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ZFYVE26/SPASTIZIN

A close link between complicated hereditary spastic paraparesis and autophagy

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Defective autophagy is associated with neurodegenerative disorders including Alzheimer, Parkinson and Huntington diseases, amyotrophic lateral sclerosis and SCA (spinocerebellar ataxias). Autophagy defects were detected also in SPG49, a complicated form of hereditary spastic paraparesis (cHSP) associated with mutations in the *TECPR2* gene, suggesting a role of autophagy also in this heterogeneous group of neurodegenerative diseases. We recently found defective autophagy in SPG15, another HSP subtype associated with mutations in the *ZFYVE26/SPG15* gene. Patient-derived cells (fibroblasts/lymphoblasts) carrying different ZFYVE26 mutations show accumulation of immature autophagosomes and increased MAP1LC3B-II and SQSTM1/p62 levels. These findings indicate that ZFYVE26 is a key determinant of autophagosome maturation, which is impaired when the protein is defective or absent. Replication of these findings in primary neurons supports the relevance of defective autophagy in SPG15-related neurodegeneration.

Keywords: complicated hereditary spastic paraparesis, SPG15, autophagy, autophagosome maturation, ZFYVE26/SPASTIZIN, BECN1/Beclin 1

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Autophagy is a conserved intracellular catabolic process that delivers cytoplasmic constituents to lysosomes for degradation and recycling through the formation of double-membrane vacuoles, termed autophagosomes. Identified originally as an adaptive response, autophagy is now recognized as a key player in the pathogenesis of different types of disorders including neurodegenerative diseases. The role of autophagy in the heterogeneous group of neurodegenerative diseases termed hereditary spastic paraparesis (HSP) was recently proposed based on the data obtained in patient fibroblasts carrying a *TECPR2* mutation associated with SPG49, a complicated form of HSP. HSP are characterized by progressive spasticity and weakness at the lower limbs due to retrograde axonal degeneration of the corticospinal tracts, and can be classified either as pure or complicated. Patients with "pure" HSP display isolated pyramidal signs, whereas complex forms may show variable combinations of neurological and non-neurological signs and symptoms in addition to spasticity.

We recently reported that ZFYVE26, involved in SPG15, a different form of cHSP, plays a key role in autophagy. ZFYVE26 is a large protein of 2539 amino acid residues and contains a FYVE domain that confers to the protein binding affinity to the membrane-associated phosphatidylinositol 3-phosphate (PtdIns3P). ZFYVE26 has a diffuse cytoplasmic distribution and partially colocalizes with early endosomes, the endoplasmic reticulum, microtubules, and vesicles. It localizes also to the midbody during cytokinesis. ZFYVE26 interacts with BECN1, a subunit of the class III phosphatidylinositol 3-kinase (PIK3C3/VPS34) complex and it is supposed to recruit BECN1 to the midbody, thereby promoting cytokinesis. Alongside cytokinesis BECN1 is implicated in autophagy by promoting the formation of the BECN1-PIK3C3/VPS34-PIK3R4/VPS15 core complex and by regulating PIK3C3 activity through the interaction with several proteins, such

as ATG14, UVRAG, and KIAA0226/RUBICON. The binding of ATG14 to the core complex regulates PtdIns3P synthesis and autophagosome formation, whereas the binding of UVRAG or KIAA0226 promotes or inhibits autophagosome maturation, respectively. We found that ZFYVE26 interacts with BECN1, PIK3C3, KIAA0226, and UVRAG, but not with ATG14, suggesting that ZFYVE26 interacts with the BECN1 complexes containing UVRAG and KIAA0226, but not with the complex containing ATG14.

The differential interaction with the BECN1 complexes was supported further by ZFYVE26 subcellular distribution, which overlaps that of KIAA0226 and UVRAG, and differs from that of ATG14. ZFYVE26, as seen with KIAA0226 and UVRAG, localizes to endosomes and not to autophagosomes or phagophores. However, different from UVRAG and KIAA0226, ZFYVE26 does not localize to lysosomes. We then tested the effects of ZFYVE26 pathogenic mutations on protein interactions in basal and autophagy-inducing conditions using SPG15 patient-derived fibroblasts and lymphoblasts bearing either missense mutations (p.L243P, p.I508N) or truncating mutations (p.S1312X, p.R1209fsX, in which ZFYVE26 is not expressed).

In L243P and I508N *SPG15*-mutated cells the anti-ZFYVE26 antibody co-immunoprecipitates only a minimal amount of BECN1 with respect to controls and does not co-immunoprecipitate PIK3C3, KIAA0226, and UVRAG. In S1312X *SPG15*-mutated cells, which lack the ZFYVE26 protein, the anti-ZFYVE26 antibody fails to co-immunoprecipitate BECN1, PIK3C3, KIAA0226, and UVRAG, as expected. However, in the *SPG15*-mutated cells the anti-BECN1 antibody is still able to co-immunoprecipitate its interactor proteins, indicating that ZFYVE26 mutations have no effect on the formation of the BECN1-UVRAG-KIAA0226 complex. ZFYVE26 interactions in control and mutated cells were confirmed also by immunofluorescence.

We then analyzed the role of ZFYVE26 in autophagy and the effect of its mutations on this process. We analyzed autophagosome number in control and mutated cells by immunofluorescence using the autophagosomal marker MAP1LC3B. ZFYVE26-mutated fibroblasts show an accumulation of autophagosomes. This correlates with increased MAP1LC3B-II and SQSTM1 expression levels. Nevertheless, no defect in autophagy induction was found by checking several markers (BECN1, MTOR phosphorylation, ATG7) or by bafilomycin A_1 treatment to monitor flux.

The effect of the truncating mutations, leading to ZFYVE26 depletion, observed in fibroblasts and lymphoblasts was replicated by ZFYVE26 silencing in HeLa cells and murine hippocampal neurons, where depletion of ZFYVE26 induces an increased accumulation of autophagosomes.

Reduced colocalization of MAP1LC3B and LAMP1 in patients' fibroblasts compared with controls in basal and autophagy-inducing conditions indicates impairment in the autophagosome-to-lysosome fusion step. Of note, in ZFYVE26-mutated cells autophagosome maturation is not completely blocked, since treatment of L243P, I508N, R1209fsX, and S1312X cells with bafilomycin A_1 induces a further increase in MAP1LC3B-II levels compared with untreated L243P, I508N, R1209fsX, and S1312X cells. The ultrastructural analysis of patients' fibroblasts revealing the presence of immature autophagosomes definitely confirmed the impairment of the autophagosome-lysosome fusion process in these cells.

Our observations clearly indicate that ZFYVE26 is required for generating mature autophagosomes. Based on ZFYVE26 localization data and on the presence of a PtdIns3P-binding FYVE domain we suggest that a possible function of ZFYVE26 in autophagy is to recruit BECN1 and its binding partners UVRAG and KIAA0226 on structures enriched in PtdIns3P that are involved in autophagosomes maturation. The fact that ZFYVE26 mutations do not alter the interaction between BECN1 and the components of the complexes, and the evidence that KIAA0226-associated structures are enriched in PtdIns3P support this hypothesis. Alternatively, ZFYVE26 could collaborate with KIAA0226 in sequestering UVRAG in the KIAA0226-UVRAG-BECN1 complex; ZFYVE26 mutations or depletion could perturb the equilibrium between the 2 complexes involved in maturation, thereby affecting this process. Overall these findings identify ZFYVE26 as a novel key protein in the autophagic pathway, strengthening the role of autophagy in the neurodegenerative processes underlying cHSP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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