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COMMENTARY

C9ORF72 is a GDP/GTP exchange factor for Rab8 and Rab39 and regulates autophagy

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ABSTRACT

Amyotrophic Lateral Sclerosis and Frontotemporal Dementia (ALS-FTD) are devastating neurodegenerative disease affecting motoneurons from the spinal chord and neurons from the frontal and temporal cortex, respectively. The most common genetic cause for ALS-FTD is an expansion of GGGGCC repeats within the first intron of the C9ORF72 gene. However, little is known on the function of C9ORF72. Recently, other and we found that C9ORF72 forms a stable complex with the SMCR8 and WDR41 proteins. This complex acts as a GDP/GTP exchange factor for the small RAB GTPases Rab8a and Rab39b. Since Rab8 and Rab39 are involved in macroautophagy, we tested the role of C9ORF72 in this mechanism. Decrease expression of C9ORF72 in neuronal cultures leads to autophagy dysfunction characterized by accumulation of aggregates of p62/SQSTM1. However, loss of C9ORF72 expression does not cause major neuronal cell death, suggesting that a second stress may be required to promote cell toxicity. Intermediate size of polyglutamine repeats within Ataxin-2 (ATXN2) is an important genetic modifier of ALS-FTD. We found that decrease expression of C9ORF72 synergizes the toxicity and aggregation of ATXN2 with intermediate size of polyglutamine (30Q). Overall, our data suggest that reduce expression of C9ORF72 causes suboptimal autophagy that sensitizes neurons to a second stress. These data suggest that reduce expression of C9ORF72 may partly contribute to ALS-FTD pathogenesis.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal motoneuronal neurodegenerative disorder for which no effective treatment is available. With an estimated incidence of 2.6/100,000 per year and a lifetime risk of 1:350 for male and 1:450 for female, Amyotrophic lateral sclerosis (ALS) is the third most common neurodegenerative disease worldwide and the most frequent motor neuron disease. ALS is mainly characterized by the massive degeneration of both upper (UMN) and lower (LMN) motor neurons in the cerebral cortex and spinal cord, which rapidly leads in most patients to paralysis and death due to denervation of the respiratory muscles. Frontotemporal dementia (FTD) is the second most common presenile dementia after Alzheimer disease. Increasing evidences indicate that FTD and ALS pathologies form a continuum of neurological diseases, which share a common pathological background. First, ALS and FTD patients have an overlap of clinical symptoms, since approximately 15% of FTD patients have motor dysfunction meeting the criteria of ALS and 15% to 30% of ALS patients have FTD. Also, histopathological analyses demonstrate that ALS and FTD patients share common histopathological markers with accumulation of cytoplasmic aggregates of phosphorylated and cleaved transactive response DNA-binding protein 43 (TDP-43) in the vast majority of ALS patients and in the most common Tau-negative pathological subtype of FTD.[31] Finally, the concept that FTD and ALS represent a clinicopathological spectrum of disease is confirmed by genetic evidences as mutations in *TARDBP* (encoding TDP-43 protein), *UBQLN2, FUS* and, topic of this highlight, *C9ORF72*, lead to co-occurrence of ALS-FTD. Importantly, 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 forms and 1–7% of sporadic ALS-FTD patients in Northern Europe and North America.[9,35] The GGGGCC expansion is located between 2 5 prime non-coding exons of C9ORF72, which encodes a poorly characterized protein. Three main non-exclusive mechanistic models of C9ORF72-mediated ALS have been proposed (review in ref. 17)

First, various studies indicate that expanded GGGGCC repeats are transcribed both in the sense and the antisense

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strands, forming nuclear aggregates of RNA containing expanded GGGGCC or GGCCCC repeats. Such mutant RNA may bind and titrate specific RNA binding proteins, including hnRNP-A3, Pur-α, ADARB2, hnRNP-H, and Nucleolin.[1,10,18] However, whether the recruitment of these RNA binding proteins leads to titration and loss of their function remains to be determined.

The second potential mechanism for neurotoxicity of expansions is a form of non-canonical protein translation termed repeat-associated, non-ATG (RAN) translation.[48] Extensive studies have now established that sense and antisense RNA containing the expanded repeats are RAN translated in all 6 sense and antisense frames, resulting in expression of 5 different di-peptide repeats containing proteins (DPRs), which form inclusions throughout the brain of patients with C9-ALS/FTD.[2,16,30,49] These DPRs were also identified in mice expressing expanded GGG GCC repeats;[4] Peters et al., 2015; O'Rourke et al., 2015;[27] Jiang et al., 2016), and were found to be toxic in neuronal cell cultures and in Drosophila models through alteration of the nucleocytoplasmic transport.[14,22,40,47]

Third, several studies consistently found reduced levels of C9ORF72 transcripts in GGGGCC expanded-repeats carriers, suggesting a possible loss-of-function disease mechanism.[1,9,35,42,43] Haploinsufficiency of C9ORF72 is to be anticipated if the hexanucleotide-expanded genomic DNA promotes epigenetics modification and impairs C9ORF72 transcription. However, the absence of neuronal phenotypes in mouse depleted of C9orf72 expression in brain or in neurons,[23,24] as well as the absence of ALS/FTD patients with null alleles or misense mutations in C9ORF72, argue against a loss-of-function of C9ORF72 as the sole or main cause of ALS-FTD. Nevertheless, evidences of locomotion deficit in zebrafish with reduced expression of C9ORF72[7] and the correlation of decreased C9ORF72 mRNA expression with decreased patient survival[43] suggest that reduced expression of C9ORF72 may partly contribute to ALS-FTD pathogenesis.

C9ORF72 in complex with SMCR8 regulates autophagy through Rab8 and Rab39

Despite many major recent advances, little is known on the normal molecular and cellular functions of C9ORF72. Other and we found that C9ORF72 forms a complex with 2 proteins, SMCR8 and WDR41 of unknown functions.[37,39,45] Bioinformatics analysis identified that both C9ORF72 and SMCR8 contain DENN (Differentially Expressed in Normal and Neoplastic cells) domains characteristic of Rab GDP/GTP exchange factors (GEFs).[26,46] Consistent with these predictions, we found that SMCR8 interacts with various RAB GTPases and that the C9ORF72-SMCR8 complex promotes *in vitro* GDP/GTP exchange for the Rab8a and Rab39b. Since Rab8 and Rab39 are involved in macroautophagy (Pilli et al., 2012;[36,38] and as SMCR8 was identified in proteomic analysis of autophagy network,[3] we investigated whether C9ORF72 was regulating autophagy. Depletion of C9ORF72 expression by shRNA and/or siRNA in transformed neuronal cells or in primary cultures of cortical neurons of E18 mouse embryo has a deleterious role on autophagy, with notable accumulation of unresolved aggregates of the autophagy receptor P62/SQSTM1. A function of C9ORF72 in autophagy is consistent with a previous report of LC3B alteration in C9ORF72 siRNA-depleted neuronal cells,[12] but also with the increased accumulation of P62 and the susceptibility to autophagy inhibitors observed in cultures of human neurons derived from iPS cells of ALS-FTD patients carrier of an GGGGCC expansion in C9ORF72.[1,8] Furthermore, the recent demonstration that siRNA-mediated depletion of C9ORF72 leads to a decrease formation of LC3B-positive vesicles conclusively pinpoints a role of C9ORF72 in the initiation of autophagy.[44] Finally, a role of the C9ORF72-SMCR8 complex in autophagy is also strengthened by the observation that this complex interacts with 2 kinases regulating autophagy, ULK1 and TBK1[3,37,39,44] Our in vitro phosphorylation assays indicate that both ULK1 and TBK1 kinases phosphorylate SMCR8, but not C9ORF72. Interestingly, a mutant of SMCR8 threonine 796 in aspartic acid, which mimics a constitutive phosphorylation of SMCR8 by TBK1, is able to correct autophagy dysfunctions caused by siRNA-mediated reduced expression of either SMCR8 or TBK1. This may be relevant to disease pathogenesis as loss of function mutations in TBK1 cause ALS-FTD.[6,15] Furthermore, TBK1 is known to phosphorylate P62 and OPTN, 2 autophagy receptors also found muted in rare case of ALS.[28] In that aspect, we found that Rab39b interacts with P62 and confirmed previous observations[19] (Pilli et al., 2012) that OPTN interacts with Rab8a. These results support a model where P62 or OPTN autophagy receptors act as essential hubs to gather specific Rab GTPases with there specific GEF effectors and kinase regulators to initiate autophagy precisely at the site of ubiquitinated protein aggregates, dysfunctional organelles or intracellular pathogens (Fig. 1). A model supported by the recent report of the importance of TBK1 recruitment to dysfunctional mitochondria to phosphorylate P62 or OPTN and initiate mitophagy.[20,25,29] Finally, we found that a mutant form of Rab39b, which is locked in its GTP conformation and does not consequently require any GEF activity, can rescue autophagy dysfunction caused by siRNA-mediated loss of C9ORF72-SMCR8 or of its upstream kinase regulator, TBK1. These

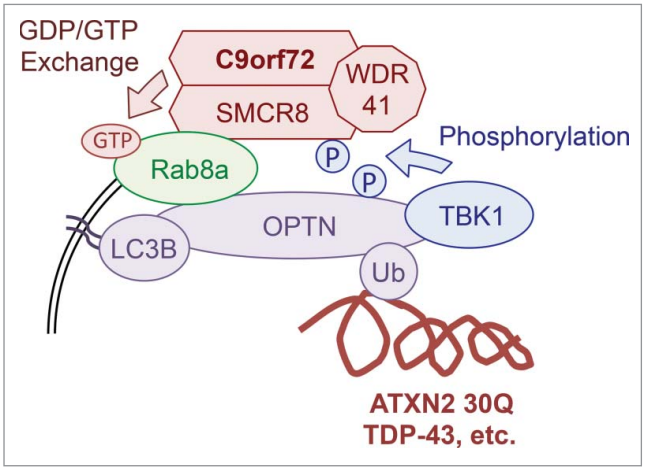

Figure 1. Tentative model of C9ORF72 role in autophagy. C9ORF72 forms a complex with the SMCR8 and WDR41 proteins. This complex acts as a GDP/GTP exchange factor for the small GTPases Rab8a that interacts with the autophagy receptor Optineurin (OPTN), or for Rab39b that interacts with P62, an autophagy receptors alike to OPTN. SMCR8 is phosphorylated and potentially activated by the TBK1 kinase, which also interacts with OPTN. Optineurin bridges ubiquitinated proteins to the autophagosome membrane through the LC3B proteins. Recruitment of Rab8a and TBK1 by OPTN allows initiation of autophagy at the precise site of protein aggregate, dysfunctional organelles or intracellular pathogen. However, it remains to determine the function of Rab8a or RAb39b in autophagy, notably whether Rab8a or Rab39b may promote autophagosome membrane elongation or fusion.

results suggest that TBK1, C9ORF72-SMCR8 complex and RAB39b belong to a common pathway regulating autophagy in neuronal cells.

Loss of C9ORF72 synergizes ATXN2 polyQ toxicity

While reduce expression of C9ORF72 causes a partial dysfunction of autophagy, this was not sufficient to trigger overt neuronal cell death in neuronal cultures. This is consistent with the absence of neurodegeneration in mouse depleted of C9orf72 expression in brain or in neurons.[23,24] Thus, we hypothesized that a second stress might be required to trigger neuronal cell loss upon C9ORF72 depletion. Intermediate size of 27 to 33 glutamines in Ataxin-2 increases the risk of ALS-FTD.[11] Importantly, we found that decrease expression of C9ORF72 synergizes the toxicity of Ataxin-2 with 30 glutamines both in mammalian neuronal cell cultures and in zebrafish embryos. Importantly, this synergic or double hit model of toxicity is consistent with the absence of ALS/FTD patients with null or misense mutations in C9ORF72, while there is increasing genetic evidences of oligogenicity in ALS-FTD.[13,42] Also, this synergic toxicity appears specific to Ataxin-2 since reduced expression of C9ORF72 does not accentuate aggregation of Ataxin-3 or huntingtin with expanded polyglutamine repeats.[37] This is rather unexpected, as these polyglutamine-containing proteins are cleared by autophagy. However, it is possible that the little effect of C9ORF72 loss on the aggregation of Ataxin-3 or huntingtin that we observed is due to the inherent limitation of *in vitro* cell cultures and the short time frame of our study. Alternatively, the aggregations of Htt or Ataxin-3 with expanded polyglutamine repeats may have reach a maximum in our cell culture and cannot be enhanced further. Thus, an effect of C9ORF72 depletion on polyglutamine-containing proteins remains to be tested in animal model and/or on a longer time period of analysis.

Conclusion and perspectives

Expansion of GGGGCC repeats within the first intron of the C9ORF72 gene is the prime cause of ALS-FTD, but little is known on the molecular function of C9ORF72. Recent evidences indicate that C9ORF72 interacts with SMCR8 and that this complex regulates autophagy.[3,12,37,39,44,45] However, the precise role of C9ORF72 or SMCR8 in the autophagy pathway remains to be determined. C9ORF72 and SMCR8 in complex or in isolation interact with various Rab GTPases, including Rab8 and Rab39 as well as Rab1, Rab5, Rab7 and Rab11,[12,44] but it is unclear which Rab would regulate which autophagy steps in which tissue or cellular type. Furthermore, Rab GTPases act in cascades where the passage from one Rab to the next requires the recruitment of Rab GAP and GEF effectors to the upstream and downstream Rab GTPases, respectively. Thus, it remains to determine the importance of C9ORF72-SMCR8 in such Rab cascade as well as the identity of such Rab GTPases. In that aspect, the recent demonstration that Rab1 may be the initial Rab GTPase recruiting C9ORF72 to promote GDP/GTP exchange and thus activation of a downstream Rab GTPase is especially exciting.[44] It is also highly possible that C9ORF72 and SMCR8 in complex or in isolation bind specific Rab GTPases involved in other cellular process than autophagy. In that aspect, functions of C9ORF72 in endocytosis and lysosomal pathways have been recently suggested.[5,12,33,39] Next, we found that SMCR8 is phosphorylated by ULK1 and TBK1. Also, SMCR8 is found phosphorylated by AMPK and mTOR kinases in large proteomic screen. However, the physiological consequences of SMCR8 phosphorylation by ULK1, TBK1, AMPK or mTOR kinases remain to be explored. Finally, reduce expression of C9ORF72 is not overly toxic by itself but synergizes the toxicity of Ataxin-2 with intermediate size of polyglutamine. This synergic model supports a 2 hit hypothesis in ALS-FTD and partly explains why the sole loss of C9ORF72 is not sufficient to cause massive neuronal cell death in cell culture or in knockout mouse models. However, it remains to test validity of this model in mammals,

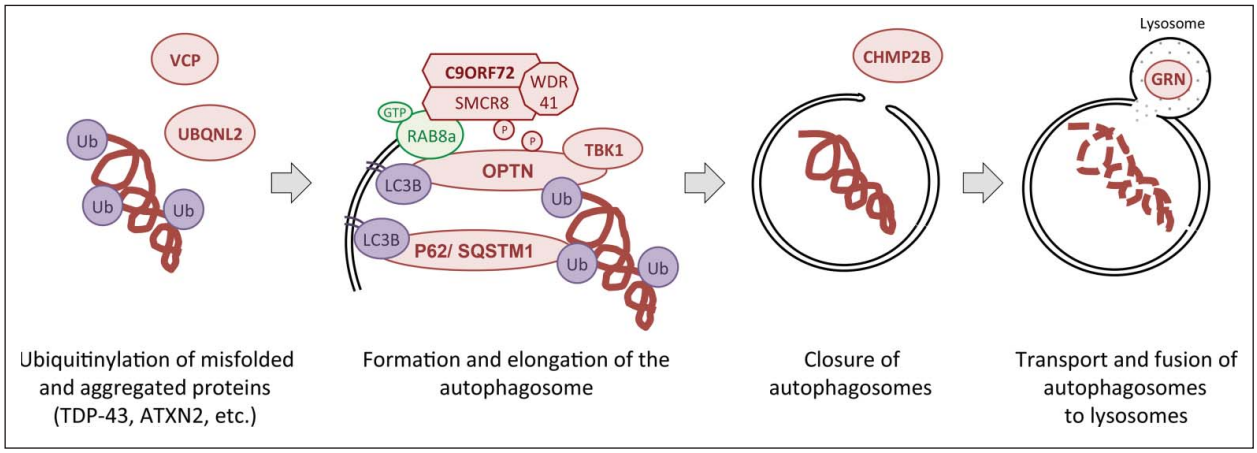

Figure 2. Various genes muted in ALS-FTD regulate endo-lysosomal and protein degradation pathways. The kinase TBK1 activates autophagy through phosphorylation of the autophagy receptors P62 and OPTN. P62, encoded by the SQSTM1 gene, and OPTN bridge ubiquitinated proteins to be degraded with lipidated LC3B proteins that are bound to the autophagy membrane. The Valosin-Containing protein (VCP) is an ATPase that unfolds substrate ubiquitinated protein complex allowing the released proteins to be degraded by the proteasome or by autophagy. The ubiquitin-like protein Ubiquilin-2 (UBQLN2) functionally links the ubiquitination machinery to the proteasome and autophagy machineries. Charged multivesicular body protein 2b (CHMP2B) belongs to the Endosomal Sorting Complex III (ESCRT-III) required for closure of membrane vesicles and notably of multivesicular bodies and autophagosomes. Finally, progranulin (GRN) is localized to the lysosome, which is the journey's end of endosomes and autophagosomes. Mutation in TBK1, SQSTM1, OPTN, UBQLN2, VCP cause ALS-FTD, while haploinsufficiency mutations of GRN causes FTD.

notably by testing whether expression of Ataxin-2 with intermediate size of polyglutamine promotes neuronal cell death in C9ORF72 knockout mice. Similarly, it remains to explore whether reduced expression of C9ORF72 may synergize other cellular stress such as RAN translation of expanded GGGGCC repeats into toxic DPRs. Furthermore, as Rab39b interacts with P62 that is involved in the autophagic clearance of protein aggregates, while Rab8 interacts with OPTN that is involved in the autophagy of dysfunctional mitochondria,[20,25,29] it will be exciting to test whether C9ORF72 is also involved in mitophagy. Last but not least, it is striking to note that various mutations causing ALS-FTD are found in genes involved in protein clearance pathways, including *UBQLN2, CHMP2B, VCP, OPTN, SQSTM1* and *TBK1*. Thus, our work linking decrease expression of C9ORF72 to suboptimal autophagy may provide further support to compromised protein-clearance mechanisms in ALS-FTD (Fig. 2). Whether suboptimal protein degradation may contribute to disease pathogenesis in ALS-FTD is an exciting area that remains to be fully explored.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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