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# Rapamycin-induced autophagy aggravates pathology and weakness in a mouse model of VCP-associated myopathy

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**Keywords:** rapamycin, MTOR, autophagy, VCP, myopathy

**Abbreviations:** FTD, frontotemporal dementia; IBM, inclusion body myopathy; PDB, Paget disease of the bone; ALS, amyotrophic lateral sclerosis; VCP, valosin-containing protein; MTOR, mechanistic target of rapamycin; EIF4EBP1, eukaryotic translation initiation factor 4E binding protein 1; LC3, microtubule-associated protein 1 light chain 3; SQSTM1, sequestosome 1; RPS6, ribosomal protein S6; RPS6KB1, ribosomal protein S6 kinase, 70kDa, polypeptide 1; LAMP2, lysosomal-associated membrane protein 2; AMPK, AMP-activated protein kinase; DBE-Q, N2N4-dibenzylquinazoline-2,4-diamine; RHEB, Ras homolog enriched in brain; IGF1, insulin-like growth factor 1

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**P**athological phenotypes in inclusion body myopathy (IBM) associated with Paget disease of the bone (PDB), frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (IBMPFD/ALS) include defective autophagosome and endosome maturation that result in vacuolation, weakness and muscle atrophy. The link between autophagy and IBMPFD/ALS pathobiology has been poorly understood. We examined the AKT-FOXO3 and MTOR pathways to characterize the regulation of autophagy in IBMPFD/ALS mouse muscle. We identified a defect in MTOR signaling that results in enhanced autophagosome biogenesis. Modulating MTOR signaling may therefore be a viable therapeutic target in IBMPFD/ALS.

Mutations in valosin containing protein (VCP) cause IBM associated with PDB, FTD and ALS. Prominent pathological phenotypes in VCP-associated disease include muscle weakness, which manifests in ~90% of patients. Moreover, vacuoles in IBMPFD/ALS patient skeletal muscle contain autophagic and endocytic markers, such as SQSTM1/p62, LC3 and LAMP2. That autophagosome and endosome maturation is disrupted in IBMPFD/ALS is a serious concern because autophagic degradation is critical for cellular homeostasis. We reasoned that defects in regulatory components, such as MTOR, which control autophagy, may contribute to pathogenesis of IBMPFD/ALS. The R155H mutation is the most common IBMPFD mutation, and the majority of patients with IBMPFD/ALS bear VCP mutations in this residue. We made use of a mouse

model that expresses the VCP-R155H mutation (VCP-RH) and recapitulates the myopathy component of IBMPFD/ALS to characterize the regulation of the MTOR-autophagy pathway with respect to IBMPFD/ALS pathogenesis.

In an effort to further understand autophagic dysfunction in IBMPFD/ALS, we examined the AKT-FOXO3 and MTOR pathways, which regulate autophagy in skeletal muscle, and found that basal levels of phosphorylated AKT<sup>Ser473</sup> and phosphorylated FOXO3<sup>Ser253</sup> are normal in VCP-RH mice when compared with control mice and mice overexpressing the wild-type VCP transgene (VCP-WT mice). This observation was further confirmed by expression of downstream targets of FOXO3, which were found to be unchanged. However, when compared with control and VCP-WT mice, the levels of phosphorylated forms of direct targets of MTOR are reduced in two different VCP-RH mouse lines. These findings suggest that MTOR signaling independent of AKT is defective in VCP-RH-expressing mouse muscle. Consistent with the fact that the MTOR direct targets EIF4EBP1 and RPS6KB1/p70S6 kinase 1 regulate protein translation, we showed that basal global protein translation is reduced in VCP-RH-expressing mouse muscle. MTOR phosphorylates ULK1, inhibiting autophagosome formation. Diminished MTOR activity thus leads to an increase in autophagosome formation. By blocking autophagosome degradation *in vivo* with colchicine we confirmed that VCP-RH have enhanced autophagosome biogenesis as compared with control and VCP-WT mouse muscle.

Having established that MTOR signaling is defective in IBMPFD/ALS, we further inhibited MTOR by treating mice every other day for 21 d with rapamycin, a well-established autophagy inducer. Surprisingly, compared with control and VCP-WT mice, VCP-RH mice develop a further decrease in muscle strength, concomitant with a significant increase in serum creatine kinase levels as well as the number of vacuolated and atrophic fibers in response to rapamycin treatment. Similarly, autophagic substrates accumulate more in VCP-RH mice following rapamycin treatment. These findings suggest that chronic inhibition of MTOR, which results in enhanced autophagosome biogenesis, significantly worsens the already existing muscle degenerative phenotype in VCP-RH mice. MTOR activity is responsive to upstream environmental cues such as insulin or IGF1, nutrients, and energy changes. Which of these signaling pathways underlies MTOR dysfunction in the setting of VCP mutations? To address this question, we showed that phosphorylation of AKT, a central player in the insulin signaling pathway, is stimulated by insulin in both VCP-WT and VCP-RH mice. Furthermore, we found that activation of AMPK, a master sensor/gauge for cellular energy, is unaffected in VCP-RH mice. However, treating mice with leucine i.p. after overnight starvation diminishes MTOR activation in VCP-RH mice compared with VCP-WT mice. Complementary experiments using U20S

cells demonstrated that a potent VCP inhibitor, N<sub>2</sub>,N<sub>4</sub>-dibenzylquinazoline-2,4-diamine (DBeQ), decreases both basal and nutrient-stimulated phosphorylation of MTOR targets similar to the effects of rapamycin. These findings, therefore, harmonize the *in vivo* data.

What are the chances and promises of restoring MTOR activity in IBMPFD/ALS? The unexpected observation that MTOR dysfunction aggravates weakness and pathology in IBMPFD/ALS led to the proposition that restoration of MTOR activity in the setting of this disease may provide clues to therapeutic strategies. MTOR signaling requires translocation of MTOR to an acidic late endosomal compartment that contains RHEB, and the integrity of the endo-lysosomal system is necessary for this process. Inhibition of VCP activity and IBMPFD/ALS mutant VCP expression disrupts endo-lysosomal trafficking resulting in enlarged nonacidic late endosomes. To this end, we suspected that aberrant endo-lysosomes may fail to localize RHEB or redistribute MTOR upon nutrient stimulation. With this in mind, we transfected GFP or constitutively active RHEB (caRheb-N153T-Flag) into U20S cells in the setting of DBeQ treatment and into stable VCP mutant cell lines including VCP-E578Q and VCP-R155H. Although restoration of the phosphorylation of RPS6 is less robust in the setting of DBeQ treatment and VCP-E578Q, the restoration in VCP-R155H is comparable to the VCP-WT cells. These

data, coupled with the fact that RHEB expression can increase the myofiber size when transiently expressed, established an emerging hypothesis that activation of MTOR by RHEB may be a viable and commendable therapeutic approach for IBMPFD/ALS. To further lend credence to this hypothesis, we demonstrated that transient expression of the caRheb-N153T-Flag plasmid, by electroporation, into mouse tibialis anterior muscle is capable of activating MTOR and recovering myofiber size in VCP-RH mice. Thus, our recommendations for future studies toward obtaining a finer picture of the outcome of restoring MTOR activity in IBMPFD/ALS hinge on a closer inspection of prolonged RHEB expression to determine whether it can abrogate weakness, vacuolation and inclusion body formation in VCP-IBM mice.

In conclusion, our data, which provide evidence that MTOR signaling is disrupted in IBMPFD/ALS resulting in increased autophagosome biogenesis, constitute a major leap forward in our understanding of the pathobiology of IBMPFD. Notably, chronic rapamycin treatment significantly worsens the degenerative phenotype in VCP-RH mice. Thus, the data suggest a new role for VCP in autophagy, specifically, that VCP regulates MTOR signaling.

**Disclosure of Potential Conflicts of Interest**  
No potential conflicts of interest were disclosed.