Farber Institute for Neuroscience Faculty Papers

Farber Institute for Neuroscience

10-11-2016

Cell-to-Cell Transmission of Dipeptide Repeat Proteins Linked to C9orf72-ALS/FTD.

Thomas Westergard
Jefferson Weinberg ALS Center, Vickie and Jack Farber Institute for Neuroscience, Department of Neuroscience, Thomas Jefferson University

Brigid K Jensen
Jefferson Weinberg ALS Center, Vickie and Jack Farber Institute for Neuroscience, Department of Neuroscience, Thomas Jefferson University

Xinmei Wen
Jefferson Weinberg ALS Center, Vickie and Jack Farber Institute for Neuroscience, Department of Neuroscience, Thomas Jefferson University

Jingli Cai
Farber Institute for Neurosciences, Thomas Jefferson University

Elizabeth Kropf
JM Cell Center, Vickie and Jack Farber Institute for Neuroscience, Department of Neuroscience, Thomas Jefferson University

Follow this and additional works at: https://jdc.jefferson.edu/farberneursofp

Part of the Other Medical Sciences Commons

Let us know how access to this document benefits you

See next page for additional authors

Recommended Citation

Westergard, Thomas; Jensen, Brigid K; Wen, Xinmei; Cai, Jingli; Kropf, Elizabeth; Iacovitti, Lorraine; Pasinelli, Piera; and Trotti, Davide, "Cell-to-Cell Transmission of Dipeptide Repeat Proteins Linked to C9orf72-ALS/FTD." (2016). *Farber Institute for Neuroscience Faculty Papers.* Paper 27.
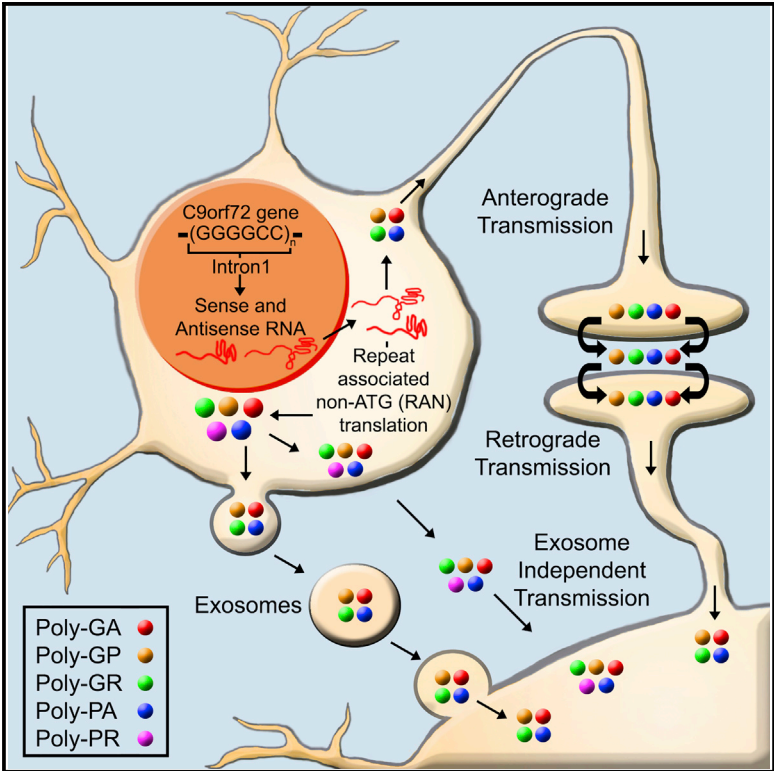https://jdc.jefferson.edu/farberneursofp/27

Authors

Thomas Westergard, Brigid K Jensen, Xinmei Wen, Jingli Cai, Elizabeth Kropf, Lorraine Iacovitti, Piera Pasinelli, and Davide Trotti

Cell Reports

Cell-to-Cell Transmission of Dipeptide Repeat Proteins Linked to *C9orf72*-ALS/FTD

Graphical Abstract

Authors

Thomas Westergard, Brigid K. Jensen, Xinmei Wen, ..., Lorraine Iacovitti, Piera Pasinelli, Davide Trotti

Correspondence

davide.trotti@jefferson.edu

In Brief

Westergard et al. examine the cell-to-cell spread of dipeptide repeat proteins related to C9orf72-ALS/FTD in vitro and in animal models. They suggest that transcellular transmission may explain the clustered expression pattern seen in human post-mortem CNS areas, as well as the progressive neurodegeneration of these diseases.

Highlights

- Cell-to-cell transmission of DPRs is evident in vitro

- C9orf72-ALS patient-derived spinal motor neurons exhibit DPR transmission

- Exosomes containing DPRs are one modality for transmission

- Exosome-independent transmission is the primary mechanism for poly(PR) spread

CrossMark

CellPress

Cell-to-Cell Transmission of Dipeptide Repeat Proteins Linked to *C9orf72*-ALS/FTD

Thomas Westergard,[1] Brigid K. Jensen,[1] Xinmei Wen,[1] Jingli Cai,[2] Elizabeth Kropf,[2] Lorraine Iacovitti,[2] Piera Pasinelli,[1] and Davide Trotti[1,3,*]

[1]Jefferson Weinberg ALS Center, Vickie and Jack Farber Institute for Neuroscience, Department of Neuroscience, Thomas Jefferson University, 900 Walnut Street, Philadelphia, PA 19107, USA
[2]Stem Cell Center, Vickie and Jack Farber Institute for Neuroscience, Department of Neuroscience, Thomas Jefferson University, 900 Walnut Street, Philadelphia, PA 19107, USA
[3]Lead Contact
*Correspondence: davide.trotti@jefferson.edu
http://dx.doi.org/10.1016/j.celrep.2016.09.032

SUMMARY

Aberrant hexanucleotide repeat expansions in *C9orf72* are the most common genetic change underlying amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). RNA transcripts containing these expansions undergo repeat-associated non-ATG translation (RAN-T) to form five dipeptide repeat proteins (DPRs). DPRs are found as aggregates throughout the CNS of C9orf72-ALS/FTD patients, and some cause degeneration when expressed in vitro in neuronal cultures and in vivo in animal models. The spread of characteristic disease-related proteins drives the progression of pathology in many neurodegenerative diseases. While DPR toxic mechanisms continue to be investigated, the potential for DPRs to spread has yet to be determined. Using different experimental cell culture platforms, including spinal motor neurons derived from induced pluripotent stem cells from C9orf72-ALS patients, we found evidence for cell-to-cell spreading of DPRs via exosome-dependent and exosome-independent pathways, which may be relevant to disease.

INTRODUCTION

Abnormal intronic hexanucleotide (GGGGCC/CCCCGG) repeat expansions (HREs) in the *C9orf72* gene are the most common genetic cause for both amyotrophic lateral sclerosis (ALS), a motor neuron degenerative disease, and frontotemporal dementia (FTD), a form of dementia characterized by selective deterioration of the frontal and temporal lobes (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The HREs result in three potential pathogenic hallmarks of disease. First, decreased C9orf72 mRNA expression levels in patients suggest a loss-of-function mechanism (Ciura et al., 2013; Therrien et al., 2013). Second, RNA transcripts from the HREs potentially gain a toxic function by sequestering RNA-binding proteins in foci (Gendron et al., 2014) and/or inhibiting transcription through the formation of

RNA-DNA hybrids (Gitler and Tsuiji, 2016). Lastly, both sense and antisense RNA transcripts can undergo non-canonical repeat-associated non-ATG translation (RAN-T), generating five potentially toxic dipeptide repeat protein species (DPRs): poly(glycine-alanine) [poly(GA)], poly(glycine-proline) [poly(GP)], poly(glycine-arginine) [poly(GR)], poly(proline-alanine) [poly(PA)], and poly(proline-arginine) [poly(PR)] (Gitler and Tsuiji, 2016).

DPR inclusions were reported in different CNS areas of C9orf72-ALS/FTD patients (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013a, 2013b; Zu et al., 2013). In addition, pervasive DPR pathology is found during presentation of initial symptoms of disease, preceding onset of pathology such as TDP-43 inclusions (Baborie et al., 2015; Proudfoot et al., 2014). DPRs alter cellular functions and induce toxicity in different ways in various models. In primary neurons and fly models, the arginine-rich DPRs display the highest toxicity. Poly(GR), which localizes in the cytoplasm and aggregates in the nucleus, and poly(PR), which exclusively aggregates in the nucleus, trigger nucleolar stress, nuclear transport defects, RNA processing alterations, and protein mislocalization (Gitler and Tsuiji, 2016). Poly(GA) is also toxic through proteasome impairment, aggregation of Unc119, and impairment of HR23 and nucleocytoplasmic transport proteins (May et al., 2014; Zhang et al., 2014, 2016). In contrast, marginal and no toxicity have been associated with poly(GP) and poly(PA), respectively (Wen et al., 2014; Zu et al., 2013). These findings have solidified the importance of DPR pathology in C9orf72-ALS/FTD.

The progression of many neurodegenerative diseases, including ALS, is thought to be driven by cell-to-cell transmission of disease-related proteins, which leads to seeded aggregation and template-directed misfolding. A growing body of evidence has uncovered a propensity for disease-relevant proteins such as poly-glutamine, α-synuclein, β-amyloid, SOD1, and TDP-43 to be transmitted from cell to cell and to seed template nucleation (Feiler et al., 2015; Gallegos et al., 2015; Kanouchi et al., 2012; Nath et al., 2012; Ren et al., 2009; Silverman et al., 2016). Mechanisms of transmission involve secretion of exosomes (Bellingham et al., 2012; Danzer et al., 2012; Pant et al., 2012; Saman et al., 2012), tunneling nanotubes, hemichannels between cells, exocytosis and endocytosis of proteins, and phagocytosis of infected cells or cellular debris (Costanzo and Zurzolo, 2013; Gallegos et al., 2015). These
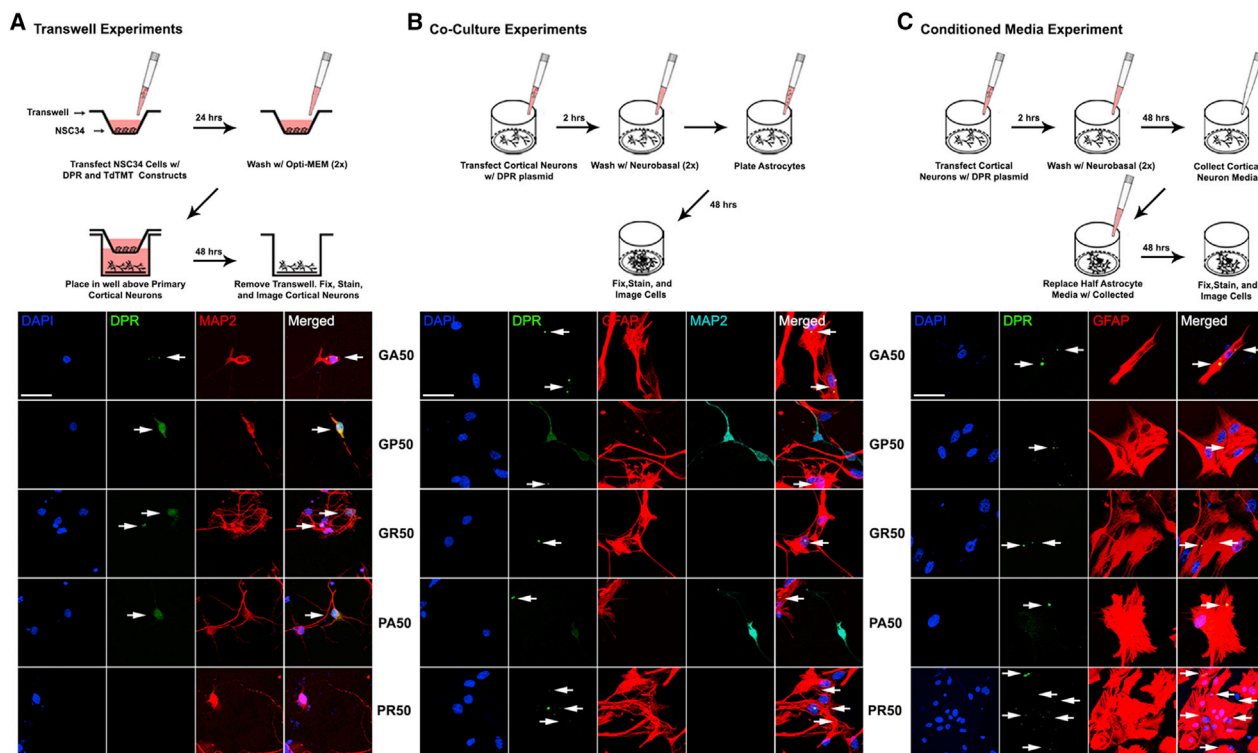
Figure 1. In Vitro Transmission of DPRs from NSC34 Cells to Cortical Neurons and from Cortical Neurons to Astrocytes
(A–C) Schematics of experimental workflow.
(A) Bottom: transfected NSC34 cells transmitted DPRs to cortical neurons in a transwell system. All DPR species were detected in cortical neurons besides poly(PR). Nuclei were stained with DAPI (blue), green represents DPRs, and red represents MAP2⁺ neurons.
(B) Bottom: representative confocal images show transmission of all DPR species (GFP tagged) from cortical neurons to astrocytes. Cells were stained with DAPI (blue for nuclei), DPRs (GFP, shown in green), GFAP (red), and MAP2 (cyan). There was negligible MAP2 staining in GA, GR, and PR groups, suggestive of extensive neuronal death.
(C) Bottom: cells were stained with DAPI (blue for nuclei), DPRs (GFP, shown in green), and GFAP (red) for astrocytes. Small or low fluorescent aggregates are marked by arrows. Scale bars, 50 μm.

protein-spreading modalities are commonly interpreted as a mechanism underlying the progressive nature of many neurodegenerative diseases.

Although progress is being made on mechanisms behind DPR toxicity, their potential to transmit between cells is untested thus far. The potential for DPR transmission was hinted at for a low-repeat-length, synthetic poly(GA) using N2a neuroblastoma cells (Chang et al., 2016). Poly(GP) has also been detected in patients' cerebrospinal fluid (CSF), suggesting active secretion (Su et al., 2014). In addition, neuropathological analysis of C9orf72-ALS/FTD autopsy brains demonstrated two types of aggregation and localization patterns for cells containing DPRs: high-density clusters and isolated cells (Zu et al., 2013). This not only suggests specific foci in which RAN-T occurs but also raises the hypothesis that DPRs produced in these foci could spread to neighboring areas. Furthermore, DPRs are detected as insoluble aggregates in human tissue, and most form aggregates in vitro, a common pathological hallmark of disease-relevant proteins found to spread (Brettschneider et al., 2015). Using live-cell microscopy, we observed that toxic poly(PR) aggregates persist in the dish long after the expressing neurons broke up and died (Wen

et al., 2014), again suggesting that these aggregates might be taken up by other cells. Based on these lines of evidence, we hypothesized that DPRs could undergo cell-to-cell transmission between CNS-resident cell types. Using a variety of cell culture platforms, we report here evidence for exosome-dependent and exosome-independent cell-to-cell transmission for all C9-DPRs.

RESULTS

DPRs Can Be Transmitted to Neurons and Glia Cells
To test in vitro the hypothesis that DPRs are transmissible, we used constructs encoding GFP-tagged poly(GA)$_{50}$, poly(GP)$_{50}$, poly(GR)$_{50}$, poly(PA)$_{50}$, and poly(PR)$_{50}$ dipeptides (Wen et al., 2014). Our first approach was to use motor neuron-like NSC34 cells cultured in transwells on a mesh surface (0.4 μm pores) (Figure 1A) and transfected with GFP or GFP-DPR encoding constructs. NSC34 cells were extensively washed twenty-four hr later and the transwells were placed into wells plated with mature primary cortical neurons (DIV10). Forty-eight hr later, confocal microscopy analysis revealed the presence of DPRs in cortical

neurons, suggesting that transmission occurred. Staining was absent from the GFP-only encoding group, indicating that the acquisition of DPRs was not due to unintended transfection from residual construct particles (Figure S1A). GA showed distinct cytoplasmic aggregates in recipient cortical neurons (~7% of total cortical neurons), whereas GR nuclear and cytoplasmic aggregates, in addition to diffuse localization, were detected in 6% of cortical neurons. GP and PA displayed both diffuse localization and cytoplasmic aggregates in recipient cortical neurons (~3% of total cortical neurons). There was no significant evidence of PR transmission to cortical neurons (Figure S1D).

It was reported that neurodegenerative disease-relevant proteins, such as TDP-43, propagate to both neuron and glial cells (Brettschneider et al., 2015). In addition, DPRs have been observed in ependymal and subependymal glial cells (Schludi et al., 2015). Seeking additional evidence of DPR transmission, we tested whether transmission of the different DPRs could occur between neurons and glial cells. Cortical neurons were transfected with GFP or GFP-DPR constructs and thoroughly washed 24 hr later when untreated primary astrocytes were seeded in the culture plate. Confocal microscopy analysis performed 48 hr later revealed DPR localization within astrocytes (Figure 1B), with absent GFP-only staining in astrocytes (Figure S1B). Quantification of DPR transmission revealed that in the co-culture cell-contact model, the percentage of cell-containing DPRs was ~2-fold higher compared to that in the transwell experiments (Figure S1E). GA presented as cytoplasmic aggregates, whereas GR and PR displayed as both cytoplasmic and nuclear aggregates. GP and PA exhibited both cytoplasmic aggregates and diffuse localization in astrocytes.

Using a different experimental approach, conditioned medium (CM) from GFP or GFP-DPR expressing cortical neurons was transferred to cultured astrocytes. Neuron-to-astrocyte transmission was again observed, with similar DPR localization patterns in the recipient cells as reported in co-cultures (Figure 1C) and absent GFP-only transmission (Figure S1C). However, quantification of transmission was lower (with 6%–8% of astrocytes displaying GA-, GR-, and PR-positive aggregates and ~4% of astrocytes displaying cytoplasmic aggregates and diffuse localization of GP and PA), as reported in the co-culture cell-contact model, but it was similar to the transwell model (Figure S1F). DPR transmission via CM transfer also occurred to other glia cell types, such as microglia and mature oligodendrocytes (Figure S2). These observations provide further evidence for DPR transmission between CNS cell types.

Anterograde and Retrograde Transmission of DPRs
DPR aggregates are found throughout different CNS areas in high-density clusters and isolated cells (Zu et al., 2013). In a compartmentalized microfluidic culture system, both anterograde transmission and retrograde transmission of TDP-43 have been demonstrated. This could explain the complex variable distribution of TDP-43 pathology in axonally connected distant brain regions (Feiler et al., 2015). We used a similar in vitro approach to test whether DPRs can potentially spread between neuronal networks. Cortical neurons were cultured in one of the microfluidic chambers (chamber 1), and their neurites

were made to extend across microgrooves to contact cortical neurons cultured in the opposite chamber (chamber 2) (Figure 2A). Axon projection directionality (chamber 1 to chamber 2) was driven by applying higher hydrostatic pressure in chamber 1. Once the microfluidic culture was established, higher hydrostatic pressure was applied to chamber 2 to impede anterograde transfer of solution and material, and the projecting cortical neurons in chamber 1 were transfected with DPR-encoding plasmids. Forty-eight hr post-transfection, DPRs were detected in cortical neurons of chamber 2 (Figure 2B), indicating anterograde transfer of DPRs had occurred via the connecting neurites. GA-, GP-, and PA-containing neurons displayed cytoplasmic aggregates, while GR-containing neurons had both cytoplasmic and nuclear aggregates. We found no evidence for PR transmission, consistent with previous evidence with the transwell platform. Retrograde transmission was observed using a similar setup but with the absence of cortical neurons in chamber 2 (Figure 2C). Upon establishment of the microfluidic culture, CM from DPR-expressing NSC34 cells was transferred into chamber 2 while applying higher hydrostatic pressure in chamber 1 to block retrograde transfer of solution and material. Forty-eight hr later, all DPRs except PR were detected in cortical neurons in chamber 1 (Figure 2D).

Exosome-Dependent Transmission of DPRs
Cell-to-cell transmission of proteins may occur via different modalities, such as regulated secretion by exocytosis or release from dying cells (Costanzo and Zurzolo, 2013). Packaging and release of microvesicles known as exosomes were implicated as a primary mode of transmission of proteins relevant in neurodegenerative disease (Bellingham et al., 2012; Danzer et al., 2012; Saman et al., 2012; Silverman et al., 2016). We demonstrated in the transwell and microfluidic systems that direct cell contact was not required for inter-cellular spreading of DPRs, suggesting that neuronal cells are also capable of releasing DPRs. To determine whether cell-contact-independent DPR transmission implicates release of DPR-containing exosomes, NSC34 cells were first transfected with DPR- or GFP-encoding constructs, and then 48 hr later, exosome particles were isolated and purified from the culture medium. Purity of the exosomal fraction was assessed by the enrichment of specific exosomal markers and absence of other vesicular types, such as those derived from the Golgi (GM130), ER (calnexin), and mitochondria (cytochrome C) (Figure 3A; Figure S3). The presence of DPRs in exosomes was assessed by dot-blot analysis (Figure 3A; Figure S3). DPR immunopositive signals relative to markers of exosomes (i.e., flotillin, TSG101, and CD63) (Bellingham et al., 2012) revealed enrichment of GA, intermediate levels of PA and GR, low levels of GP, and very low to undetectable levels of PR.

To verify the transmission potential of DPR-containing exosomes, primary cortical neurons were incubated with DPR-enriched exosomal fractions isolated from the CM of transfected NSC34 cells (Figure 3B). For comparison, a separate group of cortical neurons was incubated with unprocessed CM. In both conditions, GA, GP, GR, and PA were present with varying rates in the cortical neurons, primarily as cytoplasmic aggregates, indicating that neuronal uptake had occurred (Figure 3C; Figures S3B and S3C). Significant transmission of PR was only observed
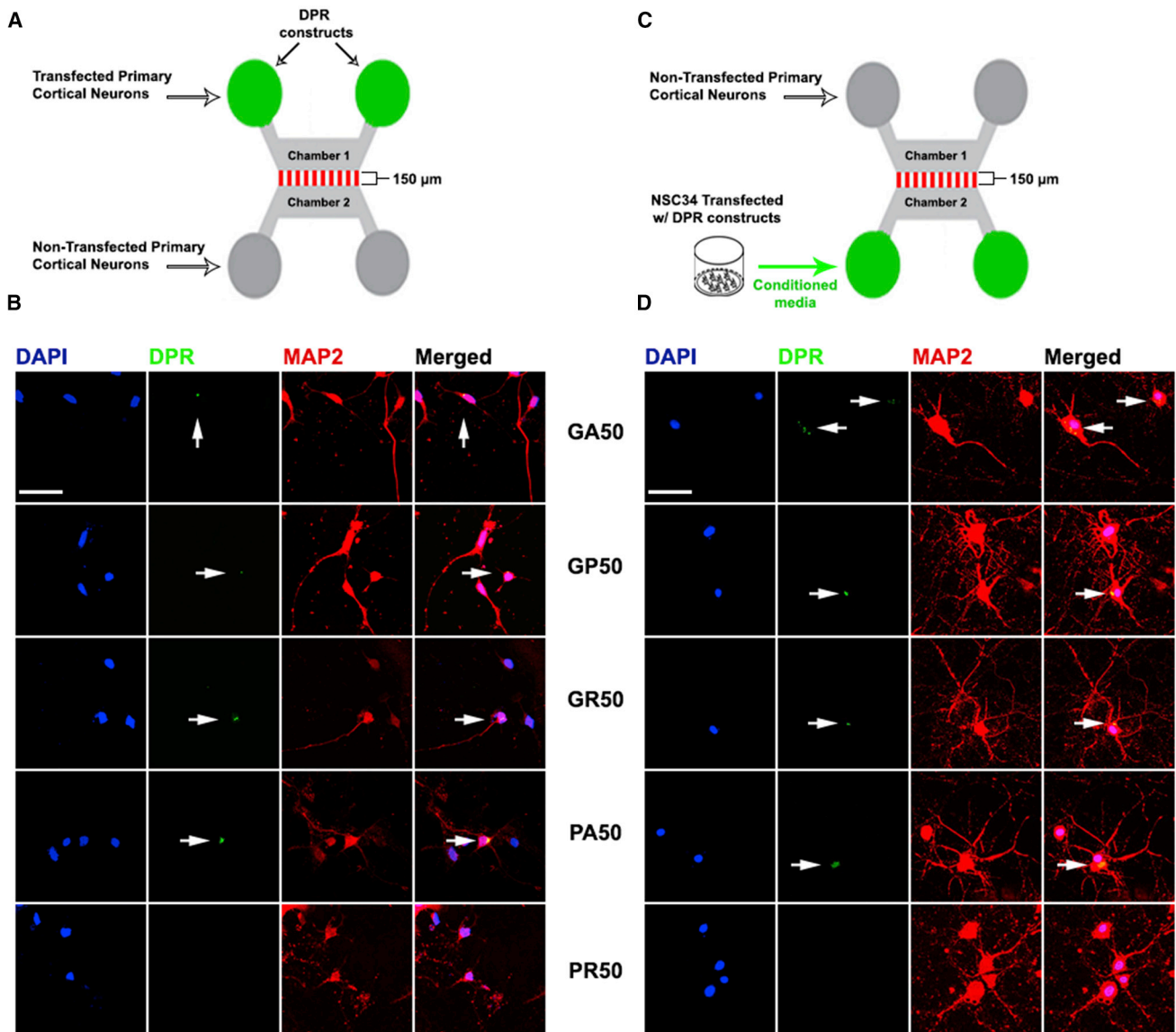
Figure 2. Anterograde and Retrograde Transmission of DPRs
(A and C) Schematics of the experimental design.
(B) Transfected cortical neurons in chamber 1 transmitted DPRs to cortical neurons in chamber 2.
(D) CM of DPR-expressing NSC34 cells added to chamber 2 transmitted DPRs to cortical neurons in chamber 1.
All DPRs were detected in cortical neurons, except for PR. DAPI (blue), DPRs (green), and MAP2 (red) are shown. DPR aggregates are marked by arrows. Scale bars, 50 μm.

from crude CM exposure, whereas incubation of neurons with the respective exosomal fraction showed no significant PR transmission.

Human iPSC-Derived sMNs Exhibit Cell-to-Cell Transmission of DPRs

To establish whether DPR transmission also occurred in human neurons, the co-culture and CM experimental designs previously described were implemented by using human induced pluripotent stem cell (iPSC)-derived spinal motor neurons (sMNs). C9orf72 (two different iPSC lines, Target ALS) and control (one iPSC line,

Wicell) iPSC lines were differentiated into sMNs using a modified version of a previously described protocol (Figure S4) (Maury et al., 2015). Most differentiated neurons are ChAT and Hb9 positive. Control sMNs were transduced with a lentivirus expressing GFP under a cytomegalovirus (CMV) promoter for identification. We found evidence of expression of the sense strand DPRs (GA, GR, and GP) in C9orf72 sMNs (Figures 4Aa, 4Ac, and 4Ae). Expression of anti-sense DPRs (PA and PR) in sMNs was not detected under our culturing condition; hence, we focused our analysis on GA, GR, and GP. In the co-culture system, C9orf72 sMNs were co-cultured with GFP-expressing control sMNs and further
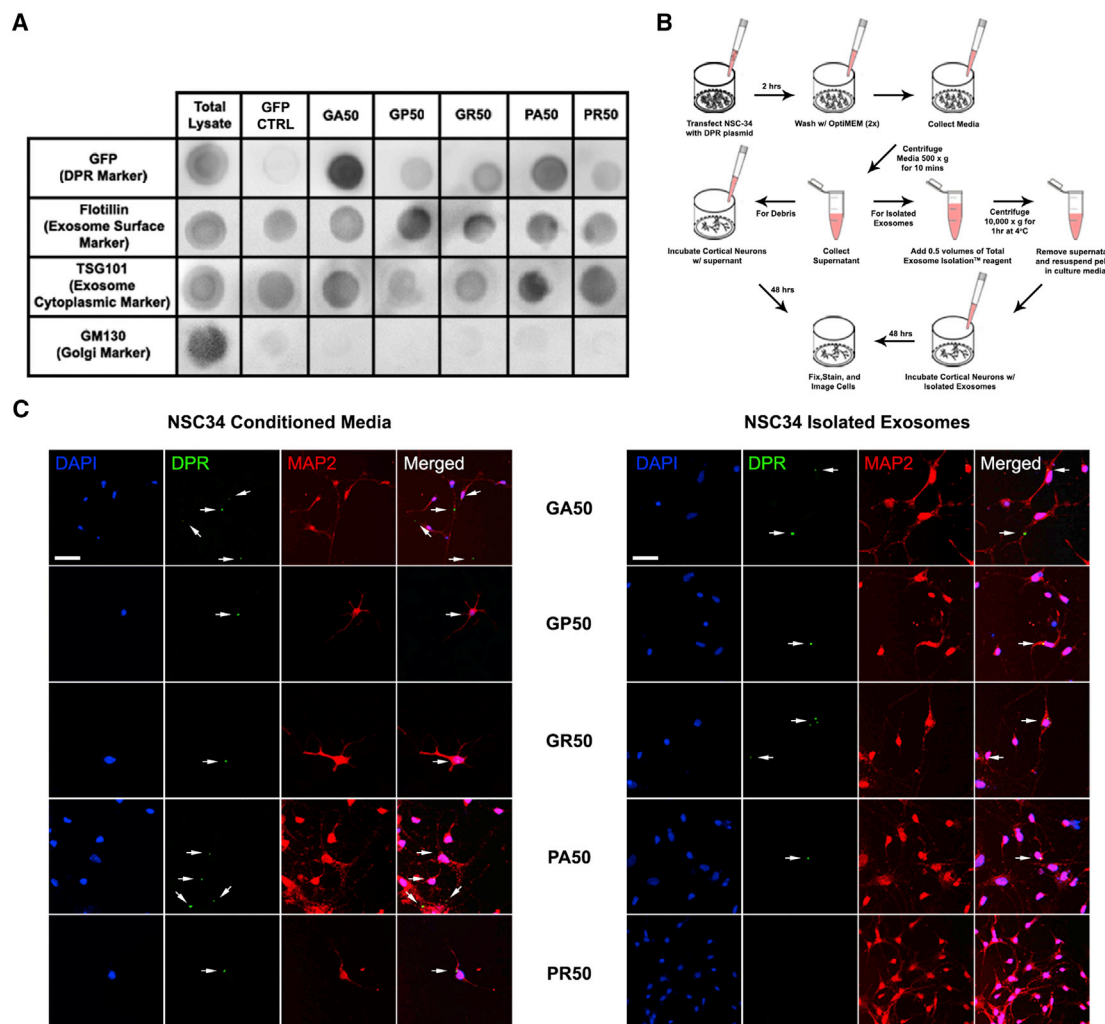
Figure 3. Exosome-Dependent and Exosome-Independent Transmission of DPRs

(A) Representative dot blot of the exosomal fraction isolated from CMs of NSC34 cells transfected with different DPR-encoding constructs. DPRs were detected by GFP immunostaining. Flotillin and TSG101 were used as markers of the exosomal fraction. GM130 was used as marker of other membrane vesicle types.

(B) Experimental workflow to compare DPR transmission from the exosomal fraction and total CMs.

(C) Left: representative confocal images show transmission of DPRs to cortical neurons through CMs. Right: representative images show transmission of DPRs, except PR, to cortical neurons through isolated exosomes.

DAPI (blue), DPRs (green), and MAP2 (red) are shown. DPR aggregates are marked by arrows. Scale bars, 50 μm.

matured to day 22. Staining for the sense strand DPRs, which have a higher concentration in vivo, revealed the presence of GA and GR aggregates in GFP-labeled sMNs (control motor neurons normally negative for their presence), indicating transmission has occurred from the C9orf72 sMNs (Figures 4Ab, 4Ad, and 4Af). No detectable transmission occurred in the case of GP. Similarly, C9orf72 sMN CM applied to GFP-expressing control sMNs revealed the presence of GA and GR aggregates, but the absence of GP staining, in control sMNs (Figure 4B).

DISCUSSION

The tendency for disease-relevant proteins to spread across the CNS has begun to be reported in neurodegenerative diseases

(Costanzo and Zurzolo, 2013), shaping our understanding of their progressive nature. In ALS, TDP-43 and SOD1 spread inter-cellularly and promote seeding activity, which leads to the misfolding of native protein species within the recipient cells (Feiler et al., 2015; Kanouchi et al., 2012; Silverman et al., 2016). C9-RAN-T DPRs are key pathogenic mediators of C9orf72-linked ALS/FTD (Gitler and Tsuiji, 2016). In patients, DPRs have been reported throughout the CNS, including the spinal cord, frontal cortex, motor cortex, hippocampus, and cerebellum (Schipper et al., 2015). Studies show toxicity and cellular dysfunctions resulting from some of these DPRs; however, their ability to transmit between cells has yet to be described. Neuropathological analysis of post-mortem tissues from C9orf72-ALS patients showed that the expression of DPR aggregates has a
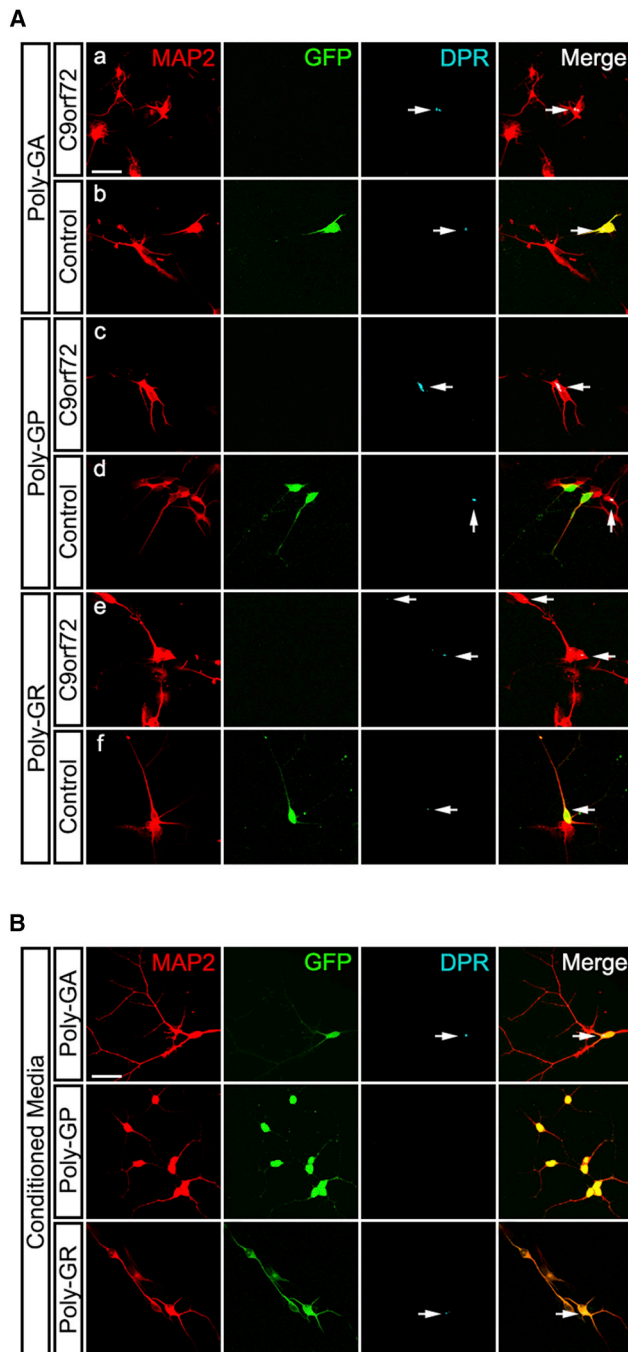
A

B

Figure 4. DPRs Transmit in iPSC-Derived sMNs
(A) Representative confocal images show transmission of sense DPR species in co-cultures of control and C9orf72 iPSC-derived sMNs. DPR production was verified in C9orf72 sMNs (a, c, and e). Both C9orf72 iPSC lines provide similar results. Control sMNs, identified by GFP (green), showed the presence of GA, GR, and GP, which are not normally present (b, d, and f). MAP2 (red) and DPRs (cyan) are shown.
(B) Representative confocal images show transmission of sense DPR species through CMs of C9orf72 sMNs. Green represents transduced control sMNs, red represents MAP2+ neurons, and cyan represents DPRs.
Scale bars, 50 μm.

distinct clustered pattern (Zu et al., 2013). This might suggest not only the existence of focal areas in which RAN-T occurs but also that, once made, DPRs can progressively pollute adjacent cells and spread. Here, we have demonstrated intercellular transmittance of DPRs by employing various in vitro cell culture platforms and have gained initial insights into possible modalities of this process.

We first explored DPR transmission between neurons and from neuron to glia. All DPR species transmitted from cell to cell, with disparate localization patterns and frequency of transmission. When NSC34 cells are transfected with equal amounts of cDNA, the arginine-rich DPRs are normally less efficiently expressed than the other DPRs (Wen et al., 2014). This lesser expression is likely due to the arginine-rich DPR effect on protein translation (Gitler and Tsuiji, 2016). Therefore, quantification of frequency of transmission among different DPRs does not accurately reflect differences in transmission efficiency intrinsic to the different DPR species, but it is meant only to compare transmission efficiency among different experimental paradigms. In the cases of GA, GR, and PR co-culture experiments, when astrocytes were assessed for DPR presence, there were virtually no transfected and were sparse non-transfected neurons remaining on the dish. Although circumstantial, this evidence suggests the establishment of a possible toxic environment produced as a result of changes in DPR-receiving astrocytes. Nevertheless, this evidence does not fully support the notion that transmission of DPRs from one cell to another is equivalent to propagation of pathology in vivo, which likely requires template nucleation. More follow-up experiments will be needed to establish a cause-effect relationship in the DPR transmission process. Furthermore, retrograde transmission and anterograde transmission were observed for most DPRs, suggesting spread between connecting neurons in different CNS regions.

Ectopic expression of DPRs by lipofection of cells in culture can lead to their overabundance, potentially forcing a transmission event that otherwise would not occur. However, the pathophysiological relevance of this process was confirmed by the observation of transmission of DPRs in iPSC-derived sMNs from C9orf72 patients, which endogenously produce DPRs.

Cell-to-cell transmission can occur through various mechanisms, such as phagocytosis, endocytosis, and exocytosis, tunneling nanotubes, hemichannels, or microvesicle release (Costanzo and Zurzolo, 2013). Direct cell-to-cell interaction was not a requirement for DPR transmission, though cellular contact was sufficient and did enhance transmission of some DPR species. We explored transmission through release of DPRs using transwell and microfluidic culturing systems. In both, DPR transmission to cortical neurons was observed except for PR, which surprisingly displayed rare to absent transmission. In addition, anterograde transmission and retrograde transmission seen in microfluidic platforms suggest that it can occur through axon terminals, similarly to TDP-43 (Feiler et al., 2015). In these systems, GP and PA displayed cytoplasmic aggregates in the receiving neurons. In vitro, GP and PA display diffuse localization (Wen et al., 2014). However, GP and PA are present in aggregates in C9orf72-ALS/FTD human tissue (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013a, 2013b; Zu et al., 2013). This raises the possibility that GP and PA could

undergo transmission in vivo and have a different toxic profile than their diffuse in vitro counterparts.

Exosomes have been implicated as a primary mode for cell-to-cell transmission in many neurodegenerative diseases (Danzer et al., 2012; Saman et al., 2012; Silverman et al., 2016). Exosomes are vesicles (50–150 nm) released by most mammalian cells that contain an assortment of macromolecules such as mRNAs, microRNAs (miRNAs), bioactive lipids, and proteins and play a role in health and disease (Pant et al., 2012; Raposo and Stoorvogel, 2013). We found that exosomes have the capability to incorporate DPRs, although PR accumulation was negligible compared to the others. Consequently, isolated exosomes transmit DPRs to cortical neurons. PR was primarily transmitted in an exosome-independent manner, likely because of its strong nuclear localization propensity and highly toxic profile. However, the role and modalities of an exosome-independent transmission for PR need to be fully explored.

Although the precise mechanisms involved in release and cellular uptake of DPRs still need to be investigated, we have begun to establish the involvement of exosomes in DPR transmission. Important questions are being investigated. For example, can DPRs transfer across synapses in vivo, and can this account for the propagation of pathology along neural networks? As current studies are extended and augmented by new findings, it would seem that stopping the spread of DPRs, and other disease-relevant proteins, could provide an interesting and novel therapeutic target. Because DPRs initiate a cascade of toxic cellular dysfunctions, preventing this cell-to-cell transmission may salvage remaining healthy cells and halt disease progression.

EXPERIMENTAL PROCEDURES

Microfluidic Chambers

Two-chamber microfluidic devices were assembled using Colorfrost Plus Slides as a support platform. Slides were UV sterilized, coated with poly-D-lysine, and placed into secondary containment dishes. Following a rinse with 70% ethanol, Standard Neuron Device (150 μm microgroove barrier; Xona Microfluidic cat. SND150) was applied to the slides. Then, 300 μL of neuronal growth medium was added to each set of plating wells connected by a channel, observing by eye that the channel became filled with media. Primary rat cortical neurons (20,000 cells/150 μL) were seeded into each well. The following day, total growth medium was increased to 500 or 250 μL to establish hydrostatic pressure differential in the two chambers. This ensured unidirectional growth through the microgrooves during neurite and axon extension. At the time of treatment, hydrostatic pressure was reverted to prevent cross-over of transfection reagents.

Exosome Isolation

Exosomes from NSC34 cells were prepared using the Total Exosome Isolation kit (Thermo Fisher Scientific #4478359) or through ultracentrifugation, as described previously (Théry et al., 2006). Both techniques were used initially to ensure the highest and a purer yield of exosomes. There was no significant difference between the yields, as determined via dot blot.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.032.

REFERENCES

Ash, P.E., Bieniek, K.F., Gendron, T.F., Caulfield, T., Lin, W.L., Dejesus-Hernandez, M., van Blitterswijk, M.M., Jansen-West, K., Paul, J.W., 3rd, Rademakers, R., et al. (2013). Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. Neuron 77, 639–646.

Baborie, A., Griffiths, T.D., Jaros, E., Perry, R., McKeith, I.G., Burn, D.J., Masuda-Suzukake, M., Hasegawa, M., Rollinson, S., Pickering-Brown, S., et al. (2015). Accumulation of dipeptide repeat proteins predates that of TDP-43 in frontotemporal lobar degeneration associated with hexanucleotide repeat expansions in C9ORF72 gene. Neuropathol. Appl. Neurobiol. 41, 601–612.

Bellingham, S.A., Guo, B.B., Coleman, B.M., and Hill, A.F. (2012). Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? Front. Physiol. 3, 124.

Brettschneider, J., Del Tredici, K., Lee, V.M., and Trojanowski, J.Q. (2015). Spreading of pathology in neurodegenerative diseases: a focus on human studies. Nat. Rev. Neurosci. 16, 109–120.

Chang, Y.J., Jeng, U.S., Chiang, Y.L., Hwang, I.S., and Chen, Y.R. (2016). The glycine-alanine dipeptide repeat from C9orf72 hexanucleotide expansions forms toxic amyloids possessing cell-to-cell transmission properties. J. Biol. Chem. 291, 4903–4911.

Ciura, S., Lattante, S., Le Ber, I., Latouche, M., Tostivint, H., Brice, A., and Kabashi, E. (2013). Loss of function of C9orf72 causes motor deficits in a zebrafish model of amyotrophic lateral sclerosis. Ann. Neurol. 74, 180–187.

Costanzo, M., and Zurzolo, C. (2013). The cell biology of prion-like spread of protein aggregates: mechanisms and implication in neurodegeneration. Biochem. J. 452, 1–17.

Danzer, K.M., Kranich, L.R., Ruf, W.P., Cagsal-Getkin, O., Winslow, A.R., Zhu, L., Vanderburg, C.R., and McLean, P.J. (2012). Exosomal cell-to-cell transmission of alpha synuclein oligomers. Mol. Neurodegener. 7, 42.

DeJesus-Hernandez, M., Mackenzie, I.R., Boeve, B.F., Boxer, A.L., Baker, M., Rutherford, N.J., Nicholson, A.M., Finch, N.A., Flynn, H., Adamson, J., et al. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. Neuron 72, 245–256.

Feiler, M.S., Strobel, B., Freischmidt, A., Helferich, A.M., Kappel, J., Brewer, B.M., Li, D., Thal, D.R., Walther, P., Ludolph, A.C., et al. (2015). TDP-43 is intercellularly transmitted across axon terminals. J. Cell Biol. 211, 897–911.

Gallegos, S., Pacheco, C., Peters, C., Opazo, C.M., and Aguayo, L.G. (2015). Features of alpha-synuclein that could explain the progression and irreversibility of Parkinson's disease. Front. Neurosci. 9, 59.

Gendron, T.F., Bieniek, K.F., Zhang, Y.J., Jansen-West, K., Ash, P.E., Caulfield, T., Daughrity, L., Dunmore, J.H., Castanedes-Casey, M., Chew, J., et al. (2013). Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form nuclear RNA foci and undergo repeat-associated non-ATG translation in c9FTD/ALS. Acta Neuropathol. *126*, 829–844.

Gendron, T.F., Belzil, V.V., Zhang, Y.J., and Petrucelli, L. (2014). Mechanisms of toxicity in C9FTLD/ALS. Acta Neuropathol. *127*, 359–376.

Gitler, A.D., and Tsuiji, H. (2016). There has been an awakening: emerging mechanisms of C9orf72 mutations in FTD/ALS. Brain Res. *1647*, 19–29.

Kanouchi, T., Ohkubo, T., and Yokota, T. (2012). Can regional spreading of amyotrophic lateral sclerosis motor symptoms be explained by prion-like propagation? J. Neurol. Neurosurg. Psychiatry *83*, 739–745.

Maury, Y., Côme, J., Piskorowski, R.A., Salah-Mohellibi, N., Chevaleyre, V., Peschanski, M., Martinat, C., and Nedelec, S. (2015). Combinatorial analysis of developmental cues efficiently converts human pluripotent stem cells into multiple neuronal subtypes. Nat. Biotechnol. *33*, 89–96.

May, S., Hornburg, D., Schludi, M.H., Arzberger, T., Rentzsch, K., Schwenk, B.M., Grässer, F.A., Mori, K., Kremmer, E., Banzhaf-Strathmann, J., et al. (2014). C9orf72 FTLD/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal toxicity and Unc119 sequestration. Acta Neuropathol. *128*, 485–503.

Mori, K., Arzberger, T., Grässer, F.A., Gijselinck, I., May, S., Rentzsch, K., Weng, S.M., Schludi, M.H., van der Zee, J., Cruts, M., et al. (2013a). Bidirectional transcripts of the expanded C9orf72 hexanucleotide repeat are translated into aggregating dipeptide repeat proteins. Acta Neuropathol. *126*, 881–893.

Mori, K., Weng, S.M., Arzberger, T., May, S., Rentzsch, K., Kremmer, E., Schmid, B., Kretzschmar, H.A., Cruts, M., Van Broeckhoven, C., et al. (2013b). The C9orf72 GGGGCC repeat is translated into aggregating dipeptide-repeat proteins in FTLD/ALS. Science *339*, 1335–1338.

Nath, S., Agholme, L., Kurudenkandy, F.R., Granseth, B., Marcusson, J., and Hallbeck, M. (2012). Spreading of neurodegenerative pathology via neuron-to-neuron transmission of β-amyloid. J. Neurosci. *32*, 8767–8777.

Pant, S., Hilton, H., and Burczynski, M.E. (2012). The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. Biochem. Pharmacol. *83*, 1484–1494.

Proudfoot, M., Gutowski, N.J., Edbauer, D., Hilton, D.A., Stephens, M., Rankin, J., and Mackenzie, I.R. (2014). Early dipeptide repeat pathology in a frontotemporal dementia kindred with C9ORF72 mutation and intellectual disability. Acta Neuropathol. *127*, 451–458.

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. J. Cell Biol. *200*, 373–383.

Ren, P.H., Lauckner, J.E., Kachirskaia, I., Heuser, J.E., Melki, R., and Kopito, R.R. (2009). Cytoplasmic penetration and persistent infection of mammalian cells by polyglutamine aggregates. Nat. Cell Biol. *11*, 219–225.

Renton, A.E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J.R., Schymick, J.C., Laaksovirta, H., van Swieten, J.C., Myllykangas, L., et al.; ITALSGEN Consortium (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. Neuron *72*, 257–268.

Saman, S., Kim, W., Raya, M., Visnick, Y., Miro, S., Saman, S., Jackson, B., McKee, A.C., Alvarez, V.E., Lee, N.C., and Hall, G.F. (2012). Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. J. Biol. Chem. *287*, 3842–3849.

Schipper, L.J., Raaphorst, J., Aronica, E., Baas, F., de Haan, R., de Visser, M., and Troost, D. (2015). Prevalence of brain and spinal cord inclusions, including dipeptide repeat proteins, in patients with the C9ORF72 hexanucleotide repeat expansion: a systematic neuropathological review. Neuropathol. Appl. Neurobiol. Published online September 16, 2015. http://dx.doi.org/10.1111/nan.12284.

Schludi, M.H., May, S., Grässer, F.A., Rentzsch, K., Kremmer, E., Küpper, C., Klopstock, T., Arzberger, T., and Edbauer, D.German Consortium for Frontotemporal Lobar Degeneration; Bavarian Brain Banking Alliance (2015). Distribution of dipeptide repeat proteins in cellular models and C9orf72 mutation cases suggests link to transcriptional silencing. Acta Neuropathol. *130*, 537–555.

Silverman, J.M., Fernando, S.M., Grad, L.I., Hill, A.F., Turner, B.J., Yerbury, J.J., and Cashman, N.R. (2016). Disease mechanisms in ALS: misfolded SOD1 transferred through exosome-dependent and exosome-independent pathways. Cell. Mol. Neurobiol. *36*, 377–381.

Su, Z., Zhang, Y., Gendron, T.F., Bauer, P.O., Chew, J., Yang, W.Y., Fostvedt, E., Jansen-West, K., Belzil, V.V., Desaro, P., et al. (2014). Discovery of a biomarker and lead small molecules to target r(GGGGCC)-associated defects in c9FTD/ALS. Neuron *83*, 1043–1050.

Therrien, M., Rouleau, G.A., Dion, P.A., and Parker, J.A. (2013). Deletion of C9ORF72 results in motor neuron degeneration and stress sensitivity in C. elegans. PLoS ONE *8*, e83450.

Théry, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr. Protoc. Cell Biol. *30*, 3.22.1–3.22.29.

Wen, X., Tan, W., Westergard, T., Krishnamurthy, K., Markandaiah, S.S., Shi, Y., Lin, S., Shneider, N.A., Monaghan, J., Pandey, U.B., et al. (2014). Antisense proline-arginine RAN dipeptides linked to C9ORF72-ALS/FTD form toxic nuclear aggregates that initiate in vitro and in vivo neuronal death. Neuron *84*, 1213–1225.

Zhang, Y.J., Jansen-West, K., Xu, Y.F., Gendron, T.F., Bieniek, K.F., Lin, W.L., Sasaguri, H., Caulfield, T., Hubbard, J., Daughrity, L., et al. (2014). Aggregation-prone c9FTD/ALS poly(GA) RAN-translated proteins cause neurotoxicity by inducing ER stress. Acta Neuropathol. *128*, 505–524.

Zhang, Y.J., Gendron, T.F., Grima, J.C., Sasaguri, H., Jansen-West, K., Xu, Y.F., Katzman, R.B., Gass, J., Murray, M.E., Shinohara, M., et al. (2016). C9ORF72 poly(GA) aggregates sequester and impair HR23 and nucleocytoplasmic transport proteins. Nat. Neurosci. *19*, 668–677.

Zu, T., Liu, Y., Bañez-Coronel, M., Reid, T., Pletnikova, O., Lewis, J., Miller, T.M., Harms, M.B., Falchook, A.E., Subramony, S.H., et al. (2013). RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. Proc. Natl. Acad. Sci. USA *110*, E4968–E4977.