplasmic transport due to sequestration of the THOC complex and RNA binding proteins (31). Since GA_{15} peptides also form typical amyloid fibrils (14) we speculated that poly-GA may also impair nucleocytoplasmic transport.

Therefore, we tested whether poly-GA, poly-GR and poly-PR affect the nuclear import of TDP-43, because cytoplasmic TDP-43 aggregation may ultimately trigger neurodegeneration in *C9orf72* cases. We analysed cytoplasmic mislocalization of endogenous TDP-43 and of a reporter containing the bipartite classical nuclear localization signal (NLS) of TDP-43. This signal has been shown to mediate TDP-43 nuclear import of TDP-43 via the importin-α/β pathway (32–34). To elucidate the mechanism of impaired nuclear import of TDP-43, we redirected the cytoplasmic poly-GA aggregates into the nucleus and performed rescue experiments using key components of the nuclear import machinery.

Results

Poly-GA causes mislocalization of a TDP-43 NLS reporter

To test functional consequences of DPR expression on nucleocytoplasmic transport of TDP-43 in HeLa cells, we generated a fluorescence-based reporter containing RFP fused with the nuclear localization signal (NLS) of TDP-43, a well characterised bipartite NLS (33). Upon co-expression of RFP-NLS_{TDP} with GFP, RFP-NLS_{TDP} almost exclusively localised to the nucleus in HeLa cells in interphase, as expected (Fig. 1A first row). In contrast, many cells with GA_{149}-GFP inclusions showed significant levels of the RFP-reporter in the cytoplasm suggesting impaired nuclear import mediated by this classical NLS (Fig. 1A, second row, arrows). Quantitative analysis using manual counting confirmed significant mislocalization of the reporter in ∼40% of inclusion bearing cells compared to ∼5% of GFP expressing cells (Fig.1B). In contrast, only about 20% of cells with compact GFP-GR_{149} or PR_{175}-GFP inclusions showed enhanced cytoplasmic RFP-NLS_{TDP} localization (Fig. 1A, third and fourth row). While GA_{149}-GFP and GFP-GR_{149} were expressed at a similar level, the weaker effect of poly-PR may be due to the lower expression of PR_{175}-GFP (Supplementary Material, Fig. S1A). Using automated image analysis with the Columbus Acapella system, we confirmed mislocalization of the RFP-NLS_{TDP} reporter in poly-GA expressing cells by comparing the mean cytoplasmic RFP fluorescence in double transfected cells (Fig. 1C). Moreover, we used differential centrifugation to separate the cytosolic and nuclear fraction of the RFP-NLS_{TDP} reporter in DPR expressing cells. As expected, the majority of RFP-NLS_{TDP} was in the histone 3 positive nuclear fraction. However, the cytosolic fraction of RFP-NLS_{TDP} was increased in the GA_{149}-GFP cotransfected HeLa cells (Fig. 2A and B). Immunostaining revealed no cytoplasmic mislocalization of endogenous TDP-43 in GFP-DPR expressing HeLa cells 2 days after transfection (data not shown).

While importin-α/β recognises the NLS of TDP-43, transportin (TNPO) binds to so-called PY-NLS motifs to initiate the nuclear import of other proteins, such as hnRNPA1 (29,35). To test whether DPR proteins could also inhibit this import pathway, we used a second reporter (RFP-NLS_{PY}) containing the well characterised PY-NLS of hnRNPA1 (Supplementary Material, Fig. S1B and C). The nuclear localization of this reporter was not affected by expression of poly-GA, -GR, or -PR. Thus, DPR proteins mainly affect the nuclear import through the classical importin α/β pathway. In particular, poly-GA inclusions inhibit nuclear import of a TDP-43 NLS-containing reporter protein more severely than poly-GR.

Poly-GA inhibits nuclear import of p65

To analyse the effect of poly-GA on the importin α/β-mediated nuclear import on an endogenous protein, we investigated the nuclear translocation of the transcription factor p65 (also known as RelA or NFκB3) in response to TNFα stimulation in HeLa cells. In ∼90% of the unstimulated cells expressing GFP or GA_{149}-GFP, p65 is restricted to the cytoplasm (Fig. 3A first and third row, quantification in Fig. 3B). Upon TNFα stimulation (4 ng/ml, 30 min), p65 translocated into the nucleus of ∼80% of the cells expressing GFP (Fig. 3A, second row). In contrast, only

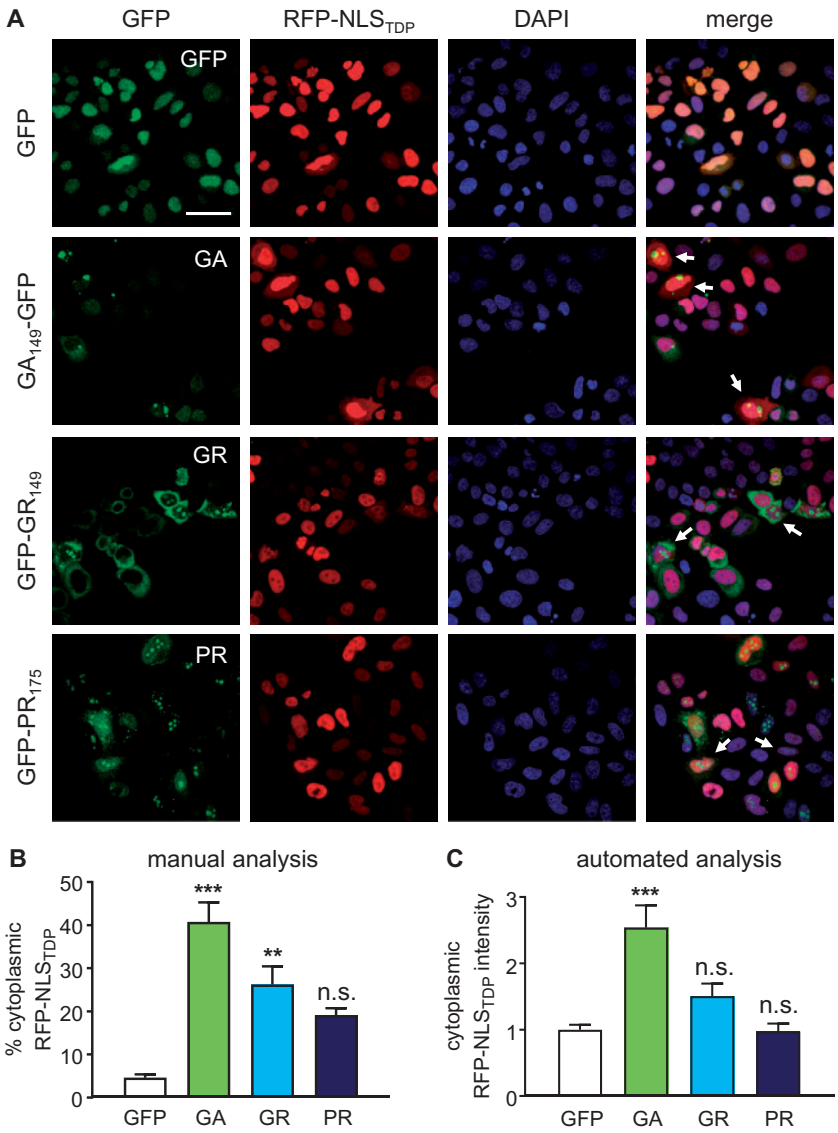

Figure 1. poly-GA reduces the activity of the TDP-43 nuclear localization signal. HeLa cells were cotransfected with RFP fused to the nuclear localization signal of TDP-43 and GFP or GFP-tagged DPR expression vectors. **(A)** Images show RFP and GFP fluorescence of cells stained with DAPI to visualise nuclei. Note that many cells with compact poly-GA inclusions (arrows) show significant levels of RFP-NLS$_{TDP}$ in the cytoplasm. **(B)** Manual quantification of the percentage of cells showing cytoplasmic mislocalization of the RFP-NLS$_{TDP}$ reporter in cells co-expressing GFP or DPR-GFP inclusions. $n = 4$–5 biological replicates, each containing 38–344 double positive cells from 3 to 6 tile scans each. Mean ± SEM. One way ANOVA with Dunnett's post-test, *** denotes $P<0.001$, * denotes $P<0.05$). Scale bar denotes 50 μm. **(C)** Automatic image analysis of reporter localization in HeLa cells coexpressing GFP or GFP-tagged DPR. The mean level of cytoplasmic of RFP-NLS$_{TDP}$ is shown. $n = 7$–8 tiles scans containing 994–2009 cells per group from 5 independent experiments. Mean ± SEM. One-way ANOVA with Dunnett's post-test, *** denotes $P<0.001$.

46% of the cells with GA$_{149}$-GFP inclusions show nuclear translocation of p65 (Fig. 3A, fourth row). Subcellular fractionation corroborates impaired nuclear import of p65 upon TNFα stimulation in poly-GA expressing cells on a biochemical level (Supplementary Material, Fig. S2). Thus, poly-GA aggregation in HeLa cells can directly inhibit nuclear import of p65 and possibly other signalling factors which may affect the function and survival of inclusion bearing neurons.

Poly-GA enhances TDP-43 localization into cytoplasmic granules in neurons

Next, we analysed how DPR aggregates affect the nuclear import in hippocampal neurons. Normally, TDP-43 is predominantly nuclear, but a small fraction of TDP-43 is found in cytoplasmic RNA transport granules (36). We expressed GFP-tagged DPR proteins using lentivirus and analysed the localization of endogenous TDP-43 in hippocampal neurons. Consistent with previous reports, none of the DPR proteins led to dramatic mislocalization or even aggregation of TDP-43 in the cytoplasm. However, poly-GA expression consistently enhanced cytoplasmic TDP-43 granules (arrows in Fig. 4A). Such cytoplasmic TDP-43 granules were present in about 80-90% of neurons expressing poly-GA, compared to ~35% in the GFP control (Fig. 4B). While poly-GR had no effect on TDP-43 localization in this assay, poly-PR expression also promoted the formation of cytoplasmic TDP-43 granules found in ~50% of the neurons. This finding was confirmed by automated analysis of the ratio of cytoplasmic to nuclear TDP-43 (Fig. 4C).

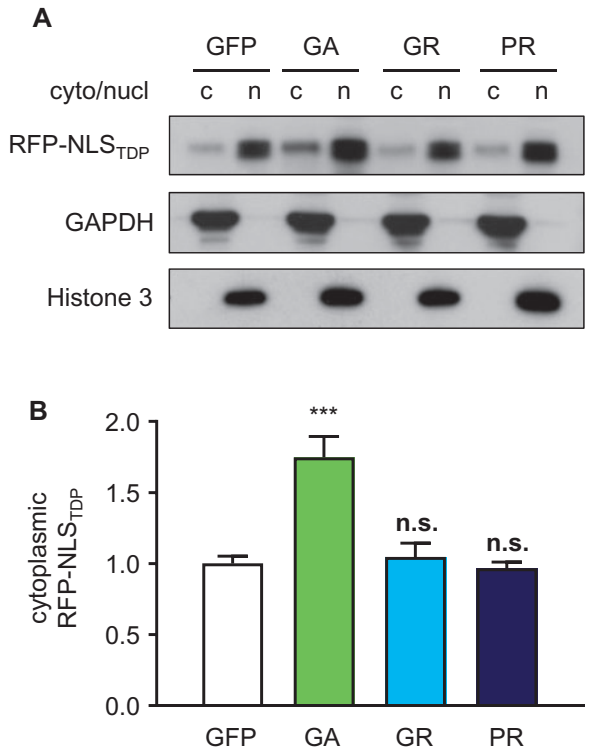

Figure 2. poly-GA induces cytoplasmic mislocalization of the RFP-NLS$_{TDP}$ reporter. HeLa cells cotransfected with RFP-NLS$_{TDP}$ and GFP or GFP-tagged DPR expression vectors were subjected to subcellular fractionation. **(A)** Immunoblot of cytoplasmic and nuclear fractions with the indicated antibodies. GAPDH and histone 3 are used as markers for cytoplasm and nucleus, respectively. **(B)** Quantification of cytoplasmic RFP-NLS$_{TDP}$ from $n = 4$ biological replicates. Mean ± SEM. One way ANOVA with Dunnett's post-test, *** denotes $P<0.001$.

We noticed no significant colocalization of any DPR species with TDP-43. In order to determine the nature of the cytoplasmic TDP-43 granules in poly-GA-expressing neurons, we analysed colocalization with stress granules and lysosomes. Cytoplasmic TDP-43 partially localised in LAMP1-positive lysosomes, but not the stress granule marker TIAR (Supplementary Material, Fig. S3).

Thus, poly-GA inhibits the nuclear localization of TDP-43 in neurons consistent with the results using the RFP-NLS$_{TDP}$ reporter in HeLa cells.

Rerouting of poly-GA to the nucleus prevents cytoplasmic TDP-43 mislocalization

As artificial β-sheet proteins only inhibit nuclear import when they aggregate in the cytosol, but not in the nucleus (31), we asked whether the localization of poly-GA aggregates is critical for their effects on TDP-43 import. Thus, we fused poly-GA with the NLS of the SV40 large T antigen to shift aggregate formation from the cytosol to the nucleus.

Indeed, in neurons about 40% of the GA$_{175}$-NLS inclusions were found in the nucleus, although many cells still bore residual cytoplasmic inclusion indicating incomplete nuclear import of GA$_{175}$-NLS (Fig. 5A). While neurons with cytoplasmic GA$_{175}$-NLS aggregates often contain cytoplasmic TDP-43 granules, cells with pure nuclear GA$_{175}$-NLS aggregation showed lower level of TDP-43 mislocalization comparable to controls (Fig. 5B).

GA$_{149}$-GFP-NLS expressing HeLa cells showed less often cytoplasmic mislocalization of the RFP-NLS$_{TDP}$ reporter than GA$_{149}$-GFP expressing cells (Fig. 5C, compare Fig. 1). While 45% of cells with residual cytoplasmic GA$_{149}$-GFP-NLS aggregates showed cytoplasmic mislocalization of the RFP-NLS$_{TDP}$ reporter, only 17% of cells with GA$_{149}$-GFP-NLS inclusions restricted to the nucleus showed cytoplasmic RFP-NLS$_{TDP}$ fluorescence (Fig. 5D). Thus, only cytoplasmic poly-GA aggregates disturb nuclear import of TDP-43 similar to the findings with artificial β-sheet proteins (31).

Overexpression of importin-α and nuclear pore components restores nucleocytoplasmic transport in poly-GA expressing cells

Since (GGGGCC)$_n$ RNA and poly-GR/PR toxicity has been rescued by Ran and RanGAP1 overexpression in drosophila and yeast (26–28,37), we asked whether it would also restore nuclear import of TDP-43 in poly-GA expressing cells. However, co-transfection of Ran or RanGAP1 did not affect poly-GA induced cytoplasmic mislocalization of the RFP-NLS$_{TDP}$ reporter (Supplementary Material, Fig. S4) despite robust expression of Ran and RanGAP1 in HeLa cells (Supplementary Material, Fig. S5A). In contrast to a previous report (30), we also found no specific colocalization of poly-GA aggregates with endogenous Ran or RanGAP1 (Supplementary Material, Fig. S5B).

We therefore tested importin-α (isoforms KPNA3 and KPNA4), the cytoplasmic receptor for the TDP-43 NLS (32), and crucial factors for nuclear import of TDP-43, such as CSE1L/CAS (which helps to recycle importin-α from the nucleus to the cytoplasm after releasing its cargo) and the nuclear pore complex components NUP54 and NUP62 (32). KPNA4 and NUP62 have also been linked to nucleocytoplasmic transport deficits in various *C9orf72* model systems (26–28,37). Co-expression of these proteins in the GFP control cells partially reduced cytoplasmic localization of the RFP-NLS$_{TDP}$ reporter, however, without reaching statistical significance (Fig. 6A and B). In contrast, co-expression of these factors in GA$_{149}$-GFP transfected cells significantly reduced mislocalization of the reporter. Importantly, expression of KPNA3, NUP54 and NUP62 fully restored nuclear localization of the RFP-NLS$_{TDP}$ reporter to control levels. Thus, our data suggest that poly-GA interferes with the nuclear transport machinery without affecting the Ran-GTP gradient.

Discussion

The expanded *C9orf72* repeat RNA and arginine-rich DPR proteins poly-GR/PR have been previously linked to impaired nucleocytoplasmic transport, but surprisingly poly-GA, the most abundant DPR species, has not been analysed (26–28,37). Here we show that cytoplasmic aggregates of poly-GA inhibit nuclear import of TDP-43 to an even greater extent than poly-GR/PR. This defect is rescued by overexpression of several nuclear transport components. Our data point to a specific inhibition of the import-α/β pathway by cytoplasmic poly-GA aggregates, because rerouting poly-GA aggregates to the nucleus also restored nuclear import of TDP-43.

Poly-GA inhibits nuclear import of TDP-43

Since (GA)$_{15}$ but not 15-mers of the other DPR species adopt an amyloid conformation (14) and synthetic β-sheet proteins can inhibit nuclear transport (31), we analysed the effect of poly-GA

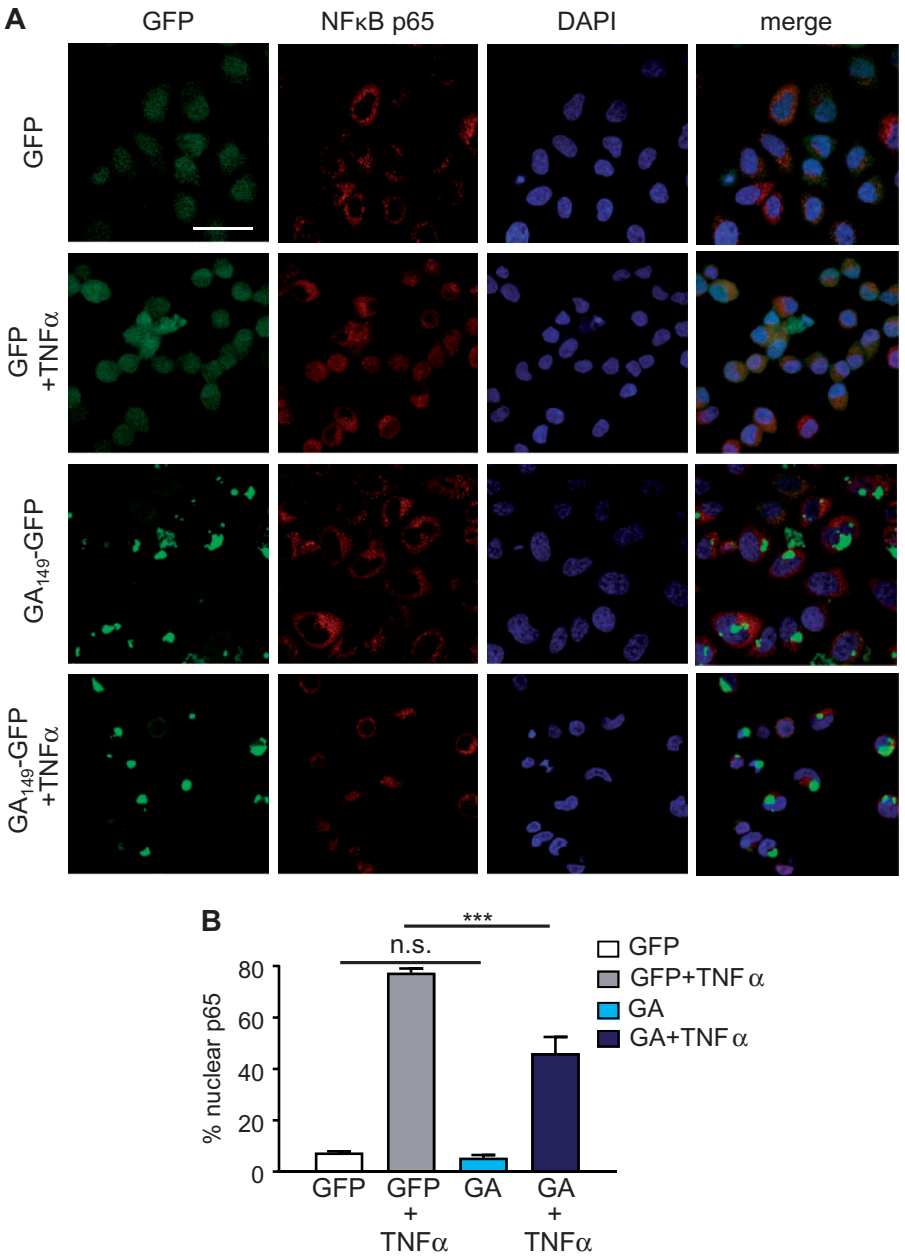

Figure 3. poly-GA inhibits nuclear import of p65. HeLa cells transfected with GFP or GA_{149}-GFP expression vectors were treated with TNFα (4 ng/ml) for 30 min to induce nuclear import of p65/RelA. **(A)** Immunofluorescence images showing p65 and GFP/GA_{149}-GFP. Nuclei were labelled with DAPI. Single confocal planes are shown. **(B)** Quantification shows the fraction of cells with nuclear p65 staining depending on the presence of diffuse or aggregated GFP or GA_{149}-GFP (n=4 experiments, each counting 66–303 double positive cells in 3–6 images, Mean ± SEM. One way ANOVA with Dunnett's post-test, *** denotes $P<0.001$, * denotes $P<0.05$). Scale bar denotes 50 μm.

on nuclear import comparing it to poly-GR and poly-PR. In contrast to the previous studies, we focused on the nuclear import of TDP-43, because the cytoplasmic mislocalization and aggregation of this nuclear RNA binding protein seem to be a crucial trigger of neurodegeneration in ALS/FTD (13,38). Poly-GA had a more dramatic effect on nuclear import of TDP-43 than poly-GR/PR in HeLa cells and in primary neurons (Figs 1 and 3). 40% of HeLa cells containing compact poly-GA inclusion showed reduced nuclear import of an RFP reporter containing the TDP-43 NLS. In primary neurons, even 80% of cells with poly-GA inclusions showed cytoplasmic TDP-43 granules compared to 35% in controls after one week of expression. While one group reported partial colocalization of TDP-43 with cytoplasmic poly-GR and poly-PR aggregates in HEK293 cells (39), no other publication showed direct effects of the repeat RNA or individual DPR species on TDP-43 localization, phosphorylation or aggregation in cellular models (18–21). So far only two *C9orf72* models reproduced significant TDP-43 pathology, however, without elucidating the mechanism. A subset of fast progressing BAC transgenic mice showed TDP-43 aggregates in the areas of neurodegeneration (25). In the AAV-based $(GGGGCC)_{66}$ expressing mouse model TDP-43 aggregates were predominantly in neurons showing co-aggregates of poly-GA and poly-GR at 6 months of age (22). Therefore, the subtle TDP-43 mislocalization seen after

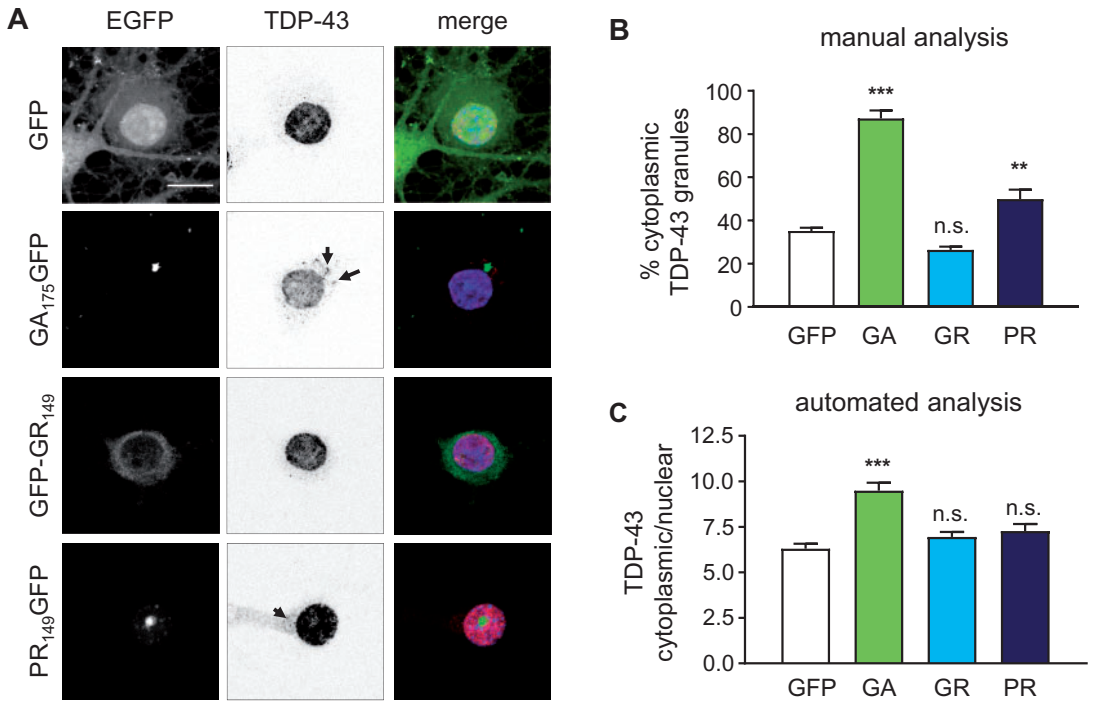

Figure 4. poly-GA inclusions induce partial TDP-43 mislocalization. Primary hippocampal neurons were transduced with GFP or GFP-tagged DPR proteins (DIV6 + 7) and endogenous TDP-43 localization was analysed by immunofluorescence. **(A)** Immunostaining shows enhanced punctate staining in the cytoplasm of neurons expressing cytoplasmic poly-GA inclusions and diffuse cytoplasmic TDP-43 mislocalization in poly-PR expressing cells. The TDP-43 panel is inverted to better visualise cytoplasmic TDP-43 granules (arrows). Scale bar denotes 15 μm. **(B)** Manual quantification of the fraction of neurons containing cytoplasmic TDP-43 granules in cells containing DPR aggregates compared to GFP control. Note that quantification was done from TDP-43 images taken under identical settings. $n = 10$ tile scans per condition with 12–51 cells per image. Mean ± SEM. One way ANOVA with Dunnett's post-test, *** denotes $P<0.001$, ** denotes $P<0.01$. **(C)** Automatic quantification of ratio of cytoplasmic to nuclear TDP-43 in GFP or GFP-DPR transduced neurons. $n = 20$-30 tile scans containing 32–80 neurons from 3 independent experiments. Mean ± SEM. One way ANOVA with Dunnett's post-test, *** denotes $P<0.001$.

1 week in our cellular poly-GA model might be a precursor for further aggregation. In primary neurons, only poly-GA and poly-PR, but not poly-GR affected TDP-43 localization, which is consistent with the weaker toxicity of poly-GR compared to poly-PR (20,27). A potential confound is the weaker expression of poly-PR compared to poly-GA in our system. However, since poly-GA aggregates are over 100-fold more abundant than poly-PR aggregates we favour a crucial role of poly-GA in patients (40).

While none of the DPR proteins affected the localization of a reporter containing a transportin-dependent PY-NLS (Supplementary Material, Fig. S1), poly-GA also inhibited TNFα induced nuclear import of endogenous p65 in HeLa cells suggesting a broad effect on importin α/β dependent nuclear import (Fig. 2 and Supplementary Material, Fig. S2). Impaired NF-κB signalling in neurons may affect neurogenesis and synaptic plasticity (41,42). Thus, impaired nucleocytoplasmic transport due to poly-GA cytoplasmic inclusions may have implications for *C9orf72* ALS/FTD pathogenesis beyond contributing to TDP-43 pathology.

How do DPRs affect nucleocytoplasmic transport?

Several mechanisms for impaired nucleocytoplasmic transport in *C9orf72* models have been proposed. GGGGCC RNA expressed as an exon and poly-GR/PR have been reported to disturb the Ran-GTP gradient presumably through direct binding of RanGAP1 (26). In the repeat RNA expressing flies these effects were rescued by overexpression of Ran, RanGAP1 and importin-α (26) or components of the nuclear pore complex (28). In contrast, intronic expression of the repeat RNA as in patients causes no toxicity in flies (43). In yeast and flies, poly-PR toxicity can be rescued by promoting formation of the nucleocytoplasmic Ran-GTP gradient and overexpression of several importins (27,37), but the primary cause of the effect remains unknown.

We found no evidence for altered Ran localization and coaggregation of RanGAP1 with poly-GA in our cellular models (Supplementary Material, Fig. S5), while others had reported partial colocalization of poly-GA and RanGAP1 in mice (19). In line with these findings, overexpression of Ran or RanGAP1 did not restore the impaired import of the RFP-NLS reporter in our system (Supplementary Material, Fig. S4). However, overexpression of importin-α (KPNA3), the receptor for the classical NLS of both TDP-43 and p65 (32,44), restored nuclear import mediated by the TDP-43 NLS. Moreover, overexpression of two nuclear pore components (NUP54 and NUP62) fully rescued nuclear localization of the reporter. Interestingly, NUP54 and NUP62 were previously shown to be essential for nuclear import of TDP-43 (32) and NUP62 knockdown enhances $(PR)_{25}$ toxicity in flies (37).

In patients, about 10% of poly-GA inclusions are intranuclear and their pathological relevance has been unclear (40). We show that rerouting poly-GA aggregation into the nucleus by adding an NLS prevents the toxic effects on nuclear import suggesting that the factors responsible for the nuclear transport deficit are mainly localised in the cytoplasm. Our data are in line with the findings on synthetic β-sheet proteins (31), although their interactome is vastly different from the poly-GA interactome (18). Cytoplasmic poly-Q aggregates inhibit nuclear

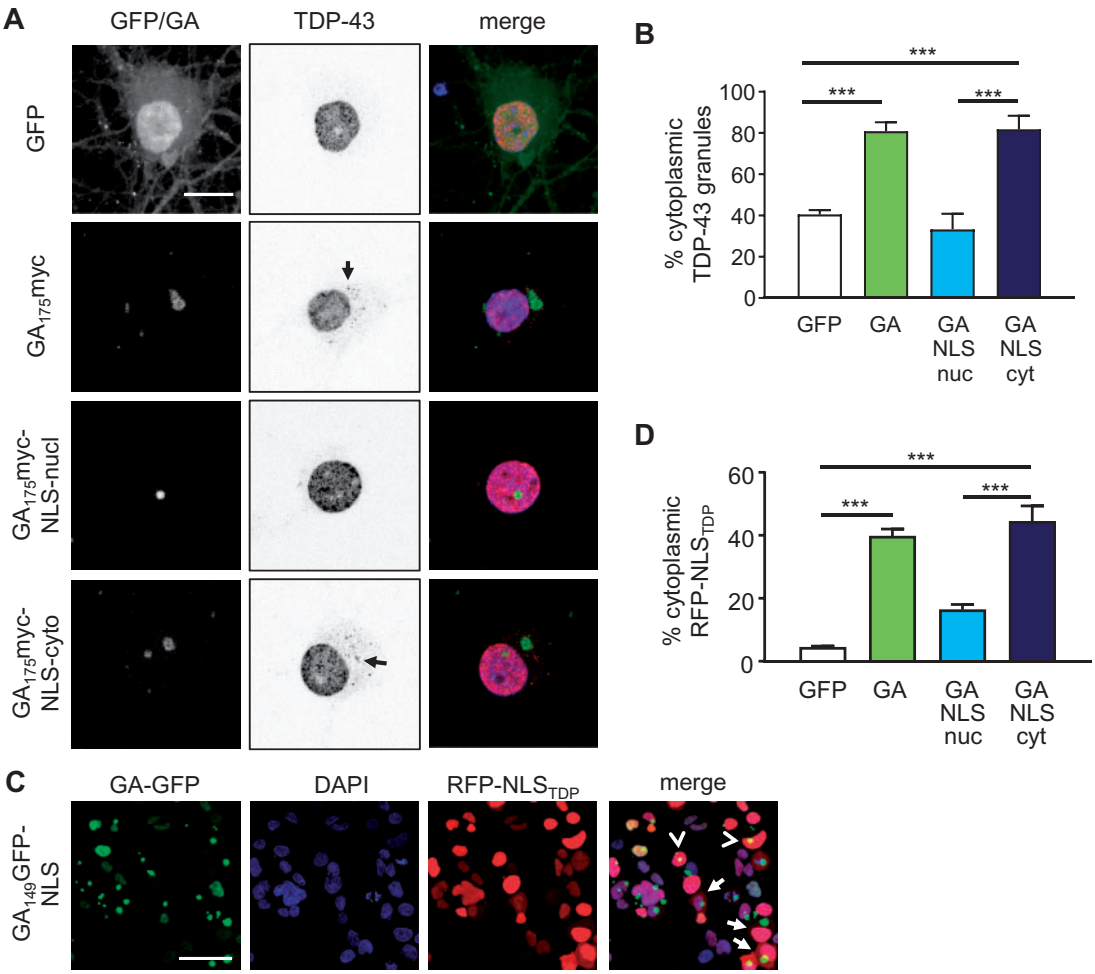

Figure 5. Only cytoplasmic poly-GA inclusions affect TDP-43 localization. **(A,B)** Primary hippocampal neurons were transduced with GFP or GA_{175}-myc constructs (DIV6 + 7) and endogenous TDP-43 localization was analysed by immunofluorescence. The NLS of SV40 large T was used to reroute poly-GA aggregates to the nucleus. **(A)** Immunostaining of representative cells shows partial cytoplasmic TDP-43 mislocalization (arrows). The TDP-43 panel is inverted to better visualise cytoplasmic TDP-43 granules. Scale bar denotes 15 μm. **(B)** Quantification of neurons showing cytoplasmic TDP-43 granules. GA_{175}-NLS transduced cells were separated according to the localization of poly-GA inclusions in the nucleus or cytoplasm ($n = 10$ tile scans per condition with 19–50 cells per image. Mean ± SEM, one way ANOVA with Dunnett's post-test, *** denotes $P<0.001$). **(C,D)** HeLa cells were transfected with RFP-NLS together with GA_{149}-GFP-NLS as in Figure 1. Data was pooled from four experiments together. **(C)** Immunofluorescence showing RFP-NLS mislocalization in cells with cytoplasmic poly-GA aggregates (arrows) and reduced mislocalization in cells with nuclear poly-GA aggregates (arrowheads). Scale bar denotes 50 μm. **(D)** Quantification of RFP-NLS mislocalization from the groups in Figure 4C ($n = 4$ experiments, 27–252 double positive cells per replicate from 2 to 4 images. Mean ± SEM. One way ANOVA with Dunnett's post-test, *** denotes $P<0.001$).

degradation of misfolded cytoplasmic proteins by sequestration of chaperones in yeast (45), suggesting that intranuclear DPR aggregates found in patients might also be targeted for degradation, while cytoplasmic aggregates inhibit nuclear import of TDP-43 and other proteins (31).

Implications for C9orf72 ALS/FTLD

Although gain-of-function mechanisms clearly trigger TDP-43 pathology and neurodegeneration in model systems (4,22,25), neither RNA foci nor any of the DPR species correlates strongly with TDP-43 pathology in patients (13,40). These data argue for synergistic effects of repeat RNA and different DPR species on TDP-43 aggregation possibly involving non-cell-autonomous effects. Two recent studies suggest that poly-GA may be transmitted between cells similar to tau and β-synuclein (14,15,46,47). These non-cell-autonomous effects may explain the slow transition from a prodromal DPR-only stage to the clinical stage of ALS/FTD with additional TDP-43 pathology (2). Our data show for the first time a direct link between poly-GA, the main aggregating protein in *C9orf72* patients, and TDP-43 mislocalization and further highlights the role of nucleocytoplasmic transport in *C9orf72* pathogenesis.

Materials and Methods

Antibodies and reagents

TDP-43 (CAC-TIP-TD-P09, Cosmo bio Tokyo, Japan), myc (9E10, Santa Cruz Biotechnology, Dallas, TX, USA), HA (3F10, Sigma), NF-κB p65 (D14E12, Cell Signaling Technology, Danvers, MA, USA), Ran (ab53775, Abcam, Cambridge, UK), RanGAP1 (ab92360, Abcam), Anti-Histone H3 (ab10799, Abcam), LAMP1 (Ly1C6, Enzo life sciences, Farmingdale, NY, USA), TIAR (BD life sciences, Durham, NC, USA), Tumor Necrosis Factor alpha, human recombinant (rHuTNFα, 50435.50, Biomol, Hamburg, Germany),

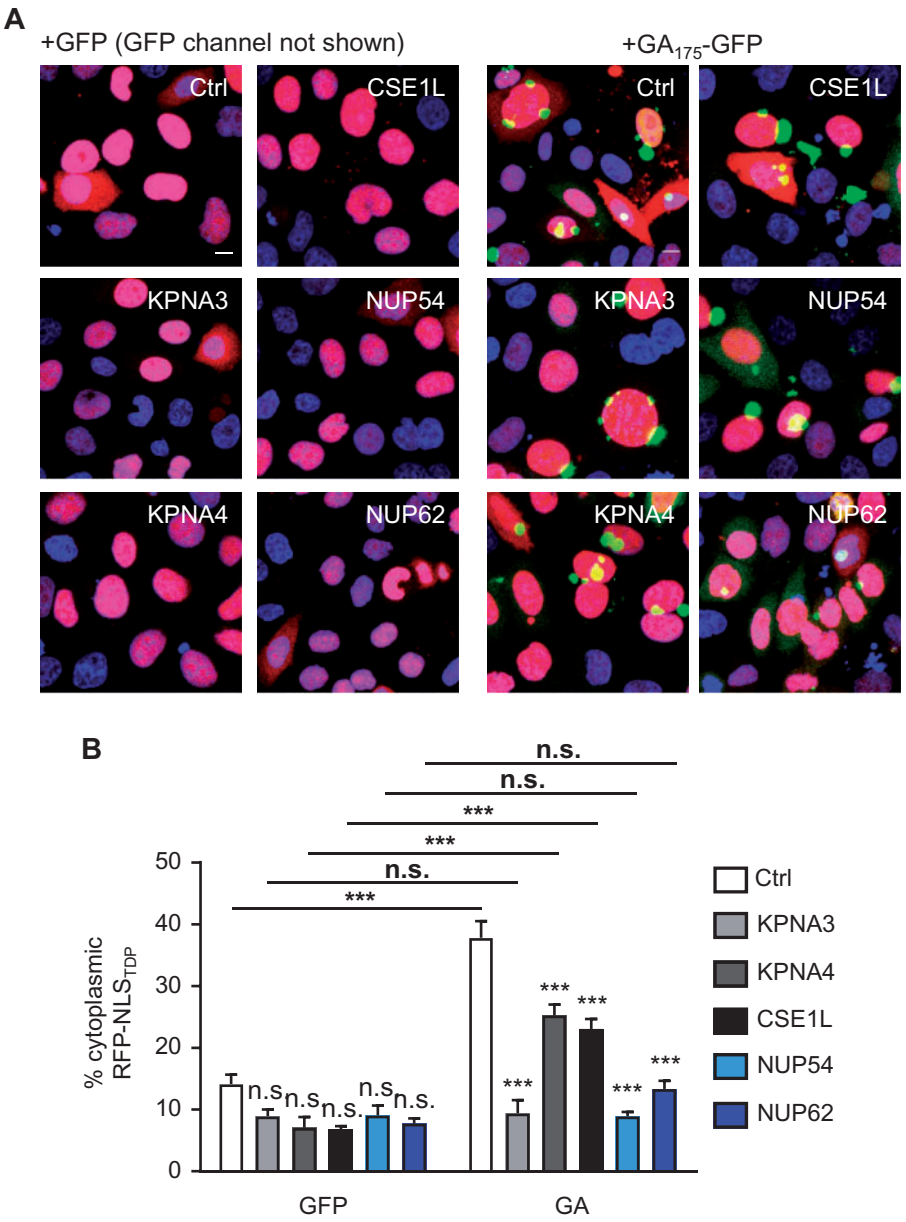

Figure 6. Overexpression of importin-α and nucleoporins restores nuclear import in poly-GA expressing HeLa cells. HeLa cells were cotransfected with RFP fused to the nuclear localization signal of TDP-43 together with GFP or GA_{149}-GFP and the indicated expression constructs or an empty vector control. **(A)** Images show RFP and GFP fluorescence of cells stained with DAPI to visualise nuclei. In the control GFP fluorescence is not depicted to allow better view of the cytoplasmic RFP- NLS_{TDP} reporter in the merge. Note that cells overexpressing nucleoporins show RFP-NLS_{TDP} less frequently in the cytoplasm compared to controls. **(B)** Quantification of the fraction of cells showing cytoplasmic mislocalization of the RFP-NLS_{TDP} reporter for cells co-expressing GFP or GA_{149}-GFP. Co-expression of the indicated proteins had no significant effect in GFP-expressing cells, but significantly reduced cytoplasmic mislocalization in GA_{149}-GFP expressing cells compared to the respective controls ($n = 7$ experiments, counting 13–152 double positive cells from 2 to 3 images, Mean ± SEM. One way ANOVA with Tukey post-test, *** denotes $p < 0.001$). Scale bar denotes 20 μm.

GAPDH (AM4300, ThermoFisher Scientific, Waltham, MA, USA). Anti-GFP (N86/8, UC Davis/NeuroMab Facility, UC Davis, CA, USA), Anti-tRFP antibody (AB233, Evrogen, Russian Federation).

Plasmids, transfection and viral packaging

Synthetic DPR constructs containing an ATG start codon for lentiviral expression (synapsin promoter) and transient transfection (EF1 promoter) were described before (18,40). Additionally, we generated tagRFP and iRFP670 tagged variants to allow multi-channel imaging. The NLS of human TDP-43 (PKDNKRKMDETDASSAVKVKRA, position 78–99) or hnRNP A1 (60 C-terminal amino acids from human) were fused to the tagRFP C-terminus. To reroute poly-GA to the nucleus, the SV40 Large T-antigen (PKKKRKV) was fused to the C-terminus of GA_{175}-myc and GA_{149}-GFP. Rescue experiments were performed using HA-tagged KPNA3, KNPA4, CSE1L, NUP54 and NUP62, Ran and RanGAP1 (cloned from human cDNA) driven by the human Ubiquitin promoter. HeLa cells were transfected with Lipofectamine 2000 (Life Technologies) for immunostaining and immunoblotting. Lentivirus was packaged in HEK293FT cells using the VSV-G/pSPAX2 system as described before (18).

Neuron culture, immunostaining and quantification

Primary hippocampal neurons were cultured from embryonic day 19 rats and infected with lentivirus as described previously (18). Transduction of the primary neurons with specified lentiviruses was performed at day 6 or 7 in vitro (DIV6/DIV7). 7 days after transduction the neurons were fixed with 4% paraformaldehyde, permeabilised (0.2% Triton X-100, 50 mM NH_4Cl in PBS) and blocked for 30 min (2% fetal bovine serum, 2% serum albumin, 0.2% fish gelatin in PBS). After 1h incubation in primary antibody solution (diluted in blocking buffer) at room temperature, coverslips were washed and finally incubated in Alexa-coupled secondary antibody solution. Heat shock was performed for 1h at 44 °C at 5% CO_2.

Fractionation

Subcellular fractionation of p65 was performed as described (48). To analyse the cytoplasmic fraction of RFP-NLS_{TDP} reporter we homogenised HeLa cells in hypotonic buffer (10 mM MOPS, pH 7.0, 10 mM KCl, with protease inhibitors) using a tight fitting homogenizer (30 strokes). The nuclear fraction was pelleted by centrifugation (1,000 g for 15 min at 4 °C). Other membranous compartments were cleared from the supernatant by further centrifugation (100,000g for 1 h at 4 °C) to yield the cytoplasmic fraction.

Microscopy and image analysis

Images were taken at the confocal laser scanning microscope LSM710 from Zeiss with a Plan Apochromat oil immersion objective (40x, NA 1.4). If possible images were taken blind to the experimental condition. Most images were taken as tile scans to avoid bias for the area selection for quantification. For each experiment, data from several images were averaged. The RFP-NLS reporters and TDP-43 were imaged using identical settings for all groups. Images were manually analysed using Metamorph Software and ImageJ (version 1.49g) without intensity or size thresholding. Statistical analysis was done in GraphPad Prism (version 7.01) using one way ANOVA.

Automatic image analysis was executed with Columbus Acapella version 2.4.1 (PerkinElmer). Nucleus candidate objects were detected on the basis of the DNA stain by using "Find Nuclei C" (Area $> 30\,\mu m^2$, Split Factor: 7.0, Individual Threshold 0.4, Contrast > 0.45). Dead and mitotic nuclei were rejected by applying a linear classifier with the "Select Population" function. The classification was based on following nucleus features: Area, Roundness, Haralick Homogeneity, Haralick Correlation. The training set consisted of ~60 manually selected nuclei across all populations. For all selected nuclei, cell region was defined by growing the nucleus region for 6 μm with morphological dilation. We selected GFP and GFP-DPR positive cells by setting a threshold on the mean intensity in the nucleus region. From this selection, RFP positive cells were selected by a second threshold on the basis of the RFP channel. We defined different thresholds for HeLa and neuronal cells. However, thresholds were kept constantly across all sub populations. For HeLa cells, we analysed the mean RFP-NLS_{TDP} intensity in cytoplasm. For neurons, we quantified the mean cytoplasmic and nuclear TDP-43 intensity and determined the cytoplasmic/nuclear TDP-43 ratio for each cell. Averaged results from one image were treated as $n = 1$ for the statistical analysis.

Supplementary Material

Supplementary Material is available at *HMG* online.

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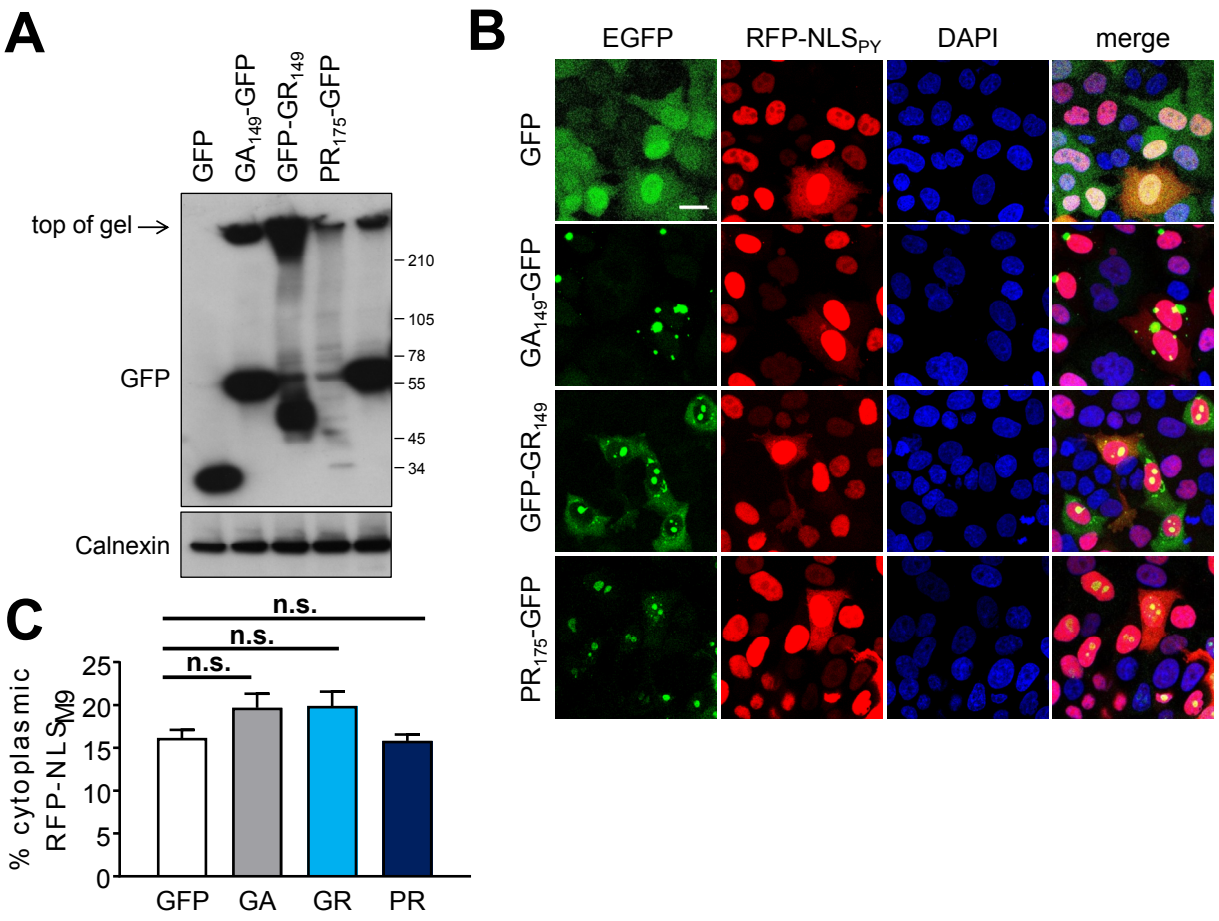

Figure S1: DPR proteins have no effect on the localization of a PY-NLS

(A) HeLa cells transfected with the GFP or GFP-DPR fusion proteins were analyzed by immunoblotting with the indicated antibodies to show comparable expression levels of GA_{175}-GFP and GFP-GR_{149}. By immunoblotting PR_{175}-GFP expression seems weaker, although the fluorescence intensity is similar (same settings for poly-GR and poly-PR in Fig. 1A and S1B). **(B,C)** HeLa cells were cotransfected with RFP-fused to the PY nuclear localization signal of hnRNPA1 and GFP or GFP-tagged DPR expression vectors. **(B)** Images show RFP and GFP fluorescence of cells stained with DAPI to visualize nuclei. Note that DPR expression does not alter localization of RFP-NLS_{PY}. Scale bar denotes 20 µm. **(C)** Quantification of cytoplasmic mislocalization of the RFP-NLS_{PY} reporter for cells co-expressing GFP or DPR-GFP inclusions. n=6 replicates counting 37-192 double positive cells from 2-3 images. Mean ± SEM. One way ANOVA shows no significant differences).

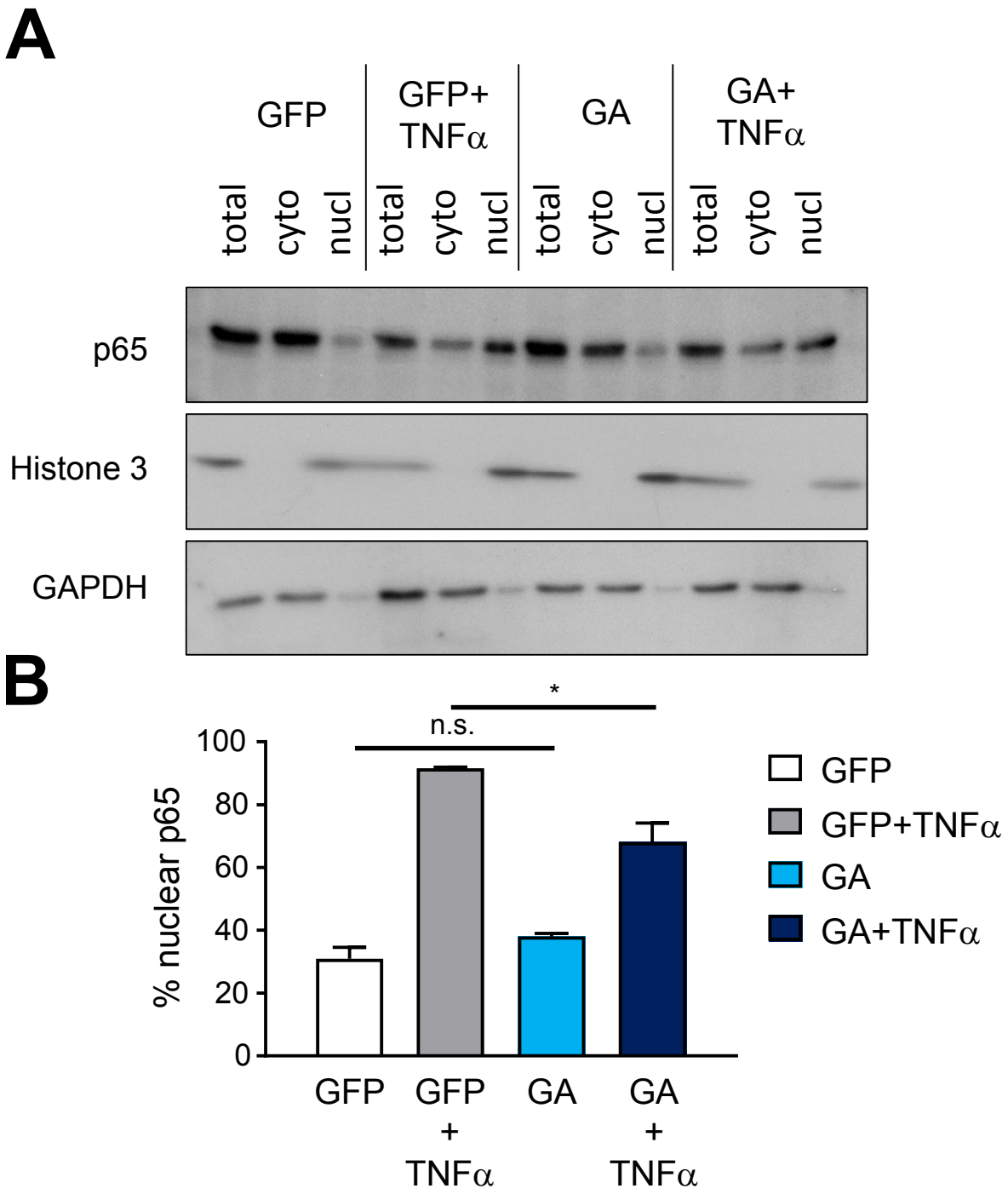

Figure S2: poly-GA inhibits nuclear import of p65

HeLa cells transfected with GFP or GA_{149}-GFP expression vectors were treated with TNFα (4 ng/ml) for 30 min to induce nuclear import of p65/RelA as in Fig. 2. **(A)** Immunoblotting of subcellular fractions with the indicated antibodies. **(B)** Quantification of the nuclear fraction of p65 relative to the total cellular levels. n=3 replicates, mean ± SEM, one way ANOVA with Dunnett's post-test, * denotes $p<0.05$.

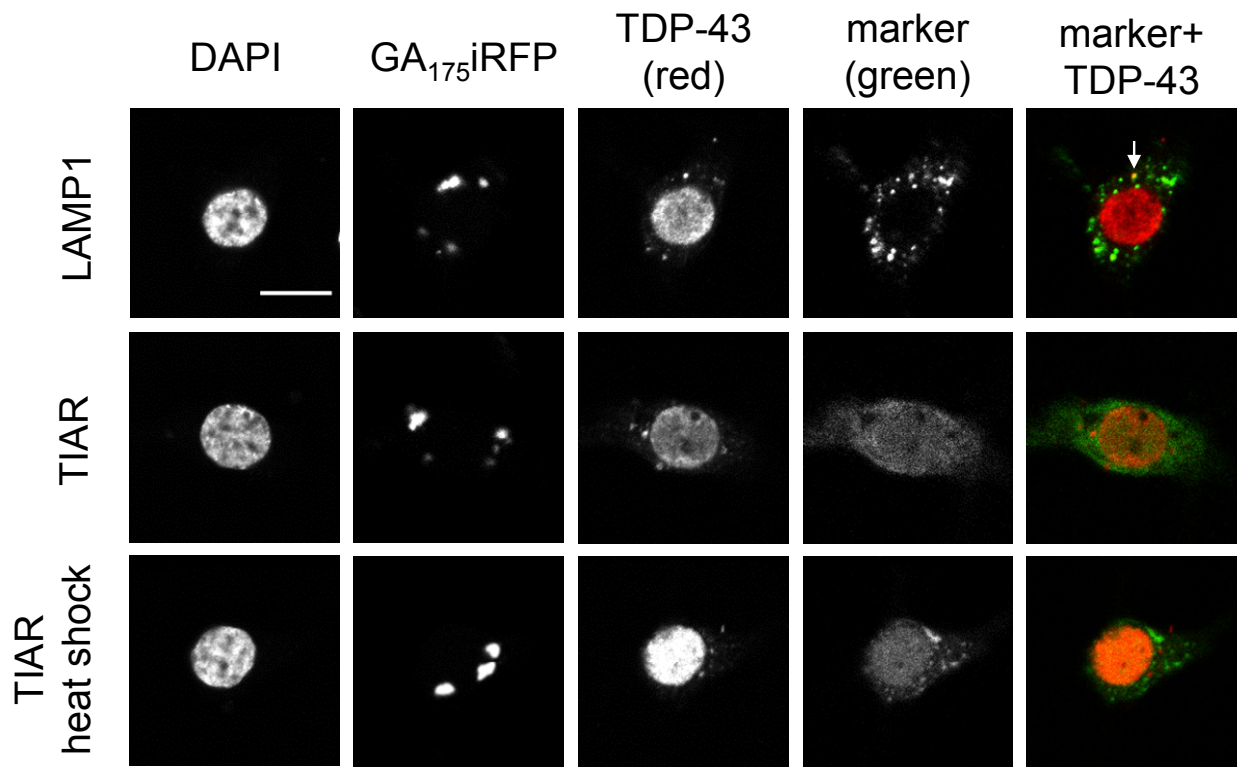

Figure S3: Cytoplasmic TDP-43 granules partially colocalize with LAMP1

Primary neurons were transduced with GA-iRFP expressing lentivirus (DIV7+7). Immunostaining shows partial co-staining of cytoplasmic TDP-43 granules with the lysosomal marker LAMP1 (arrow), but not with the stress granule marker TIAR in poly-GA expressing neurons (with or without 1 h 44ºC heat shock). Single confocal planes are shown. Markers are stained in green, TDP-43 is stained in red. DAPI stains nuclei. Scale bar denotes 15 µm.

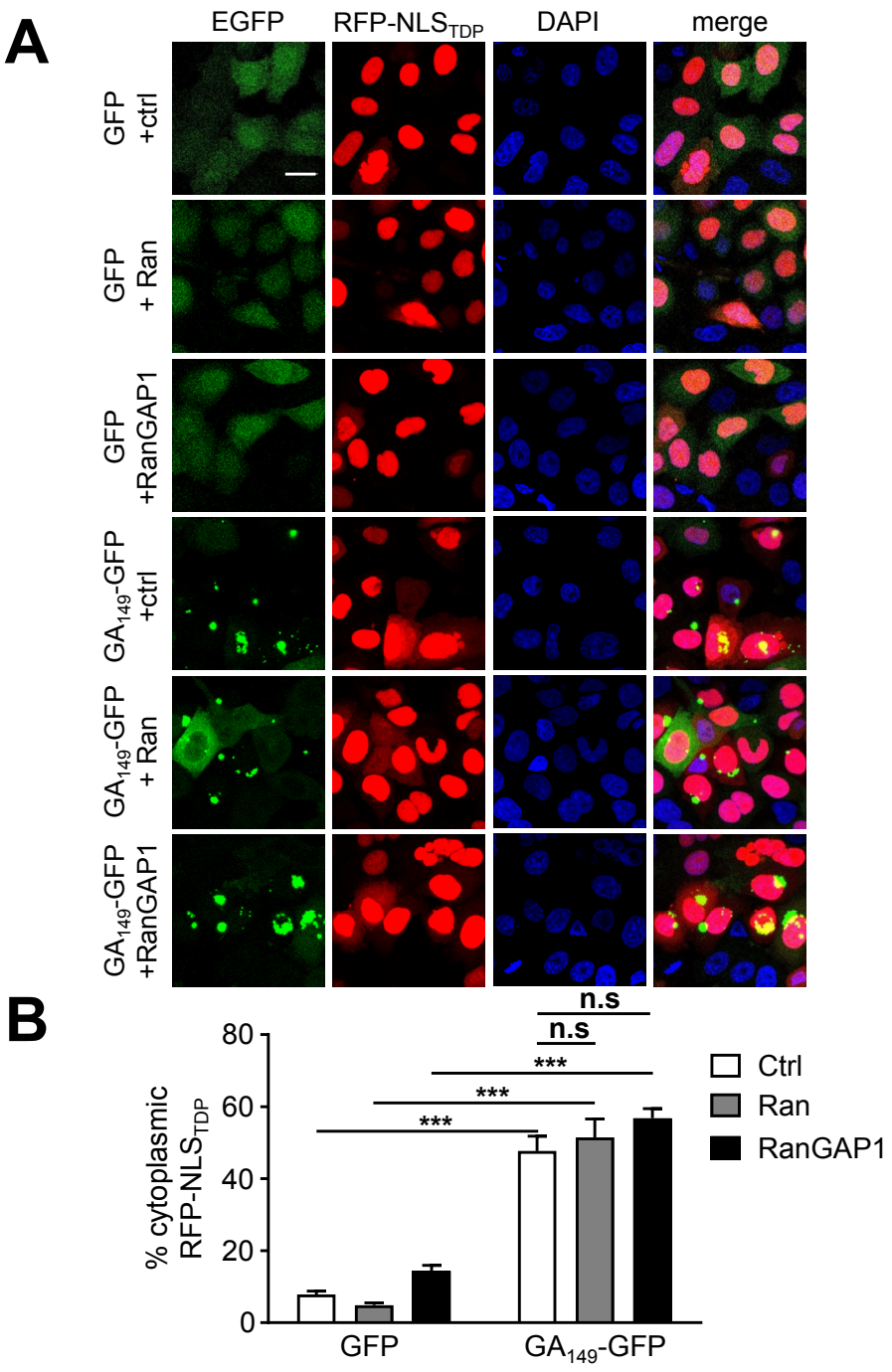

Figure S4: Ran and RanGAP1 have no effect on nuclear import in poly-GA expressing HeLa cells

HeLa cells were cotransfected with RFP fused to the nuclear localization signal of TDP-43, GFP or GA_{149}-GFP and HA-Ran, HA-RanGAP1 or control vector. **(A)** Images show RFP and GFP fluorescence of cells stained with DAPI to visualize nuclei. **(B)** Quantification of cytoplasmic mislocalization of the RFP-NLS_{TDP} reporter in GFP-positive cells. n=4 experiments, counting 27-427 double positive cells from 3-5 images. Mean ± SEM. One way ANOVA with Tukey post-test, *** denotes $p<0.001$, * denotes $p<0.05$). Scale bar denotes 20 µm.

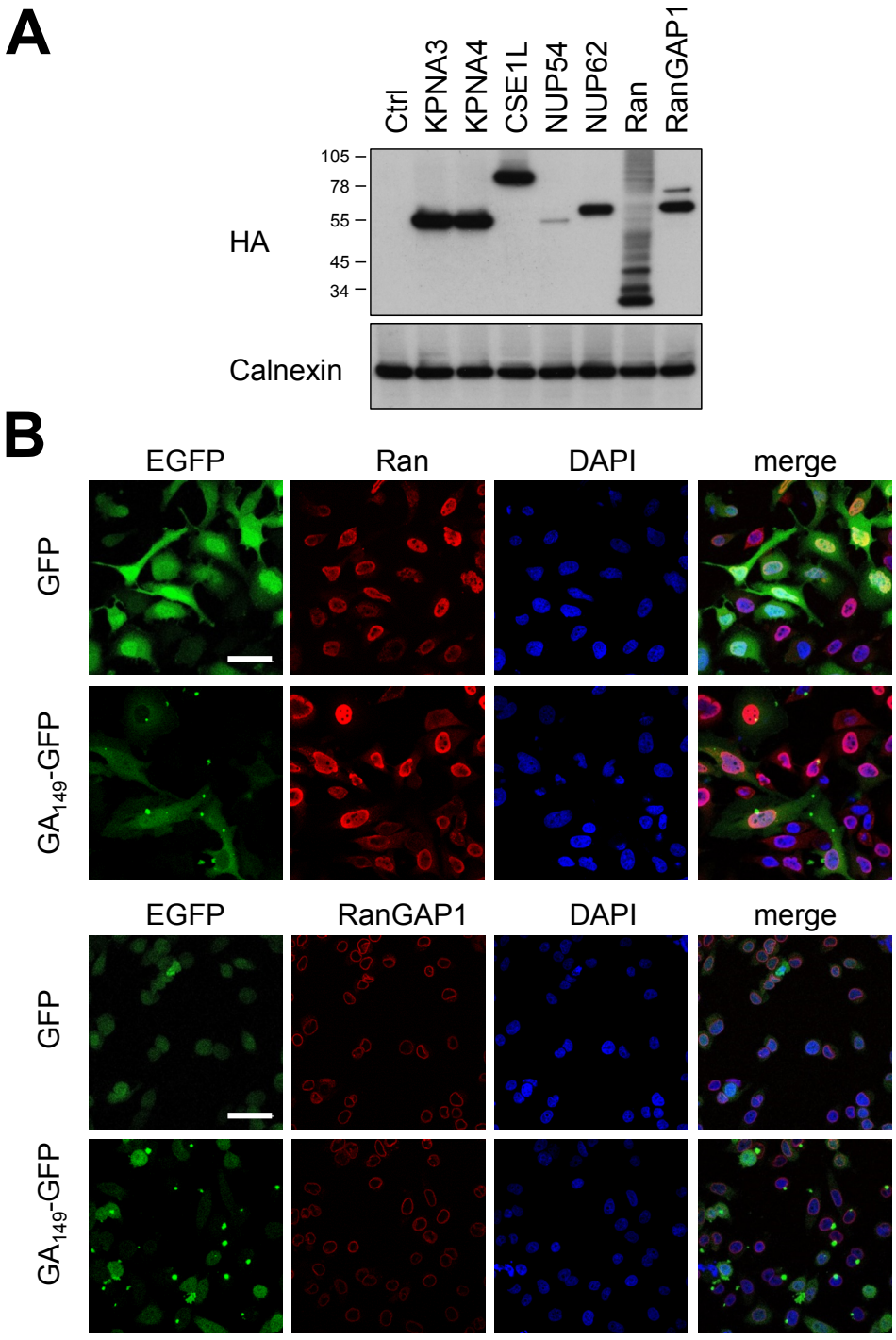

Figure S5: poly-GA has no effect on Ran and RanGAP1 localization

(A) HeLa cells were transfected with HA-tagged KPNA3, KPNA4, CSE1L, NUP54, NUP62, Ran, RanGAP1 or vector control. Immunoblotting shows comparable expression levels of the rescue constructs. **(B)** Immunostaining shows similar expression pattern of endogenous Ran and RanGAP1 in HeLa cells expressing GA_{149}-GFP or GFP control. Images show a single confocal plane. DAPI stains nuclei. Scale bar denotes 50 µm.

5.2. Cell-to-cell transmission of *C9orf72* poly-(Gly-Ala) triggers key features of ALS/FTD

In 5.1. I have shown that poly-GA inhibits nuclear import of TDP-43, but in patients poly-GA and TDP-43 pathology mostly occur in different cells. Identifying the molecular mechanisms might help to develop effective therapeutic approaches.

Cell-to-cell transmission of tau in AD, α-syn in PD, and Htt in HD have been shown through different cellular mechanisms (Costanzo and Zurzolo 2013, Jucker and Walker 2018). Spreading of DPRs between cells has been shown by three groups, including us *in vitro* (Chang, Jeng et al. 2016, Westergard, Jensen et al. 2016, Zhou, Lehmer et al. 2017). Thus, in **Research Article 2**, I tested the hypothesis that non-cell-autonomous effects of DPRs could cause TDP-43 pathology in neighboring cells. To dissect the molecular mechanisms I investigated effects of transmitted poly-GA on ubiquitin proteasome system and nucleocytoplasmic transport.

My data showed poly-GA inclusions triggered cytoplasmic TDP-43 mislocalization non-cell-autonomously. This effect was mitigated by anti-GA antibodies that block poly-GA transmission between cells. In addition, I showed poly-GA can affect TDP-43 pathology by impairing proteasomal function in receiver cells. Boosting proteasomal function using rolipram administration or overexpression of PSMD11 partially rescued the effects of poly-GA on TDP-43.

Overall, these findings suggest a significant role for proteasomal inhibition caused by poly-GA which could eventually trigger TDP-43 pathology in *C9orf72* ALS/FTD and highlights the therapeutic potential of proteasome activation and antibody therapy.

Author contribution: performed most cell biological and biochemical experiments, image acquisition, analyzed most data from the experiments, generated reagents, contributed to designing the study, and writing parts of the manuscript (please see section 11 for further details).

Article

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Cell-to-cell transmission of *C9orf72* poly-(Gly-Ala) triggers key features of ALS/FTD

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Abstract

The *C9orf72* repeat expansion causes amyotrophic lateral sclerosis and frontotemporal dementia, but the poor correlation between *C9orf72*-specific pathology and TDP-43 pathology linked to neurodegeneration hinders targeted therapeutic development. Here, we addressed the role of the aggregating dipeptide repeat proteins resulting from unconventional translation of the repeat in all reading frames. Poly-GA promoted cytoplasmic mislocalization and aggregation of TDP-43 non-cell-autonomously, and anti-GA antibodies ameliorated TDP-43 mislocalization in both donor and receiver cells. Cell-to-cell transmission of poly-GA inhibited proteasome function in neighboring cells. Importantly, proteasome inhibition led to the accumulation of TDP-43 ubiquitinated within the nuclear localization signal (NLS) at lysine 95. Mutagenesis of this ubiquitination site completely blocked poly-GA-dependent mislocalization of TDP-43. Boosting proteasome function with rolipram reduced both poly-GA and TDP-43 aggregation. Our data from cell lines, primary neurons, transgenic mice, and patient tissue suggest that poly-GA promotes TDP-43 aggregation by inhibiting the proteasome cell-autonomously and non-cell-autonomously, which can be prevented by inhibiting poly-GA transmission with antibodies or boosting proteasome activity with rolipram.

Keywords antibody therapy; *C9orf72*; neurodegeneration; nucleocytoplasmic transport; proteasome

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

Neuronal cytoplasmic aggregates of the nuclear RNA-binding protein TDP-43 are the key feature of sporadic amyotrophic lateral sclerosis (ALS) and define a large subgroup of frontotemporal dementia (FTD) neuropathologically (Geser *et al*, 2009; Ling *et al*, 2013; Scotter *et al*, 2015; Prasad *et al*, 2019). TDP-43 undergoes constitutive nucleocytoplasmic shuttling mediated by a bipartite nuclear localization signal (NLS) and diffusion-driven nuclear egress (Winton *et al*, 2008; Ederle *et al*, 2018) and active export (Aksu *et al*, 2018; Archbold *et al*, 2018). Normally, TDP-43 is located predominantly in the nucleus and regulates expression, splicing, and polyadenylation of hundreds of target genes (Polymenidou *et al*, 2011; Tollervey *et al*, 2011). In ALS/FTD, cytoplasmic TDP-43 aggregates are strongly correlated with neurodegeneration and contain ubiquitinated C-terminal fragments (CTFs) that show characteristic hyperphosphorylation (Neumann *et al*, 2006; Geser *et al*, 2009; Igaz *et al*, 2009; Zhang *et al*, 2009). Cytoplasmic TDP-43 aggregation most likely causes neurodegeneration through a combination of direct toxicity and loss of function due to nuclear clearance of TDP-43 in affected cells (Gendron & Petrucelli, 2011; Walker *et al*, 2015; Ederle & Dormann, 2017; Prasad *et al*, 2019). The discovery of genetic mutations that cause familial ALS and/or FTD with TDP-43 pathology similar to sporadic cases has highlighted the role of the ubiquitin–proteasome system (e.g., *UBQLN2*, *VCP*, *SQSTM1*) and the autophagy pathway (e.g., *C9orf72*, *TBK1*, *OPTN*) in pathogenesis (Ling *et al*, 2013; Scotter *et al*, 2015; Gotzl *et al*, 2016; Gao *et al*, 2017). However, apart from rare aggregation-enhancing mutations directly in the TDP-43 encoding gene, the cause of the pathological TDP-43 mislocalization and aggregation in familial and sporadic cases remains elusive (Ederle & Dormann, 2017; Prasad *et al*, 2019).

The most common pathogenic mutation found in about 10% of all ALS/FTD patients is a massive $(GGGGCC)_n$ repeat expansion in

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the first intron of *C9orf72* (DeJesus-Hernandez *et al*, 2011; Renton *et al*, 2011). In addition to the typical TDP-43 inclusion pathology, *C9orf72* cases show nuclear foci of sense and antisense repeat RNA transcripts and unique aggregates of dipeptide repeat (DPR) proteins resulting from unconventional non-ATG translation of the expanded repeat into poly-GA/-GP/-GR/-PA and poly-PR (Edbauer & Haass, 2016). Moreover, C9orf72 protein expression from the mutant allele is reduced (Frick *et al*, 2018; Saberi *et al*, 2018). Multiple downstream effects of these three proposed pathomechanisms have been reported, but none of the *C9orf72*-specific pathologies correlates reproducibly with TDP-43 pathology and neurodegeneration in end-stage tissue (Mackenzie *et al*, 2013, 2015; DeJesus-Hernandez *et al*, 2017). Since all of the mutation-specific effects occur many years or even decades prior to disease onset (Vatsavayai *et al*, 2016), chronic and possibly non-cell-autonomous effects that synergistically trigger disease once compensatory mechanisms fail seem the most likely explanation (Edbauer & Haass, 2016). We and others have reported cell-to-cell transmission of DPRs suggesting they may have non-cell-autonomous effects (Westergard *et al*, 2016; Zhou *et al*, 2017). In *C9orf72* animal models, the most robust TDP-43 pathology has so far been reported upon viral expression of the $(GGGGCC)_n$ repeat at high levels (Chew *et al*, 2015) and to a lesser extent in one of the BAC transgenic *C9orf72* mouse lines (Liu *et al*, 2016) suggesting gain-of-function mechanisms are most important. Modest TDP-43 pathology has been observed in transgenic poly-GA mouse models (Zhang *et al*, 2016; Schludi *et al*, 2017). In cellular systems, poly-GA expression has been linked to subtle TDP-43 mislocalization (Khosravi *et al*, 2017) and phosphorylation (Nonaka *et al*, 2018). Using cryo-electron tomography, we have shown that poly-GA inclusions consist of amyloid-like twisted ribbons that sequester large amounts of proteasomes stalled in an otherwise rare transition state (Guo *et al*, 2018b). Proteasome inhibitors promote TDP-43 pathology *in vitro* (van Eersel *et al*, 2011), but DPR and TDP-43 inclusions rarely occur within the same cell in patients (Mori *et al*, 2013).

Cell-to-cell transmission of cytoplasmic Tau and α-synuclein aggregates drives stereotypic spreading of these pathologies during the progression of Alzheimer's and Parkinson's disease, respectively (Jucker & Walker, 2018). Therefore, we asked whether non-cell-autonomous effects of DPRs could trigger TDP-43 pathology in neighboring cells focusing on effects on the proteasome and nucleo-cytoplasmic transport. Using co-culture assays and antibody treatment to inhibit cell-to-cell transmission, we discovered that poly-GA inhibits the proteasome non-cell-autonomously. Although the proteasome shows high constitutive activity that is largely limited by substrate availability in most cell types, activity can be boosted by rolipram through the cAMP/protein kinase A-depended phosphorylation of PSMD11 (Lokireddy *et al*, 2015). We show that chemical and genetic proteasomal activation rescues poly-GA-induced mislocalization of TDP-43 caused by ubiquitination within the TDP-43 NLS.

Results

Cell-to-cell transmission of poly-GA causes cytoplasmic mislocalization of TDP-43

Among the DPR proteins, poly-GA has been most robustly linked to TDP-43 aggregation *in vitro*, although the mechanism is still unknown (Schludi *et al*, 2015; Khosravi *et al*, 2017; Lee *et al*, 2017; Nonaka *et al*, 2018; Solomon *et al*, 2018). We co-expressed all DPRs with the aggregation-prone CTF of TDP-43 tagged with RFP. CTFs of TDP-43 constitute the major aggregating TDP-43 species in patient tissue and are generated by caspase cleavage (Neumann *et al*, 2006; Igaz *et al*, 2009; Zhang *et al*, 2009). Co-expression of GA_{175}-GFP but not the other GFP-tagged DPR species increased aggregation of TDP-CTF (Appendix Fig S1A–D) without affecting turn-over of TDP-43-CTF (Appendix Fig S1E and F) similar to the previous reports (Nonaka *et al*, 2018). In addition, we quantified mislocalization of endogenous TDP-43 in anterior horn motor neurons of our transgenic mice expressing GA_{149}-CFP (Fig EV1A and B). Consistent with the increased phosphorylation of TDP-43 at the disease-associated residue S409/410 (Schludi *et al*, 2017), we detected enhanced levels of cytoplasmic TDP-43 in the spinal cord of poly-GA transgenic mice without detectable proteolytic cleavage (Fig EV1A–C). In this mouse model, TDP-43 mislocalization is mostly seen in ChAT-positive motor neurons where poly-GA expression is most prominent, while the posterior horn shows no overt changes (Fig EV1D). A fully automated analysis pipeline revealed that poly-GA-positive neurons in the frontal cortex of *C9orf72* FTLD cases show higher frequency of cytoplasmic mislocalization of TDP-43 than neurons without poly-GA aggregates (Fig EV1E and F).

Since TDP-43 and poly-GA only occasionally co-aggregate in patient tissue, we investigated potential non-cell-autonomous effects of poly-GA in a neuronal co-culture system. We transduced primary rat hippocampal neurons growing on coverslips with either GFP or GA_{175}-GFP ("donor cells"). Four days later, we transferred the coverslips with extensively washed donor cells into a new well containing untreated primary neurons ("receiver cells") separated by ~ 1-mm spacers (Fig 1A) and co-cultured donor and receiver cells for another 4 days. Immunofluorescence of the donor cells showed enhanced cytoplasmic localization of TDP-43 in GA_{175}-GFP-transduced compared with GFP-transduced donor cells (Fig 1B and C and Appendix Fig S2A) as we had reported previously (Khosravi *et al*, 2017). Automated quantification of the number of poly-GA aggregates per cell (2.23 ± 0.18 [mean ± SD] in the donor compartment vs 0.70 ± 0.22 in the receiver compartment) showed robust transmission of GA_{175}-GFP aggregates between neurons consistent with previous results (Westergard *et al*, 2016; Zhou *et al*, 2017). Using automated image analysis of single confocal sections, we compared cytoplasmic TDP-43 in GFP-positive and GFP-negative cells (Khosravi *et al*, 2017). Strikingly, cytoplasmic TDP-43 expression was not only enhanced in cells taking up visible GA_{175}-GFP aggregates but also enhanced in neurons without obvious GA_{175}-GFP, both on donor and receiver coverslips (Fig 1B and Appendix Fig S2A red arrows, and Fig 1C). Thus, poly-GA release from transduced neurons leads to TDP-43 mislocalization in neighboring neurons presumably even by uptake of small amounts of soluble or aggregated poly-GA. At the time scale of our experiments, no large TDP-43 aggregates or proteolytic processing was detected (Fig 1B and Appendix Fig S2B). To exclude unspecific effects due to DPR toxicity, we repeated the experiment with expression of arginine-rich DPR proteins poly-GR and poly-PR that show stronger acute toxicity in most model systems (Wen *et al*, 2014). However, expression of poly-GR/PR did not alter TDP-43 localization in either the donor or receiver compartment (Appendix Fig S2C and D).

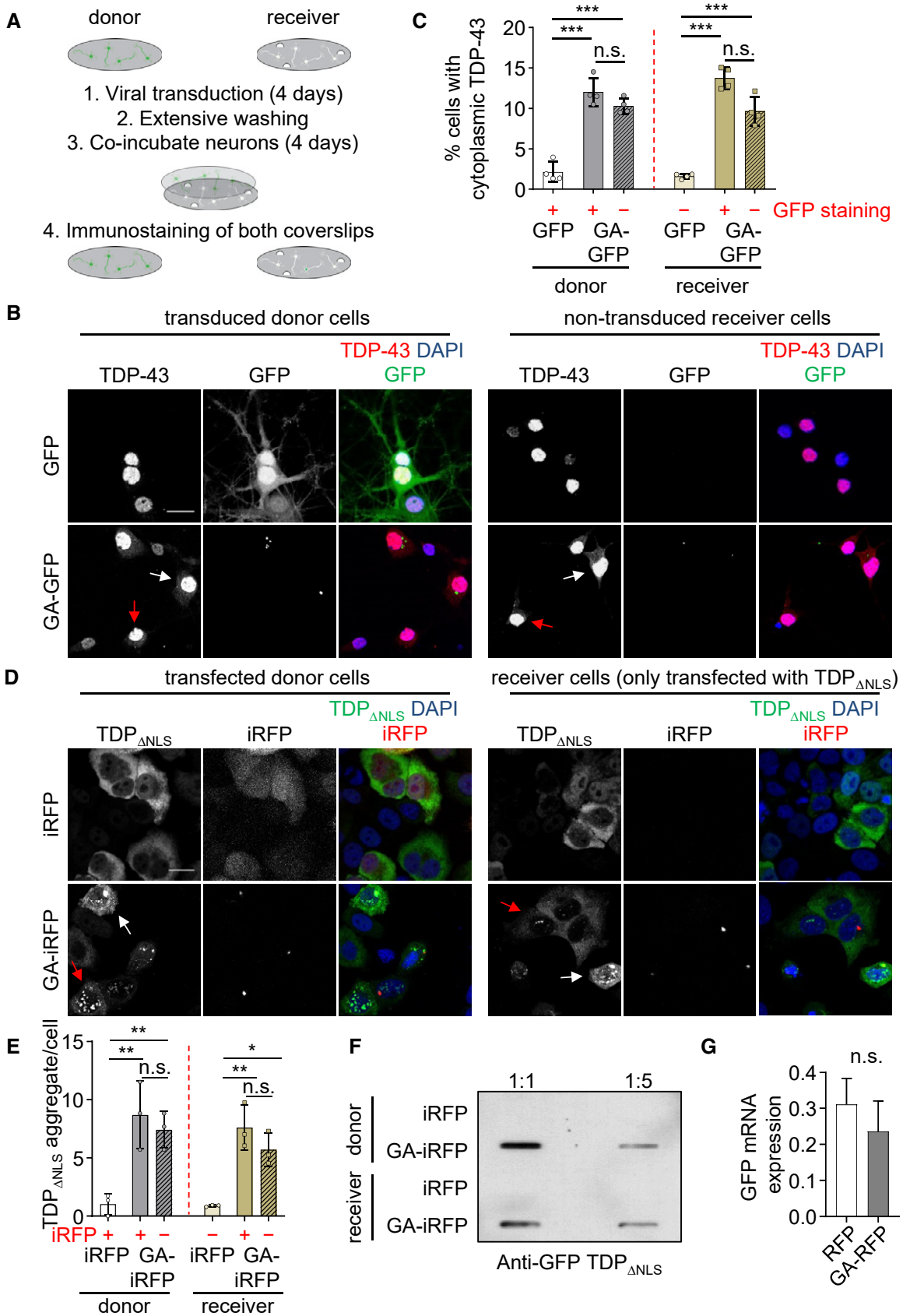
A
donor
receiver
1. Viral transduction (4 days)
2. Extensive washing
3. Co-incubate neurons (4 days)
4. Immunostaining of both coverslips
C
% cells with cytoplasmic TDP-43
***
***
n.s.
***
***
n.s.
GFP staining
GFP
GA-GFP
donor
receiver
B
transduced donor cells
non-transduced receiver cells
TDP-43 DAPI
GFP
TDP-43
GFP
GFP
GA-GFP
D
transfected donor cells
receiver cells (only transfected with TDP ΔNLS)
TDP ΔNLS DAPI
iRFP
TDP ΔNLS
iRFP
iRFP
GA-iRFP
E
TDP ΔNLS aggregate/cell
**
**
n.s.
*
**
n.s.
iRFP
GA-iRFP
donor
receiver
F
1:1
1:5
donor
receiver
iRFP
GA-iRFP
iRFP
GA-iRFP
Anti-GFP TDP ΔNLS
G
n.s.
GFP mRNA expression
RFP
GA-RFP

Figure 1.

Figure 1. Cell-to-cell transmission of poly-GA causes cytoplasmic mislocalization of TDP-43.

A–C Primary hippocampal neurons were transduced with GFP or GA_{175}-GFP (DIV4 + 4) and co-cultured with naïve primary neurons for 4 days. Endogenous TDP-43 and poly-GA aggregates in donor and receiver coverslips were analyzed by immunofluorescence. (A) Schematic representation of co-culture experiments. (B) Cytoplasmic TDP-43 immunostaining is elevated not only in poly-GA-transduced neurons, but also in the non-transduced receiver cells. White and red arrows indicate cells with cytoplasmic TDP-43 in GFP-positive and GFP-negative cells, respectively. (C) Automated quantification of cells with cytoplasmic TDP-43 in GFP- or GA_{175}-GFP-transduced (donor) and non-transduced (receiver) neurons. Cells with and without GFP signal were counted separately (indicated by +/−). Two groups (GFP-negative donor and GFP-positive receiver) were excluded due to very high GFP transduction rate and very low GFP transmission rate. n = 4 biological replicates. In total, 283 donor GFP, 273 donor GA_{175}-GFP, 284 receiver GFP, and 266 receiver GA_{175}-GFP cells were analyzed. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ***$P < 0.001$.

D–G Co-culture model in HeLa cells transfected with iRFP or GA_{175}-iRFP in the donor compartment and TDP-$43_{\Delta NLS}$-GFP in donor and receiver compartments. (D) Immunofluorescence staining and (E) automatic quantification of TDP-$43_{\Delta NLS}$ aggregate number per cell, (F) in addition to filter trap assay of SDS-insoluble TDP-$43_{\Delta NLS}$-GFP aggregates compared in iRFP- or GA_{175}-iRFP-transfected cells. In (E) n = 3 biological replicates with 368 donor iRFP, 251 donor GA_{175}-iRFP, 430 receiver iRFP, and 328 GA_{175}-iRFP cells were analyzed. Cells with and without GFP signal were analyzed separately (indicated by +/−). White and red arrows indicate cells with cytoplasmic TDP-43 in GFP-positive and GFP-negative cells, respectively. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. (G) GFP mRNA expression levels were measured by qPCR. RNA levels were normalized to GAPDH, β-actin, and β2-microglobulin mRNA. Bar graphs of mean ± SD. Unpaired two-tailed t-test with Welch's correction. *$P < 0.05$, and **$P < 0.01$.

Data information: Scale bars denote 20 μm. Additional larger fields of view for Fig 1B and D are shown in Appendix Fig S2A and E.
Source data are available online for this figure.

To differentiate the effect of poly-GA on nucleocytoplasmic transport and aggregation of TDP-43, we analyzed receiver cells expressing GFP-tagged TDP-43 lacking the nuclear localization signal (ΔNLS). Since TDP-$43_{\Delta NLS}$ is highly toxic to primary neurons as shown in mouse models (Walker *et al*, 2015), we conducted these co-culture experiments in HeLa cells. Donor cells were co-transfected with TDP-$43_{\Delta NLS}$-GFP and either iRFP or GA_{175}-iRFP, while receiver cells were transfected only with GFP-tagged TDP-$43_{\Delta NLS}$. Twenty-four hours after separate transfection, the washed coverslips were co-cultured for another 24 h before analysis of poly-GA and TDP-43 fluorescence. Strikingly, both co-expression of poly-GA and co-culture with poly-GA-expressing cells resulted in partial cytoplasmic aggregation of TDP-$43_{\Delta NLS}$-GFP suggesting poly-GA has profound cell-autonomous and non-cell-autonomous effects on TDP-43 solubility even in cells without detectable poly-GA inclusions (Fig 1D and E, Appendix Fig S2E). About 10–20% of poly-GA inclusions also contained TDP-43-ΔNLS. In addition, we confirmed that poly-GA induced GFP-TDP-$43_{\Delta NLS}$ aggregation using a filter trap assay of cell extracts from the donor and receiver cells (Fig 1F). These effects are not caused by enhanced mRNA expression (Fig 1G). Thus, transmission of small amounts of poly-GA may trigger TDP-43 mislocalization in cells without obvious DPR pathology.

Anti-GA antibodies block the non-cell-autonomous effects of poly-GA on TDP-43

Cell-to-cell transmission of aggregating proteins is a potential target for therapeutic antibodies, and we have previously shown that monoclonal antibodies can inhibit transmission of poly-GA (Zhou *et al*, 2017). We asked whether anti-GA antibodies would also inhibit poly-GA-dependent TDP-43 mislocalization. Thus, we added an anti-GA antibody (clone 5F2) or purified mouse IgG as control to the co-culture model from Fig 1A and analyzed TDP-43 localization in the poly-GA-transduced donor compartment and the non-transduced receiver compartment (Fig EV2). Importantly, anti-GA treatment reduced cytoplasmic TDP-43 levels in 5F2 treated neurons in both the donor and receiver compartments (Fig EV2A and B). Immunoblotting confirmed reduction in poly-GA in both compartments (Fig EV2C).

To exclude potential indirect effects due to unknown factors secreted from the donor cells in response to poly-GA expression beyond mere toxicity (compare Appendix Fig S2C and D), we repeated the experiments using anti-GA immunodepletion of conditioned medium from poly-GA expressing neurons (Fig 2). Consistent with the co-culture experiments, supernatant of GA_{175}-GFP-transduced cells induced TDP-43 mislocalization in receiver cells compared with GFP supernatant (Fig 2A and B). Moreover, immunodepletion with anti-GA (clone 5F2) prevented poly-GA uptake in receiver cells and strongly reduced TDP-43 mislocalization compared to depletion with control IgG, suggesting that the effects on TDP-43 are directly mediated by poly-GA released from donor cells into the conditioned media. Poly-GA immunoblotting and immunoassays confirmed successful precipitation and nearly complete clearance of poly-GA (and anti-GA antibodies) from the conditioned supernatant using 5F2 antibody (Fig 2C and D).

Taken together, anti-GA antibodies reduce poly-GA aggregation and transmission as well as cytoplasmic mislocalization of TDP-43. Immunodepletion corroborates the direct effects of transmitted poly-GA on TDP-43 in receiver cells.

Poly-GA inhibits the proteasome cell-autonomously and non-cell-autonomously

To investigate the mechanism of poly-GA on TDP-43 mislocalization and aggregation, we investigated the effects of poly-GA on the proteasome. Cryo-electron tomography has revealed that poly-GA inclusions sequester large amounts of stalled proteasomes (Guo *et al*, 2018b). Here, we confirmed enrichment of the proteasome subunit PSMC4 by immunofluorescence in GA_{149}-CFP transgenic mice and *C9orf72* ALS/FTD patients (Fig 3A and B) as well as in poly-GA-expressing HeLa cells and primary neurons (Fig EV3A and B). Moreover, only expression of poly-GA, but not the other DPR species, promoted accumulation of high-molecular weight ubiquitin species in HEK293 cells (Fig EV3C and D). To address non-cell-autonomous effects, we interrogated proteasome function in donor and receiver cells using the Ub_{G76V}-GFP reporter, which accumulates upon proteasome inhibition (Dantuma *et al*, 2000). Thus, we co-transfected HeLa cells with Ub_{G76V}-GFP and the donor

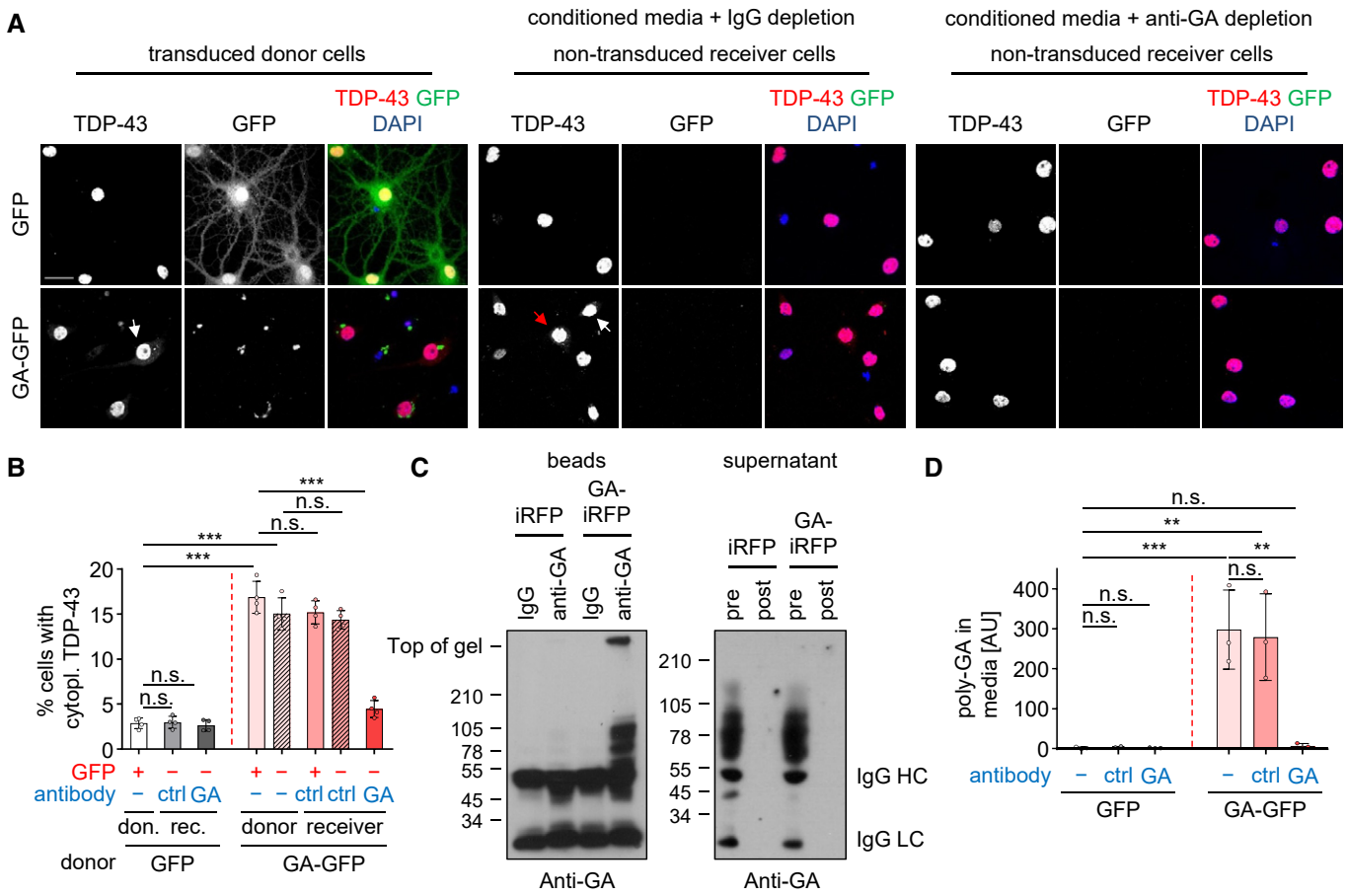

Figure 2. Anti-GA immunodepletion in conditioned media prevents the non-cell-autonomous effects of poly-GA on TDP-43.

Rat primary hippocampal neurons were transduced with GFP or GA_{175}-GFP. Two days after transduction, neurons were washed three times every 2 h with conditioned media and then incubated for another 2 days. Cell supernatant was collected 2 days later and immunodepleted with either control IgG or anti-GA antibody-coupled beads. The immunodepleted supernatants were then collected, equilibrated to 37°C, and finally put on receiver cells for 4 days.

A Confocal imaging showed anti-GA antibody treatment reduces poly-GA aggregates and TDP-43 mislocalization in hippocampal neurons. White and red arrows show cells with cytoplasmic TDP-43 in GFP-positive and GFP-negative cells, respectively. Scale bar denotes 30 μm.

B Automated quantification of cells with cytoplasmic TDP-43 in GFP- or GA_{175}-GFP-transduced (donor) and non-transduced (receiver) neurons. Four groups were excluded due to very high GFP transduction rate (GFP-negative donor) and very low GFP transmission rate (GFP-positive receiver with IgG and anti-GA) and complete prevention of GA-RFP transmission of anti-GA immunodepletion (GA-GFP receiver with anti-GA). n = 3 biological replicates. In total, 280 donor GFP, 284 receiver GFP with IgG, 317 receiver GFP with anti-GA, 277 donor GA_{175}-GFP, 294 receiver GA_{175}-GFP with IgG, and 311 receiver GA_{175}-GFP with anti-GA cells were analyzed. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ***$P < 0.001$.

C Immunoblotting of poly-GA immunoprecipitated from conditioned media using antibody-coupled beads and antibody leftover pre- and post-immunoprecipitation in the conditioned media.

D Poly-GA levels in conditioned media before and after immunodepletion with control IgG and anti-GA antibody were determined by immunoassay. n = 3 biological replicates. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. **$P < 0.01$, and ***$P < 0.001$.

Source data are available online for this figure.

compartment additionally with iRFP or GA_{175}-iRFP. Strikingly, poly-GA expression strongly increased Ub_{G76V}-GFP levels in both the GA_{175}-iRFP-transduced donor cells and the receiver cells as measured by Western blot without affecting mRNA expression of the reporter (Fig 3C–E). Flow cytometry using a HEK293 reporter line stably expressing Ub_{G76V}-GFP confirmed that the mean Ub_{G76V}-GFP fluorescence was significantly increased in cells co-cultured with GA_{175}-RFP-expressing cells, compared to cells co-cultured with RFP alone (Fig EV3E–G). Uptake of GA_{175}-RFP was detectable in over 10% of receiving cells compared to < 1% uptake in receiving cells co-cultured with RFP (Fig EV3E–G), which is consistent with previous reports (Westergard *et al*, 2016; Zhou *et al*, 2017). Differential analysis of GA_{175}-RFP-positive vs GA_{175}-RFP-negative receiver cells showed that Ub_{G76V}-GFP levels were much higher in the cells with clear GA_{175}-RFP uptake than RFP-negative receiver cells (Fig EV3H). Importantly, conditioned media from GA_{175}-RFP-transduced cells also mediated poly-GA transmission and induced Ub_{G76V}-GFP levels in receiver cells, which was completely rescued by immunodepletion of poly-GA with our monoclonal antibody (Fig 3F and G, compare Fig 2).

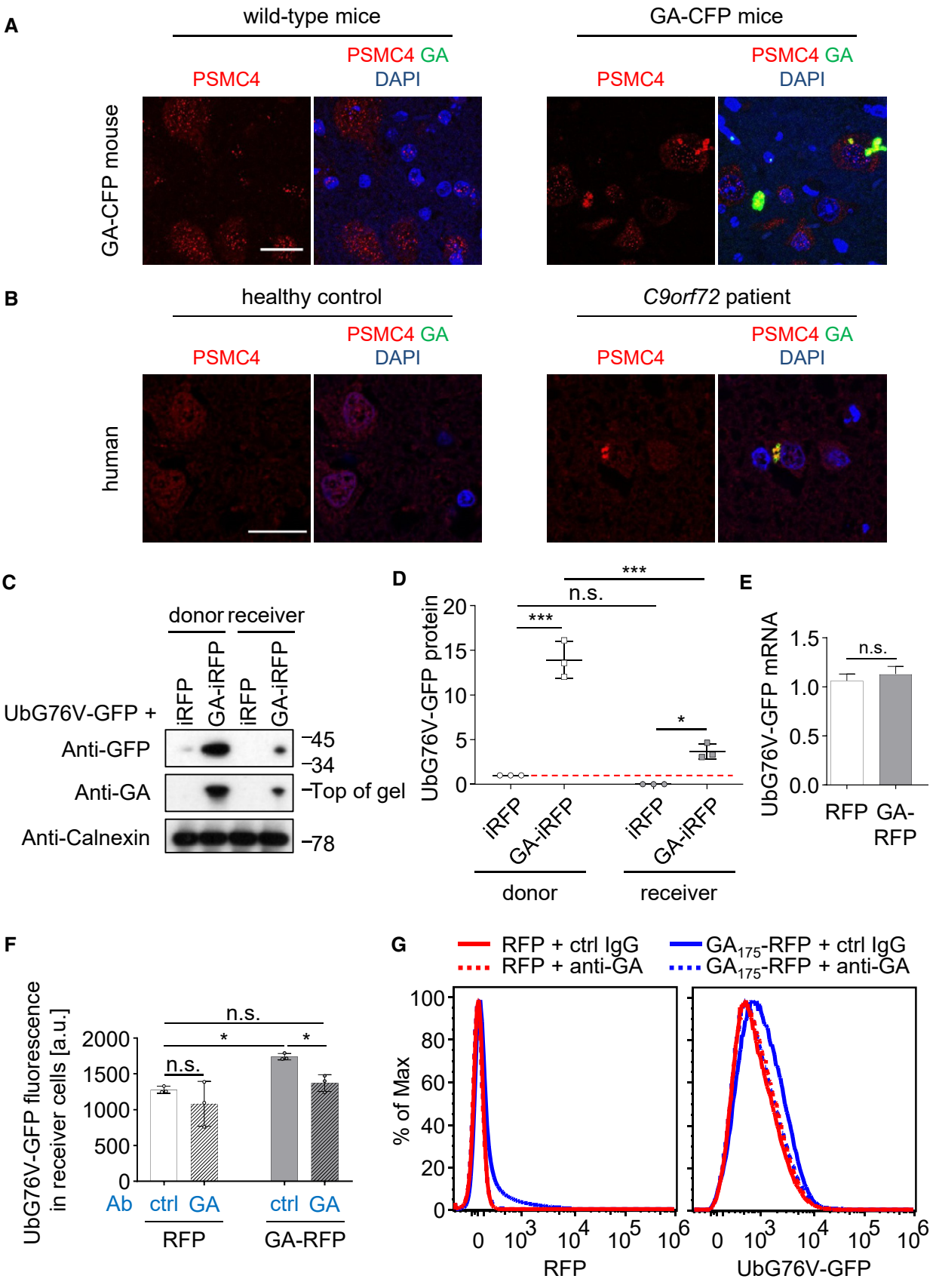

Figure 3.

Figure 3. Poly-GA inhibits the proteasome cell-autonomously and non-cell-autonomously.

A, B Double immunofluorescence of the proteasome subunit PSMC4 and poly-GA inclusions in spinal cord of GA_{149}-CFP transgenic mouse and cortex of a *C9orf72* patient compared with controls. Scale bar denotes 20 μm.

C, D Co-culture model of HeLa cells transfected with iRFP or GA_{175}-iRFP in the donor compartment and an Ub_{G76V}-GFP proteostasis reporter in donor and receiver compartments (48 h). (C) Separate analysis of both compartments by immunoblot and (D) immunoblot quantification. For quantitative analysis of immunoblots, Ub_{G76V}-GFP was normalized to calnexin. $n = 3$ biological replicates. Scatter plot with mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. Red dashed line indicates the control's expression level. $^{*}P < 0.05$, $^{***}P < 0.001$.

E GFP mRNA expression levels were measured by qPCR. RNA levels were normalized to GAPDH, β-actin, and β2-microglobulin mRNA. Bar graphs of mean ± SD. $n = 3$ biological replicates. Unpaired two-tailed *t*-test with Welch's correction.

F, G Flow cytometry analysis of a Ub_{G76V}-GFP reporter cell line incubated 48 h with conditioned media from RFP or GA_{175}-RFP-transfected cells upon immunodepletion of poly-GA or control depletion using unspecific IgG. $n = 3$ biological replicates. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. $^{*}P < 0.05$. (G) Comparisons of the corresponding histograms for compensated RFP and Ub_{G76V}-GFP fluorescence from one representative experiment that shows specific transmission of GA_{175}-RFP associated with accumulation of Ub_{G76V}-GFP in cells incubated with GA_{175}-RFP conditioned media. For flow cytometry analysis of co-culture experiments, see Fig EV3E–H.

Source data are available online for this figure.

Taken together, this suggests that cell-to-cell transmission of small amounts of poly-GA is sufficient to induce significant proteasome inhibition in neighboring cells within a short time frame.

Rolipram rescues poly-GA-dependent TDP-43 mislocalization and aggregation by boosting proteasome activity

To test whether proteasome inhibition triggers TDP-43 mislocalization to the cytoplasm upon poly-GA expression, we transduced rat primary hippocampal neurons with GFP or GA_{175}-GFP and additionally inhibited the proteasome using MG132 or stimulated proteasomal activity using rolipram (Fig 4A). Automated image analysis revealed that MG132 treatment (10 μM, 16 h) significantly increased cytoplasmic TDP-43 levels compared with the vehicle control in both GFP and GA_{175}-GFP expressing neurons. Strikingly, rolipram treatment (30 μM, 16 h) reduced cytoplasmic TDP-43 levels in the GA_{175}-GFP-transduced neurons (Fig 4A and B).

Immunoblotting confirmed that MG132 and rolipram treatment had little effect on the GFP control, but increased or decreased GA_{175}-GFP levels, respectively (Fig 4C). In addition, we used a filter trap assay to quantify the levels of SDS-insoluble poly-GA aggregates upon proteasome manipulation in primary neurons relative to control (Fig 4D and E). While MG132 significantly enhanced GA_{175}-GFP aggregation, rolipram reduced poly-GA aggregates. Immunofluorescence confirmed that MG132 and rolipram also affected aggregate number accordingly (Appendix Fig S3A and B). In HeLa cells, poly-GA expression also increased the levels of high-molecular weight species of co-expressed HA-ubiquitin similar to proteasome inhibition using MG132, which was rescued by rolipram (Appendix Fig S3C and D). Moreover, the Ub_{G76V}-GFP reporter confirmed that overall proteostasis was improved upon rolipram treatment (Appendix Fig S3E and F). To test whether proteasomal activation with rolipram can also rescue poly-GA-induced TDP-43 aggregation, we co-transfected HeLa cells with RFP-TDP-CTF and GFP or GA_{175}-GFP, treated them with MG132 or rolipram, and analyzed protein aggregation by filter trap (Fig 4F and G). Both proteasome inhibition by MG132 and expression of GA_{175}-GFP significantly increased the amount of TDP-CTF inclusions compared with the GFP control (Fig 4G). In contrast, rolipram strongly reduced poly-GA-dependent RFP-TDP-CTF aggregation. Similarly, rolipram also reduced poly-GA-induced aggregation of TDP-43-ΔNLS in a filter trap assay (Fig 4H and I). Thus, proteasome activation prevents the formation or promotes the clearance of poly-GA aggregates and reduces the cytoplasmic mislocalization and aggregation of TDP-43 in cells with residual poly-GA aggregates.

Boosting proteasomal activity prevents poly-GA-induced cytoplasmic accumulation of TDP-43

We speculated that poly-GA-induced proteasomal inhibition might directly contribute to the cytoplasmic mislocalization of TDP-43. To test the effects of poly-GA on TDP-43 localization independent of aggregation, we used RFP fused to the NLS of TDP-43 as a nuclear import reporter in HeLa cells (Khosravi *et al*, 2017). Co-transfection of the RFP-TDP-43-NLS reporter with GA_{175}-GFP increased cytoplasmic reporter levels in inclusion-bearing cells compared with the GFP control as measured by automated quantification (Fig 5A–C). Rolipram (30 μM, 16 h) did not affect localization of the RFP-TDP-43 NLS in GFP-transfected cells, but largely prevented poly-GA-induced cytoplasmic mislocalization of the reporter (Fig 5A and B). This effect was phenocopied by overexpression of PSMD11 (Fig 5D–F), which is known to enhance proteasome assembly and activity and is the direct target of rolipram (Vilchez *et al*, 2012; Lokireddy *et al*, 2015). Similar to primary neurons (Appendix Fig S3A and B), rolipram also reduced the number of GA_{175}-GFP inclusions in HeLa cells consistent with stimulated proteasomal degradation of poly-GA (Fig 5G–I). Neither rolipram treatment nor PSMD11 transfection altered the mRNA levels of the RFP-TDP-43-NLS reporter (Fig 5C, F and I). To test whether the effects of proteasome activation result from improved clearance of cytoplasmic TDP-43 or improved nuclear import, we added rolipram (30 μM) to reporter cells treated with 10 μM ivermectin, an inhibitor of the importin-α/β pathway (Wagstaff *et al*, 2011). Rolipram reduced reporter mislocalization even under these conditions suggesting that it mainly enhances degradation of cytoplasmic TDP-43 (Appendix Fig S4). Together, these findings suggest that proteasome inhibition by poly-GA promotes cytoplasmic mislocalization of TDP-43 by affecting the NLS of TDP-43, which can be prevented by proteasome activation.

Lysine 95 is critical for the inhibition of nuclear import of TDP-43 by poly-GA

Since proteasomal activation rescues nuclear import of the TDP-43 NLS reporter, we speculated that poly-GA expression might inhibit

import via ubiquitination within the TDP-43 NLS. Indeed, several previous proteome-wide mass spectrometry studies had identified ubiquitination sites within the TDP-43 NLS at lysine 84 and lysine 95 (Fig 6A) (Kim *et al*, 2011; Lumpkin *et al*, 2017; Akimov *et al*, 2018). To test the role of both residues in nuclear import of TDP-43, we generated RFP-based reporters containing lysine-to-alanine and

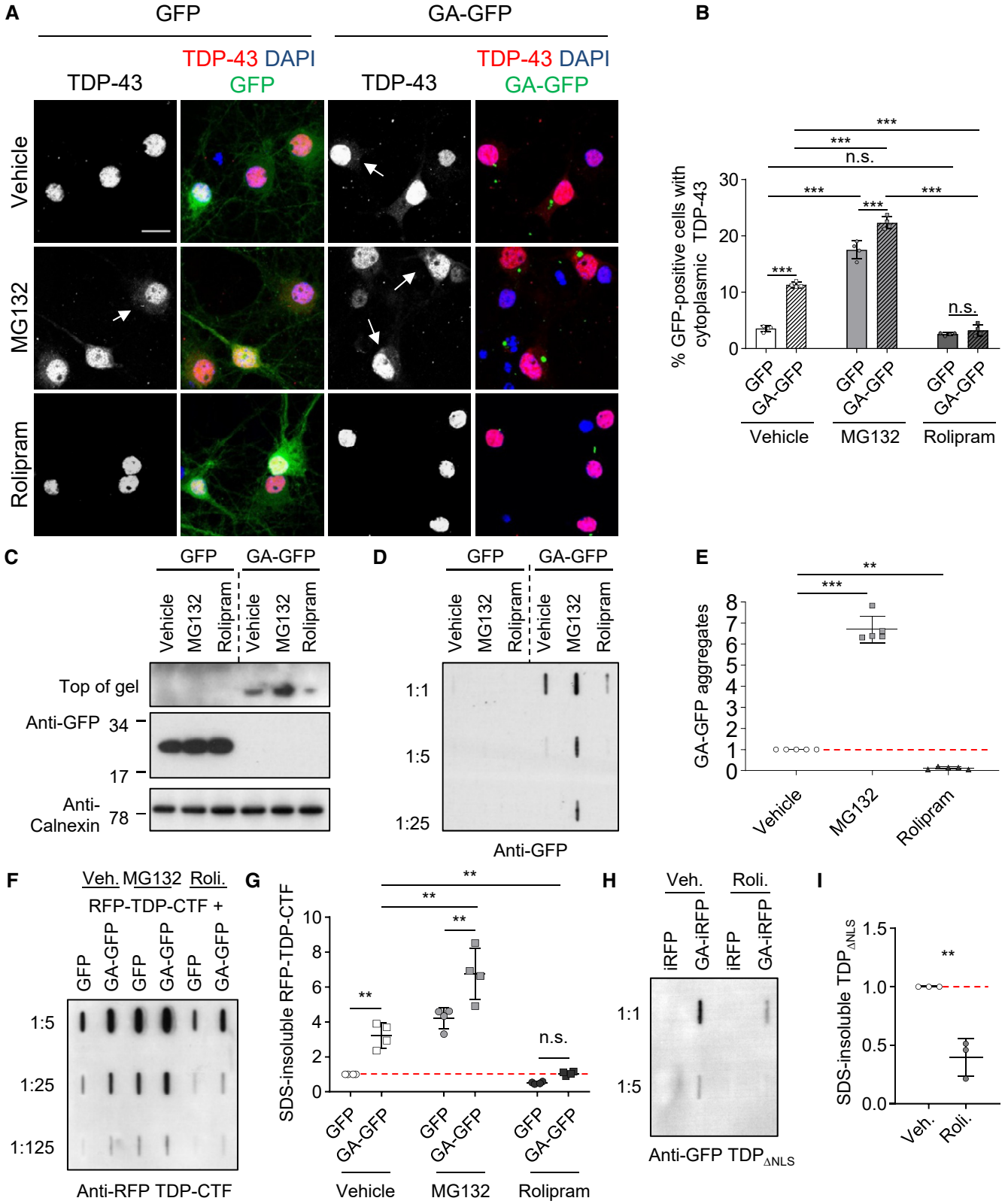

Figure 4.

Figure 4. Rolipram rescues poly-GA-dependent TDP-43 mislocalization and aggregation by boosting proteasome activity.

A–E Primary hippocampal neurons were transduced with GFP or GA_{175}-GFP after 4 days *in vitro*, incubated for 7 days (DIV 4 + 7), and treated with vehicle (DMSO), MG132 (10 μM), or rolipram (30 μM) for 16 h. (A) Immunofluorescence reveals enhanced cytoplasmic TDP-43 levels in neurons with poly-GA aggregates or treated with MG132. Arrows mark punctate TDP-43 staining. Rolipram treatment reduced cytoplasmic TDP-43 in GA_{175}-GFP neurons. Scale bar denotes 20 μm. (B) Automated quantification of cells with cytoplasmic TDP-43 in GFP- or GA_{175}-GFP-transduced neurons. n = 4 biological replicates. In total, 462 GFP and 371 GA_{175}-GFP cells treated with vehicle, and 386 GFP and 529 GA_{175}-GFP cells treated with MG132, and 513 GFP and 434 GA_{175}-GFP cells treated with rolipram were analyzed. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. (C) Immunoblot to show effects of MG132 and rolipram on GA_{175}-GFP and GFP expression. (D and E) Filter trap assay with quantification of SDS-insoluble aggregated GA_{175}-GFP. n = 5 biological replicates. Scatter plot with mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

F, G HeLa cells were co-transfected with RFP-TDP-CTF and GFP or GA_{175}-GFP for 2 days. For the final 16 h, cells were treated with rolipram (30 μM) or MG132 (10 μM). Filter trap assay of SDS-insoluble TDP-CTF aggregates quantified by densitometry. n = 4 biological replicates. Scatter dot plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. See also Appendix Fig S3.

H, I HeLa cells were co-transfected with TDP-$43_{\Delta NLS}$-GFP and iRFP or GA_{175}-iRFP for 2 days. For the final 16 h, cells were treated with either vehicle or rolipram (30 μM). Filter trap assay of SDS-insoluble TDP-$43_{\Delta NLS}$-GFP aggregates quantified by densitometry. n = 3 biological replicates. Scatter dot plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

Data information: $^{**}P < 0.01$, and $^{***}P < 0.001$. Red dashed line indicates the control's expression level.
Source data are available online for this figure.

lysine-to-arginine mutations at these sites to block ubiquitination while either removing or maintaining the positive charge. All constructs were expressed at comparable levels (Fig 6B) and showed similar turn-over in a cycloheximide experiment (Appendix Fig S5A and B). Next, we assessed the nuclear import efficacy of the mutant NLS reporters in HeLa cells co-transfected with GA_{175}-GFP or GFP control. Compared to the wild-type, K84A and K84R mutations largely prevented nuclear import of the RFP reporter even in the absence of poly-GA indicating K84 is crucial for the function of the TDP-43 NLS, which precludes the analysis of poly-GA-specific effects on K84 in this assay (Fig 6C and D). However, both K95A and K95R mutants were imported to the nucleus as efficiently as the wild-type NLS in cells co-transfected with GFP (Fig 6C and D), suggesting a positive charge at this position is not required for NLS activity. In striking contrast to the wild-type NLS, the K95A and K95R reporters remained largely nuclear even in inclusion-bearing cells (Fig 6C and D), indicating that lack of this putative ubiquitination site protects the TDP-43 NLS from the inhibitory effect of poly-GA. MG132 treatment phenocopied the effects of poly-GA on wild-type and mutant reporters, suggesting that proteasome inhibition is a main driver of reporter mislocalization. Importantly, K95 mutations also prevented poly-GA-dependent cytoplasmic mislocalization of full-length TDP-43 without affecting overall TDP-43 clearance (Appendix Fig S5C–F).

The TDP-43 NLS acts via the classical nuclear import receptor importin-α (Winton *et al*, 2008; Nishimura *et al*, 2010). To test how poly-GA interferes with this pathway, we performed co-immunoprecipitation of the GFP-NLS reporter constructs with endogenous importin-α5/KPNA1 in HeLa cells co-expressing iRFP or GA_{175}-iRFP (Fig 6E and F). The wild-type GFP-NLS and the K95A mutant co-immunoprecipitated KPNA1 under control conditions, whereas the K84A mutation severely impaired binding to the import receptor independent of poly-GA consistent with poor nuclear import. Moreover, poly-GA co-expression reduced KPNA1 binding to the wild-type GFP-NLS but not to the K95A construct that was resistant to poly-GA induced mislocalization (compare Fig 6C and D). Similarly, poly-GA reduced binding of full-length TDP-43 to importin-α5/KPNA1, which was blocked by the K95A mutation (Fig EV4). Taken together, poly-GA-induced ubiquitination or other post-translational modifications at K95 are likely inhibiting the nuclear import of TDP-43.

Poly-GA induced poly-ubiquitination of TDP-43 within the NLS at lysine 95

To test whether poly-GA induced ubiquitination within the TDP-43 NLS, we co-transfected HeLa cells with the GFP-NLS_{TDP} reporters, poly-GA and HA-ubiquitin, and analyzed the amount of ubiquitin chains in GFP-NLS_{TDP} immunoprecipitates (Fig 7). HA immunoblotting clearly showed poly-ubiquitination of the wild-type GFP-NLS reporter compared to control immunoprecipitates from cells without HA-ubiquitin expression (Fig 7A). Importantly, poly-ubiquitination of the wild-type NLS reporter increased upon poly-GA expression. In contrast, basal ubiquitination of the K95A reporter was much lower than wild-type and did not increase upon poly-GA co-expression suggesting that K95 (and not K84) is the main ubiquitination site within the TDP-43 NLS. The proteasome inhibitor MG132 induced accumulation of poly-ubiquitinated wild-type but not K95A reporter (Fig 7A and B). In contrast, rolipram reduced basal ubiquitination of the wild-type reporters to the level of the K95A mutant. Finally, introducing the K95A mutation into full-length TDP-43 largely prevented the poly-GA-induced accumulation of ubiquitinated TDP-43 (Fig EV5). Together, these data indicate that poly-GA-mediated proteasome inhibition leads to the cytoplasmic accumulation of TDP-43 NLS ubiquitinated predominantly at K95, and this mislocalized TDP-43 can be effectively cleared by boosting proteasome activity.

Discussion

Dysfunction of the ubiquitin–proteasome system has been reported for many neurodegenerative diseases, but actual sequestration and proteasome stalling has so far been detected only for poly-GA (Guo *et al*, 2018b). Here, we show in a co-culture model that poly-GA inhibits the proteasome and promotes TDP-43 mislocalization and aggregation even in neighboring cells that uptake only small amounts of poly-GA. TDP-43 mislocalization by poly-GA is mediated by ubiquitination at lysine 95 within the NLS, which inhibits binding to importin-α. We show that inhibiting poly-GA transmission with antibodies and chemically activating the proteasome with rolipram ameliorate both poly-GA and TDP-43 pathology and may thus break the pathogenic cascade in *C9orf72* patients.

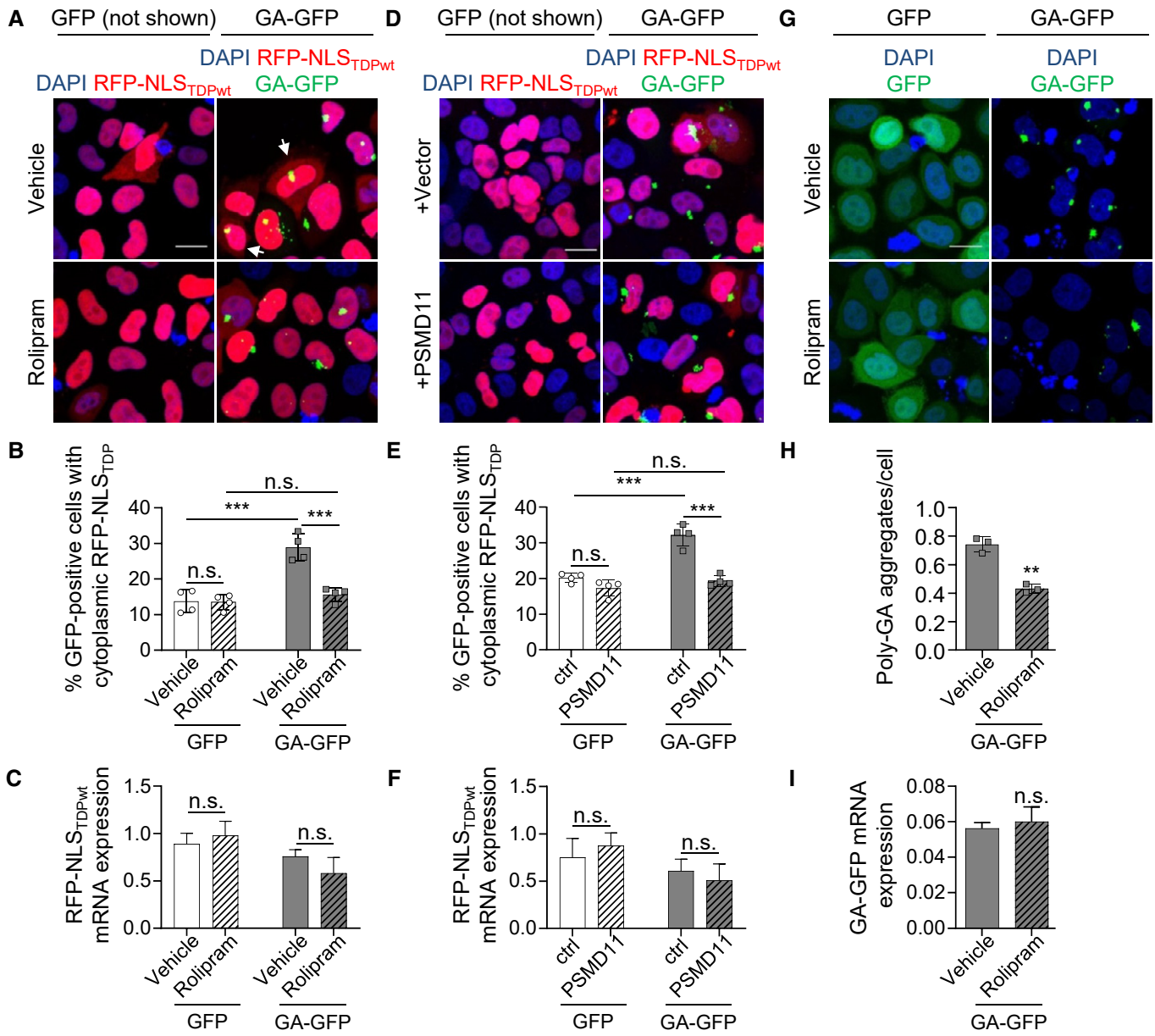

Figure 5. Boosting proteasomal activity prevents poly-GA-induced cytoplasmic accumulation of TDP-43.

A–C HeLa cells were co-transfected with an RFP-based TDP-NLS reporter and GFP or GA_{175}-GFP. Twenty-four hours after transfection, cells were treated with rolipram (30 μM) for 16 h. In the immunofluorescence, GFP is not shown because diffuse GFP expression would hide the cytoplasmic RFP reporter. White arrows indicate cells with cytoplasmic TDP-43. (B) Automated quantification of cells with cytoplasmic TDP-NLS reporter in GFP- and GA_{175}-GFP-positive cells. n = 4 biological replicates. In total, 345 GFP and 386 GA_{175}-GFP cells treated with vehicle, and 371 GFP and 404 GA_{175}-GFP cells treated with rolipram were analyzed. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. (C) RFP-NLS_{TDPwt} mRNA expression levels were measured by qPCR. n = 3 biological replicates. Bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

D–F HeLa cells were co-transfected with the RFP-based TDP-NLS reporter, GFP or GA_{175}-GFP, and PSMD11 or empty vector. Image analysis as in (A). n = 4 biological replicates. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. 354 GFP and 330 GA_{175}-GFP cells with vector, and 367 GFP and 369 GA_{175}-GFP cells with PSMD11 in total were analyzed. (F) RFP-NLS_{TDPwt} mRNA expression levels were measured by qPCR. n = 3 biological replicates. Bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

G–I Immunofluorescence of HeLa cells transfected with GFP or GA_{175}-GFP showing reduced poly-GA aggregation upon rolipram treatment (30 μM, 16 h). (H) Automated quantification of poly-GA aggregate number per cell. n = 3 biological replicates. In total, 223 cells treated with vehicle and 286 cells treated with rolipram were analyzed. Scatter plot with bar graphs of mean ± SD. Unpaired two-tailed t-test with Welch's correction. (I) GA-GFP mRNA expression levels were measured by qPCR. n = 3 biological replicates. Bar graphs of mean ± SD. Unpaired two-tailed t-test with Welch's correction.

Data information: Scale bars in immunofluorescent figures denote 20 μm. $^{**}P < 0.01$, $^{***}P < 0.001$.
Source data are available online for this figure.

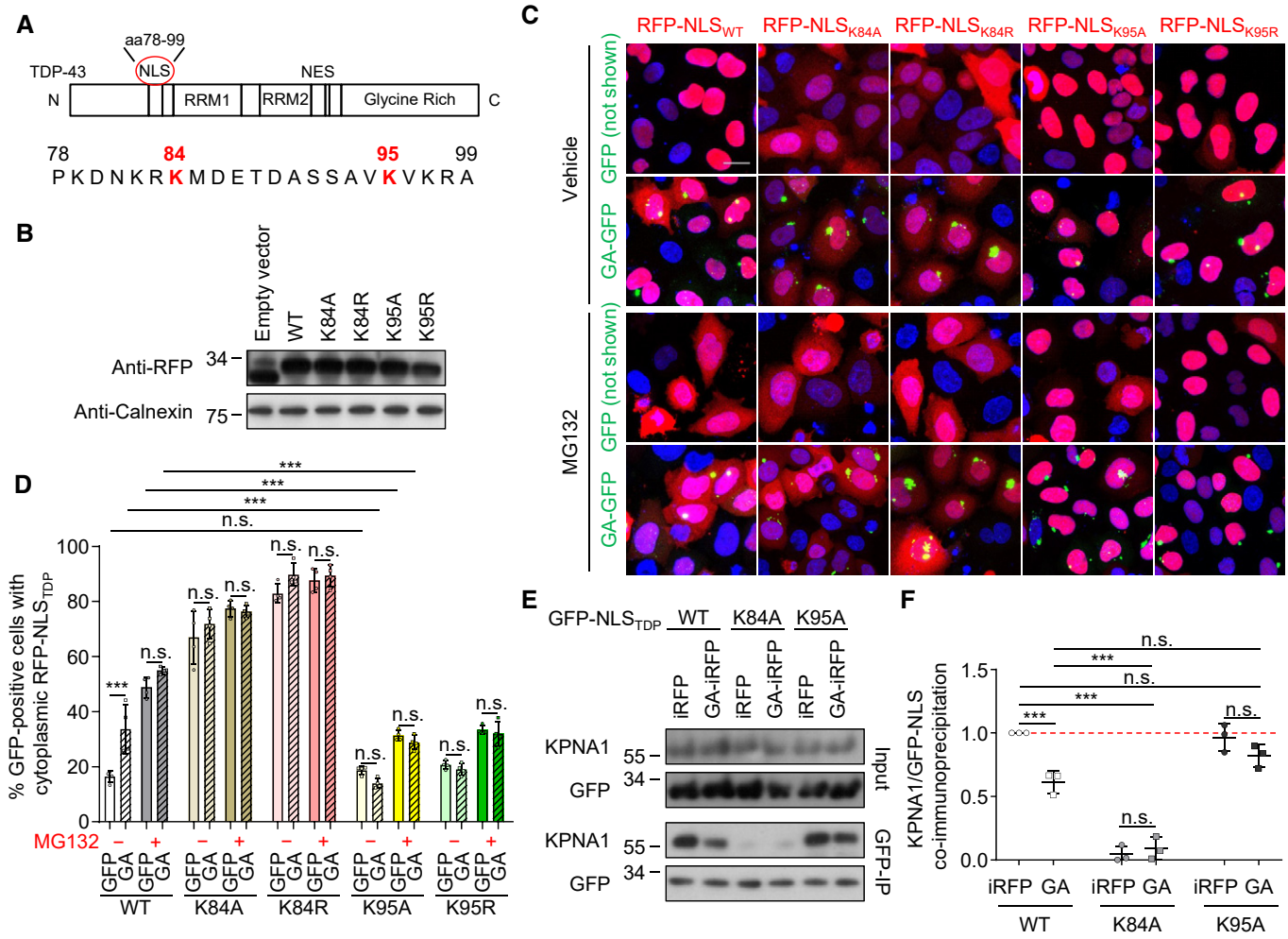

Figure 6. Lysine 95 is critical for the inhibition of nuclear import of TDP-43 by poly-GA.

A Domain structure of TDP-43 and location of the bipartite NLS at positions 78–99 (Winton *et al*, 2008). Known ubiquitination sites listed on www.phosphosite.org at K84 and K95 are highlighted.

B Immunoblot of HeLa cells transfected with RFP-based TDP-NLS wild type (WT) or mutants (K84A, K84R, K95A, K95R).

C, D HeLa cells were co-transfected with the indicated TDP-NLS reporters as well as GFP or GA_{175}-GFP, and treated with MG132 (10 μM) or vehicle for 16 h. (D) Automated quantification of RFP-NLS reporters in GFP-positive cells. Note that K84A and K84R block overall import, while K95A and K95R allow import but are resistant to inhibition by poly-GA. n = 4 biological replicates. The total number of cells analyzed per group was (from left to right) 667, 581, 789, 783, 809, 708, 628, 721, 938, 557, 857, 861, 886, 699,789, 539, 636, 577, 638, and 870. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ***$P < 0.001$. Scale bar denotes 20 μm.

E, F HeLa cells were co-transfected with the indicated GFP-TDP-NLS reporters and iRFP or GA_{175}-iRFP. Cell lysates were immunoprecipitated with anti-GFP and immunoblotted with indicated antibodies to detect co-immunoprecipitation of the TDP-43 NLS with importin-α5/KPNA1 nuclear import receptor. (F) Quantification of KPNA1 levels normalized to total GFP-NLS_{TDP} reporter levels in anti-GFP immunoprecipitates. n = 3 biological replicates. Scatter plot with mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ***$P < 0.001$. See also Fig EV4. Red dashed line indicates the control's expression level.

Source data are available online for this figure.

Proteasome activation reduces poly-GA and TDP-43 aggregate formation

Proteasome inhibition is known to promote TDP-43 aggregation *in vitro* (Igaz *et al*, 2009), but it is unclear whether this mechanism occurs in patients and how it would be triggered only in motoneurons and/or the frontotemporal cortex. Here, we analyzed cell-autonomous and non-cell-autonomous effects of poly-GA on the proteasome. We show that poly-GA aggregates partially sequester the proteasome in *C9orf72* ALS/FTD patients and a GA_{175}-CFP expressing mouse model, which confirms our *in vitro* data (Guo *et al*, 2018b). Moreover, poly-GA expression promotes cytoplasmic mislocalization of endogenous TDP-43 in our mouse model. In primary neurons, MG132 treatment or poly-GA expression acutely triggers cytoplasmic mislocalization of endogenous TDP-43 and an RFP-NLS_{TDP} reporter, suggesting that proteasome impairment is sufficient to inhibit nuclear import of TDP-43. Consistent with previous reports, only poly-GA but not the other DPR species promoted

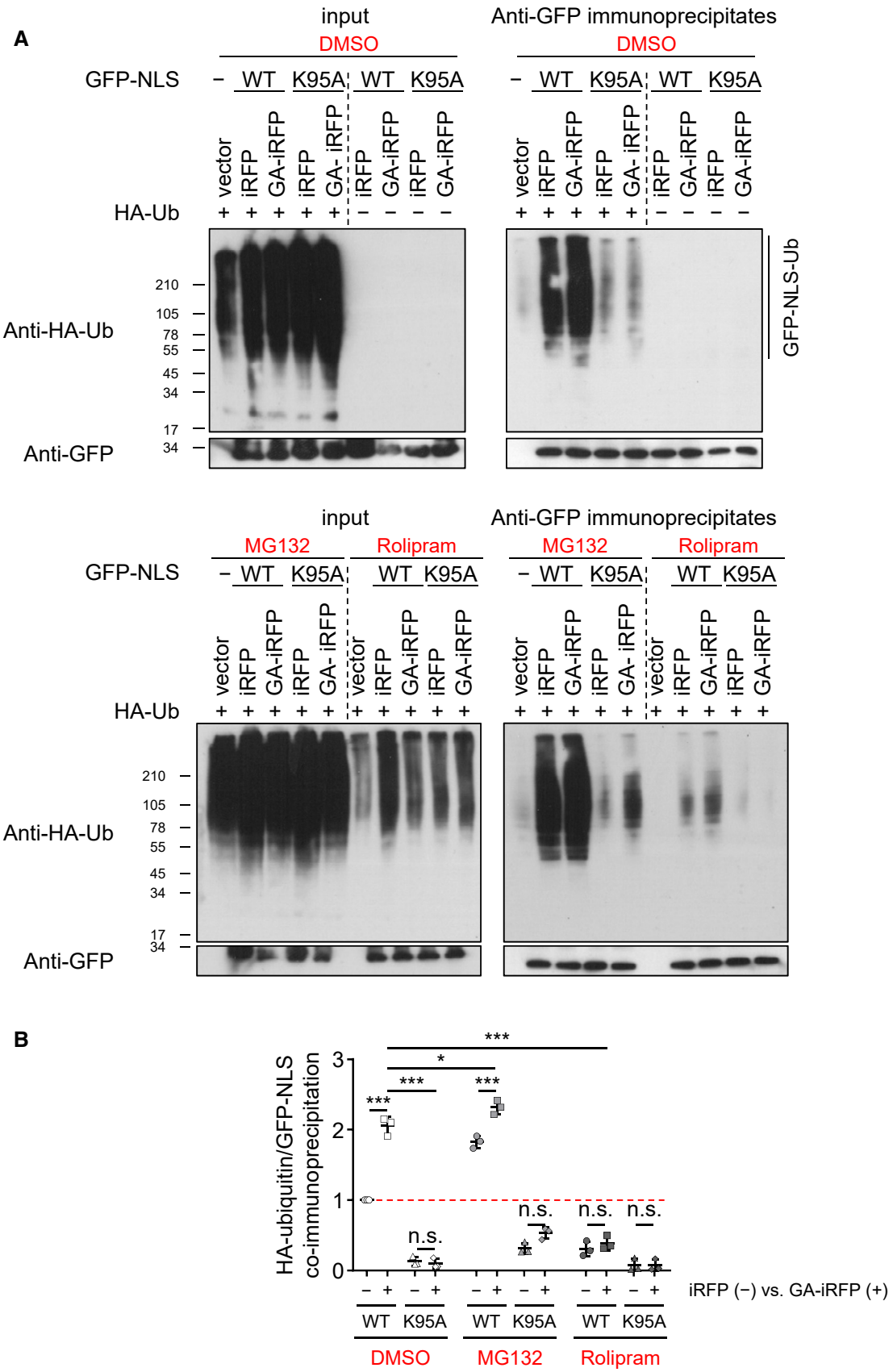

Figure 7.

Figure 7. Poly-GA induces poly-ubiquitination of TDP-43 within the NLS at lysine 95.

HeLa cells were co-transfected with wild-type or K95A GFP-TDP-NLS, HA-ubiquitin, and iRFP or GA_{175}-iRFP. Twenty-four hours after transfection, cells were treated with rolipram (30 μM), MG132 (10 μM), or DMSO (vehicle) for 16 h. Lysates were immunoprecipitated with Protein G beads coupled with anti-GFP antibody.

A Immunoblotting of input (left panels) and anti-GFP immunoprecipitates (right panels) to show GFP reporter levels and poly-ubiquitination.

B Quantification of HA-ubiquitin levels normalized to total $GFP\text{-}NLS_{TDP}$ reporter levels in anti-GFP immunoprecipitates. $n = 3$ biological replicates. Scatter plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. $^{*}P < 0.05$, and $^{***}P < 0.001$. Red dashed line indicates the control's expression level.

Source data are available online for this figure.

aggregation of a C-terminal TDP-43 fragment (Khosravi *et al*, 2017; Nonaka *et al*, 2018). Recent data from a primate model of TDP-43 pathology suggest that rodent caspases cleave TDP-43 less efficiently to generate aggregation-prone CTFs, which may explain the absence of large TDP-43 aggregates in mouse models and primary neurons (Yin *et al*, 2019).

We tested the activation of the proteasome chemically using rolipram or genetically by overexpressing PSMD11 to ameliorate poly-GA toxicity. The PDE4 inhibitor rolipram leads to PSMD11 activation via serine-14 phosphorylation by PKA, which boosts proteasome assembly (Lokireddy *et al*, 2015). In addition, proteasomes from rolipram-treated cells have a higher ATPase activity, suggesting that substrate processing is enhanced (Lokireddy *et al*, 2015). The short side chains of poly-GA may impair translocation into the catalytic subunit of the proteasome as has been shown for glycine/alanine-rich sequences of EBNA1 (Levitskaya *et al*, 1997; Kraut, 2013). PSMD11 phosphorylation may promote translocation efficacy to allow degradation of poly-GA. Moreover, enhanced degradation of soluble poly-GA forms may reduce aggregate formation. In other disease contexts, activating the proteasome in a transgenic Tau mouse model reduced Tau levels and improved cognition (Myeku *et al*, 2016). Proteasome activation was shown to promote degradation of full-length TDP-43, SOD1 and FUS *in vitro* (Lokireddy *et al*, 2015). In our experiments, rolipram treatment had no effect on basal TDP-43 localization but prevented cytoplasmic mislocalization in poly-GA-expressing cells. In addition, rolipram reduced the aggregation of poly-GA and TDP-43 CTFs. Therefore, we analyzed the effects of rolipram in all immunofluorescence assays only in cells containing visible poly-GA inclusions to exclude confounding effects. Our findings are most consistent with the model that rolipram activates both poly-GA and TDP-43 degradation and additionally inhibits poly-GA-dependent effects on TDP-43.

Non-cell-autonomous effects of poly-GA on TDP-43

Most neuropathological studies in end-stage tissue found no correlation between reduced *C9orf72* expression, RNA foci or the five DPR species and neurodegeneration (Mackenzie *et al*, 2013, 2015; Schludi *et al*, 2015; DeJesus-Hernandez *et al*, 2017). We and others reported cell-to-cell transmission of DPRs (Westergard *et al*, 2016; Zhou *et al*, 2017) and uptake of synthetic poly-GA aggregates is neurotoxic (Chang *et al*, 2016; Flores *et al*, 2016), but downstream effects were unknown. DPR inclusions were reported to cluster within human tissue which may support paracrine effects (Zu *et al*, 2013).

Here, we show by using fluorescent reporters that poly-GA affects proteasome function as well as TDP-43 localization and aggregation even in neighboring cells that do not contain obvious poly-GA aggregates, which may explain the poor regional overlap of DPRs and TDP-43 pathology in patients. We propose that some neuron populations (e.g., in cerebellum) are very efficient at non-canonical translation of the expanded *C9orf72* repeat, without being overly susceptible to their toxicity (e.g., due to higher basal proteasome activity), while motoneurons express only low levels of DPRs, but may be highly susceptible to proteasomal inhibition (Tashiro *et al*, 2012) by uptake of soluble or aggregated poly-GA. Our data show that anti-GA antibodies can break this cascade at least in cultured cells by blocking transmission of DPRs. Immunodepletion of poly-GA from conditioned media completely prevented mislocalization of TDP-43 in receiver cells, suggesting that *in vitro* the effects are mainly driven by released poly-GA. We cannot exclude that poly-GA expression triggers additional indirect effects *in vivo*, for example by directly releasing other molecules that promote TDP-43 aggregation in neighboring cells or triggering release of such factors from glial cells. Our findings provide mechanistic insights into very recent active and passive antibody therapy approaches in *C9orf72* mouse models by us and others (Nguyen *et al*, 2020; Zhou *et al*, 2020). Nguyen *et al* (2020) also reported that anti-GA antibodies partially restore proteasome function in poly-GA-expressing cells and show that antibodies clear poly-GA via the proteasome and autophagy pathway depending on the intracellular Fc-receptor TRIM21. Moreover, boosting proteasome function in donor and receiver cells with small molecules such as rolipram may overcome poly-GA-induced proteasome impairment and lead to clearance of ubiquitinated substrates such as TDP-43.

TDP-43 ubiquitination regulates nuclear import

Driving TDP-43 to the cytoplasm promotes its aggregation and is highly toxic, potentially through both gain- and loss-of-function mechanisms (Ederle & Dormann, 2017; Prasad *et al*, 2019). We provide ample evidence that ubiquitination at K95 inhibits its NLS function, possibly through steric hindrance of importin-α binding. Surprisingly, mutagenizing K95 of the bipartite NLS to alanine or arginine preserves activity but prevents ubiquitination and poly-GA-mediated inhibition of nuclear import. In addition, we confirm reduced binding of wild-type but not K95A to importin-α upon poly-GA expression. Consistent with the data by Hans *et al* (2018), K84 mutants completely block NLS activity even in the absence of poly-GA, and it is conceivable that ubiquitination at K84 may also inhibit nuclear import (Kim *et al*, 2011; Lumpkin *et al*, 2017; Akimov *et al*, 2018). However, removing K95 largely prevented ubiquitination in our assays, suggesting that K95 is the main ubiquitination site within the TDP-43 NLS. Interestingly, a recent study linked K95 ubiquitination to pathological phosphorylation at S409/410 (Hans *et al*, 2018). Boosting proteasome

activity in poly-GA-expressing cells may allow more efficient degradation of TDP-43 ubiquitinated at K95 (and other sites) and thus prevent accumulation of cytoplasmic TDP-43. A similar inhibition of nuclear transport by ubiquitination within an NLS has been described for p53 (Marchenko *et al*, 2010) and CCTα (Chen & Mallampalli, 2009), suggesting that this may be a common regulatory mechanism. Since poly-GA also promotes TDP-43-ΔNLS and TDP-CTF accumulation, ubiquitination or other post-translational modification at additional sites may also favor aggregation or liquid–liquid phase separation (Ederle & Dormann, 2017; Prasad *et al*, 2019).

So far, nucleocytoplasmic transport defects in *C9orf72* ALS/FTD have been mostly attributed to a direct effect of the repeat RNA and/or poly-GR/PR on the nuclear pore involving phase separation, but clear or even preferential effects on nuclear import of TDP-43 have not been reported (Freibaum *et al*, 2015; Jovicic *et al*, 2015; Zhang *et al*, 2015; Boeynaems *et al*, 2016). Moreover, poly-PR expression promotes recruitment of TDP-43 in stress granules upon arsenite treatment, but is ~100-fold less abundant than poly-GA in patients (Mackenzie *et al*, 2015; Boeynaems *et al*, 2017). Cytoplasmic TDP-43 aggregates further inhibit nucleocytoplasmic transport, which may trigger a vicious cycle (Chou *et al*, 2018; Solomon *et al*, 2018). We speculate that the combined effect of proteasome inhibition by poly-GA specifically on the TDP-43 NLS and a (subtle) general transport deficit caused by ubiquitous low-level expression of the repeat RNA and rare poly-PR cause the preferential mislocalization and aggregation of TDP-43 in *C9orf72* ALS/FTD patients. Recent findings on the role of TNPO1 as a chaperone for FUS (Guo *et al*, 2018a; Hofweber *et al*, 2018) suggest that the proteins affected by dysfunction of multiple pathways may be most sensitive to impairment of nucleocytoplasmic transport, when most other cargos are still trafficked normally.

Summary

Together, this work links the UPS dysfunction due to poly-GA aggregation with the deficits in nucleocytoplasmic transport recently reported in *C9orf72* FTD/ALS and other neurodegenerative diseases. Among the DPR proteins, poly-GA is the key driver of TDP-43 pathology in *C9orf72* disease, although it is not sufficient to trigger full pathology by itself in mouse models, which may be explained by additional impact of other DPR species, the repeat RNA itself, haploinsufficiency, or poor caspase cleavage of TDP-43 in rodents (Yin *et al*, 2019). Our work indicates that boosting proteasome activity or targeting poly-GA with antibodies may be a promising therapeutic strategy because it reduces not only poly-GA aggregation but also TDP-43 mislocalization and aggregation.

Materials and Methods

Plasmids, transfection, and viral packaging

Synthetic expression constructs containing an ATG start codon in pEF6 backbone (EF1 promoter) for transient transfection or in FhSynW backbone (human synapsin promoter) for lentiviral expression were described before (May *et al*, 2014; Schludi *et al*, 2015). Here, we additionally generated variants tagged with iRFP670 (Shcherbakova & Verkhusha, 2013).

We fused the NLS of human TDP-43 (PKDNKRKMDETDAS SAVKVKRA, position 78–99) to the C-terminus of GFP or tagRFP-T2 (abbreviated as RFP throughout the manuscript; gift from Michael Davidson) in FUW2 backbone as described previously (Khosravi *et al*, 2017). Similar constructs containing mutations of lysine 84 (K84) or lysine 95 (K95) to alanine or arginine were cloned using synthetic oligonucleotides. Human TDP-43 C-terminal (amino acids 220–414, CTF) fragments were generated by PCR and fused to the C-terminus of tagRFP-T2 in FUW2 backbone.

The Ub_{G76V}-GFP reporter for the ubiquitin–proteasome system (Dantuma *et al*, 2000) was subcloned into the FUW2 vector. Full-length TDP-43 and ΔNLS (K95A/K97A/R98A as described before (Winton *et al*, 2008)) were fused to the C-terminus of GFP in the FUW2 vector.

Lentivirus was packaged in HEK293FT cells (Life Technologies) as previously described (Guo *et al*, 2018b).

Antibodies

TDP-43 (Cosmo Bio Co, TIP-TD-P09), TDP-43 (Proteintech, 10782-2-AP), TDP-43 phospho-S409/410 (Cosmo Bio Co., Ltd, TIP-PTD-P02), TDP-43 (C-terminal; Proteintech, 12892-1-AP), ChAT (Merck, AB144P), GFP (UC Davis/NIH NeuroMab Facility, N86/8 and N86/38), PSMC4 (Bethyl Laboratories, A303-850A and A303-849A), tagRFP (Thermo Fisher Scientific, R10367), KPNA1 clone 114-E12 (Thermo Fisher Scientific, 37-0800), calnexin (Enzo Life Sciences, ADI-SPA-860-F), HA 3F10 (Merck, 11867423001), GA 5F2 (Mackenzie *et al*, 2013), control IgG from mouse serum (Merck, I5381), and HCS CellMask™ Deep Red Stain (Thermo Fisher Scientific, H32721) were used.

Primary neuron culture and immunofluorescence

Primary hippocampal neuron cultures were prepared from embryonic day 19 rats as described previously (Guo *et al*, 2018b). Primary neurons were then plated on sterilized poly-D-lysine-coated coverslips. For co-culture experiments, three 1- to 2-mm dots of melted paraffin were spotted on the coverslips as a spacer. Then, primary neurons transduced on separate coverslips (DIV4 + 4) were extensively washed with media and put face to face for another 4 days of incubation in fresh media. For antibody treatment in neuronal co-cultures, primary neurons on coverslips were transduced (DIV4 + 4) and washed with media and incubated face to face with non-transduced cells for 4 days, followed by 7 days of treatment with IgG control and anti-GA antibody.

HeLa cell culture and transfection

HeLa cells were cultured in DMEM, high glucose, GlutaMAX ™ Supplement containing 10% FCS and 1% penicillin/streptomycin together with MEM Non-Essential Amino Acids Solution at 37°C with 5% CO_2. HeLa cells were transfected using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer's instructions, followed by 24- to 48-h incubation at 37°C with 5% CO_2. For co-culture experiments, HeLa cells were transfected separately on two sets of coverslips with paraffin spacers for 24 h. After extensive washing with media, both coverslips were placed face to face and incubated for another 24 h.

C9orf72 patients

We selected nine *C9orf72* cases from the Brain Bank München Regina Feederle and stained frontal cortex sections for GA (Helmholtz Zentrum, 1A12) and TDP-43 (Proteintech, 10782-2-AP). One case was excluded from analysis due to extremely poor DAPI staining that precluded quantification of the frequency of poly-GA and cytoplasmic TDP-43.

Transgenic mice

Generation and characterization of Thy1-GA_{149}-CFP (abbreviated as GA-CFP) mice was reported previously (Schludi *et al*, 2017). Expression of GA_{149}-CFP was driven by Thy1.2 promoter. GA-CFP transgenic mice were kept in the C57BL/6N background. Animal handling was performed in accordance with animal law of the Government of Upper Bavaria, Germany. Animals were housed in standard cages with *ad libitum* access to food and water in pathogen-free facility on a 12-h day/night cycle. Six transgenic (four male and two female) mice and three littermates (two male and one female) were analyzed. Manual image analysis was performed blinded to the genotype.

Immunofluorescence and confocal imaging

For immunofluorescence analysis, cells were fixed with 4% paraformaldehyde and 4% sucrose for 10 min at RT and incubated with the indicated antibodies in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 450 mM NaCl, 16 mM sodium phosphate pH 7.4) and washed with PBS. All antibodies are listed in the key resources table.

For endogenous TDP-43 staining, primary hippocampal neurons (DIV4 + 7) were fixed with 4% paraformaldehyde, then permeabilized (0.2% Triton X-100, 50 mM NH_4Cl in PBS), and blocked for 30 min (2% fetal bovine serum, 2% serum albumin, 0.2% fish gelatin in PBS) and incubated with antibodies in the same buffer. In both protocols, the primary antibodies were incubated overnight at 4°C and the secondary antibodies for 1 h at room temperature.

For mouse experiments, 8- to 12-month-old mice were euthanized with CO_2 followed by cervical dislocation. Postmortem spinal cord was formalin fixated for 24 h, decalcificated with 5% formic acid for 48 h, and embedded in paraffin. Immunofluorescence staining was performed on 5-μm-thick paraffin sections as described previously (Schludi *et al*, 2017).

LSM710 confocal laser scanning system (Carl Zeiss) with Plan-APOCHROMAT 10X/NA 0.45 (420640-9900) or oil immersion 40×/NA 1.4 (420762-9900) objectives equipped with the ZEN 2011 software package (black edition, Zeiss) was used for acquiring images. For all analyses, at least three images per group were taken blind to the experimental condition at 1,024 × 1,024 pixel resolution. For z-stacked imaging, images were taken with z-step size of 0.8 μm at 5–7 μm thickness.

Automated image analysis

To quantify the fraction of cells with cytoplasmic signaling of endogenous TDP-43 staining in neurons, or RFP-NLS_{TDP} in HeLa, together with TDP-CTF intensity in HeLa cells, Columbus Acapella version 2.6.0 (PerkinElmer) was used as described before (Khosravi *et al*, 2017). Nucleus objects were detected based on DNA staining "Find Nuclei" (area > 30 μm^2, common threshold 0.10, split factor 7.0, individual threshold 0.4, contrast 0.45). In order to reject dead and mitotic nuclei, intensity properties were calculated at a standard method (mean and coefficient variance selected at quantile fraction 50%) by linear classification with the "select Population" function. Morphology of the nuclei was calculated by area, roundness, and Haralick features (including Haralick contrast, Haralick correlation, Haralick sum variance, Haralick homogeneity selected). The training set composed of $\sim$ 60 manually selected nuclei across all populations. For all selected nuclei, cell region was determined by expanding the nucleus region for 6 μm with morphological dilation. We selected the GFP/GFP-DPR-positive cells by laying a threshold on the mean intensity in the nucleus region. From this selection, we selected RFP-positive cells by setting a second threshold based on the RFP channel. We determined different thresholds for HeLa cells and primary neurons, while thresholds were maintained constant for all subpopulations. We analyzed the mean of cytoplasmic and nuclear HA-TDP-43/RFP-TDP-NLS intensities, and cytoplasmic-to-nuclear ratio and finally determined percentage of GFP-positive cells with cytoplasmic TDP-43/RFP-TDP-NLS. Average results from two tile images per experiment were treated as $n = 1$ for the statistical analysis.

The aggregate/cell ratio was quantified using Image J (version 1.52i). The Otsu image threshold was determined automatically, followed by binary water shedding. Finally, particles with 2–18 μm diameter were counted. For DAPI channel, particles > 30 μm with circularity factor 0.7–1.00 were identified as nuclei to determine the cell number.

For TDP-43 analysis from patient brains, we used a Leica fluorescent microscope (LAS X software) and imaged 50 fields of view per case in the gray matter, in manually determined grid patterns separated by 1 mm in each direction. Using CellProfiler (3.0.0), we identified DAPI-stained nuclei, cytoplasmic TDP-43 signal, bright TDP-43 inclusions, and GA aggregates. We removed the majority of glial nuclei from analysis using thresholds that identified the brightest, smallest nuclei. We summed the total nuclei, GA, and TDP-43 signals for all images per case and calculated the % GA-positive and GA-negative cells with cytoplasmic TDP-43 signal.

Western blot and filter trap assays

For Western blot analysis, cells were lysed on ice in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA) supplemented with 0.2 mg/ml DNase in PBS and protease and phosphatase inhibitors. Lysates were then centrifuged at 1,000 *g* for 10 min at 4°C or 15 min at 18,000 *g* 4°C depending on experiments. Protein concentration was adjusted according to measurements using a BCA assay (Interchim). After adding 4× Laemmli buffer (Bio-Rad) containing 2-mercaptoethanol, samples were denatured at 95°C for 10 min and loaded on Novex 10–20% Tris-Tricine gels (Life Technologies).

For filter tap, cells were lysed on ice in Triton buffer (1% Triton X-100, 15 mM $MgCl_2$ in PBS) supplemented with 0.2 mg/ml DNase and protease inhibitor (Mori *et al*, 2013). Lysates were centrifuged at 13,000 rpm 4°C 30 min. Pellets were resuspended in SDS buffer

(2% SDS in 100 mM Tris pH 7) and incubated for 2 h at RT. Samples were then filtered through a nitrocellulose membrane (0.2 μm pore). Membranes were then blocked with 2% I-Block (Thermo Scientific) according to the manufacturer's instructions and detected with antibodies as indicated. Immunoblots were analyzed by using Fiji software. Immunoblot lanes were first detected by rectangle tool and then plotted, followed by peak labeling.

Immunoprecipitation assay

HeLa cells were lysed in 2% Triton X-100, 0.75 M NaCl, 1 mM KH_2PO_4, and 3 mM Na_2HPO_4 supplemented with Benzonase Nuclease (6.7 U/ml) and protease inhibitors. 40 μl Protein G Sepharose beads were coupled with 3.96 mg/ml anti-GFP antibody for 1 h at 4°C. Lysed samples were cleared by centrifugation (1,000 *g* for 5 min), and 10% of the supernatant was taken out as input. The remainder was incubated with GFP-coupled beads overnight at 4°C, followed by extensive washing (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5% glycerol).

Anti-GA immunodepletion and immunoassay

For immunodepletion experiments, 50 μl Protein G Dynabeads were coupled with 10 μg anti-GA (5F2) or control IgG antibodies for 1 h at RT followed by three washing steps with PBS. Cell supernatant was incubated with antibody-coupled beads for 3 h at RT. Supernatant was then collected, equilibrated to 37°C, and added to receiver cells for 96 h. To confirm immunodepletion, washed beads (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5% glycerol) were analyzed by immunoblotting and aliquots of the supernatant were analyzed by immunoassay on the Meso Scale platform (MSD) as described (Zhou *et al*, 2017). Briefly, streptavidin plates (MSD Gold 96-well streptavidin) were coated with biotinylated 5F2 antibody (capture antibody, 1:1,000) in PBS overnight. After washing and blocking, the plates were then incubated with media for 2 h at RT on a shaking platform. Plates were washed three times and incubated with MSD sulfo-tag-labeled 5F2 antibody (detection antibody, 1:1,000) for 2 h at RT on a shaking platform followed by three final washing steps. The plates were measured shortly after adding 100 μl MSD Read Buffer T. MESO QuickPlex SQ 120 instrument was used to detect the electrochemical signal. Data are shown in arbitrary units after background correction.

Flow cytometry

HEK293 cells stably expressing Ub_{G76V}-GFP (Dantuma *et al*, 2000; De Smet *et al*, 2017) were transfected with the indicated constructs for co-culture assays or incubated with conditioned media from GA_{175}-RFP- or RFP-expressing cells for 48 h. Subsequently, receiver cells were harvested and analyzed by flow cytometry for GFP and RFP fluorescence using an Attune NxT Cytometric Analyser (Thermo Fisher) at the Imaging Facility of the Max Planck Institute of Biochemistry, Martinsried. Fluorescence was detected using the following settings: GFP Ex 488 nm, Em 530/30 nm, tagRFP Ex 561 nm, and Em 586/15 nm. At least 500,000 cells were analyzed per sample. Fluorescence intensities were corrected for spectral overlap using HEK293 cells expressing single fluorophores, and compensated flow cytometry data were further analyzed using FlowJo software (version 9.9; Tree Star).

RNA isolation and quantitative RT–PCR

HeLa cells were transfected and incubated for 48 h. Next, RNA isolation was performed using the QIAshredder and RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. To generate cDNA, the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used with random hexamer primers. CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) was used to perform RT-qPCR. The following primers were used: EGFP (Mr04097229_mr, Thermo Fisher Scientific), ACTB (Hs01060665_g1, Thermo Fisher Scientific), B2M (4326319E, Thermo Fisher Scientific), GAPDH (Hs02758991_g1, Thermo Fisher Scientific), and tagRFP (PrimerQuest Tool and Supply, Integrated DNA Technologies). Signals were normalized to ACTB, GAPDH, and B2M with the Bio-Rad CFX Manager Software (Bio-Rad Laboratories) by using the ΔΔCT method.

Statistical analyses

Statistical analysis was done in GraphPad Prism (version 7.01) using one-way ANOVA with Tukey's multiple comparisons test. Family-wise significance and confidence level were set at 0.05 (95% confidence interval). For experiments with only two groups, unpaired two-tailed *t*-test with 95% confidence level was performed. For comparison of cytoplasmic TDP-43 in poly-GA-positive vs poly-GA-negative cells within patients, a paired two-tailed *t*-test was used.

Data availability

Source data are provided with the manuscript.

Expanded View for this article is available online.

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Author contributions
BK performed most cell biological and biochemical experiments. KDL, HH, and TA provided and analyzed human samples. QZ, NM, and MM provided and analyzed mouse samples. HR performed qPCR analysis. FF and MSH performed flow cytometry analysis. BK, DF, and HR generated reagents. MC performed immunoassays. FUH and DE acquired funding. FUH and MSH supervised research and contributed to writing. DE designed the study, supervised research, and wrote the manuscript. All authors discussed the data and the manuscript.

Conflict of interest

D.E. holds a patent on "Dipeptide-repeat proteins as therapeutic target in neurodegenerative diseases with hexanucleotide repeat expansion" (EP2948777 and US10066007).

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Expanded View Figures

Figure EV1. Poly-GA induces cytoplasmic TDP-43 mislocalization.

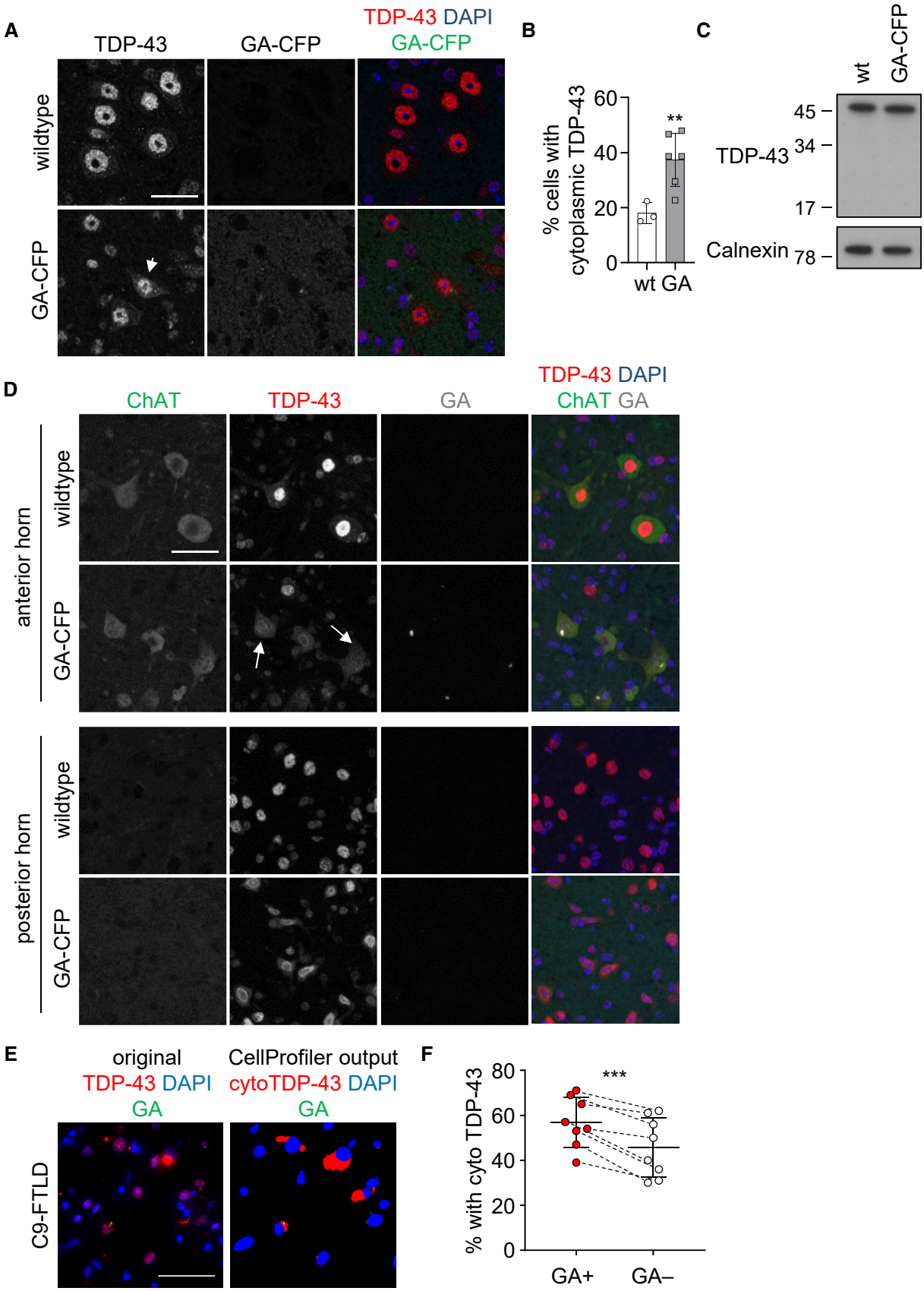
A, B Immunofluorescence analysis of endogenous TDP-43 in the anterior horn of the spinal cord of GA_{149}-CFP transgenic mice 8–12 months of age (Schludi *et al*, 2017). Single confocal sections are shown in (A). Arrow indicates neuron with cytoplasmic TDP-43 punctae. (B) Manual quantification of neurons with cytoplasmic TDP-43 in the anterior horn. To allow blinded quantification, poly-GA expression was not taken into account. Scatter plot with bar graphs of mean ± SD. Statistical analysis using unpaired *t*-test and Welch's correction (three wild-type and six transgenic animals).

C Immunoblotting of three wild-type and three GA_{149}-CFP transgenic mice spinal cord 8 months of age. Immunoblotting of one wild-type and one GA_{149}-CFP transgenic mouse spinal cord is shown. Proteolytic processing of TDP-43 was not detected in both genotypes.

D Immunofluorescence analysis of endogenous TDP-43 in large ChAT-positive motoneurons in the anterior and posterior horns of the spinal cord of GA_{149}-CFP transgenic mice 8–12 months of age (Schludi *et al*, 2017). Maximum intensity projections are shown. Arrow indicates neurons with cytoplasmic TDP-43 punctae.

E, F Automated analysis of cytoplasmic mislocalization of TDP-43 in frontal cortex of *C9orf72* FTLD patients. Representative raw image and the resulting CellProfiler mask (see Materials and Methods for details). Poly-GA-positive neurons were significantly more likely to have detectable cytoplasmic TDP-43 than neighboring poly-GA-negative neurons (paired *t*-test $t(7) = 5.58$, partial $\eta^2 = 0.816$, mean ± SD).

Data information: $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars: 50 μm.
Source data are available online for this figure.


A
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C
wt
GA-CFP
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17
TDP-43
Calnexin
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D
ChAT
TDP-43
GA
TDP-43 DAPI
ChAT GA
anterior horn
wildtype
GA-CFP
posterior horn
wildtype
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Figure EV1.

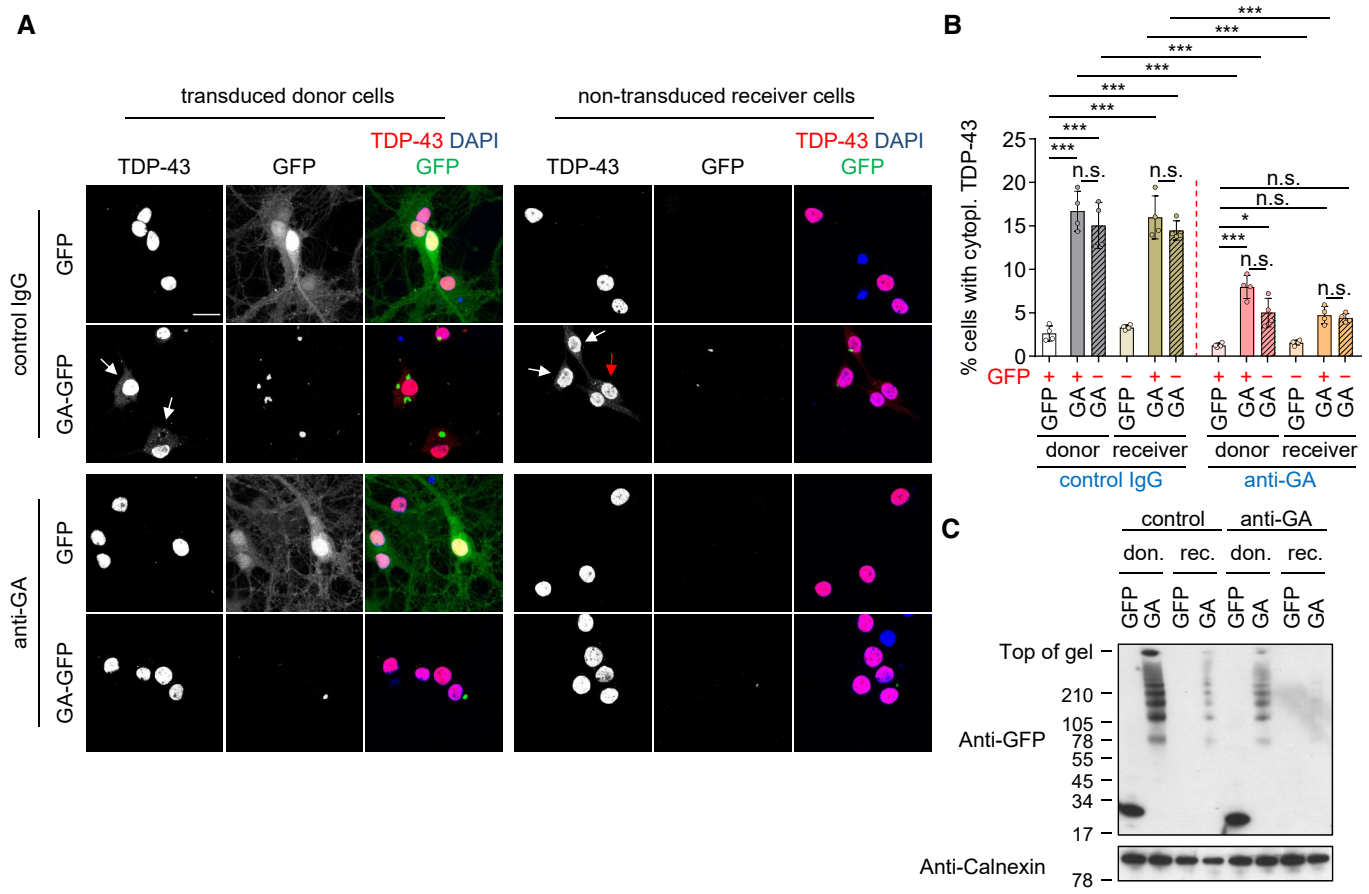

Figure EV2. Anti-GA antibodies block the non-cell-autonomous effects of poly-GA on TDP-43 in a co-culture assay.

Primary hippocampal neurons were transduced with GFP or GA_{175}-GFP (DIV4 + 4) and treated with IgG control and anti-GA (5F2) antibody.

A Confocal imaging revealed that anti-GA antibody treatment reduces Poly-GA-induced cytoplasmic mislocalization of TDP-43 in hippocampal neurons. White and red arrows show cells with cytoplasmic TDP-43 in GFP-positive and GFP-negative cells, respectively. Scale bar denotes 20 μm.

B Automated quantification of cells with cytoplasmic TDP-43 in GFP or GA_{175}-GFP-transduced cells. Cells with and without GFP signal were analyzed separately (indicated by +/−). As in Fig 1C, GFP-negative donor and GFP-positive receiver cells were excluded due to high transduction and low transmission rate of GFP. $n = 4$ biological replicates. Scatter plot with bar graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. *$P < 0.05$, and ***$P < 0.001$.

C Immunoblotting shows reduced poly-GA expression upon anti-GA antibody treatment.

Source data are available online for this figure.

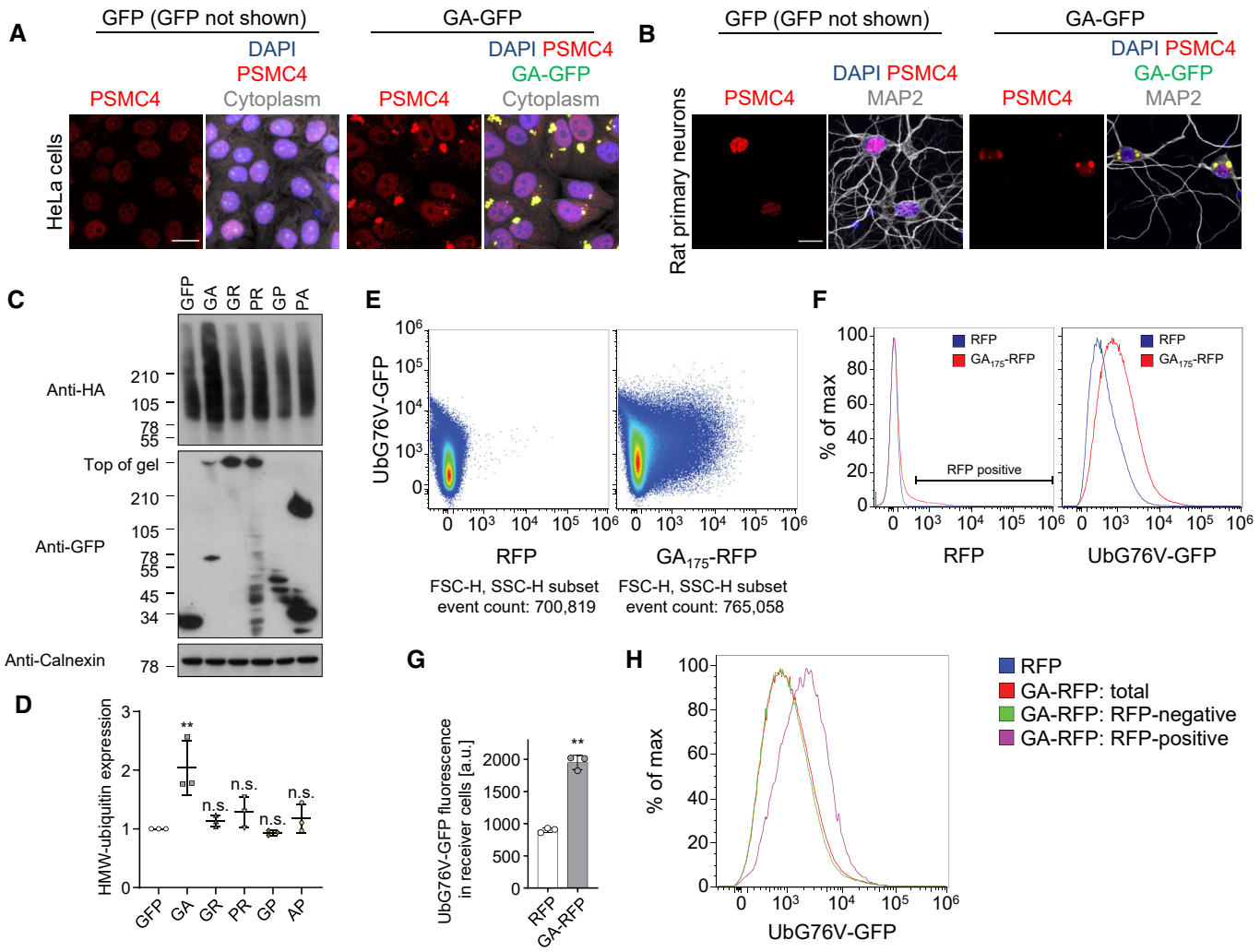

Figure EV3. Poly-GA inclusions sequester the proteasome.

A, B Immunofluorescence of the proteasome subunit PSMC4 and poly-GA inclusions in GA_{175}-GFP-transfected HeLa cells and GA_{175}-GFP-transduced rat primary neurons. To confirm cell viability, the cytoplasm of HeLa cells was stained with HCS CellMask™ Deep Red Stain and neuronal dendrites were labeled with MAP2. Scale bar denotes 20 μm.

C, D Immunoblots of HeLa cells that were co-transfected with HA-ubiquitin and GFP, GA_{175}-GFP, GFP-GR_{149}, PR_{175}-GFP, GFP-GP_{47}, and PA_{175}-GFP and incubated for 48 h and analyzed by densitometry. Scatter plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. $^{**}P < 0.01$.

E–H Flow cytometry analysis of non-cell-autonomous proteasome inhibition using HEK293 Ub_{G76V}-GFP reporter cells co-cultured for 48 h with GA_{175}-RFP- or RFP-transfected cells. (E) Two-color scatter plots, presented as pseudo-color density plots, with compensated RFP fluorescence plotted on the *x*-axis and compensated GFP fluorescence on the *y*-axis. A representative experiment out of three independent repeats is shown. (F) Comparisons of the corresponding histograms for compensated RFP and Ub_{G76V}-GFP fluorescence from one representative experiment that shows specific transmission of GA_{175}-RFP associated with accumulation of Ub_{G76V}-GFP in cells co-cultured with GA_{175}-RFP. (G) Accumulation of Ub_{G76V}-GFP signal in non-transfected receiver cells that were co-incubated for 48 h with GA_{175}-RFP-transfected donor cells compared with RFP control. $n = 3$ biological replicates. Scatter plot with bar graphs of mean ± SD. Unpaired two-tailed *t*-test with Welch's correction. $^{**}P < 0.01$. (H) Histograms for Ub_{G76V}-GFP intensity showing separate analysis of RFP-positive and RFP-negative receiver cells for the GA_{175}-RFP condition. RFP gating as indicated in (F).

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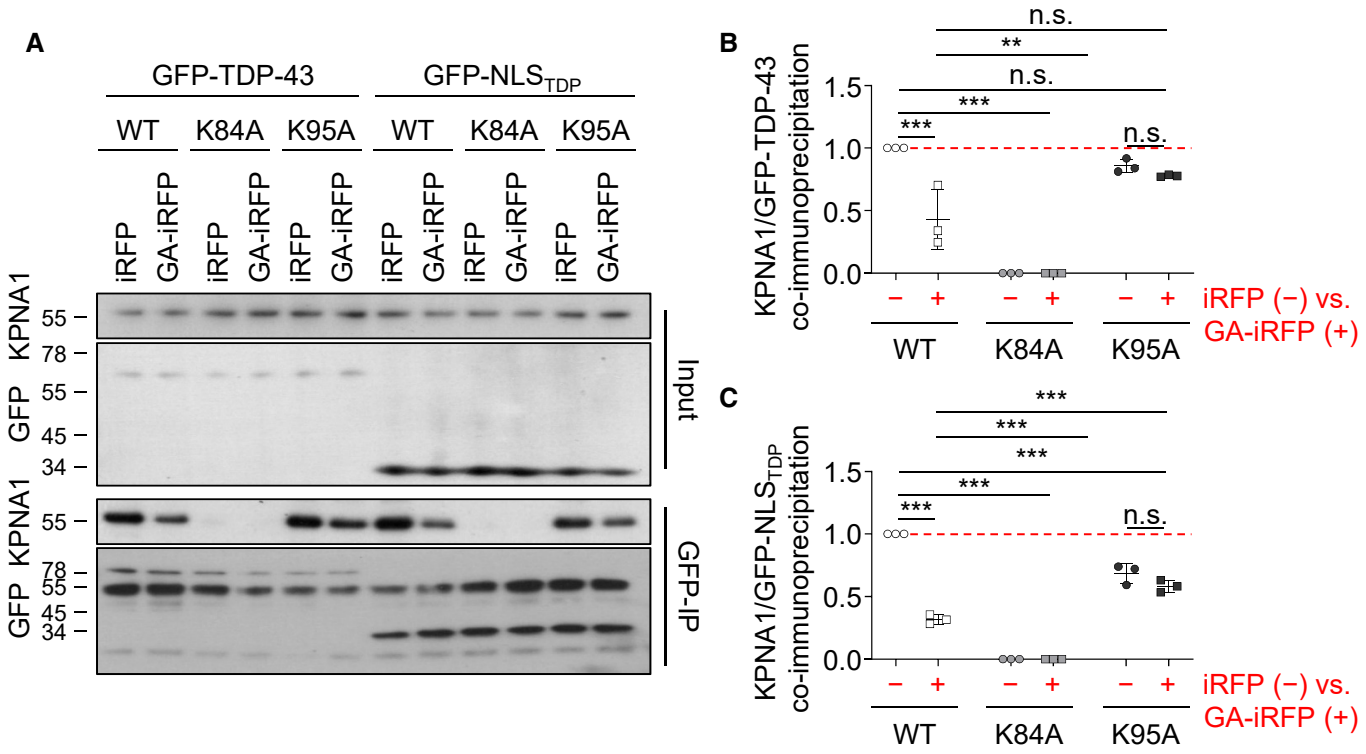

Figure EV4. Poly-GA reduces KPNA1 binding of full-length TDP-43.

A HeLa cells were co-transfected with either full-length GFP-TDP-43 (wild type, K84A, K95A) or GFP-NLS$_{TDP}$ (wild type, K84A, K95A) as well as iRFP or GA$_{175}$-iRFP and incubated for 48 h. Lysates were immunoprecipitated with anti-GFP and immunoblotted with an anti-importin-α5/KPNA1 antibody to detect binding of the nuclear import receptor. Protein expression in the input is also shown.

B, C Quantification of KPNA1 levels normalized to total GFP-TDP-43 and GFP-NLS$_{TDP}$ reporter levels in anti-GFP immunoprecipitates. n = 3 biological replicates. Scatter plot with mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. $^{**}P < 0.01$, $^{***}P < 0.001$. Red dashed line indicates the control's expression level.

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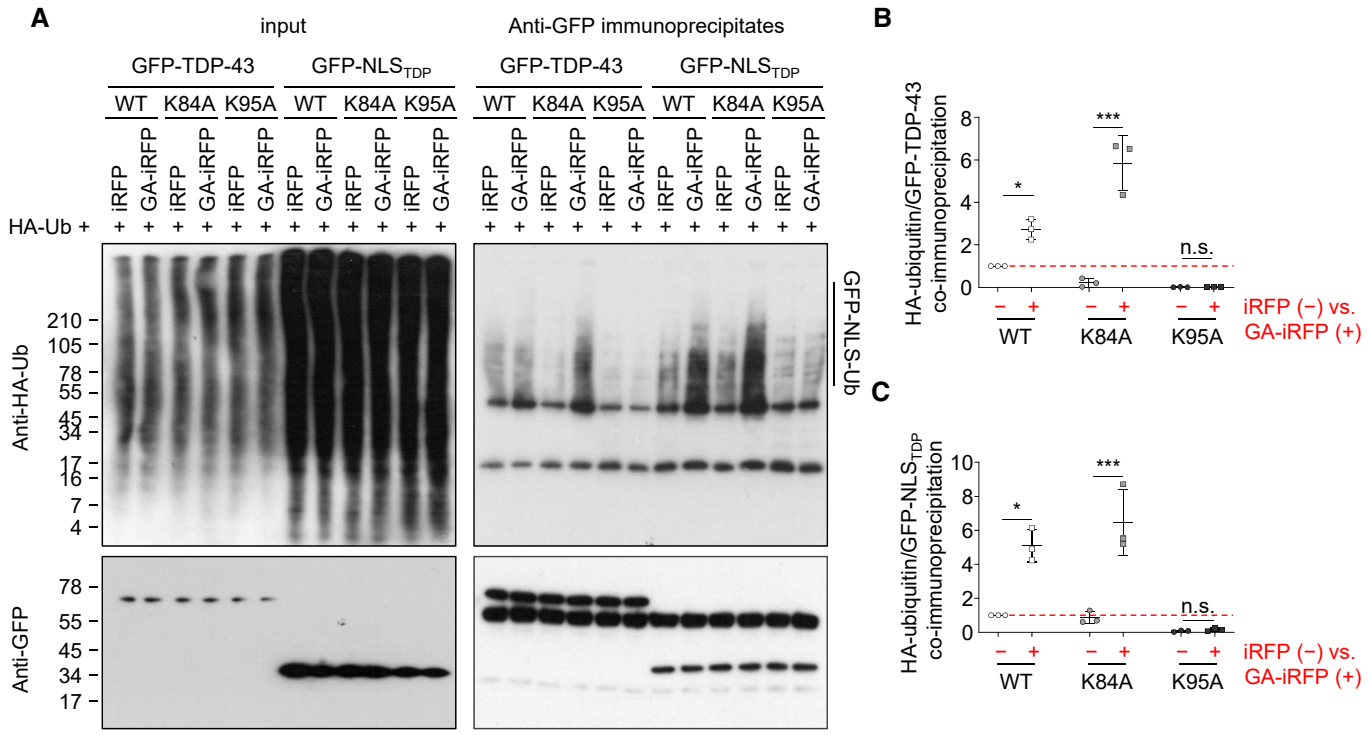

Figure EV5. Poly-GA induces poly-ubiquitination of TDP-43 at lysine 95.

A–C HeLa cells were co-transfected with either full-length GFP-TDP-43 (wild type, K84A, K95A) or GFP-NLS$_{TDP}$ (wild type, K84A, K95A) as well as HA-ubiquitin and iRFP or GA$_{175}$-iRFP, and incubated for 48 h. Lysates were immunoprecipitated with anti-GFP antibody. Immunoblotting of input (left panels) and anti-GFP immunoprecipitates (right panels) to show TDP-43 bait levels and poly-ubiquitination. (B, C) Quantification of HA-ubiquitin levels normalized to total GFP-TDP-43 and GFP-NLS$_{TDP}$ reporter levels in anti-GFP immunoprecipitates. n = 3 biological replicates. Scatter plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. *$P < 0.05$, and ***$P < 0.001$. Red dashed line indicates the control's expression level.

Source data are available online for this figure.

Cell-to-cell transmission of C9orf72 poly-(Gly-Ala) triggers key features of ALS/FTD

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Running title: Rescuing poly-GA effects on TDP-43

Keywords: Neurodegeneration / C9orf72 / proteasome / nucleocytoplasmic transport / antibody therapy

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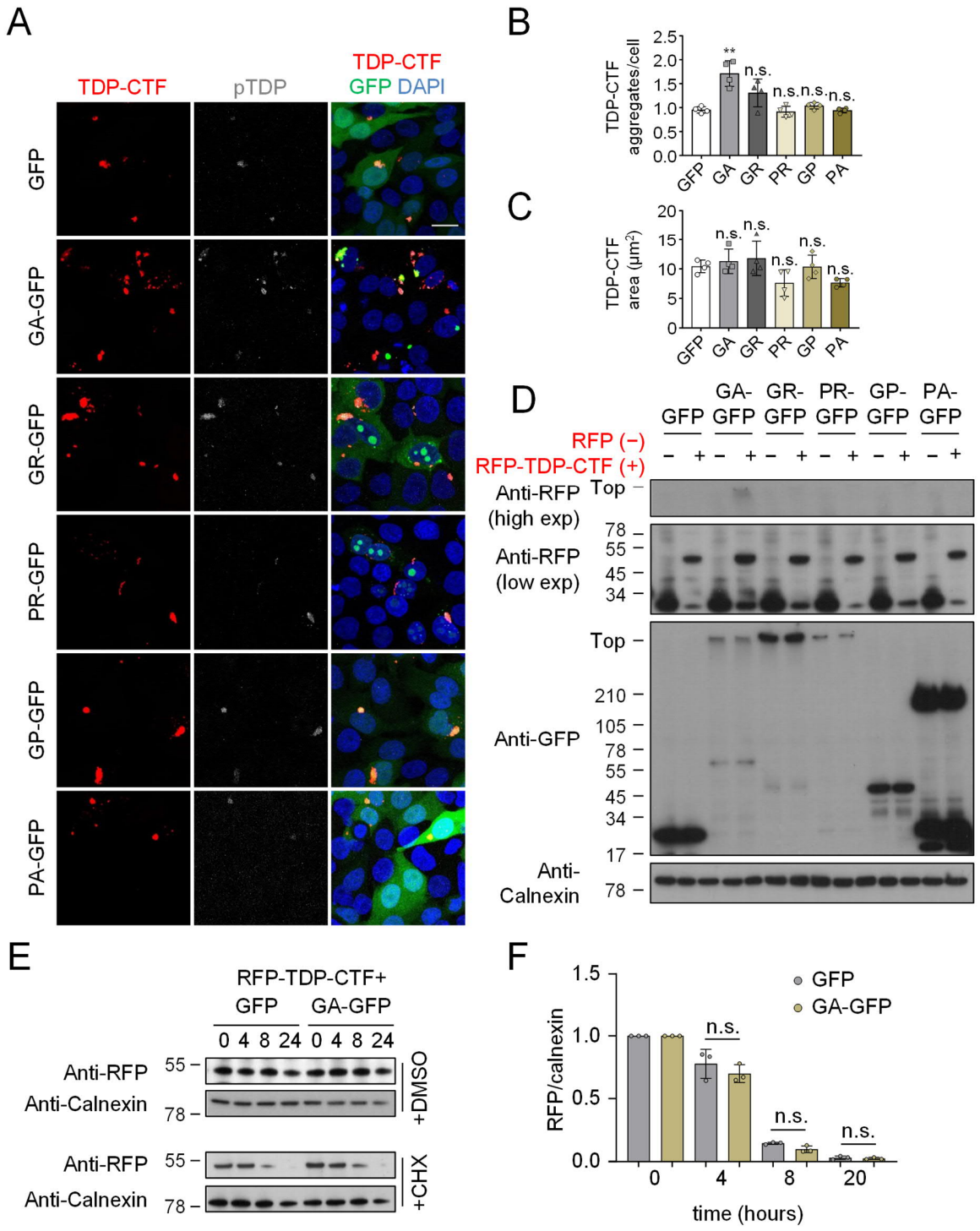
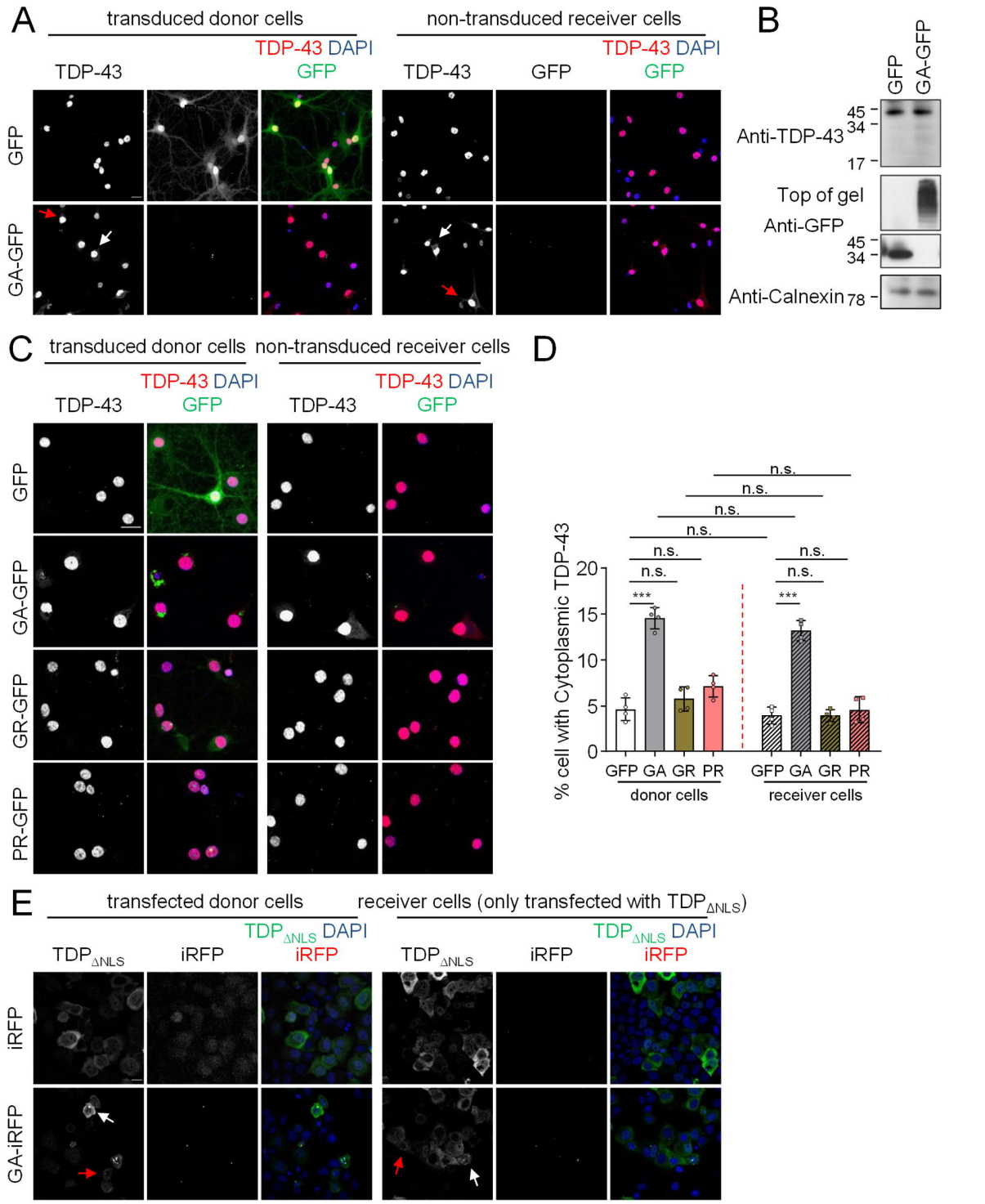


Appendix Figure S1. Poly-GA promotes TDP-43-CTF aggregation.

(A-C) HeLa cells were co-transfected with GFP or GFP-tagged DPR species and the RFP-tagged TDP-43 C-terminal fragment (TDP-CTF, amino acids 220-414) and analyzed for TDP-CTF aggregation. GA_{175}-GFP, GFP-GR_{149}, PR_{175}-GFP, GFP-GP_{47} and PA_{175}-GFP were used. (A) Immunofluorescence shows enhanced TDP-CTF aggregation upon poly-GA expression. (B) Automated quantification of aggregate number per

cell and (C) aggregate average area in μm^2. n=4 biological replicates. In total 395 GFP, 412 GA_{175}-GFP, 385 GFP-GR_{149}, 387 PR_{175}-GFP, 381 GFP-GP_{47}, and 414 PA_{175}-GFP cells with TDP-CTF aggregates were analyzed. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes $p<0.01$. (D) Immunoblotting of HeLa cells co-transfected with GFP-DPRs and RFP-TDP-CTF or RFP control. Note that GA_{175}-GFP co-expression results in TDP-43 accumulation and high-molecular weight aggregation at the top of the gel. Control cells were co-transfected with GFP-DPRs and RFP. (E-F) HeLa cells were co-transfected with RFP-TDP-CTF and GFP or GA_{175}-GFP. 24h after transfection, cells were treated with vehicle or 150 µg/ml cycloheximide (+CHX) for 0, 4, 8, and 24h. Protein turn-over was measured by immunoblotting of cell lysates. (F) Quantification of RFP-TDP-CTF protein levels normalized to calnexin. n=4 biological replicates. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.



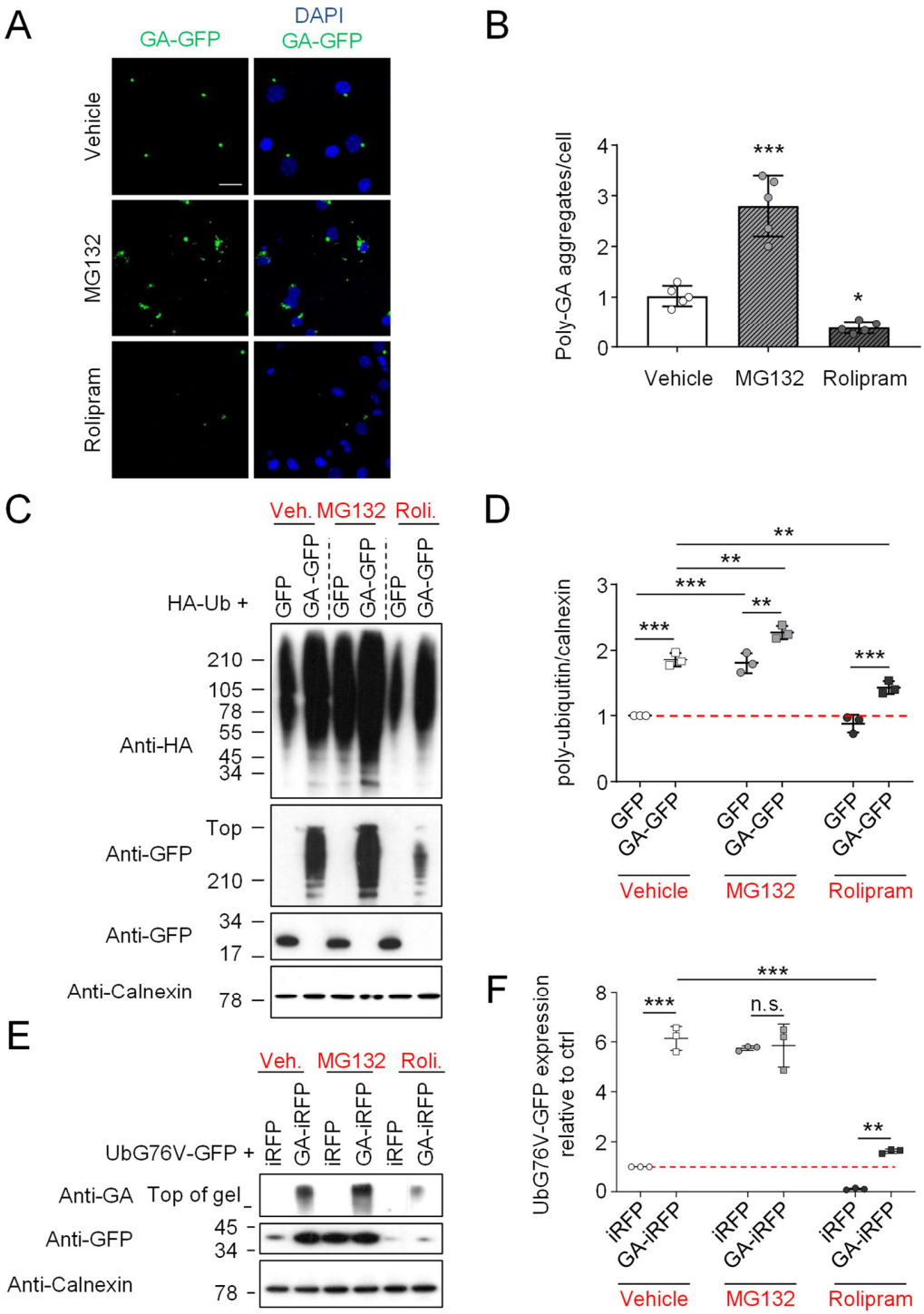
Appendix Figure S2. Cell-to-cell transmission of poly-GA causes cytoplasmic mislocalization of TDP-43.

(A) Additional images with larger fields of view from the experiments in Fig. 1B. Primary hippocampal neurons were transduced with GFP or GA_{175}-GFP (DIV4+4) and co-cultured with naïve primary neurons for 4 days. Endogenous TDP-43 and poly-GA aggregates in donor and receiver coverslips were analyzed by immunofluorescence. Cytoplasmic TDP-43 immunostaining is elevated not only in poly-GA transduced neurons, but also in the non-transduced receiver cells. White and red arrows indicate cells with

cytoplasmic TDP-43 in GFP positive and negative cells, respectively. In (B) HeLa cells were transfected with GFP or GA_{175}-GFP for 48h and immunoblotted for TDP-43. Note that there is no TDP-43 cleavage in GA_{175}-GFP expressing cells.

(C) Primary hippocampal neurons were transduced with GFP, GA_{175}-GFP, GFP-GR_{149}, and PR_{175}-GFP (DIV4+4) and co-cultured with naïve primary neurons for 4 days. Endogenous TDP-43 and poly-GA,-GR,-PR aggregates in donor and receiver coverslips were analyzed by immunofluorescence. (D) Automated quantification of cells with cytoplasmic TDP-43 in GFP or GA_{175}-GFP, GFP-GR_{149}, PR_{175}-GFP, transduced (donor), non-transduced (receiver) neurons. n=4 biological replicates. In total 334 donor GFP, 300 donor GA_{175}-GFP, 315 donor GFP-GR_{149}, 307 donor PR_{175}-GFP,322 receiver GFP and 302 receiver GA_{175}-GFP, 319 receiver GFP-GR_{149}, 294 receiver PR_{175}-GFP cells were analyzed. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. *** denotes $p<0.001$.

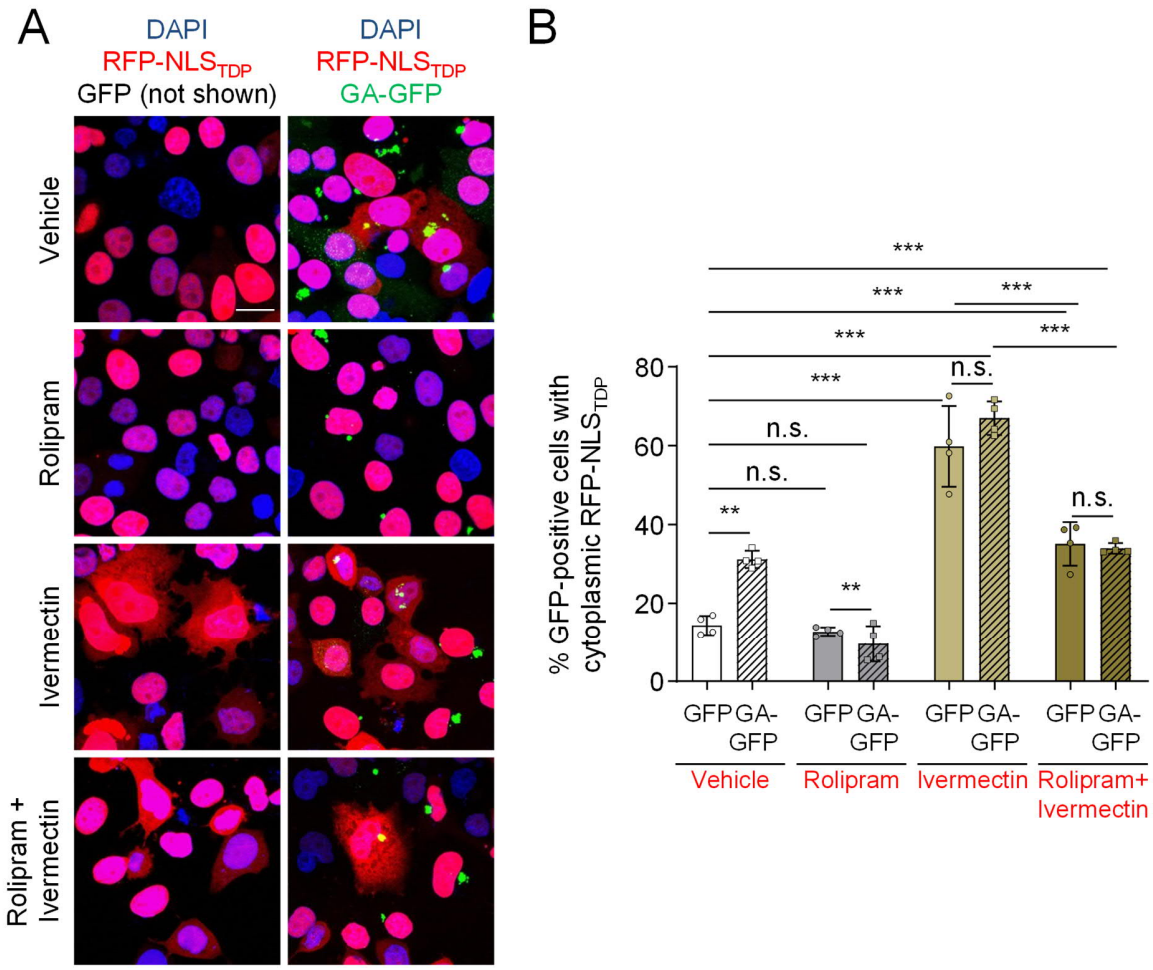
(E) Additional images with larger fields of view from the experiments in Fig. 1D. Immunofluorescence of co-culture model in HeLa cells transfected with iRFP or GA_{175}-iRFP in the donor compartment and TDP-$43_{\Delta NLS}$-GFP in donor and receiver compartment. White and red arrows indicate cells with cytoplasmic TDP-43 in GFP positive and negative cells, respectively.



Appendix Figure S3. Rolipram reduces poly-GA aggregate number and enhances proteasomal activity.

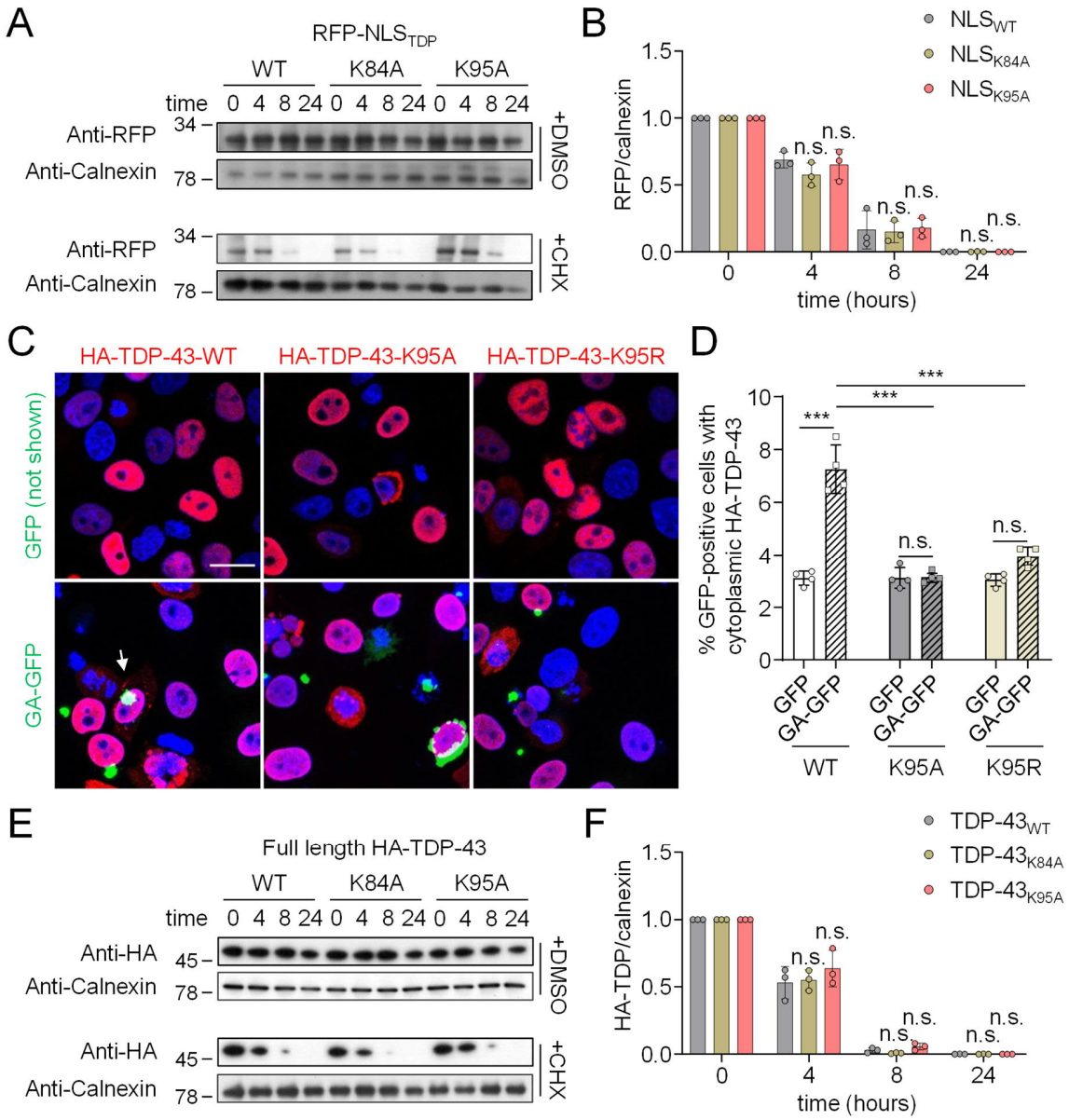
(A) Immunofluorescence of primary hippocampal neurons transduced with GFP or GA_{175}-GFP and treated with MG132 (10 µM), rolipram (30 µM) or vehicle control for 16h. (B) Automated analysis of ratio of poly-GA aggregate number to cell number. n=5 biological replicates. In total 261 cells treated with vehicle, 302 cells with MG132 and 351 cells with rolipram treatments were analyzed. Scatter plot with

bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. * denotes p<0.05, and *** denotes p<0.001. Scale bar denotes 20 µm. (C, D) Immunoblots of HeLa cells that were co-transfected with HA-Ubiquitin and GFP or GA_{175}-GFP and treated as above, and furthermore analyzed by densitometry. Scatter dot plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes p<0.01, and *** denotes p<0.001. (E,F) HeLa cells were co-transfected with Ub_{G76V}-GFP reporter and iRFP or GA_{175}-iRFP and treated as above. (D) Immunoblots of n=3 biological replicates were (E) quantified by densitometry. Scatter dot plot, mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes p<0.01, and *** denotes p<0.001.



Appendix Figure S4. Rolipram treatment promotes degradation of cytoplasmic TDP-43 RFP-NLS reporter.

(A) HeLa cells were co-transfected with RFP-NLS$_{TDP}$ reporter and GFP or GA$_{175}$-GFP. 24h after transfection, cells were treated with nuclear import inhibitor ivermectin (10 µM), proteasome activator rolipram (30 µM), or both, and analyzed by immunofluorescence. (B) Automated quantification shows cytoplasmic mislocalization of RFP-NLS reporter upon ivermectin treatment that is partially rescued by additional rolipram treatment. n=4 biological replicates. In total 337 GFP and 361 GA$_{175}$-GFP cells treated with vehicle, 383 GFP and 328 GA$_{175}$-GFP cells treated with rolipram, 337 GFP and 329 GA$_{175}$-GFP cells treated with ivermectin, 331 GFP and 340 GA$_{175}$-GFP cells treated with rolipram and ivermectin were analyzed. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test. ** denotes p<0.01, and *** denotes p<0.001.



Appendix Figure S5. TDP-43 K95 mutation blocks poly-GA induced cytoplasmic mislocalization of full length TDP-43 without affecting overall TDP-43 turnover.

(A, B) HeLa cells were transfected with RFP-NLS$_{TDP}$ wild-type, K95A or K95R. 24h after transfection, cells were treated with vehicle or 150 µg/ml cycloheximide (+CHX) for 0, 4, 8, and 24h. Protein stability was measured by immunoblotting of cell lysates. (B) Quantification of RFP-NLS$_{TDP}$ protein levels normalized to calnexin. n=4 biological replicates. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

(C) HeLa cells were co-transfected with HA-tagged full-length TDP-43 wild-type, K95A or K95R together with GFP or GA$_{175}$-GFP and analyzed by immunofluorescence. (D) Automated quantification of cells with cytoplasmic HA-TDP-43 in GFP or GA$_{175}$-GFP transfected HeLa cells. n=4 biological replicates. In total 322 GFP and 314 GA$_{175}$-GFP cells with TDP-43 WT, 291 GFP and 310 GA$_{175}$-GFP cells with TDP-43 K95A, 363 GFP and 328 GA-GFP cells with TDP-43 K95R were analyzed. Scatter plot with bar-graphs of mean ± SD.

One-way ANOVA with Tukey's multiple comparisons test. *** denotes $p<0.001$. Scale bar denotes 20 µm.

(E, F) HeLa cells were transfected with full length TDP-43 wild-type, K95A or K95R. 24h after transfection, cells were treated with vehicle or 150 µg/ml cycloheximide (+CHX) for 0, 4, 8, and 24h. Protein stability was measured by immunoblotting of cell lysates. (F) Full length TDP-43 protein levels were quantified and normalized to calnexin. n=4 biological replicates. Scatter plot with bar-graphs of mean ± SD. One-way ANOVA with Tukey's multiple comparisons test.

6. Discussion

My work for the first time indicates that cytoplasmic poly-GA inclusions, not the nuclear poly-GA aggregates, disrupt the classical nuclear import pathway of TDP-43 via the importin-α/β pathway leading to cytoplasmic accumulation of TDP-43. In addition, not only poly-GA impairs the proteasome, thereby promoting TDP-43 cytoplasmic mislocalization in the cells expressing poly-GA, but also in neighboring cells that uptake small amounts of soluble or aggregated poly-GA.

The expanded *C9orf72* hexanucleotide repeat has been so far shown to inhibit several cellular and molecular pathways, including impairment of nucleocytoplasmic shuttling, proteasome sequestration and stalling. Moreover, accumulation of cytoplasmic TDP-43, a well-known substrate for UPS, in diseased neurons is the other major pathological hallmark in ALS and FTD. How DPRs link to cytoplasmic TDP-43 aggregation and eventually causing neurodegeneration has been a question for many years. Addressing this question will help us in finding efficacious medicine to prevent or even stop the disease progression. Here I focus on the role of dipeptide repeat proteins, and provide cellular and molecular mechanisms on how poly-GA pathology among all other DPRs contributes the most to TDP-43 pathology in ALS/FTD.

6.1. Poly-GA impairs nuclear import of TDP-43

Among all five species of DPRs, poly-GA is the most abundant DPR in *C9orf72* ALS/FTD patients (Freibaum, Lu et al. 2015, Jovicic, Mertens et al. 2015, Zhang, Donnelly et al. 2015, Boeynaems, Bogaert et al. 2016). In cell and animal models, poly-GA rapidly forms cytoplasmic inclusions (Zu, Liu et al. 2013, May, Hornburg et al. 2014, Wen, Tan et al. 2014, Zhang, Jansen-West et al. 2014, Yamakawa, Ito et al. 2015). $(GA)_{15}$ but not 15-mers of the other DPR species, aggregates promptly *in vitro* and forms fibrils that are labeled with amyloid dyes (e.g. thioflavin T) and show characteristic cross-β sheet structure in synchrotron experiments (Chang, Jeng et al. 2016). Moreover, cytoplasmic inclusions of artificial β-sheet proteins and disease-associated proteins result in severe impairment of nucleocytoplasmic shuttling (Woerner, Frottin et al. 2016). In addition, cytosolic inclusions of TDP-43 in motor neurons has been identified as the pathological protein in vast majority of ALS and FTD cases (Neumann, Sampathu et al. 2006, Cairns, Neumann et al. 2007, DeJesus-Hernandez, Mackenzie et al. 2011, Murray, DeJesus-Hernandez et al. 2011, Boeve, Boylan et al. 2012, Cooper-Knock, Hewitt et al. 2012, Gijselinck, Van Langenhove et al. 2012, Hsiung, DeJesus-Hernandez et al. 2012, Mahoney, Beck et al. 2012, Simon-Sanchez, Dopper et al. 2012, Snowden, Rollinson et al. 2012, Stewart, Rutherford et al. 2012, Troakes, Maekawa et al. 2012). In other words, a significant positive correlation has been shown between the amount of TDP-43 pathology and

the degree of neurodegeneration in crucial anatomical regions, including motor cortex, frontal cortex, and spinal cord (Mackenzie, Arzberger et al. 2013) suggesting TDP-43 is a crucial trigger of neurodegeneration (Walker, Spiller et al. 2015).

Therefore, in **Research Article 1**, I analyzed poly-GA (as the most abundant DPR observed in patients and most aggregation-prone DPR in cells) (Zu, Liu et al. 2013, May, Hornburg et al. 2014, Wen, Tan et al. 2014, Zhang, Jansen-West et al. 2014, Jovicic, Mertens et al. 2015, Yamakawa, Ito et al. 2015, Zhang, Donnelly et al. 2015, Boeynaems, Bogaert et al. 2016), -GR, and -PR (reported as two most toxic DPRs in living cells (Kwon, Xiang et al. 2014, Mizielinska, Gronke et al. 2014, Wen, Tan et al. 2014, Freibaum, Lu et al. 2015, Lee, Zhang et al. 2016)) implications on nuclear import of TDP-43. Poly-GR and -PR are shown to be highly polar. Charge-charge interactions are very important for interaction with other low complexity domains (LCDs) that allow biomolecular liquid-liquid phase separation (LLPS) (Li, Banjade et al. 2012, Kwon, Xiang et al. 2014, Mizielinska, Gronke et al. 2014, Wen, Tan et al. 2014, Freibaum, Lu et al. 2015, Jovicic, Mertens et al. 2015, Tao, Wang et al. 2015, Lee, Zhang et al. 2016, Lin, Mori et al. 2016, Liu, Pattamatta et al. 2016, Mitrea and Kriwacki 2016, Banani, Lee et al. 2017, Gupta, Lan et al. 2017, Shi, Mori et al. 2017). But most importantly the link between DPRs and TDP-43 has not been shown before. Poly-GR/-PR induce stress granule (SG) assembly and engage with RNA binding proteins (RBPs) which is dependent on phosphorylation of eukaryotic translation initiation factor 2A (eIF2α) together with the presence of Ras GTPase-activating protein-binding protein 1 (G3BP) proteins (Figure 2a) (Boeynaems, Bogaert et al. 2017).

I discovered that poly-GA, compared to poly-GR/PR, had a more significant impact on TDP-43 nucleocytoplasmic transport in HeLa cells and in primary neurons. Furthermore, I showed DPR inclusions affect importin α/β-dependent protein import. While poly-GA and to a lesser extent poly-GR inhibit the import of TDP-43 NLS through classical importin α/β pathway, they had no effect on hnRNPA1 NLS which is imported to the nucleus via PY-NLS mediated by transportin (TNPO).

Hence, defects in nucleocytoplasmic transport caused by cytoplasmic poly-GA inclusions may play a role in implications for *C9or72* ALS/FTD pathogenesis beyond correlations with TDP-43 pathology.

6.2. Insights into how DPRs are linked to nucleocytoplasmic transport

Several *C9orf72* models with deficits in nucleocytoplasmic transport have been studied so far, suggesting nucleocytoplasmic transport defects may be a fundamental pathway for ALS and FTD. It is important to highlight that the contribution of amyloid-like protein aggregates to neurodegenerative disorders may be through a common mechanism, such as impairment of nucleocytoplasmic shuttling. Notably, a recent study has shown cytoplasmic mislocalization of TDP-43 in motor neurons prevents DNA double-strand break (DSB) repair, and therefore contributes to neurodegeneration in sporadic ALS (Mitra, Guerrero et al. 2019). Moreover, recent evidence suggests impaired nucleocytoplasmic transport in other neurodegenerative diseases, such as Alzheimer's disease (caused by pathological Tau) (Eftekharzadeh, Daigle et al. 2018) and Huntington's disease (caused by mutant Huntingtin (mHTT82Q) or HD RAN translation proteins) (Grima, Daigle et al. 2017).

Unbiased genetic screenings in *Drosophila* expressing poly-PR (Boeynaems, Bogaert et al. 2016), yeast (*Saccharomyces cerevisiae*) (Jovicic, Mertens et al. 2015) and human cells together with primary neurons expressing poly-GR and poly-PR (Kramer, Haney et al. 2018) revealed several interactors in the nucleocytoplasmic transport machinery to alleviate or exacerbate the disease phenotypes. In addition, poly-GA in mice has been shown to sequester nucleocytoplasmic proteins, thereby leading to abnormal distribution of proteins (Zhang, Gendron et al. 2016), although this was not confirmed in our poly-GA mouse model (Schludi, Becker et al. 2017). These studies have shown sequestered and impaired nucleoporins that form nuclear pore complexes (NPCs), most importantly in regulators of Ran-GTP cycle, that provides the energy driving nuclear transport, such as RanGAP1 (Jovicic, Mertens et al. 2015, Zhang, Donnelly et al. 2015). RanGAP1, an essential regulator of nucleocytoplasmic shuttling, was shown to bind to the $(G_4C_2)_n$ RNA and presumably poly-GR, and formed intranuclear inclusions in HRE-expressing *Drosophila*, neurons from *C9orf72* ALS patient-derived induced pluripotent stem cells (iPSC-derived neurons), and in *C9orf72* ALS case post-mortem brains (Zhang, Donnelly et al. 2015). Importantly, in mice expressing high levels of poly-GA, nuclear and cytoplasmic puncta of mislocalized RanGAP1 was shown to co-localize with poly-GA inclusions (Zhang, Gendron et al. 2016). However in consistence with studies in GA_{149}-CFP mice (Schludi, Becker et al. 2017), *C9orf72* patients (Saberi, Stauffer et al. 2018), and *C9orf72*-patient neurons (Jovicic, Mertens et al. 2015), I did not observe any differences in RanGAP1 cellular localization between controls and GA expressing HeLa cells. These data suggest that proteasome inhibition by poly-GA is more important for TDP-43 mislocalization than direct effects on the NPC machinery.

These genetic screens were used to identify toxicity modifiers, such as aggregation-prone proteins like TDP-43 in neurodegenerative diseases. TDP-43 has been described to form cytoplasmic and nuclear inclusions that are found in the majority of ALS and FTD cases (Neumann, Sampathu et al. 2006). TDP-43 is primarily nuclear, however a small fraction of TDP-43 is found in the cytoplasm and has been shown to bind numerous target mRNAs (Tollervey, Curk et al. 2011). Moreover, in mice expressing high levels of poly-GA_{50}, rare TDP-43 inclusions have been observed due to impairment of nucleocytoplasmic transport proteins (Zhang, Gendron et al. 2016). Particularly, the cytoplasmic accumulation of mutant TDP-43 has been shown to be due to differential cleavage patterns through species. Mouse Caspase-11, unlike the primate homologue caspase-4, has been discovered to be unable to cleave TDP-43 NLS and cause cytoplasmic distribution of fragmented TDP-43 (Yin, Guo et al. 2019). I have shown in primary hippocampal neurons expressing GFP-tagged DPR proteins by using lentivirus, poly-GA expression leads to increased cytoplasmic TDP-43 granules compared to poly-GR and poly-PR. Cytoplasmic TDP-43 granules that I observed in primary neurons are indeed far smaller than aggregates seen in patients, therefore we suggest slight poly-GA dependent TDP-43 mislocalization could serve as a precursor for further aggregation.

Since the cytoplasmic receptor for TDP-43 is importin α, I therefore tested overexpression of members of karyopherin-α family (KPNA3, and 4) and essential factors of nuclear pore components (CSE1L/CAS, NUP54, NUP64) in HeLa cells and observed reduced cytoplasmic TDP-43 mislocalization (Khosravi, Hartmann et al. 2017). Moreover, a study in sporadic *C9orf72* ALS/FTD cases reported nuclear depletion and cytosolic accumulation of KPNA4, a member of importin α family, that is a cytoplasmic receptor for TDP-43 (Solomon, Stepto et al. 2018).

Taken altogether, there is strong evidence indicating nucleocytoplasmic transport is impaired in *C9orf72* ALS/FTD, leading to the sequestration of transport machinery and altered cytoplasmic RBP accumulation and aggregation, including TDP-43.

6.3. Non-cell-autonomous effects of poly-GA via UPS dysfunction may explain poor regional correlation between DPRs and TDP-43 pathology in patients

Previous studies from our group and others have addressed differences in DPR species attributions to clinicopathological subtypes of *C9orf72* cases (van Blitterswijk, DeJesus-Hernandez et al. 2013, Schludi, May et al. 2015). Schludi et al. has found poly-GA inclusions were more abundant in cerebellar granular cell layer, while poly-PR inclusions, despite being rare throughout the brain, were significantly more abundant in the hippocampus. These data suggest a differential translation, together with the possible role of spreading and seeding of DPRs which could play a role in neurodegeneration. Furthermore, several other studies have shown that poly-GR aggregates are correlated with neurodegeneration (Saberi, Stauffer et al. 2018, Sakae, Bieniek et al. 2018, Gittings, Boeynaems et al. 2020). In addition, only poly-GA aggregates have been observed to rarely co-accumulate with TDP-43 inclusions within the same cell (Mackenzie, Arzberger et al. 2013, Mori, Weng et al. 2013). Moreover, spreading of DPRs between cells, like tau, α-syn, and Htt (Costanzo and Zurzolo 2013, Jucker and Walker 2018), has been shown in several *in vitro* studies (Chang, Jeng et al. 2016, Westergard, Jensen et al. 2016, Zhou, Lehmer et al. 2017). Therefore, in **Research Article 2**, I tested the hypothesis that non-cell-autonomous effects of DPRs could play a role in TDP-43 pathology while studying implications on ubiquitin proteasome system (UPS) and nucleocytoplasmic transport.

I show that poly-GA inclusions partly sequester the proteasome function, which is in consistence with our work *in vitro* (Guo, Lehmer et al. 2018). Induced poly-GA dependent proteasome dysfunction furthermore affects TDP-43 localization in cells with and without visible poly-GA aggregates (Figure 2b). In addition, inhibiting or activating proteasomal activity by chemicals such as MG132 (benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal), and rolipram (Figure 2c) led to induced and reduced cytoplasmic mislocalization of endogenous TDP-43 or a reporter including TDP-NLS, respectively. Previously, in primary hippocampal and cortical neurons, as well as an immortalized motor neuron cell line NSC-34, chemically inhibiting the proteasome by MG-132 and lactacystin was also shown to significantly result in inducing cytoplasmic accumulation and aggregation of TDP-43 (van Eersel, Ke et al. 2011). Moreover, rolipram has been shown to attenuate taupathy by clearance of tau aggregates and improve cognition in early-stage taupathy in a mouse model (Myeku, Clelland et al. 2016). It is important to mention that rolipram, by inhibiting phosphodiesterase 4 (PDE4), increases cAMP levels and therefore activates cAMP-dependent protein kinase (PKA) which leads to phosphorylation of Rpn6/PSMD11, a subunit of the 19S regulatory complex. This eventually increases peptidase activity of 26S proteasome to further promote the degradation of short peptide substrates, ubiquitinated, and aggregation-prone proteins (Lokireddy, Kukushkin et al. 2015).

In addition, I showed genetic manipulations in order to further activate proteasome by overexpressing 26S proteasome non-ATPase regulatory subunit D11 (PSMD11), resulted in prevention of cytoplasmic TDP-43 mislocalization, too.

Overall, my data indicate poly-GA inclusions result in impairing proteasome activity and therefore trigger further TDP-43 pathology. This effect can be rescued by chemically or genetically activating proteasome. In addition, hindering poly-GA transmission by use of antibodies *in vitro* prevents cytoplasmic TDP-43 mislocalization (Figure 2d).

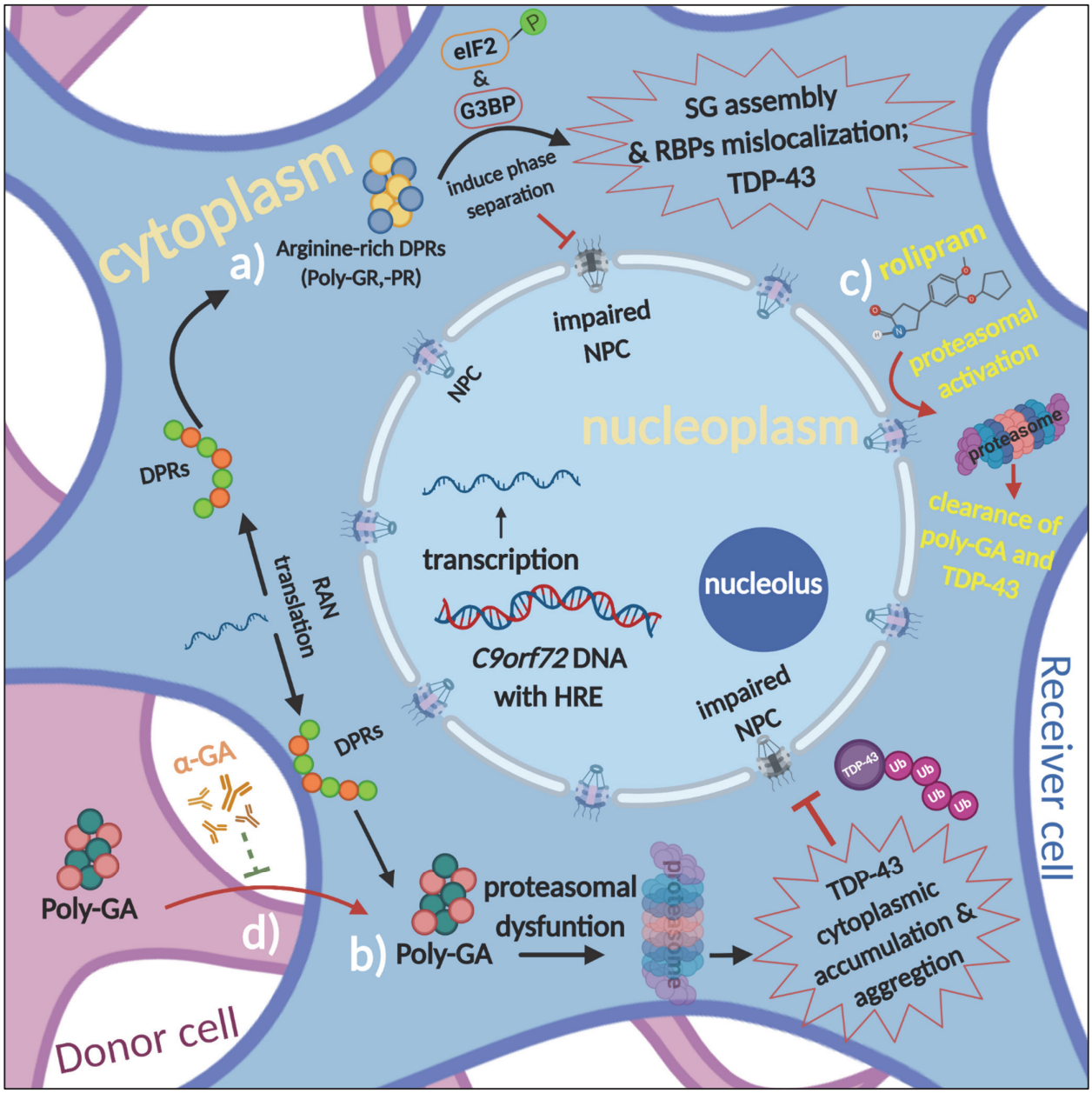


Figure2. Non-AUG translation of *C9orf72* RNA transcripts forms five different speciesof proteotoxic dipeptide repeat proteins (DPRs). DPRs accumulate in the brain and spinal cord and have been shown to contribute to disease pathogenesis. (a) Poly-GR and poly-PR promote phase-separation of RNA binding proteins (RBPs) and induce stress granule (SG) assembly depending on phosphorylation of eukaryotic translation initiation factor 2A (eIF2α) together with the presence of Ras GTPase-activating protein-binding protein 1 (G3BP) proteins. (b) Poly-GA promotes cytoplasmic mislocalization of TDP-43 via non-cell autonomous impairment of proteasome and ubiquitylation of TDP-43 within its nuclear localization signal (NLS). This results in accumulation and aggregation of cytoplasmic TDP-43. (c) Proteasome activation by chemicals such as rolipram leads to clearing poly-GA and TDP-43 pathology. (d) Anti-GA antibodies can block poly-GA transmission between cells and therefore result in diminished TDP-43 mislocalization.

6.4. Poly-GA-induced ubiquitination leads to inhibition of TDP-43 nuclear import

To answer the question on how proteasomal activation rescues nuclear import of TDP-43, I decided to investigate whether poly-GA expression inhibits import via ubiquitination within TDP-43 NLS. Previous proteome-wide mass spectrometry profiling studies have revealed lysine 84 and lysine 95 as ubiquitination sites within TDP-43 NLS (Kim, Bennett et al. 2011, Lumpkin, Gu et al. 2017, Akimov, Barrio-Hernandez et al. 2018).

I have shown that poly-GA leads to accumulation of TDP-43 poly-ubiquitinated within the bipartite NLS at lysine 95 (K95). Furthermore, I have shown that poly-ubiquitination likely inhibits importin-α binding, and mislocalized TDP-43 can be largely cleared by boosting proteasome activity. K95 mutations to alanine or arginine revealed reduced poly-GA dependent ubiquitination and rescued import deficits. Interestingly, I confirmed that poly-GA expression causes diminished binding of wild-type NLS but not K95A mutant to importin-α. Others have shown that the K95A mutant within TDP-43 NLS dramatically decreased Ser-409/410 phosphorylation, connecting TDP-43 ubiquitinylation and pathological phosphorylation (Hans, Eckert et al. 2018). Moreover, I have shown K95 mutations in both TDP-43 NLS reporter and full length TDP-43 prevent poly-GA dependent mislocalization of TDP-43 without affecting TDP-43 clearance. A similar impairment of nucleocytoplasmic transport by ubiquitination of NLS has been reported for tumor protein p53 (Marchenko, Hanel et al. 2010), and cytidylyltransferase (CCTα) (Chen and Mallampalli 2009).

Overall, my data suggest that proteasome inhibition leading to cytoplasmic accumulation of TDP-43 predominantly occurs at K95 within TDP-43 NLS.

6.5. Potential future therapies

My data from immunodepletion experiments indicated that anti-GA antibodies at least *in vitro* prevent the non-cell-autonomous effects of poly-GA on TDP-43 mislocalization by blocking poly-GA transmission. We cannot exclude the fact that poly-GA toxicity triggers additional effects such as secretion of other molecules that promote TDP-43 aggregation in neighboring cells or resulting in the release of such factors from glial cells. However, recent *in vivo* data from our group and others show that active and passive antibody therapy is beneficial in *C9orf72* mouse models (Nguyen, Montrasio et al. 2020, Zhou, Mareljic et al. 2020).

Our group has recently shown a safe active poly-GA vaccination with ovalbumin-$(GA)_{10}$ that can significantly decrease poly-GA inclusions together with improving motor function in a

C9orf72 mouse model. Importantly, immunized mice have shown lowered levels of cytoplasmic TDP-43 pathology (Zhou, Mareljic et al. 2020).

Moreover, human recombinant α-GA_1 antibody treatment extend survival of *C9orf72* BAC transgenic mice and reduced co-aggregation of proteasome and autophagy proteins to GA aggregates (Nguyen, Montrasio et al. 2020). These effects depend on the cytosolic Fc receptor tripartite motif 21 (TRIM21). Nguyen et al. showed that anti-GA antibodies are unable to clear poly-GA in heterozygous knockout TRIM21 cells ($TRIM21^{+/-}$), while in wild-type TRIM21 cells ($TRIM21^{+/+}$) anti-GA antibodies result in ~ 50% reduction of poly-GA and PSMC4, the 26S proteasome subunit ATPase 4/regulatory subunit 6B, co-localized inclusions.

In conclusion, this thesis project has provided mechanistic insights on how poly-GA causes TDP-43 pathology via proteasome inhibition and cell-to-cell transmission. I have shown that blocking poly-GA transmission by antibody treatment or chemically boosting proteasome function with rolipram alleviate poly-GA and TDP-43 pathology and may lead to future therapies for *C9orf72* patients.

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9. List of publications

2020

Khosravi B, LaClair KD, Riemenschneider H, Zhou Q, Frottin F, Mareljic N, Czuppa M, Farny D, Hartmann H, Michaelsen M, Arzberger T , Hartl FU, Hipp MS, Edbauer D. Cell-to-cell transmission of *C9orf72* poly-(Gly-Ala) triggers key features of ALS/FTD. The EMBO Journal 2020 Apr 15;39(8):e102811. (*IF 2018: 11.2*)

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Angaji SA, Aminian G, **Khosravi B**, Taran M. Serial Analysis of Gene Expression (SAGE): Its modification and application in human disease. International Journal of Biosciences 2014 Jul pg. 49-60. (*IF: not available*)

10. Affidavit

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation "**POLY-GA TRIGGERS TDP-43 PATHOLOGY BY INHIBITING THE PROTEASOME AND NUCLEOCYTOPLASMIC IMPORT IN *C9ORF72* ALS/FTD**" selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation "**POLY-GA TRIGGERS TDP-43 PATHOLOGY BY INHIBITING THE PROTEASOME AND NUCLEOCYTOPLASMIC IMPORT IN *C9ORF72* ALS/FTD**" is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, 26.11.2020

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Bahram Khosravi

11. Declaration of author contributions

Authors contributed to the manuscripts are as follows:

Research article 1:

Khosravi B*, Hartmann H*, May S, Möhl C, Ederle H, Michaelsen M, Schludi MH, Dormann D, Edbauer D. **Cytoplasmic poly-GA aggregates impair nuclear import of TDP-43 in *C9orf72* ALS/FTLD.** BK and HH performed cell biological and biochemical experiments, image acquisition and data analysis, generated reagents, and wrote parts of the manuscript. SM, HE, MM, MHC helped with experimental design and provided assistance with experiments. CM developed algorithms for data analysis. DD supervised research and contributed to writing. DE designed the study, supervised research, and wrote the manuscript. All authors discussed the data and the manuscript and edited the paper.

Research article 2:

Khosravi B, LaClair KD, Riemenschneider H, Zhou Q, Frottin F, Mareljic N, Czuppa M, Farny D, Hartmann H, Michaelsen M, Arzberger T , Hartl FU, Hipp MS, Edbauer D. **Cell-to-cell transmission of *C9orf72* poly-(Gly-Ala) triggers key features of ALS/FTD. BK performed most cell biological and biochemical experiments.** KDL, HH, and TA provided and analyzed human samples. QZ, NM, and MM provided and analyzed mouse samples. HR performed qPCR analysis. FF and MSH performed flow cytometry analysis. BK, DF, and HR generated reagents. MC performed immunoassays. FUH and DE acquired funding. FUH and MSH supervised research and contributed to writing. DE designed the study, supervised research, and wrote the manuscript. All authors discussed the data and the manuscript and edited the paper.

Herewith, I confirm the contributions to the manuscripts.

München, 26.11.2020

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Bahram Khosravi	Prof. Dr. Dieter Edbauer (supervisor)	Hannelore Hartmann (Shared co-first author)