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# The most prevalent genetic cause of ALS-FTD, C9orf72 synergizes the toxicity of ATXN2 intermediate polyglutamine repeats through the autophagy pathway

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**ABSTRACT**

The most common genetic cause for amyotrophic lateral sclerosis and frontotemporal dementia (ALS-FTD) is repeat expansion of a hexanucleotide sequence (GGGGCC) within the *C9orf72* genomic sequence. To elucidate the functional role of C9orf72 in disease pathogenesis, we identified certain molecular interactors of this factor. We determined that C9orf72 exists in a complex with SMCR8 and WDR41 and that this complex acts as a GDP/GTP exchange factor for RAB8 and RAB39, 2 RAB GTPases involved in macroautophagy/autophagy. Consequently, C9orf72 depletion in neuronal cultures leads to accumulation of unresolved aggregates of SQSTM1/p62 and phosphorylated TARDBP/TDP-43. However, C9orf72 reduction does not lead to major neuronal toxicity, suggesting that a second stress may be required to induce neuronal cell death. An intermediate size of polyglutamine repeats within ATXN2 is an important genetic modifier of ALS-FTD. We found that coexpression of intermediate polyglutamine repeats (30Q) of ATXN2 combined with C9orf72 depletion increases the aggregation of ATXN2 and neuronal toxicity. These results were confirmed in zebrafish embryos where partial C9orf72 knockdown along with intermediate (but not normal) repeat expansions in ATXN2 causes locomotion deficits and abnormal axonal projections from spinal motor neurons. These results demonstrate that C9orf72 plays an important role in the autophagy pathway while genetically interacting with another major genetic risk factor, ATXN2, to contribute to ALS-FTD pathogenesis.

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Amyotrophic lateral sclerosis (ALS), a neurodegenerative disease affecting motor neurons, is the third most common neurodegenerative disease affecting one in 50,000 people. Frontotemporal dementia (FTD), which affects neurons from the frontal and temporal cortex, is the second most common presenile dementia after Alzheimer disease. An expansion of hundreds to thousands of GGGGCC repeats within the first intron of the *C9orf72* gene represents the most common inherited cause for ALS and FTD accounting for 20–60% of familial patients and 1–7% of sporadic patients for these disorders in Northern Europe and North America. Major effort has been dedicated over the past 5 y to unravel the mechanism of toxicity through which C9orf72 repeat expansion leads to neurodegeneration. Notably, noncanonical translation, termed repeat-associated non-ATG (RAN) translation, of these expanded GGGGCC repeats generates repeated dipeptide chains that are toxic in neuronal cultures as well as in yeast, *Drosophila*, and mice.

In parallel, pathologically expanded GGGGCC repeats were shown to affect C9orf72 expression with reduced transcript and protein levels consistently measured in pathological tissue obtained from ALS and FTD patients. Therefore, C9orf72 haploinsufficiency may also participate in neuronal degeneration in ALS-FTD. In line with this evidence, reduced levels of C9orf72 in *C. elegans* and zebrafish, but not in mice, trigger specific phenotypic features associated with motor neuron degeneration. Importantly, very little is yet known about the role of C9orf72, and a better comprehension of its function will be essential to understand its implication in ALS-FTD. To ascertain the molecular and cellular function for C9orf72, we applied an initial proteomic approach to identify its partners. Tandem tag immunoprecipitation revealed that C9orf72 exists in a complex with 2 proteins, SMCR8 and WDR41. These results were confirmed by co-immunoprecipitation experiments and by reconstituting this complex with recombinant proteins from baculovirus-infected insect cells. Both SMCR8 and C9orf72 contain DENN domains, which are typical of GDP/GTP exchange factors (GEFs) for small RAB GTPases. Indeed, in vitro the complex formed by C9orf72, SMCR8 and WDR41 promotes

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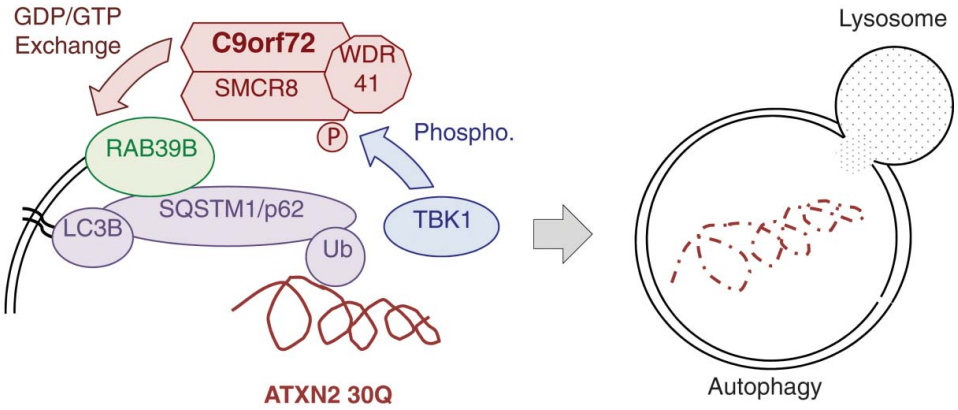


**Figure 1.** Tentative model of C9orf72 function. C9orf72 forms a complex with the SMCR8 and WDR41 proteins and acts as a GDP/GTP exchange factor for the small RAB GTPase RAB39B. SMCR8 is phosphorylated and potentially activated by the TBK1 kinase. The partially reduced expression of C9orf72 partly impairs autophagy but does not cause massive neuronal cell death. In contrast, reduced expression of C9orf72 synergizes the aggregation and toxicity of ATXN2 with intermediate length polyglutamine repeats. These results suggest a double-hit hypothesis for ALS-FTD.

GDP/GTP exchange for the small GTPases RAB8A and RAB39B. Since RAB8 and RAB39 are involved in macroautophagy and since SMCR8 was found in proteomic analysis of the autophagy network, we investigated whether C9orf72 was regulating macroautophagy. Knockdown of C9orf72 by shRNA and/or siRNA in neuronal primary cultures derived from mouse frontal cortex demonstrate that a partial depletion of C9orf72 has a deleterious effect on autophagy, with notable accumulation of unresolved aggregates of the autophagy receptor SQSTM1. In contrast, the deleterious effect of the loss of C9orf72 on the lipidation of LC3B was rather mild. Also, we found that the C9orf72 complex associates with the autophagy receptors OPTN and SQSTM1, probably through indirect interaction via RAB8 or RAB39 or other proteins, yet to be identified. Note that mutations in OPTN and SQSTM1 cause ALS-FTD and that SQSTM1-positive aggregates are observed in ALS-FTD patients with expansion of GGGGCC repeats in C9orf72. The role of the C9orf72 complex in autophagy was further strengthened as 2 kinases regulating autophagy, the ULK1 and TBK1 kinases, were shown to phosphorylate SMCR8. The TBK1 phosphorylation of SMCR8 is important to neuronal cells as expression of a SMCR8 form mimicking TBK1-constitutive phosphorylation restores the autophagy imbalance due to depletion of SMCR8 or TBK1. Again, the link with ALS is patent as loss-of-function mutations in TBK1 were recently shown to cause ALS-FTD.

Importantly, the autophagy deficiency observed in neuronal cultures upon knockdown of C9orf72 or TBK1 can be rescued upon co-expression of a constitutively active GTP-locked form of RAB39B that does not require GEF activity. Of interest, loss-of-function mutations of RAB39B cause mental retardation associated with early onset Parkinson disease. Therefore, our results indicate that interaction of C9orf72 with TBK1-phosphorylated SMCR8 and their association and consequent activation of RAB39B has an important role for autophagy in neurons as shown in Figure 1.

Furthermore, our data indicate that decreased expression of C9orf72 leads to some accumulation of TARDBP aggregates, which is a pathological hallmark of ALS-FTD. Therefore, reduced expression of C9orf72 in neuronal cultures recapitulates some of the pathological features of C9orf72-associated ALS-FTD. However, autophagy deregulation by C9orf72 loss of function was not sufficient to trigger neuronal cell death in mammalian neuronal cultures. Therefore, we hypothesized that an extra cellular stressor was required to trigger neurodegeneration upon C9orf72 loss of function.

ATXN2 (ataxin 2) with intermediate polyglutamine (polyQ) repeats represents the most important risk factor for ALS-FTD, and we have recently demonstrated that C9orf72 repeat expansions coincide with intermediate size of polyQ in ALS-FTD patients. Of interest to disease pathogenesis, partial C9orf72 depletion coupled with intermediate polyQ repeats (30x) leads to increased ATXN2 aggregation and neuronal cell death. This epistatic interaction appears specific for ATXN2 intermediate polyQ repeats, because C9orf72 loss of function does not accentuate toxicity of other proteins harboring polyQ repeats (ATXN3, HTT) or other ALS-related mutants (FUS and SOD1), at least in neuronal culture and in the time frame of our study. Importantly, the genetic interaction described in neuronal cultures was confirmed in vivo in zebrafish embryos where partial C9orf72 knockdown coupled with intermediate (30x), but not normal-size (22x), polyQ repeats of ATXN2 is associated with swimming deficits and aberrant axonal projections from spinal motor neurons (Fig. 1).

The exact regulatory role of C9orf72 complex in the autophagy pathway and the molecular mechanism through which C9orf72 partial loss of function accentuates the toxicity of ATXN2 intermediate repeats remain to be established. Similarly, the consequences of SMCR8 phosphorylation by ULK1 and TBK1 or other kinases remain to be ascertained. Also, the synergic toxicity observed could be caused by independent molecular cascades that converge on protein degradation through the autophagosome. In the model that we propose, increased polyQ size renders ATXN2 more stable and potentially less available to degradation. This is aggravated

by C9orf72 depletion through a deregulation of the autophagosome capacity. Overall, our study provides a better understanding of the function of C9orf72, therefore opening new perspectives on its potential role in ALS-FTD pathogenesis.

## Abbreviations

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| ATXN2 | ataxin 2 |
| C9orf72 | chromosome 9 open reading frame 72 |
| polyQ | polyglutamine |
| SQSTM1/p62 | sequestosome 1 |
| SMCR8 | Smith-Magenis syndrome chromosome region, candidate 8 homolog |
| TARDBP/TDP-43 | TAR DNA binding protein |
| TBK1 | tank binding kinase 1 |
| WDR41 | WD repeat domain 41 |

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No potential conflicts of interest were disclosed.

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